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SURFACE DISTRIBUTION OF THE FATTY ACID SIDE CHAINS OF PHOSPHATIDYLETHANOLAMINE IN MIXED PHOSPHOLIPID VESICLES

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SUMMARY

A method has been developed for the selective determination of the fatty acid side chain distribution associated with the amino containing phospholipids located in the inner and outer surfaces of membranes. Using sonicated phosphatidylethanolamine/phosphatidylcholine vesicles as a model, the analysis consists of selective labeling of the outer surface amino groups with the membrane impermeable reagent 2,4,6-trinitrobenzenesulfonic acid. Outer and inner surface phosphatidylethanolamine fractions are separated by thin-layer chromatography. Analysis of methyl esters derived from these two fractions, by gas-liquid chromatography, yields the fatty acid side chain distribution. Our results show that there is no mol fraction dependence of the incorporation of any specific fatty acid side chains of egg yolk phosphatidylethanolamine into the vesicle or any preferential distribution of these side chains in the inner or outer vesicle surface. The surface distribution of the egg yolk phosphatidylethanolamine molecules in these vesicles appears to be determined by the head group packing requirements and not the fatty acid side chain composition.

INTRODUCTION

Detailed information concerning the surface distribution of molecular components in both model membranes and biological membranes has recently been obtained. This information has dealt primarily with the localization of proteins and various classes of lipids in either the inner or the outer membrane surface by chemical labeling of reactive side chains [1–3], by the utilization of hydrolytic enzymes [4, 5], by the application of shift reagents in ^{1}H-NMR experiments [6, 7] and by ^{31}P-NMR studies [7, 11]. In model systems, such measurements have shown that the phospholipid surface distribution is dependent on both the head group charge [7] and its cross sectional area [2] or combinations of both effects [11]. A more subtle level of information concerns the distribution of fatty acids associated with the various classes of phospholipids in the two surfaces of the bilayer. A model system consisting of mixed phosphatidylethanolamine/phosphatidylcholine vesicles has been used to study this

question. Our results indicate that there is no preferential occupancy of any of the egg yolk phosphatidylethanolamine fatty acid side chains in either the inner or the outer vesicle surface.

MATERIALS AND METHODS

Lipid purification and vesicle preparation and labeling

Chromatographically pure phosphatidylethanolamine and phosphatidylcholine derived from hen egg yolk were employed in this study. Phospholipid purification, lipid concentration determination by phosphate analysis, vesicle formation, labeling of outer surface amino groups of phosphatidylethanolamine and determination of phosphatidylethanolamine mol fraction have all been described previously [8].

Trinitrophenyl derivatives of the amino groups of outer surface phosphatidylethanolamine molecules were formed by reaction of 2,4,6-trinitrobenzenesulfonic acid with mixed phosphatidylethanolamine/phosphatidylcholine vesicles in aqueous bicarbonate buffer, pH 8.5. The labeling reaction is terminated by acidification of the reaction of the reaction mixture, with 2 ml of 0.5 M HCl in 46 % propanol, the final volume being 4 ml. This solution is then extracted with 12 ml of chloroform/methanol (2/1, v/v) containing 20 mg % of butylated hydroxytoluene. The chloroform layer was washed three times with 2 ml of water, and the washes discarded. The chloroform layer was taken to dryness on a rotary evaporator at reduced nitrogen pressure. The lipid sample was taken up in benzene and spotted, by streaking, on a 20×20 cm Silica H plate, 0.7 mm thick. The plate was developed in chloroform/acetone/methanol/acetic acid/water (36 : 48 : 12 : 12 : 6, v/v). After development, a score mark was made parallel to the edge of the plate in the direction of development so as to delineate a 20 mm lane; the plate was placed on a plexiglass frame, over an iodine tank, such that only the 20 mm lane was exposed to the iodine vapors. This procedure allowed the visualization of the individual lipid spots on the plate. Parallel regions on the unexposed region of the plate were scraped and placed in 15-ml screw capped vials. In low mol fraction samples, unlabeled phosphatidylethanolamine was visualized in the 20-mm lane with ninhydrin.

Methyl ester formation

Methyl esters of the phospholipid fatty acid side chains were formed by addition of 10 ml of 3 % (w/w) HCl (formed by adding acetyl chloride, obtained from Applied Science, to dry methanol) to the vials containing the silica gel powder derived from the thin-layer chromatography experiment. After being placed under an argon atmosphere and vortexed, the sample was heated for 1 h at 60 °C. An addition of 96 μg of pentadecanoic acid per μmol of phosphatidylethanolamine transesterified was made to each vial as an internal standard for the mass of fatty acid side chain. After cooling, the samples were transferred to 18-ml test tubes and 3 ml of 5 % NaCl solution added to each tube. The mixture is then extracted with 5 ml of pentane, pesticide grade, by vortexing. The sample is allowed to stand until phase separation occurs and the upper pentane layer is drawn off with a pasteur pipette. The pentane extraction is repeated twice using 5 ml and then 3 ml. The pooled pentane fractions are taken to dryness with a stream of nitrogen and the methyl esters dissolved in 20 μl of Lipopure hexane (Applied Sciences Labs, Inc.).

Gas-liquid chromatography

Analysis of fatty acid methyl ester compositions were carried out on a Shimadzu GC 4BPT gas chromatograph fitted with a thermal conductivity detector. 10 % Silar 10 C adsorbant on 100/120 mesh Gas Chrom Q Support (Applied Sciences Labs, Inc., State College, Pennsylvania) packed in stainless steel columns (3 mm internal diameter, 4 mm external diameter) 3 m long were employed in these studies. The column temperature was programmed to operate isothermically at 145 °C for 17 min and to then initiate a temperature rise to 210 °C at a rate of 3 °C/min. A carrier gas flow rate of 40 ml/min of He was employed. Samples injected usually contained the fatty acid methyl esters derived from 0.2 μM of phosphatidylethanolamine in a maximum of 4 μl of lipopure hexane (Applied Sciences Labs, Inc.).

The mass of methyl ester present in each sample is determined by reference to the integrated area of the pentadecanoic acid standard which was added to each sample [9]. Areas of peaks in the chromatographic profiles were estimated by multiplying the amplitude of the peak by its half bandwidth.

RESULTS AND DISCUSSION

The surface distribution of phospholipid classes can be expected to depend on at least three factors: the net charge on the phospholipid head group, the cross sectional area of the head group, and the length and degree of unsaturation of the fatty acid side chains. Reports have appeared which implicate both charge [7] and cross sectional area [8] in inducing head group asymmetry across phospholipid bilayers in sonicated vesicles. Recent experiments employing ^{1}H-NMR and ^{31}P-NMR show that in vesicles formed from mixtures of phosphatidylcholine with phosphatidylserine, phosphatidylinositol and phosphatidic acid, the non-choline-containing, charged phospholipids all distribute preferentially towards the inner vesicle surface [11]; this is in good agreement with my own results for the surface distribution of phosphatidylserine in mixed phosphatidylserine/phosphatidylcholine vesicles (Litman, B. J., unpublished). These experiments with charged phospholipids indicate that the major physical force in determining the surface distribution of these molecules in vesicle systems is the head group packing requirement and that charge effects are a secondary perturbation, which is manifested most strongly when the cross-sectional areas of the phospholipid head groups of the lipids in the vesicle bilayer are close to equivalent, such as in the case of phosphatidylglycerol/phosphatidylcholine vesicles. [7].

Since the free volume behind the cross sectional area of a head group in the outer surface of a bilayer is a decreasing function going from the surface towards the median line of the bilayer, and the free volume behind the equivalent head group in the inner vesicle surface increases going towards the median line [10], one would expect that packing considerations would lead to a preferential distribution of a particular fraction of phospholipid molecules in the interior or exterior vesicle surface, which would be dependent on the free volume associated with the hydrocarbon portion of the phospholipid molecule and hence on the degree of unsaturation and length of the fatty acid side chains. In experiments designed to obtain information concerning the surface distribution of fatty acid side chains of phosphatidylethanolamine in mixed phosphatidylethanolamine/phosphatidylcholine vesicles, the outer surface phosphati-

dylethanolamine molecules were selectively labeled by reaction with the membrane impermeable reagent 2,4,6-trinitrobenzenesulfonic acid. Thin-layer chromatographic procedures allow the separation of the phosphatidylethanolamine into a labeled and an unlabeled fraction, which corresponds to a separation of the outer and inner surface phosphatidylethanolamine molecules, respectively. Transesterification to obtain the fatty acid side chain methyl esters, followed by gas-liquid chromatography in the presence of an internal mass standard, allows one to determine the mass distribution of the various fatty acid side chains associated with the inner and outer surface phosphatidylethanolamine molecules. Experiments similar to those described above, carried out on unlabeled phosphatidylethanolamine/phosphatidylcholine vesicles, yields the total phosphatidylethanolamine fatty acid side chain distribution in the two surfaces.

The mol fraction dependence of the fatty acid side chain distribution associated with the total egg yolk phosphatidylethanolamine incorporated into the mixed phosphatidylethanolamine/phosphatidylcholine vesicles was determined at 0.11, 0.33 and 0.57 mol fraction of phosphatidylethanolamine (Table I). Since the mass percent distribution of the fatty acid side chains associated with the phosphatidylethanolamine molecules shows no dependence on the mol fraction of phosphatidylethanolamine in the vesicle and is identical to that of the stock solution from which the vesicles were formed, we conclude that there is an equal probability of incorporation into the vesicle for all molecular species of phosphatidylethanolamine. A comparison of the fatty acid side chain distribution of the inner and outer surface phosphatidylethanolamine fractions was made at the aforementioned phosphatidylethanolamine mol fractions (Table II). The results show that the fatty acid side chain distributions associated with the inner and outer surface phosphatidylethanolamine fractions are identical to each other and to the phosphatidylethanolamine stock solution over the entire mol fraction range studied; these findings demonstrate that there is an equal probability of occupancy in either vesicle surface for all the molecular species of phosphatidylethanolamine.

Each class of naturally occurring phospholipids represents a group of molecules

TABLE I

TOTAL FATTY ACID SIDE CHAIN DISTRIBUTION OF PHOSPHATIDYLETHANOLAMINE INCORPORATED IN MIXED PHOSPHATIDYLETHANOLAMINE PHOSPHATIDYLCHOLINE VESICLES*

Fatty acid	Phosphatidylethanolamine	Mol fraction of phosphatidylethanolamine		
		0.11	0.33	0.57
16 : 0	21	22	23	20
18 : 0	26	26	27	27
18 : 1	19	20	18	18
18 : 2	16	15	15	15
20 : 4	12	10	12	13
unknown	1	2	3	3
22 : 6	3	2	2	2

* Reported as mass percent of total fatty acid side chain in sample.

TABLE II

COMPARISON OF INNER AND OUTER SURFACE PHOSPHATIDYLETHANOLAMINE FATTY ACID SIDE CHAIN DISTRIBUTIONS IN MIXED PHOSPHATIDYLETHANOLAMINE PHOSPHATIDYLCHOLINE VESICLES*

Fatty acid	Mol fraction of phosphatidylethanolamine					
	0.11		0.33		0.57	
	Inner surface	Outer surface	Inner surface	Outer surface	Inner surface	Outer surface
16 : 0	26	23	23	22	22	21
18 : 0	26	27	29	29	28	27
18 : 1	20	19	19	18	18	18
18 : 2	15	16	15	15	15	16
20 : 4	9	11	10	11	12	13
unknown	2	2	2	3	3	3
22 : 6	2	2	2	2	3	3

* Reported as mass percent of total fatty acid side chain in sample.

with a common head group, but a variety of pairs of fatty acid side chains. The mass percentage composition of the egg yolk phosphatidylethanolamine employed in this study shows an average of one saturated to one unsaturated fatty acid side chain. The absence of a preferential surface distribution of the egg yolk phosphatidylethanolamine molecules suggests that a one to one pairing of saturated and unsaturated fatty acid side chains may occur in these molecules in such a fashion that no molecular species shows a preferential surface distribution that is driven by a specific side chain packing requirement of that species. Synthetic phosphatidylethanolamines, which contain either paired saturated or paired unsaturated fatty acid side chains, might be expected to form vesicles in which the effect of fatty acid chain compositions would be expressed more strongly. Initial experiments involving the mixing of dilaurylphosphatidylethanolamine, dimyristoylphosphatidylethanolamine and dipalmitoylphosphatidylethanolamine, each mixed at 0.1 mol fraction with egg yolk phosphatidylcholine, show that the saturated phosphatidylethanolamines distribute in the outer surface to a greater degree than egg yolk phosphatidylethanolamine at the same mol fraction. These results indicate that if molecular species having greatly different packing requirements for their fatty acid side chains occur in the vesicle bilayer, then these requirements may express themselves through changes in the surface distribution of these components. More extensive studies are being carried out to assess this possibility.

Our results show that there is no preferential surface distribution of the fatty acid side chains of egg yolk phosphatidylethanolamine molecules in either vesicle surface, supporting the contention that the head group packing requirements is the major determinant of the surface distribution of these molecules. They further indicate that in certain systems the fatty acid side chain composition may influence the surface distribution. The methods described herein demonstrate the feasibility of assessing the factors determining the surface distribution of amino containing phospholipids in model systems and biological membranes.

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