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Curvature and composition-dependent lipid asymmetry in phosphatidylcholine vesicles containing phosphatidylethanolamine and gangliosides

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The effect of curvature on transbilayer lipid asymmetry in vesicles is investigated using vesicles of different sizes (30–140 nm) prepared by sonication and polycarbonate filter extrusion techniques. The transbilayer distributions of phosphatidylethanolamine and gangliosides are measured using 2,4,6-trinitrobenzenesulphonic acid and *Clostridium perfringens* neuraminidase as non-penetrating probes, respectively. The distribution of phosphatidylethanolamine in a phosphatidylcholine/phosphatidylethanolamine (4:1, molar ratio) system is more or less symmetric and curvature seems to have little effect. However, the distribution of gangliosides in a phosphatidylcholine/ganglioside (10:1, molar ratio) system is asymmetric in favour of the outer layer in smaller vesicles, the asymmetry disappearing as the degree of curvature decreases. In a phosphatidylcholine/phosphatidylethanolamine/ganglioside (8:2:1, molar ratio) system, both phosphatidylethanolamine and gangliosides distribute asymmetrically, indicating a composition-dependent asymmetric distribution of phosphatidylethanolamine. In this system asymmetry also increases with increasing curvature. The asymmetric distribution of gangliosides in vesicles of low curvature may be due to their long headgroup and larger headgroup surface area in accordance with the theoretical predictions of Israelachvili et al. (Biochim. Biophys. Acta 470 (1977) 185–201).

Introduction

There is considerable evidence indicating an asymmetric transbilayer distribution of phospholipids and glycolipids in biological membranes [1–3]. For instance, in the plasma membrane, glycolipids are almost exclusively located on the outer layer while PA, PS, PI, and PE have a preference for the inner (cytosolic) layer. Such asymmetric distributions might reflect the functional roles of various lipids. Glycolipids have been implicated in a wide variety of cell surface recognition phenomena including cell-cell adhesion and this probably justifies their presence on the outer layer of plasma membrane [4–6]. Recent studies implicating PI metabolites as second messengers in many intracellular processes offer a functional justification for the presence of PI on the cytosolic side of the plasma membrane [7]. Though such correlations between functional roles and transbilayer distribution of lipids can be suggested for other lipids as well, the basic factors producing and/or maintaining lipid asymmetry are still unclear. Theoretical and experimental studies have implicated a variety of factors as responsible for lipid asymmetry [8,9]. These include differences in the packing of lipids in the two layers [10], specific lipid–protein interactions [11], asymmetric synthesis of lipids on membranes [12,13], asymmetric turnover of lipids on membranes [9] and differences in rates of lipid flip-flop between the two layers, mediated presumably by proteins and lipid phase changes [14–17]. It is quite likely that a combination of all or some of these factors rather than a single one might be responsible for the distribution of each lipid.

The contribution, if any, of lipid packing towards asymmetry can be easily assessed using lipid vesicles as model systems. Some studies have shown that certain lipids distribute asymmetrically in vesicles [18–22]. From

Abbreviations: PC, phosphatidylcholine (egg); PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; TNBS, 2,4,6-trinitrobenzenesulphonic acid; SUV, small unilamellar vesicle; EV_n, extrusion vesicles prepared with a polycarbonate filter of pore size n nm.

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theoretical considerations, Israelachvili et al. [8] concluded that vesicles of mixed lipid systems should be asymmetric and the extent of asymmetry is determined by the size (curvature) of the vesicle and the size of the lipid headgroup. The nature of the acyl side chain has also been implicated in the asymmetric distribution of lipids [23]. However, curvature of the vesicle is likely to be the most important factor determining lipid packing. The measurement of PE distribution in PC-PE vesicles by Nordlund et al. [24] has shown that the asymmetry of PE distribution (apparently, in favour of the outer layer) in SUV disappears in LUV prepared by ethanol injection method. Though this is in agreement with the theoretical predictions of Israelachvili et al. [8], the data are not sufficient to make a general conclusion. Further, the behaviour of a lipid preferring the outer layer of plasma membrane is not known. Hence, we have undertaken a more systematic study of the effect of curvature on the transbilayer distribution of PE and gangliosides in egg PC matrix over a wide range of vesicle sizes produced by the polycarbonate membrane extrusion technique [25,26]. A ternary system consisting of egg PC, PE and gangliosides was chosen with a view to study composition-dependent changes, if any, in the transbilayer distribution of PE and gangliosides. The results have shown both curvature- and composition-dependent changes in the transbilayer distribution of PE as well as that of gangliosides.

Materials and Methods

Materials

Egg PC (Type V), bovine brain PE, trinitrobenzenesulphonic acid (TNBS), Triton X-100, neuraminidase from *Clostridium perfringens* (Type VI), 2-thiobarbituric acid and resorcinol were obtained from Sigma Chemicals, U.S.A. Bovine brain mixed gangliosides (Folch extract) were purchased from Supelco, Canada and purified by a further Folch partitioning [27]. The purity of the mixed gangliosides thus purified as well as that of PC and PE were checked by TLC and were found to be pure. Polycarbonate membranes were from Nuclepore, Canada. Sephacryl S-500 and Sepharose 4B were products of Pharmacia Fine Chemicals. [^{3}H]Cholesterol (spec. act. 60 Ci/mmol) obtained from New England Nuclear, Canada, was purified by TLC [26].

Preparation of vesicles

A multilamellar dispersion of the mixed lipid system of the required composition (usually 4 μmol of PC, 1 μmol of PE and 0.5 μmol of gangliosides) in 3 ml of 10 mM Tris-acetate buffer (pH 7.0) containing 50 mM NaCl was prepared as described earlier [26,28]. The dispersion was sonicated and centrifuged as described earlier to obtain SUVs [26,28].

Multilamellar dispersions were frozen in liquid nitrogen and thawed in lukewarm water. The freeze-thaw cycle was repeated five times and then the dispersion was subjected to extrusion through a stacked pair of polycarbonate filters of the desired pore size (50–400 nm) with nitrogen pressure as described elsewhere [25,26]. The dispersion obtained after 12 cycles of extrusion was used for various measurements as 'extrusion vesicles' (EV). The notation EV_n is used to denote the extrusion vesicles prepared with a filter of pore size n nm.

Characterization of vesicles

The vesicle preparations were characterized by negative staining electron microscopy and gel filtration on Sephacryl S-500. The vesicle size was measured by negative staining electron microscopy as described earlier [26].

Some of the vesicle preparations labelled with traces of [^{3}H]cholesterol were subjected to gel filtration on a 46 × 1.5 cm Sephacryl S-500 column. 1-ml samples were applied and eluted with 10 mM Tris-acetate buffer (pH 7.0) containing 50 mM NaCl at a flow rate of 20 ml/h. Fractions (1 ml) were collected and the radioactivity in 100-μl aliquots was measured by liquid scintillation counting.

Measurement of PE and ganglioside distribution

The transbilayer distribution of PE in vesicles was measured by the TNBS reaction of Litman [18] as modified by Nordlund et al. [24].

The transbilayer distribution of mixed gangliosides in vesicles was measured as the extent of desialation by *Cl. perfringens* neuraminidase as described earlier with some minor modifications [22]. Briefly, an aliquot of vesicle containing 25 nmol of gangliosides was incubated with 10 munits of neuraminidase (the stock solution of neuraminidase contained 2 mg bovine serum albumin per unit of the enzyme to stabilize it [29]) in the presence and absence of 10 mM Triton X-100 at 37 °C in 10 mM Tris-acetate buffer (pH 7.0) containing 50 mM NaCl. After 5 h of incubation, the sialic acid released was measured by the thiobarbituric acid assay of Warren [30]. A blank containing everything except the enzyme was run and the reading subtracted. Under these experimental conditions, the desialation reaction is complete within 3–4 h. The ratio of sialic acid released in the absence of Triton X-100 to that in the presence of Triton X-100 gives the fraction of gangliosides exposed on the outer surface of vesicles.

Other analytical methods

The phospholipid concentration in vesicles was estimated by measuring the total lipid phosphorus according to the method of Fiske and SubbaRow [31] using Elon (*p*-methylaminophenol sulphate) as the re-

ducing agent. The concentration of gangliosides in vesicles was calculated assuming 2 mol of sialic acid/mol of ganglioside and estimating sialic acid by the resorcinol method of Svennerholm [32]. The lipid composition of typical vesicles prepared by extrusion and sonication were analyzed by these methods and was found to be the same as the input composition within experimental error limits.

Results

Lipid vesicles of different composition with PC as the matrix lipid have been prepared by sonication and polycarbonate membrane extrusion techniques for these studies. While sonicated vesicles (SUVs) have been used and characterized by numerous investigators during the last two decades, the use of extrusion vesicles has started gaining momentum only recently. The extrusion technique provides a good method for preparing vesicles of varying sizes free of detergents and organic solvents, the contaminants in vesicles prepared by detergent analysis and reverse phase evaporation methods. Such contaminants are likely to influence physico-chemical properties of vesicles as has been shown in some cases [33–35]. Hence, extrusion vesicles are ideally suited for studying lipid asymmetry and especially, the curvature dependence of asymmetry. Recently, we have used extrusion vesicles containing 50 mol% cholesterol for investigating the effect of curvature on the rate of cholesterol transfer between lipid vesicles [26]. The vesicles used in the present study are slightly different from these vesicles. The most important difference is the absence of cholesterol in the present vesicles. Also, the methods of preparation are not identical, though very similar. In the present case, vesicles were prepared as described by Mayer et al. [25]. The changes in lipid composition and procedure necessitated a partial characterization of extrusion vesicles.

The diameter of the vesicles measured from negative staining electron micrographs are given in Table I. The vesicle size for the PC/PE/ganglioside (8 : 2 : 1, molar ratio) system increases with increasing pore size of the polycarbonate filter as reported earlier for other lipid compositions [25,26]. The values are closer to those for PC alone reported by Mayer et al. [25] than to those for the PC/PS/cholesterol (4 : 1 : 5, molar ratio) system reported by us [26]. This is probably a reflection of the ability of cholesterol to increase vesicle size at high mole fractions [36]. In fact, the size of the PC-PE-ganglioside extrusion vesicles given in Table I are slightly less than those reported for vesicles of PC alone by Mayer et al. [25] and this may be due to the presence of gangliosides. Gangliosides are known to cause the formation of micelles in mixed lipid systems at concentrations more than 30 mol% of the total lipid [37,38]. The concentration used in our system (9 mol%) is well below this limit. However, the possibility of micelle formation was examined by gel filtration. Sephacryl S-500 gel filtration profiles for some of the vesicle preparations are given in Fig. 1. There is no evidence for micelle formation at all in the (8 : 2 : 1) PC/PE/ganglioside system as well as in the (10 : 1) PC/ganglioside system as there are no peaks for EV_{100} around fraction number 62 which is the eluting position for micelles (as given by ganglioside micelles). This result was also confirmed by the gel filtration profiles using a Sepharose 4B column of the same dimensions (data not shown).

The fractions (as percentage) of PE and ganglioside exposed on the outer surface of vesicles are also given in Table I. The use of TNBS and *Cl. perfringens* neur-

TABLE I

Transbilayer distribution of PE and gangliosides in vesicles of different sizes and composition

Vesicle type	Lipid composition	Pore size of the filter (nm)	Vesicle diameter [a] (nm)	% Lipid exposed on the outer surface of vesicle			
				expected [b]	PE [c]	Gang [c,d]	Gang/PE
SUV	(8:2:1) PC/PE/Gang	sonication	31 ± 15	64.5	70.6 ± 1.4	93.8 ± 1.3	1.37
EV_{50}	(8:2:1) PC/PE/Gang	50	66 ± 37	56.4	65.5 ± 1.8	73.6 ± 2.3	1.12
EV_{80}	(8:2:1) PC/PE/Gang	80	69 ± 24	56.1	66.8 ± 1.2	66.2 ± 0.4	0.99
EV_{100}	(8:2:1) PC/PE/Gang	100	89 ± 30	54.7	63.1 ± 1.4	63.8 ± 0.5	1.01
EV_{200}	(8:2:1) PC/PE/Gang	200	113 ± 40	53.7	60.1 ± 1.5	59.5 ± 0.8	0.99
EV_{400}	(8:2:1) PC/PE/Gang	400	139 ± 70	53.0	49.5 ± 2.5	48.0 ± 1.1	0.94
SUV	(4:1) PC/PE	sonication		64.5	61.1 ± 1.8	-	-
EV_{100}	(4:1) PC/PE	100		54.7	56.5 ± 2.1	-	-
SUV	(10:1) PC/Gang	sonication		64.5	-	88.1 ± 0.5	-
EV_{100}	(10:1) PC/Gang	100		54.7	-	64.6 ± 1.7	-

[a] Vesicle diameter obtained from negative staining electron microscopy and given as mean ± S.D.

[b] Calculated as the ratio of surface area of outer layer to that of inner layer.

[c] Values are given as mean ± S.E. from measurements on 3–5 different preparations, each one done in duplicate. Colorimetric estimations have an error of ±4%.

[d] Gang is the abbreviation for mixed gangliosides.

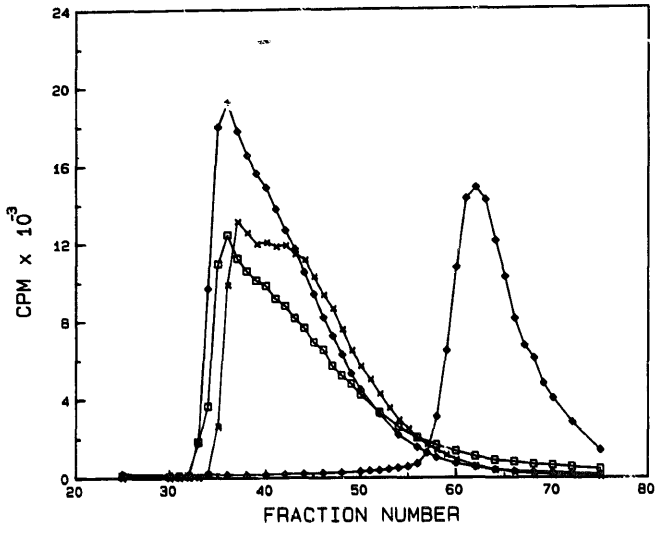

Fig. 1. Gel-filtration profiles for ganglioside micelles (◆) and EV_{100} made up of (4:1) PC/PE (□), (10:1) PC/ganglioside (◇) and (8:2:1) PC/PE/ganglioside (×). 1 ml sample was applied to a 46×1.5 cm Sephacryl S-500 column and eluted with 10 mM Tris-acetate buffer (pH 7.0) containing 50 mM NaCl at a flow rate of 20 ml/h. 1-ml fractions were collected and the radioactivity ([^{3}H]cholesterol added as tracer) in 100-μl aliquots measured.

aminidase as non-penetrating probes for the determination of PE and gangliosides, respectively, on the outer surface of lipid vesicles is well established [18,22,24]. The values in Table I indicate an increasingly asymmetric distribution of both PE and gangliosides with increasing curvature (decreasing size) of the vesicle except

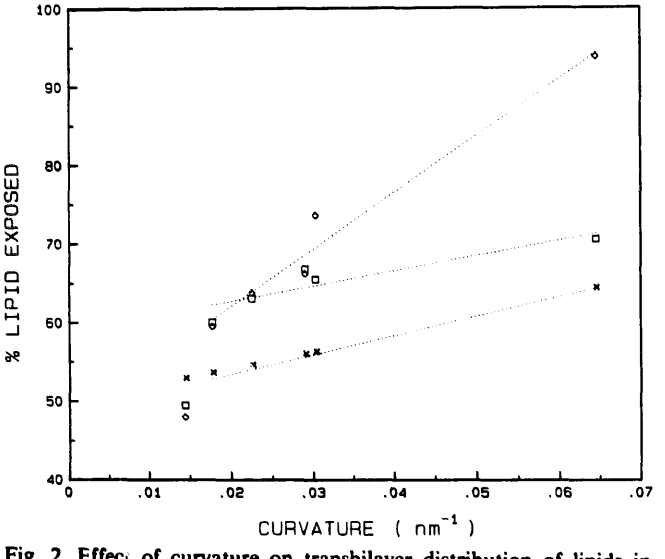

Fig. 2. Effect of curvature on transbilayer distribution of lipids in vesicles. % PE exposed (□) and % ganglioside exposed (◇) on the outer surface of (8:2:1) PC/PE/ganglioside vesicles prepared by sonication and extrusion through polycarbonate filters of different pore sizes (data from Table I). The expected symmetric distribution (×) based on curvature is also plotted. Linear fitting by the method of least squares gives correlation coefficients of 0.90, 0.98 and 1.0 for PE, ganglioside and expected distributions, respectively (EV_{400}, which may contain multilamellar vesicles, is omitted in these calculations). The dotted lines represent the fitted curves.

for the (4:1) PC/PE vesicle preparations. The effect of curvature (geometrically defined as the reciprocal of the radius) of the vesicle on the transbilayer distribution of PE and gangliosides is represented diagrammatically in Fig. 2. The % PE as well as ganglioside exposed on the outer surface increase with increasing curvature of the vesicle almost linearly. The linear fittings shown in Fig. 2 have omitted the points corresponding to EV_{400} as this preparation is likely to be contaminated by multilamellar vesicles (see Discussion).

Discussion

Asymmetry is a confusing term when it comes to lipid vesicles. There is a tendency to consider any distribution other than 50:50 as asymmetric. While this is more or less correct for biological membranes (because they are almost planar), it is not so for lipid vesicles, especially the highly curved ones such as SUVs. A simple definition for symmetric distribution may be given as the ratio of the surface areas of the outer and the inner layers of the bilayer (assuming the same surface area per headgroup on both the layers). If r is the radius of the vesicle and t the thickness of the bilayer, this ratio will be $r^2/(r-t)^2$. Assuming a bilayer thickness of 4 nm, this will give an outside/inside ratio of 2.16, i.e. 68% of total lipid exposed for SUVs of diameter 25 nm, and this is the value experimentally observed [39,40]. Similar calculations result in 54% of total lipids on the outer layer of vesicles of 100 nm size as the symmetric distribution. Thus, the symmetric distribution depends on the size of the vesicle and only deviations from this symmetric distribution can be considered asymmetric. For instance, 55–60% PE exposed on SUVs of PC containing 30–50 mol% PE should be considered asymmetric in favour of the inner layer and not in favour of the outer layer as the number may apparently suggest [18,24].

The expected symmetric distributions (calculated as above) for the vesicles prepared in the present study are tabulated in Table I and also plotted in Fig. 2. Comparing these values with the experimentally observed values does show an asymmetric distribution of both PE and gangliosides in most of the vesicles measured. Another factor to be considered for contributing to these changes in asymmetry is the lamellarity of the vesicle. Obviously, vesicles should be unilamellar. The presence of multilamellar vesicles will lead to values (% exposed) lower than the actual distribution. ^{31}P-NMR line broadening can, in principle, be used to study this factor [25]. However, we have not attempted this because asymmetric distributions of PE and gangliosides might induce an asymmetric distribution of PC as well, thereby invalidating the basic assumption of this technique [41]. Mayer et al. [25] have shown that EV_{30}, EV_{50} and EV_{100} made of PC alone are unilamellar while EV_{200} is predomi-

nantly unilamellar. Since we have followed the same method of preparation, it may be assumed that EV_{50}, EV_{80} and EV_{100} prepared by us are also unilamellar. Since the size of EV_{200} prepared by us is close to that of EV_{100} prepared by Mayer et al. [25] and the percentages of PE and gangliosides exposed on EV_{200} are greater than the expected symmetric distribution, EV_{200} should also be unilamellar. Similar considerations suggest a slight contamination of EV_{400} with multilamellar vesicles. One more factor to be considered for contributing to the differences in asymmetry is the presence of micelles in vesicle preparations, especially those containing gangliosides because of their tendency to form micelles in mixed systems [37,38]. The concentration of gangliosides in our vesicle preparations is well below the critical concentration (30 mol% ganglioside) for micelle formation and further the gel filtration profiles (Fig. 1) indicate a total absence of micelles in vesicle preparations. Thus, the differences between expected and observed distributions seen in Table I represent real lipid asymmetry.

The effect of curvature on asymmetry can be seen in Fig. 2. The difference between the experimental and expected values of distribution, which is a measure of the asymmetry, increases with increasing curvature of the vesicle for gangliosides in the (8:2:1) PC/PE/ganglioside system. A more quantitative measure of asymmetry is the ratio of the experimental to the expected distribution. For gangliosides the values (of the ratio) are 1.45, 1.31, 1.18, 1.17 and 1.11 for SUV, EV_{50}, EV_{80}, EV_{100} and EV_{200}, respectively, thus showing a continuous change in asymmetry with curvature. The limited data on the (10:1) PC/ganglioside system (Table I) also show the same effect. This curvature effect is in agreement with the theoretical predictions of Israelachvili et al. [8]. However, the PE distribution does not show a similar relationship. This difference in behaviour of gangliosides and PE can be seen more clearly from the ratio of gangliosides exposed to PE exposed in the vesicles given in Table I. An important conclusion from these data is that spontaneous asymmetric distribution of lipids can occur in membranes of very low curvature and that this is determined by the nature of the lipid molecule.

Can mixed lipid systems cause transbilayer asymmetry? The data presented here also throw some light on this aspect. Comparing binary and ternary systems having common lipid components including a matrix lipid is one of the best ways to study this. The PE distribution in the (4:1) PC/PE system is almost symmetric in SUV as well as EV_{100}. The incorporation of gangliosides into this system induces an asymmetric distribution of PE in favour of the outer monolayer in all vesicle preparations except EV_{400}. On the other hand, the distribution of gangliosides in the (10:1) PC/ganglioside system is already asymmetric in favour of the outer layer in both SUV and EV_{100}. The addition of PE does not alter this distribution in EV_{100}, but enhances the asymmetry in SUV ($P < 0.007$). Thus, the PC/PE/ganglioside system seems to exhibit composition-dependent asymmetry.

The most important observation from these studies is the asymmetric distribution of gangliosides in large vesicles. According to the theoretical treatment of Israelachvili et al. [8], mixed lipid vesicles should always be asymmetric. In binary systems, the asymmetry is determined by headgroup interactions and the lipid with a longer headgroup and greater ratio of headgroup surface area to vesicle radius (for the pure lipid) will prefer the outer layer. Both these factors should promote an asymmetric distribution of gangliosides in favour of the outer layer (on the average, a ganglioside headgroup will be 3-times longer than a PC headgroup. The average surface area of ganglioside is twice more than that of PC [6,42]. The radius of the ganglioside micelle is smaller than that of the PC vesicle [43]). The present results are in agreement with this. The magnitude of the asymmetry arising from headgroup differences is inversely related to the radius of the mixed lipid vesicle and is consistent with our findings (Fig. 2). Thus the small, but finite curvature of the large vesicles seems to be the basic factor causing asymmetry. A preferential interaction of gangliosides with PE over PC may be responsible for the composition-dependent asymmetry in the PC/PE/ganglioside system.

The physiological significance of these results is a matter of speculation. Apparently, the ganglioside asymmetry in lipid vesicles reported here parallels the asymmetry seen in plasma membranes [1,3]. However, the almost complete ganglioside asymmetry in plasma membrane may be arising from other factors. In Golgi apparatus, where they are synthesized, and in vesicles which are thought to transport them to the cell surface, gangliosides are believed to be located in the inner monolayer facing the lumen [44]. Fusion of these vesicles with the plasma membrane will lead to a reversal of the sides of vesicles resulting in the natural outward orientation of gangliosides in plasma membrane. Thus, the asymmetric synthesis of gangliosides in Golgi apparatus along with vesicular transport and the very slow flip-flop in vesicles could account for the origin of ganglioside asymmetry in plasma membranes. However, the formation of stable asymmetric membranes of low curvature reported here may be involved at least partially in maintaining this asymmetry against a possible decay by flip-flop.

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