ACTION OF PHOSPHOLIPASE A_2 ON PHOSPHOLIPID VESICLES

Preservation of the membrane permeability barrier during asymmetric bilayer degradation

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Received 27 November 1978

1. Introduction

Phospholipases have proven to be valuable tools in the study of membrane structure. Transverse distribution of phospholipids has been investigated in various membrane species by using phospholipases to attack selectively the outer half of the bilayer structure [1–7]. In these studies a major problem is to preserve the membrane permeability barrier during the action of the phospholipase. The accumulation of phospholipid degradation products may cause changes in the permeability of the membrane, possibly rendering the inner half of the bilayer accessible to the enzyme used. Indeed, several subcellular membranes from rat liver recently have been demonstrated to become permeable to macromolecules during treatment with phospholipase A_2 [4–6]. Albumin was found to protect the membranes largely against the lytic action of lysophospholipids and fatty acids [3,4]. On the other hand, erythrocytes in the absence of albumin do retain their contents during phospholipase A_2-catalyzed degradation of the lipids in the outer half of the bilayer [1].

We subjected phospholipid vesicles to the action of phospholipase A_2 and found the vesicle membranes to be degraded asymmetrically. Moreover, the entire pool of phospholipids in the outer half of the bilayer could be degraded without release of an encapsulated compound, 6-carboxyfluorescein.

Abbreviations: PC, phosphatidylcholine (lecithin); PE, phosphatidylethanolamine; NAc-PE, *N*-acetimidoylphosphatidylethanolamine; IAI, isethionyl acetimidate; 6-CF, 6-carboxyfluorescein

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2. Experimental

Egg yolk PC and lyso-PC, bovine serum albumin (essentially fatty-acid free) and pure phospholipase A_2 from bee venom were obtained from Sigma. The PC was labelled with ^{14}C as in [8]. PE was extracted from egg yolk by the method in [9] and purified by column chromatography [10] and preparative thin-layer chromatography on silicagel. IAI was from Pierce and 6-CF from Eastman Kodak. The latter compound was purified as in [11].

Phospholipid vesicles were prepared from a mixture of PC and PE (molar ratio, 1:1) by sonication with a Branson B15 probe sonifier, at 10 or 20 μmol total lipid/ml aqueous medium. During sonication suspensions were cooled with ice/water and flushed with N_2. Multilamellar vesicles were isolated from preparations sonicated during 4 min by gel filtration on Sepharose CL-4B [12]. Single-bilayer vesicles were prepared by sonication during 30 min followed by centrifugation at 105 000 × *g* in a Spinco Ti50 rotor for 60 min. Usually >90% of the phospholipid was recovered in the supernatant essentially as single-bilayer vesicles (see fig.4).

Outer monolayer PE was derivatized with IAI as in [13]. Vesicles to be used for this purpose were prepared in 50 mM NaCl. Amidination was carried

out during 15 min at 25°C in 20 mM NaCl/0.2 M $NaHCO_3$ (pH 10.0) at IAI and 50 mM and 8.0 mM lipid, respectively. Subsequently the vesicles were isolated by elution with 50 mM NaCl on Sephadex G-100 and diluted to 2.0 mM lipid. Prior to phospholipase treatment an equal vol. 50 mM Tris·HCl (pH 7.5) was added.

Incubations with phospholipase A_2 were carried out at 25°C in 50 mM NaCl/50 mM Tris·HCl (pH 7.5), unless indicated otherwise. Concentrations were: lipid, 1.0 mM; $CaCl_2$, 1.0 mM; phospholipase, 10 μg/ml. Reactions were terminated by addition of EDTA to 5.0 mM.

For permeability measurements vesicles were prepared in 0.1 M 6-CF (adjusted to pH 7.5 with NaOH) and freed from non-encapsulated dye by elution with 50 mM NaCl/50 mM Tris·HCl (pH 7.5) on Sephadex G-100 (single-bilayer vesicles) or Sepharose CL-4B (multilamellar vesicles). At the concentration of the dye used its fluorescence is strongly quenched. Owing to dilution, leakage of 6-CF from the vesicles is revealed by an increase of the fluorescence [11]. Residual fluorescence of intravesicular 6-CF was always found to be <15%. Corrections were made using data obtained from vesicles prepared with varying intravesicular 6-CF concentrations. Total encapsulated 6-CF was determined after addition of Triton X-100 to 1% (v/v). Fluorescence measurements were carried out in a Perkin-Elmer MPF 43 fluorescence spectrophotometer with excitation and emission wavelengths of 490 nm and 520 nm, respectively.

Lipid analyses were done as in [14,15], with the exception that phospholipids from IAI-treated vesicles were separated as in [7]. PC and lyso-PC were determined by radioactivity measurements [16], non-radioactive phospholipids by phosphate assay [17].

3. Results

Action of phospholipase A_2 from bee venom on single-bilayer vesicles composed of PC and PE (molar ratio, 1:1) results in a maximal level of hydrolysis of ~60% for each of the lipid species. Although considerable amounts of potentially lytic lysophospholipids are formed, encapsulated 6-CF is completely retained in the vesicles during the time the phospholipase is acting, unless albumin is added (fig.1A). This indicates that the lysophospholipids and fatty acids formed, until they are removed by albumin, remain located in the vesicle surface and thus aid to maintain the bilayer integrity. With multilamellar vesicles lipid degradation levels off at ~35% hydrolysis, while addition of albumin results in release of only 55% of the encapsulated 6-CF (fig.1B).

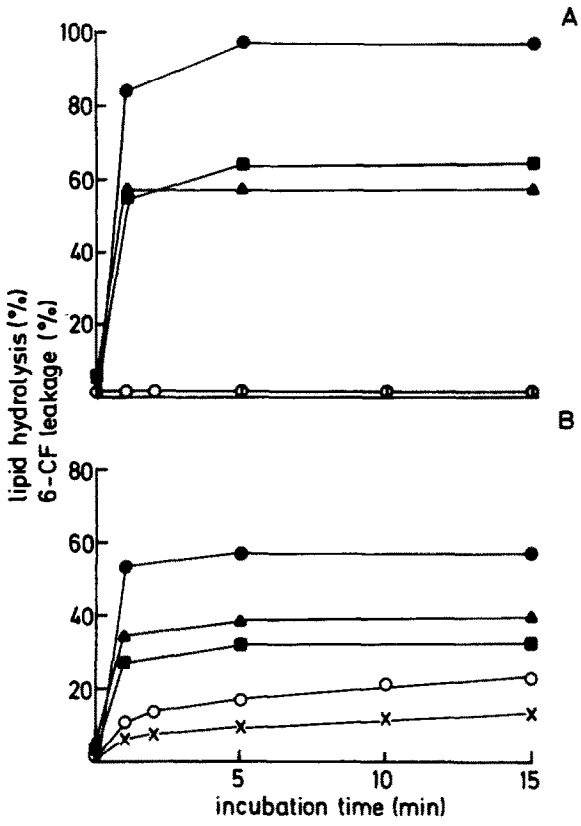

Fig.1. Action of phospholipase A_2 on PC/PE single-bilayer (A) and multilamellar (B) vesicles containing encapsulated 6-CF. At the time intervals indicated aliquots were taken for lipid analyses and fluorescence measurements. For direct fluorescence measurements samples were diluted with 50 mM NaCl/1.0 mM EDTA/50 mM Tris·HCl (pH 7.5). For measurements after albumin treatment, samples were incubated first with an equal volume of a solution of 40 mg albumin/ml NaCl/Tris-medium containing 5.0 mM EDTA and then diluted. (■) PC hydrolysis; (▲) PE hydrolysis; (●) leakage of 6-CF after addition of albumin; (○, X) leakage of 6-CF before addition of albumin (in fig.1B data designated by 'X' refer to leakage from whole vesicles and data designated by (○) to leakage from outer compartments only).

Assuming that phospholipase action is restricted to the exterior bilayer (see section 4) the albumin-induced release of 6-CF would correspond to the fraction of dye encapsulated in the outermost aqueous compartment of the vesicles. During phospholipase treatment multilamellar vesicles, contrary to single-bilayer vesicles, do loose some 6-CF before albumin is added (fig.1B).

The preservation of the bilayer permeability barrier during phospholipase action suggests that lipid hydrolysis is restricted to the outer monolayer of the lipid bilayer. This is supported by the observation that only part of the phospholipids can be degraded by the phospholipase. By following an experimental approach as in [6,7] we could substantiate this asymmetry of bilayer degradation. PC/PE single-bilayer vesicles were treated with the non-permeating amino-reagent IAI, which converts 85–90% of the PE in the outer half of the bilayer to the *N*-acetimidoyl derivative [7,13]. When such vesicles are subjected to phospholipase action the NAc-PE is degraded almost completely, whereas most of the PE remains unaffected, indicating that the bilayer is degraded asymmetrically (fig.2A). In order to exclude the possibility that NAc-PE as such influences the mode of action of the phospholipase we performed a control experiment in which vesicles were prepared from a lipid extract of IAI-treated vesicles. In such vesicles, in which the transverse distributions of PE and NAc-PE in the bilayer are supposedly the same, the phospholipase hydrolyzes ~60% of each of the three composite lipid species (fig.2B).

Since the phospholipase exclusively degrades phospholipids in the outer monolayer of the bilayer the inner monolayer remains composed entirely of non-degraded phospholipids. This may at least partly account for the stability of the vesicles during phospholipase treatment, but, in view of the difference between single-bilayer and multilamellar vesicles with respect to 6-CF leakage (fig.1), bilayer curvature may also be important in maintaining vesicle integrity. In order to explore these possibilities we first degraded the outer monolayer of PC/PE single-bilayer vesicles and then prepared new vesicles from the total lipid extract in the presence of 6-CF. Such vesicles, in which lysocompounds and fatty acids supposedly are present in both halves of the

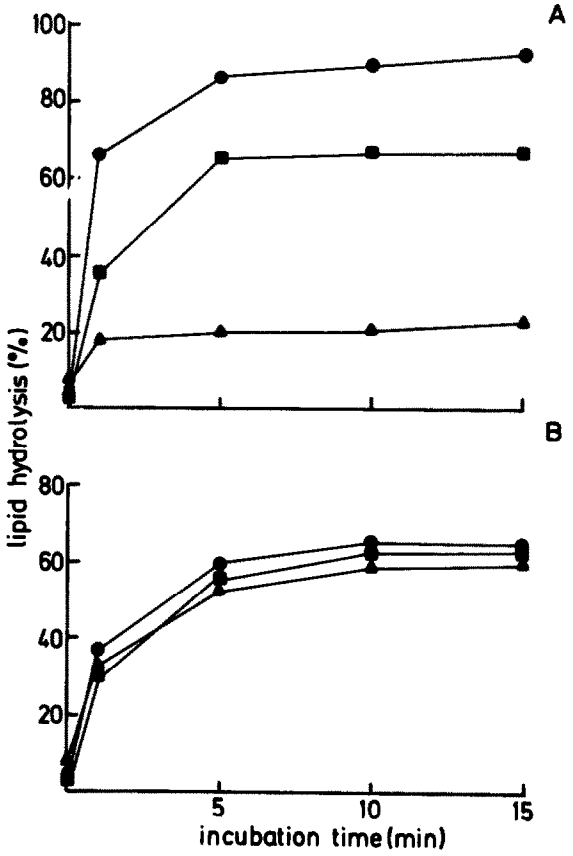

Fig.2. Action of phospholipase A_2 on IAI-treated PC/PE single-bilayer vesicles (A) and vesicles prepared from a lipid extract of IAI-treated vesicles (B). (•) NAc-PE hydrolysis; (■) PC hydrolysis; (▲) PE hydrolysis.

bilayer, are still able to entrap 6-CF though to much lower extents than PC/PE vesicles (7–10 nmol versus 27–35 nmol 6-CF/μmol total phospholipid). The rate of 6-CF leakage from these vesicles is considerably higher than that from phospholipase-treated single-bilayer vesicles, while leakage from non-treated PC/PE vesicles is very slow (fig.3). Furthermore, when we compare 6-CF leakage, after phospholipase treatment, from single-bilayer vesicles with that from the outermost compartment of multilamellar vesicles it strikes that 6-CF is released much faster from the latter, indicating a stabilizing effect of bilayer curvature.

The relatively poor encapsulation of 6-CF in vesicles made of the lysophospholipid-containing

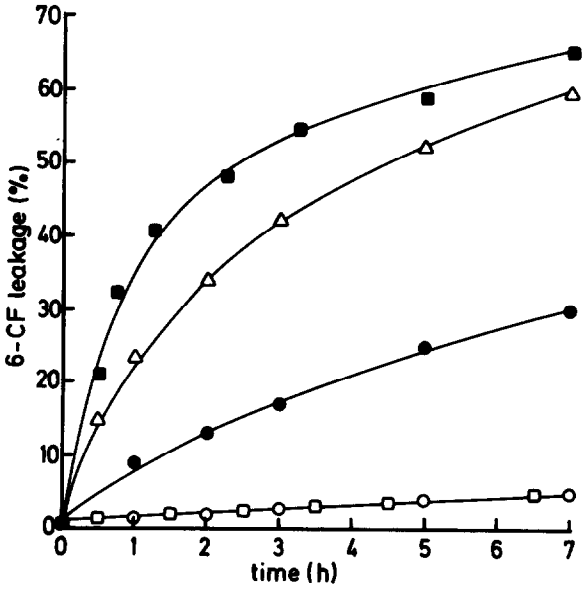

Fig.3. Leakage of 6-CF from lipid vesicles at 25°C. Circles and squares represent leakage from PC/PE single-bilayer and multilamellar vesicles, respectively. Open symbols, spontaneous leak; filled symbols, leak after a 15 min treatment with phospholipase at 25°C (data designated by (▪) refer to leakage from outer compartments only). Triangles represent leakage from single-bilayer vesicles prepared from a total lipid extract of vesicles of which the outer monolayer had been degraded with phospholipase (composition: 18% PC; 21% PE; 32% lyso-PC; 29% lyso-PE and corresponding amounts of fatty acids).

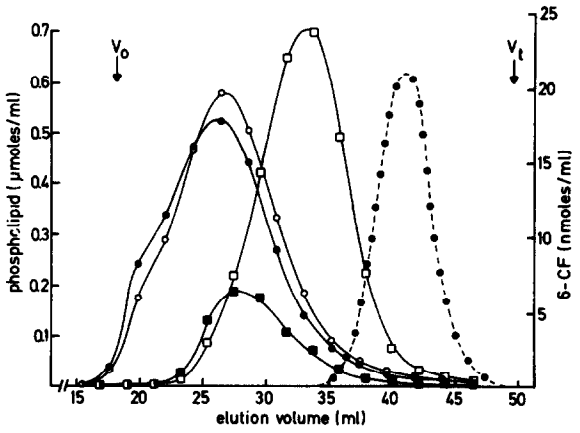

Fig.4. Fractionation on Sepharose CL-4B of PC/PE single-bilayer vesicles, lyso-PC micelles and vesicles prepared from a lipid extract containing lysophospholipids and fatty acids. Vesicles were prepared in the presence of 6-CF and freed from non-encapsulated dye on Sephadex G-100. The 6-CF-containing vesicles (~8 μmol phospholipid in 1.5 ml) were then applied to the Sepharose column (1.1 × 50 cm) and eluted with NaCl/Tris-medium. Fractions of 2.1 ml were collected and analyzed for phospholipid and 6-CF content. Circles, PC/PE vesicles; squares, lysophospholipid-containing vesicles (for preparation and composition see fig.3 legend); open symbols, phospholipid; filled symbols, 6-CF. (•—•—•) Elution profile of lyso-PC micelles. V_0 indicates the void volume, V_t the elution volume of 6-CF.

extract might be suggestive of a heterogeneous composition of the particle preparation, only part of the lipid being present as vesicular structures, the remainder constituting particles without trapping potency such as micelles. Therefore we chromatographed the preparation on Sepharose CL-4B. It appeared (fig.4) that the vesicles are intermediate in size between PC/PE single-bilayer vesicles and lyso-PC micelles, while only the larger vesicles in the preparation contain significant amounts of 6-CF. However, throughout the vesicle peak the phospholipid composition remained identical to that of the initial lipid preparation, suggesting a gross similarity of the particles involved. Therefore, the low average encapsulation of 6-CF is presumably the result of the presence of a substantial proportion of vesicles with an extremely small aqueous compartment.

4. Discussion

In agreement with [7] we observed that vesicle bilayers can be degraded asymmetrically by phospholipase A_2 (fig.2). Maximal hydrolysis then corresponds to complete degradation of the phospholipids in the outer half of the bilayer, which implies that the 'outside/inside' molar ratio for total phospholipid in the PC/PE single-bilayer vesicles we used is 1.56. PE has a relative preference for the inner monolayer, the outside/inside ratio being 1.36, while for PC the ratio is 1.77 (fig.1A). These results are in agreement with the observations in [10,18]. In multilamellar vesicles degradation levels off at 35% hydrolysis, probably representing the fraction of the phospholipids located at the vesicle surface. This would be in agreement with findings indicating that in similar vesicles 35% of the PE is exposed to a non-permeating amino-reagent (Y. Barenholz, personal communication). It can be calculated that vesicles with 35% of their phospho-

lipids exposed are composed of 3–4 bilayers and have a diameter of 60–80 nm if a thickness of 5 nm is assumed for bilayers as well as aqueous layers. The calculated diameter is in good agreement with our electron-microscopic observations on such vesicles (E. Wisse, G.L.S., unpublished).

In view of reported lytic effects of lysolecithin on artificial [19,20] as well as biological membranes [21,22] it is remarkable that vesicle bilayers containing approx. 60% lysophospholipid still exhibit barrier properties. Although, accepting permeability change as a measure of bilayer integrity, we have to conclude that lysophospholipids and fatty acids have some destabilizing effect on membrane structure (fig.3), this destabilization remains limited if the bilayer is strongly curved and lysophospholipids and fatty acids are located in one half of the bilayer only. The effect of curvature may be understood from the geometrical consideration that the stronger the bilayer curvature the tighter wedge-shaped lysophospholipids [23] can be packed in the outer monolayer. In addition, curvature might act to maintain the asymmetric lysophospholipid localization, transbilayer movements of lipids being extremely slow in small vesicles [13,24]. In this respect it should be noted that liposome lysis induced by lysolecithin is markedly facilitated around the phase transition temperature of the lipid [19,20]. Exogenous lysolecithins, acting as monomers rather than as micelles [22], are presumably incorporated in the outer half of the lipid bilayer. Within the lipid phase transition the asymmetry thus induced is likely to be counteracted by rapid transbilayer movements [25]. In view of our results this would lead to destabilization of the bilayer. Similarly, cold-induced erythrocyte lysis in the presence of exogenous lysolecithin is strongly accelerated by preincubation of the cells with the lysolecithin at 37°C [21]. Such a preincubation could very well provide the appropriate conditions for transbilayer movements of the lysolecithin to the inner monolayer.

It has been reported recently that albumin protects subcellular membranes from rat liver against the lytic action of phospholipase A_2 reaction products [3,4]. On the other hand, addition of albumin to erythrocytes [26] or vesicles (fig.1) after phospholipase treatment results in immediate collapse of the membrane structure. Since albumin will act to remove reaction products from the outer monolayer mainly rapid transbilayer movements of phospholipids, compensating for this loss of outer-monolayer lipid, could be responsible for stabilization of the rat liver membranes, whereas in erythrocytes such transbilayer movements are probably much less rapid and in vesicles even extremely slow also after phospholipase treatment.

Acknowledgements

We wish to thank Dr A. J. B. M. van Renswoude for helpful suggestions and for generously supplying purified 6-carboxyfluorescein and Rinske Kuperus for typing the manuscript.

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