

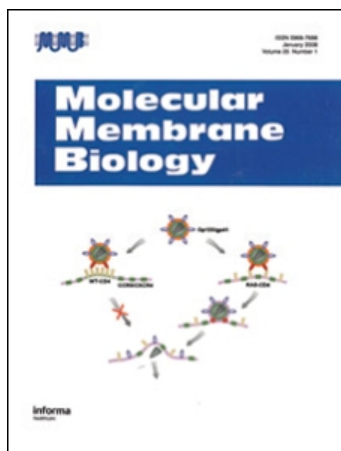
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Rapid transmembrane diffusion of ceramide and dihydroceramide spin-labelled analogues in the liquid ordered phase

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Abstract

In order to study the basic physical phenomena underlying complex lipid transbilayer movement in biological membranes, we have measured the transmembrane diffusion of spin-labelled analogues of sphingolipids in phosphatidylcholine (PC) large unilamellar vesicles in the absence or presence of cholesterol, going from a fluid (liquid disordered) l_d phase to a more viscous, liquid ordered (l_o), phase. We have found cholesterol to reduce the transverse diffusion of glucosylceramide (GlcCer) and galactosylceramide (GalCer) in a concentration-dependent manner. However, surprisingly, we could neither detect any influence of cholesterol on the rapid flip-flop of ceramide nor on the flip-flop of dihydroceramide, for which the $\tau_{1/2}$ of flip-flop remains in the order of 1 minute at 20°C in the presence of cholesterol. As a consequence of rapid flip-flop of ceramide in both the l_o and the l_d phase, ceramide is likely to distribute between the two monolayers of a membrane, and could in principle partition into segregated domains in each side of the plasma membrane of eukaryotic cells.

Keywords: *Lipid flip-flop, transmembrane asymmetry, cholesterol, ceramide, dihydroceramide, sphingolipids, liquid ordered phase, spin-labels*

Introduction

In pure lipid bilayers, the spontaneous movement of a lipid from one membrane leaflet to the other, i.e. the spontaneous transverse or transmembrane diffusion called 'flip-flop', is generally a slow phenomenon associated with long lipid lifetimes in each membrane leaflet. $\tau_{1/2}$ for phospholipids and sphingolipids ranges from several tens of minutes to several hours [1–3]. However, certain lipids of biological importance have been reported to undergo fast flip-flop ($\tau < 1$ min) at physiological temperatures. These generally correspond to lipids with a small polar moiety or a polar head-group that can be protonated. Among those are free fatty acids, cholesterol, diacylglycerides, phosphatidic acid and ceramides [4–9]. In biological membranes, active and passive lipid transbilayer movement mediated by proteins occurs in addition to the spontaneous flip-flop taking place within a pure lipid bilayer. As a consequence, the steady-state asymmetrical distribution of lipids in biological membranes is the result of lipid traffic with many different time scales involved.

Lipid movement within a bilayer highly depends on the physical state of the bilayer phase. Lateral diffusion, as well as the flip-flop rate, are decreased when lipids are embedded in a highly ordered bilayer, corresponding to a gel or a condensed phase enriched in cholesterol [10]. In the last years, much attention was paid to the hypothesis of the liquid ordered phase (l_o) coexisting with fluid, liquid disordered (l_d) domains in cell membranes, hence, forming functional lateral domains (or rafts) [11,12]. In model membranes, a l_o phase is formed by mixing an unsaturated lipid (principal constituent of the l_d phase) with high amounts of a saturated lipid and/or cholesterol at suitable proportions. Phase diagrams have been constructed for different temperatures and different proportions of such lipids [13,14]. Although the lateral diffusion of lipids is still significant in the l_o phase, it is lower in the presence of cholesterol than in a fluid l_d phase depleted of cholesterol [15,16]. A phase modification from fluid l_d to ordered l_o phase triggered by a decrease in temperature or by an increase of cholesterol content can be expected to modify the transverse diffusion as

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well, although cholesterol molecules themselves (at least cholesterol analogues) were shown to experience a fast flip-flop in lipid bilayers [6,17].

In general, the content of cholesterol in eukaryotic cellular membranes increases from the organelle membranes to the plasma membrane: The cholesterol-to-lipid molar ratio in the ER is small compared to that of the Golgi membrane, and even more so compared to the plasma membrane [18]. Cholesterol reduces membrane permeability to ions and modifies the physical properties of cell membranes by being in particular responsible for the formation of rafts [19]. Some sphingolipid precursors are synthesized in the monolayer opposite to the final product [20]. This raises the question whether sphingolipid synthesis and trafficking could be controlled by a cholesterol-regulated spontaneous transmembrane diffusion in conjunction with other translocation mechanisms involving flippases [21]. In a previous publication, we have shown that non-labelled ceramide rapidly flips between the two monolayers of a fluid lipid bilayer, as well as in the plasma membrane of erythrocytes [7]. The rapid flip flop of ceramide was determined by us using shape change of giant vesicles and was also measured independently by other techniques in other laboratories [8], and more recently by [9]. We will show in this article that the flip rate of ceramide appears to be independent of cholesterol presence, but for other sphingolipids cholesterol reduces considerably the transmembrane diffusion and therefore can be useful to stabilise a lipid asymmetry.

In the present paper, we measured the spontaneous transbilayer movement of spin-labelled sphingolipid analogues in large unilamellar vesicles (LUVs), a well-controlled physical model, commonly used for measuring lipid transbilayer movement in membranes. Variation of LUVs' lipid composition by modulation of cholesterol and sphingomyelin content permitted us to generate liquid disordered and liquid ordered phases, respectively. We measured the flip-flop of spin-labelled analogues of several sphingolipids: glucosylceramide (GlcCer), galactosylceramide (GalCer), lactosylceramide (LacCer), ceramide (Cer), dihydroceramide (dihyCer) and sphingomyelin (SM) (see Box 1). Experiments were performed at 20 and 37°C, and revealed a broad spectrum of flip-flop half-times within the sphingolipid class, as well as great differences between the effect of the bilayer physical phase. A remarkable exception was the transmembrane diffusion of ceramide and of dihydroceramide, which appeared to be independent of the lipid phase.

Indeed while the spontaneous transverse diffusion of monohexosylsphingolipids was notably decreased by the presence of cholesterol and almost stopped in

the l_o phase; surprisingly we found that the transverse diffusion of spin-labelled analogues of ceramide or of dihydroceramide are almost identical in the l_o and l_d phases, with a $\tau_{1/2}$, which remained in the order of 1 min at 20°C. If ceramides is an essential component of rafts [22], the rapid transmembrane diffusion of ceramides in the liquid ordered phase has implications in relation with the existence of lateral domains in the eukaryotic plasma membrane.

Materials and methods

Materials

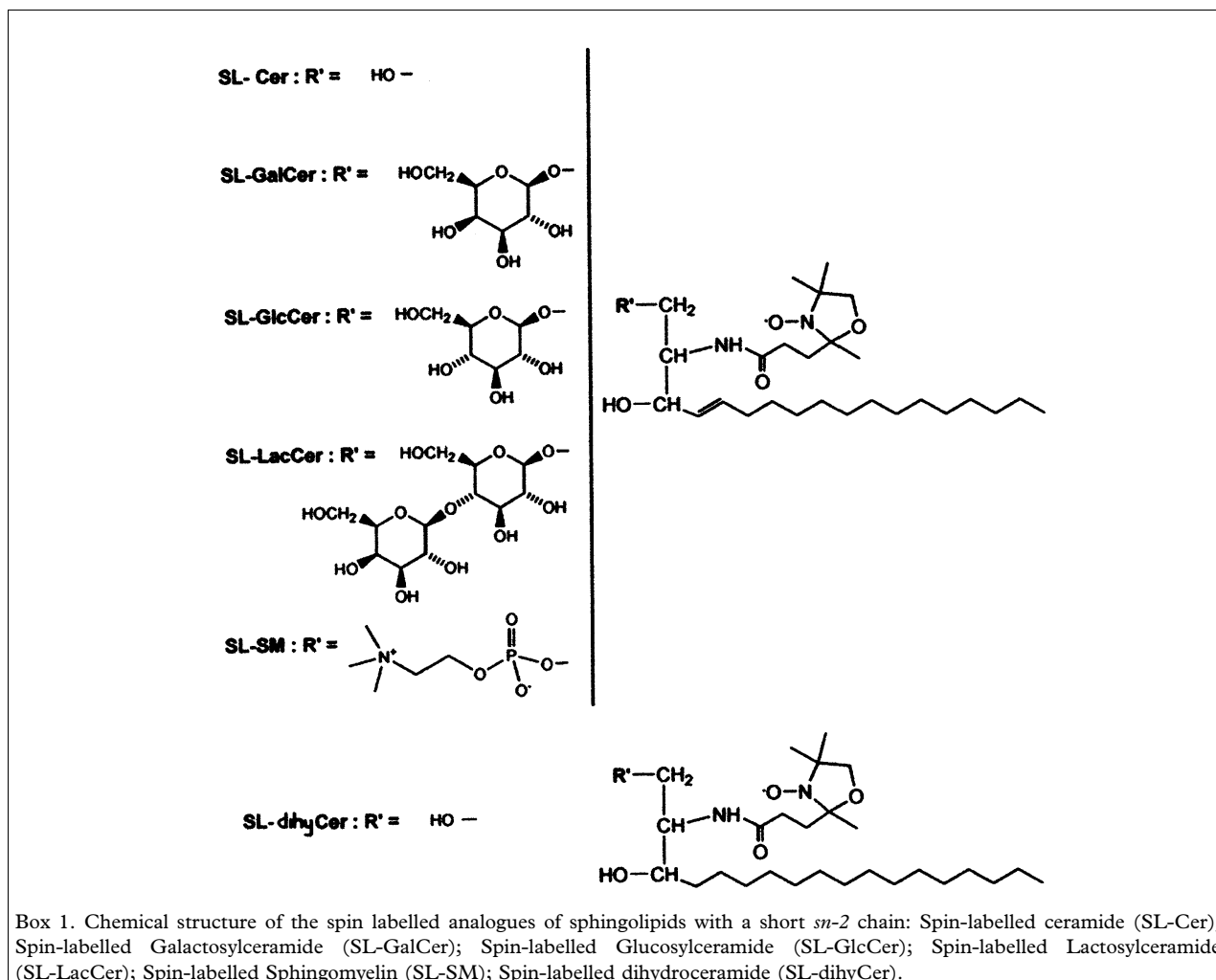
Ascorbic acid, bovine serum albumin (BSA), cholesterol, glucose, Hepes, sucrose, phospholipase C and reagents for lipid synthesis were purchased from Sigma-Aldrich. Potassium hexacyanoferrate (III) and NaCl were from Merck. KCl and MgSO₄ were obtained from Prolabo (Paris, France) and Tris-HCl from Research Organics. egg-PC, POPC, cholesterol and brain SM were bought from Avanti Polar Lipids (Alabaster, AL, USA), Polycarbonate filters from Whatman (Maidstone, UK). Solvents were from SDS. Dihydrosphingosine was bought from Sigma (USA).

Spin-labelled lipids synthesis

Chemical structures of spin-labelled lipids employed in the present investigation are shown in Box 1. Spin-labelled (0,2) [1-palmitoyl-2-(4-doxylpentanoyl)] GlcCer, GalCer, LacCer [23], SM [24] and Cer [7] were synthesized in our laboratory as described previously. Spin-labelled (0,2) [1-palmitoyl-2-(4-doxylpentanoyl)] SL-dihyCer, was synthesized by grafting 4-doxylpentanoic acid, on the amine function of the commercial dihydrosphingosine (see *Materials*) according to the method already reported [7], yielding the expected spin labelled (0,2)-dihydroceramide. Spin-labelled dihydroceramide was purified on silica gel with gradient elution (chloroform:methanol: 100:0; 99:1; 98:2; 97:3; and 95:5) yielding 80% of the pure expected product. Dihydroceramide was identified by comparing the *rf* (~0.75) on TLC (eluant:chloroform:methanol:water, 80:20:2.7) with that of the dehydrogenated homologous. Paramagnetic properties of the SL-(0,2)-dihydroceramide were checked by ESR spectroscopy and no further characterization was carried out.

Vesicle preparation

LUVs were prepared by adding buffer A (1 mM MgSO₄, 250 mM sucrose, 50 mM Tris-HCl, pH 7.3)



to a film of dried lipid (final concentration 4 mM), vortexing and four freeze-thawing cycles using liquid nitrogen, followed by repeated, sequential extrusion through 0.4 μm , 0.2 μm and 0.1 μm pore size polycarbonate filters using a Swinney 13 mm filter syringe system by Millipore (Bedford, MA, USA). LUVs in a l_d phase were prepared with egg-PC, containing a mixture of chain lengths, resembling lipid mixtures in biological membranes or POPC. In order to detect the effect of the cholesterol content on the spontaneous transbilayer movement, 25 or 44% mol of cholesterol was incorporated in egg-PC LUVs. For the l_o phase, the lipid composition of LUVs was POPC/SM/Chol in a molar ratio of 20/20/60 mol.

Measurement of SL-lipid transbilayer movement in LUVs

SL-lipid transbilayer inward movement in LUVs was determined as described by [23]. Briefly, LUVs were added to SL-lipids (final concentration 20 μM , that is, a molar fraction of 0.005 of total lipid) in buffer A and incubated on ice for 10 min to allow for

integration of the analogue into the outer membrane leaflet. LUVs were transferred to 20°C. At indicated time points, 50 μl aliquots were added to 30 μl ice-cold BSA (final concentration 5%) in buffer A and incubated on ice for 30 sec before addition of 5 μl ascorbic acid (final concentration 10 mM). The amount of SL-lipid inaccessible to reduction was immediately measured on a Bruker (Wissembourg, France) ER 200D-SRC Electron Spin Resonance (ESR) Spectrometer.

For the determination of SL-lipid transbilayer outward movement, symmetrically labelled LUVs were prepared from dry lipid films containing lipids (8 mM) and SL-lipids (final concentration in solution 5–10 μM for each lipid species). LUVs were added to a buffer containing BSA (4.4% in weight) and incubated as described above to remove the spin-labelled lipids from the outer leaflet. After addition of ascorbic acid (10 mM final concentration), the nitroxides exposed on the outer leaflet were subsequently chemically reduced. The remaining signal inaccessible to reduction was determined

in the same sample at 20°C or 37°C (indicated in the text when necessary) at indicated time points.

ESR signal analysis

SL-lipid was quantified from the full height of the midfield-peak. In the presence of ascorbic acid, the full height of the low field-peak was determined in LUV preparations using EPR3 Stellar (Mede, Italy) software.

Results

Characterization of the acyl chain order of liposomes

The analysis of the extreme splittings (Figure 1A) or the line widths (Figure 1B, 1C) of ESR spectra permit the evaluation of the mobility of a probe, attached to a lipid chain either near the polar head group or near the methyl terminal of the alkyl chains. In order to characterize the acyl chain order of lipid vesicles, we recorded ESR spectra at 20°C of three different spin labelled PC molecules (SL-PC[10,3], SL-PC[1,14], SL-PC[5,10]) which are characterized by the position of the probe on the sn-2 chain. The different analogues were incorporated in LUVs with diverse lipid compositions, corresponding to different lipid phases. Tested lipid compositions ranged from pure (egg PC or POPC) (fluid or liquid disordered phase, l_d) to POPC/SM/

Chol (liquid ordered phase, l_o), passing through intermediated compositions where l_o and l_d phases coexisted (egg-PC with 25 or 44% mol of cholesterol). When passing from a fluid l_d phase to an l_o phase by increasing the content of cholesterol, we found gradual differences in line shapes. SL-PC is expected to be inserted into the l_d phase. Correspondingly, differences in ESR line shapes were not remarkable when the two lipid phases coexisted.

For clarity, Figure 1 only shows the spectra of the three different SL-PC analogues when inserted in the l_d phase (dashed line) or in the l_o phase (continuous line). For all probe molecules, a broadening of the line spectrum was observed in the l_o phase versus the l_d phase at the same temperature revealing a clear increase in chain order. Figure 1 reveals that a lipid mixture which corresponds to the composition of the liquid ordered phase (l_o) is indeed more visquous than pure egg-PC or POPC liposomes at the same temperature (phase l_d). The ESR spectra in Figure 1A show highly deviating line shapes for the two phases. ESR spectra obtained with different spin-labelled fatty acids suggest a homogeneous ordered phase. Thus, *a priori*, the fraction of fluid l_d phase that could coexist in the l_o sample must be very small. Additionally, if the probes were clustered in a small fraction of the vesicle surface, electron spin-electron spin interactions would broaden the ESR spectra.

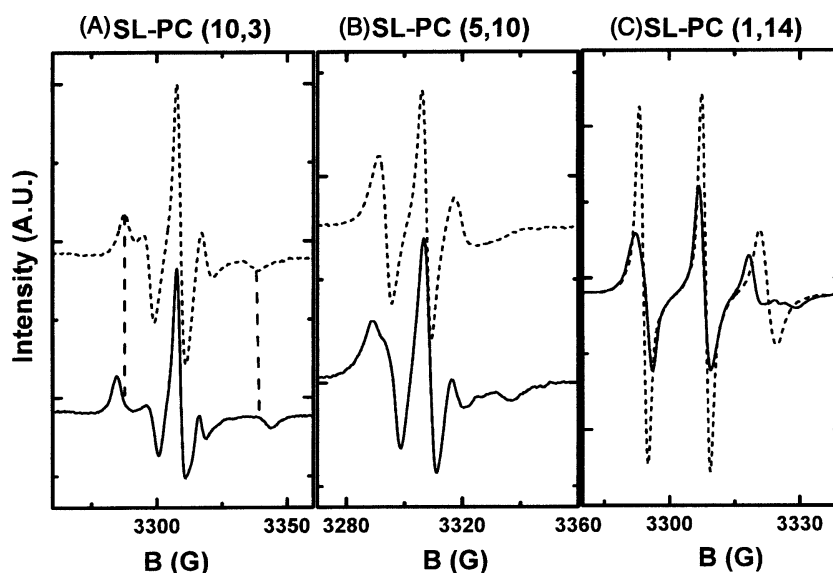


Figure 1. ESR spectra for different spin labelled phosphatidylcholines embedded in a l_d membrane (dashed line) or in l_o membrane (solid line). (A) SL-PC (10,3) (the probe is attached near the polar head group). (B) SL-PC (5,10) (the probe is attached at carbon 11 of the sn2 chain) and (C) SL-PC (1,14) (the probe is attached near the methyl terminal of the alkyl chains at carbon 15). The line broadening, indicative of a decrease on molecular motion, was systematically observed for all probes when inserted in more ordered phases. Differences in spectra line shape depends on the position of the nitroxide group on the acyl chain.

Cholesterol reduces the spontaneous transbilayer movement of monohexosylsphingolipids in a concentration-dependent manner

In a first series of experiments, the influence of cholesterol on lipid translocation was investigated for different sphingolipid analogues. The inward spontaneous transbilayer movement of SL-GlcCer (black symbols) and SL-GalCer (open symbols) was assessed in egg-PC LUVs in the absence or presence cholesterol (from 0–44 mol%) at 20°C (Figure 2A). The coexistence of two different lipid phases was confirmed by ESR spectra line shapes. The ratio of labelled lipid to egg-PC was typically 0.01 (mol ratio) to avoid spin-spin interactions between spin-labelled lipids or possible artefacts due to a high concentration of molecules with one short chain. Experimental data points obtained from the back-exchange method (see *Materials and methods*) were fitted to an exponential growth function $f = a(1 - e^{-t/\tau})$, where τ is related with the spontaneous transbilayer halftime. Halftimes were calculated from this equation.

The fact that internalization curves of SL-GlcCer and SL-GalCer do almost superimpose reflects the structural similarity of GalCer and GlcCer (Box 1). In agreement with earlier results [23], SL-monohexosylsphingolipid transbilayer movement was found

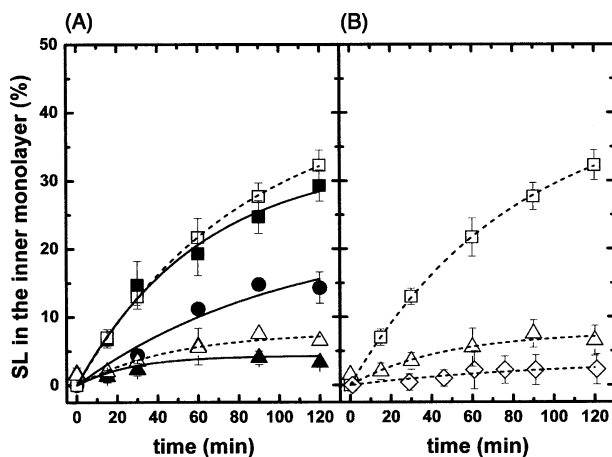


Figure 2. (A) Inward movement of SL-GlcCer (solid symbols) and SL-GalCer (open symbols) in egg-PC LUVs containing 0% (squares), 25% (circles) or 44% (triangles) cholesterol at 20°C. (B) Inward movement of SL-GalCer in (POPC/SM/Chol; 20/20/60 mol) LUVs (l_o phase, open rhombus) at 20°C. For comparison, data from A corresponding to the inward transport of SL-GalCer in more fluid phases, were also replotted (squares and triangles). After 10 min incubation of SL-lipids with LUVs on ice, tubes were transferred to 20°C. At indicated time points, samples were mixed with ice-cold BSA (final concentration 5%) and incubated on ice for 30 sec before addition of ascorbic acid (final concentration 10 mM). SL-lipid inaccessible to reduction was quantified by ESR. Results are means \pm SEM of at least $n=3$ independent experiments.

to be of the order of ~ 40 min in the continuous l_d phase (0% of cholesterol) at 20°C. $\tau_{1/2}$ was increased to ~ 270 min in vesicles containing 44% of cholesterol. The flip-flop half time in LUVs possessing an intermediate cholesterol concentration (25% mol) was ~ 90 min.

In the continuous l_o phase (POPC/SM/Chol, 20/20/60 mol), the inward spontaneous flip-flop of SL-GalCer was quasi inexistent (Figure 2B, open rhombus). For comparison, the kinetics of probe internalization in the l_d phase (egg-PC, open square) and in the $l_o + l_d$ phase (egg-PC/Chol, 56/44 mol, open triangle) are also shown. The $\tau_{1/2}$ for SL-GalCer calculated from fitting of the experimental data was increased to ~ 3000 min in the l_o phase. This numerical value is an approximation because of the very small values of the internalization measurement which prevents a rigorous determination, but it is a clear indication of a practically total immobilization of SL-GalCer within the l_o phase, and of the high interaction of SL-GalCer with cholesterol and/or SM.

A different behaviour was found for SL-LacCer and SL-SM. Figure 3A and 3B show the inward diffusion of SL-SM and SL-LacCer at 20°C in egg-PC LUVs (black symbols, l_d phase) and in a l_o phase (open symbols). The influence of cholesterol on the spontaneous flip-flop of these two molecules was difficult to detect as the transmembrane diffusion without cholesterol is already very slow for these lipids [23]. For both probes, halftimes were one two- to threefold greater in the l_o phase than in the l_d phase (shifting from ~ 800 min and ~ 400 min to ~ 1500 min and ~ 1300 min, for SL-SM and SL-LacCer, respectively).

Increase of temperature accelerates the spontaneous transbilayer movement of monohexosylsphingolipids

In order to evaluate the influence of temperature on the flip-flop rates of SL-GalCer and SL-GlcCer, we have measured the inward translocation at a temperature of 37°C, in egg-PC LUVs with or without 44% mol cholesterol. Translocation curves plotted in Figure 4A show that SL-GalCer and SL-GlcCer exhibit similar kinetic behaviour, as reported in Figure 2. In the absence of cholesterol (l_d phase), flip-flop was strongly accelerated when compared to values obtained at 20°C, yielding $\tau_{1/2}$ of 1.5 min. When the spin labelled probes were embedded in egg-PC LUVs with a high amount of cholesterol (44% mol), the half-time was also decreased almost thirty-fold, changing from 270–10 min.

When the transbilayer movement of SL-LacCer was assayed in both l_d and l_o phases (Figure 4B) at 37°C, slow transbilayer movement was obtained for

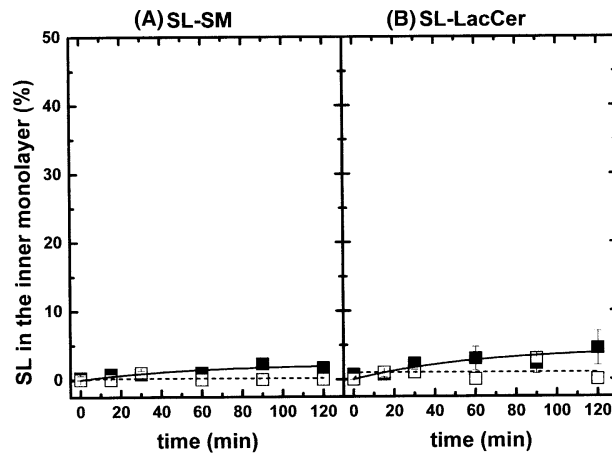


Figure 3. (A) Inward movement of SL-SM in POPC LUVs (l_d phase, solid squares) and in (POPC/SM/Chol; 20/20/60 mol) LUVs (l_o phase, open squares). Temperature was 20°C. (B) Inward movement of SL-LacCer in POPC LUVs (l_d phase, solid squares) and in (POPC/SM/Chol; 20/20/60 mol) LUVs (l_o phase, open squares). Temperature: 20°C. Results are means \pm SEM of two duplicated independent experiments.

the dihexosylsphingolipid SL-LacCer in both lipid phases, with half-times of 600 and 400 min for the l_d and l_o phase, respectively. Taking into account the uncertainty of measurements at incubation times short in relation to translocation rates, half-times found here for molecules with a slow transbilayer movement are rather similar to those found at 20°C. Once again, these results show the low ability of LacCer to undergo spontaneous transbilayer movement.

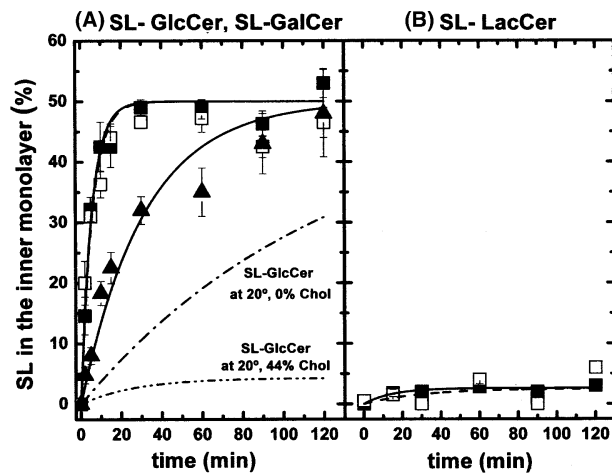


Figure 4. (A) Inward movement of SL-GlcCer (solid symbols) and SL-GalCer (open symbols) in egg-PC LUVs containing 0% (squares) or 44% (triangles) cholesterol at 37°C. For comparison, data from Figure 3A corresponding to the inward transport of SL-GlcCer at 20°C were also replotted (dashed-dot lines). (B) Inward movement of SL-LacCer in POPC LUVs (l_d phase, solid squares) and in (POPC/SM/Chol; 20/20/60 mol) LUVs (l_o phase, open squares) at 37°C.

Flip-flop of SL-Cer and SL-dihyCer is rapid in the l_o phase

We found previously that transmembrane diffusion of a spin-label ceramide is of the order of 1 min at 20°C in the fluid phase in Large Unilamellar Vesicles (LUVs) [7]. Similar transbilayer rates were obtained with spin-labelled ceramide in cholesterol containing LUVs. The outward transmembrane movement was measured actually for two different ceramide analogues: (i) A spin-labelled analogue of ceramide, and (ii) a spin-labelled analogue of dihydroceramide (see Box 1). The spin-labels were incorporated into LUVs in the l_o phase (POPC/SM/cholesterol (20/20/60% mol). Surprisingly, the transbilayer movement of ceramide, shown in Figure 5, was almost as fast in the l_o phase than in a fluid l_d phase ($\tau_{1/2} \sim 1,1$ min). Furthermore, we did not detect any meaningful difference between ceramide spin-labelled analogue and dihydroceramide spin-labelled analogue. The same values were obtained for flop and flip rates (data not shown). For the sake of clarity, all half-times $\tau_{1/2}$ of flip-flop measured for lipid analogues used here are collected in Table I.

Discussion

Validity of spin-label probes

In this article, spin-labeled sphingolipids with one short-chain bearing a nitroxide ring were used as analogues of endogenous lipids to measure the spontaneous transmembrane diffusion of lipids. The short fatty acid chain increases the water-solubility of

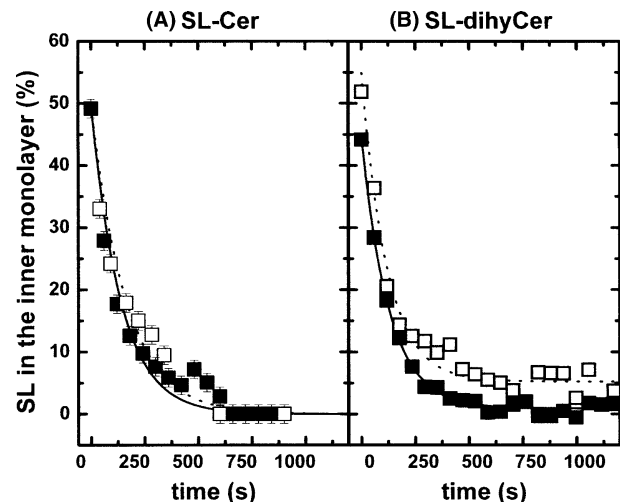


Figure 5. (A) Outward movement of SL-Cer in POPC LUVs (l_d phase, solid squares) and in (POPC/SM/Chol; 20/20/60 mol) LUVs (l_o phase, open squares). Temperature: 20°C. (B) Outward movement of SL-dihyCer in POPC LUVs (l_d phase, solid squares) and in (POPC/SM/Chol; 20/20/60 mol) LUVs (l_o phase, open squares). Temperature: 20°C.

Table I. Halftimes $\tau_{1/2}$ (in minutes) of flip-flop for sphingolipids analogues in different lipid phases at 20 and 37°C.

	20°C			37°C		
	L _d phase	L _d +L _o phase (25% Chol)	L _d +L _o phase (44% Chol)	L _o phase	L _d phase	L _d +L _o phase (44% Chol)
SL-Cer	1.1			1.1	18 seconds*	
SL-dihyCer	1.3			1.1		
SL-GalCer	40		270	~3000	1,5	10
SI-GlcCer	40	90	270		1,5	
SL-SM	~800			~1500		
SL-LacCer	~400			~1300		

*Taken from reference [7].

a lipid and consequently facilitates its introduction in a membrane. In the present case, these analogues were chosen mainly because their transmembrane orientation in a bilayer can be easily assessed: the probed lipid can either be extracted selectively from the external leaflet of a unilamellar vesicle with bovine serum albumine, and the fraction of labeled lipids present in the outer leaflet be determined by ESR spectroscopy [25], or the transmembrane lipid asymmetry can be determined *in situ* by chemical reduction of the probes exposed on the outer leaflet. Actually, the short chain is probably a bigger perturbation than the nitroxide ring. The nitroxide ring is relatively small and its steric hindrance minimal, less important than most fluorescent probes [26]. On the other hand, short chain lipids probably behave slightly differently from long chain lipids. The spontaneous diffusion of ceramide with a short C₆ chain was shown to be significantly faster than that of the C₁₆-Cer [7]. Goñi and collaborators have shown that the short chain C₂-Cer has very different properties than long chain ceramides and can destabilise a lipid bilayer [27]. Methods suitable for the quantification of the transmembrane distribution of unlabeled lipids were originally based on exogenous phospholipase attack of the cell surface [28]. The precision of this technique is limited and the *in situ* lipid degradation can cause artefacts. Recently, we have shown that the transmembrane distribution of lipids added to a giant liposome surface can be deduced from the shape of the liposomes without requiring labeled lipids [7]. However, this technique is difficult to apply in the case of a mixture of different lipid phases. It has also been reported that LUVs have slightly different properties than GUV or true biological cell membrane because of the relatively low radius of curvature [29]. For these reasons and also because this work was based on the use of lipid probes, the absolute values of $\tau_{1/2}$ may slightly differ from those in a biological cell membrane. Meanwhile, here we used a classical technique of investigation with spin-label lipids [1,25] which has proven to give at least reliable information on comparative values of the flip-flop

rates of different phospholipids [30]. Nevertheless, we do not claim that values determined for one class of lipid probe is rigorously that of the corresponding endogenous lipid.

Short chain ceramides (C₂, C₆ and C₁₂) have different properties than long chain ceramides. Thus, the spontaneous diffusion of ceramide with a short C₆ chain was shown to be slightly faster than that of the C₁₆-Cer [7], short chain C₂-Cer can destabilise a lipid bilayer [27] and more recently it has been found that short ceramides can alter the physical properties of liquid ordered domains [30]. Besides problems inherent to probes and to short chains, very fast flip-flop movements are difficult to determine by conventional ESR. One has to admit the limits of the ESR technique based on the use of aliquots removed periodically with less than 30 sec or 1 min intervals. Only stop flow ESR permits the precise measurement for $\tau_{1/2} < 1$ min. [31]. However, the results of many former studies have demonstrated that lipids with different structures, bearing one short chain with a nitroxide label, yield valuable information on the effect that the head-group can have on phospholipids transbilayer movement. [32–34].

In the following paragraphs we shall tentatively discuss the results obtained in this work in the context of the sphingolipid asymmetrical distribution in eukaryotic lipid traffic, taking into account that the cholesterol content in eukaryotic cells gradually goes from ~0% mol in the ER to 30–40% mol in the plasma membrane.

Monohexosyl sphingolipids

Although GlcCer and GalCer constitute only minor components of most mammalian membranes [35], their transbilayer mobility may affect physiological processes, namely the clear cholesterol concentration-dependent effect on the spontaneous transmembrane diffusion of SL-GalCer and SL-GlcCer. GlcCer, synthesized from Cer in the luminal cis-Golgi, is itself the precursor of LacCer in the

luminal trans-Golgi [36]. The remaining transmembrane diffusion rate for GlcCer in presence of a moderate concentration of cholesterol (as in the case of the Golgi) could lead one to speculate that GlcCer will be accessible to enzymes of LacCer synthesis. The final destination for both GlcCer and GalCer is the outer monolayer of the plasma membrane. GalCer was even found in the plasma membrane of cells in which a brefeldin A assay – stopping anterograde vesicular transport between the compartments of the Golgi and thus leading to a redistribution of the Golgi into the ER – had previously been performed [37]. A monomeric transport to the plasma membrane is hence hypothesized [38]. In this case, GalCer (and GlcCer) molecules would be found in the inner monolayer of the plasma membrane. The remaining spontaneous flip-flop of SL-GalCer (SL-GlcCer) measured at high concentrations of cholesterol (10 min at 44% cholesterol and at 37°C, see Figure 4A) should re-equilibrate the distribution in both monolayers, even for those molecules originating from vesicular traffic. However, GalCer and GlcCer are only found in the outer monolayer of the plasma membrane. Thus, the asymmetrical distribution of these lipids is likely to be maintained by a flippase [36,39,40]. MDR1 (P-gP) has been proposed to be a good candidate for this [8,21].

Lactosylceramide and sphingomyelin

A reduction of the transbilayer diffusion of SM and LacCer by cholesterol was not detected here, probably due to the very slow diffusion even in the absence of cholesterol. In fact, the time scale chosen did not allow the detection of differences that may be visible only after several hours. In previous experiments, cholesterol was shown to reduce the transbilayer movement of SL-PC in erythrocytes observed up to 18 h [32]. Both LacCer and SM are transferred from the inner leaflet of the trans-Golgi, where they are synthesized [41,42], to the outer leaflet of the plasma membrane by vesicular transport [43,44]. The extremely slow spontaneous transverse diffusion measured here ensures the passage from the luminal Golgi to the extracellular leaflet of the plasma membrane, two sides of identical topology.

SL-ceramide and SL dihydroceramide

It was proposed already [7] that the very fast transmembrane diffusion of ceramide, independently of cholesterol concentration, facilitates its location in both sides of membranes where ceramide acts as a precursor of other sphingolipids: GalCer

synthesis in the inner leaflet of ER [45]; GlcCer in the outer leaflet of the cis-Golgi [43,46,47] and the SM synthesis in the inner monolayer of the trans-Golgi [48]. As pointed out above we have not been able to evidence convincing differences between SL-ceramide and SL-dihydroceramide.

Due to their lack of effective polar headgroup, both spin-labelled lipids flip rapidly in the l_d phase (Figure 5A). More surprisingly, and contrarily to the behaviour of the monohexosylsphingolipids SL-GalCer and SL-GlcCer, the rapid transbilayer movement was not affected when the ceramide analogues were embedded in the more ordered phase l_o , with virtually identical values for SL-Cer and SL-dihyCer (Figure 5B). In order to rule out the possibility of artefacts, we have regarded several scenarios. A first hypothesis to explain the rapid transmembrane diffusion of a lipid in the l_o phase could be a lipid mixture deviating from the desired composition, in which a small percentage of segregated fluid domains could form. Ceramide might prefer such an environment, cluster and flip rapidly in the l_d phase.

An alternative possibility, rather similar in effect, would be the formation of microdomains separated by ‘defects’ lines between small homogenous microdomains in the l_o phase. Along these defects, the actual chain order state could be very different from the bulk l_o phase. However, such hypotheses would be difficult to reconcile with the slow flip-flop of GalCer. Indeed when it is incorporated in the same lipid preparation SL-Gal-Cer flipped at a much slower rate than in an l_d phase. The flip rates of spin-labelled lyso-PC, SL-PC and SL-PE, which all bear a nitroxide, are very long (several hours), even in a fluid membrane environment [32]. It can also be ruled out that, because of the ordered character of the l_o phase, SL-Cer and SL-dihyCer were excluded from the bilayer and maintained in the aqueous phase where the nitroxide would be rapidly reduced by ascorbate. This would then also occur in control experiments with spin-labelled PC, for which no rapid flip-flop of spin-labelled PC investigated by this technique has ever been reported in an ordered phase. Thus, the rapid transmembrane diffusion of SL-Cer in the l_d or l_o phases cannot be easily explained by a probe artefact or an error in the detection assay. A last possibility reported by Goñi’s laboratory consists in the ability of lipids prone to form non-lamellar structures, such as ceramide, to destabilize the lipid bilayer [27,49,50]. However, ceramides should then accelerate the transbilayer movement of other lipids in the same membrane. Yet, we found that the presence of natural ceramide in the lipid composition does not modify the flip-flop rate of SL-PC in the same LUVs [7].

We propose that this lipid scrambling could be explained by a local destabilization of the lipid bilayer upon *in situ* conversion of sphingomyelin (SM) into ceramide or upon a high asymmetrical increase of ceramide concentration by external addition of molecules, both cases would generate a high lipid mismatch between the two monolayers [50,51]. While the rapid flip-flop of ceramide and of dihydroceramide analogues in the lipid ordered phase is not easy to explain, our experiments clearly indicate that the lipid headgroup is the bottle neck that prevents the lipids from flipping. It also confirms that the so-called liquid ordered l_o phase is different from a condensed or crystalline phase since, in agreement with the data on lateral diffusion [16], it allows hydrophobic molecules to move within.

The rapid transmembrane diffusion of SL-Cer and SL-dihyCer in the l_o phase may have important biological consequences. Ceramide, is formed *in vivo* by hydrolysis of sphingomyelin in the outer monolayer of the plasma membrane and was reported to accumulate in rafts [22,52]. Because of its propensity to flip rapidly from one monolayer to the other in a bilayer, ceramide might distribute between the two monolayers of the plasma membrane and could form domains in each side of the membrane. This remark is to be taken into account in discussions about lateral domains in the plasma membrane of eukaryotic cells [9].

Conclusions

The present article reports several large amplitude effects indicating a clear difference in transmembrane diffusion between ceramides and the other sphingolipids. We believe that the results are indications of significant phenomena.

Firstly, the size of the polar headgroup is a major determinant of spontaneous transbilayer movement of sphingolipids, as shown by our results obtained with spin-labelled lipid analogues in fluid phase (l_d). Spontaneous transbilayer movement is very slow for SL-LacCer and SL-SM, which possess a large polar headgroup. Conversely, the absence of an effective polar headgroup facilitates the spontaneous transbilayer movement of ceramide and of dihydroceramide. The transbilayer diffusion of SL-Cer is at least 100 times faster than that of SL-SM or SL-LacCer. As an intermediate case, monohexosylsphingolipids with medium-sized headgroups have a $\tau_{1/2}$ values one order of magnitude larger than that of SL-Cer. These results also confirm previous measurements [23], and additionally allow direct comparison between the different sphingolipid species investigated by the same methodology.

Secondly, the spontaneous transbilayer movement of a lipid, besides depending on its own chemical structure, is affected by the physical state of its environment. We found that the spontaneous transbilayer movement of monohexosylsphingolipids decreased drastically with an increasing cholesterol content. In organelle membranes, where the cholesterol content is generally lower than in the plasma membrane, the transbilayer movement of various lipid analogues has been found to be relatively rapid [23,34,53]. Even if this movement is largely due to active transport or facilitation of transbilayer movement by one or several proteins [54], it would be conceivable to be favoured additionally by the low cholesterol content in these membranes. It can be hypothesized that the different concentrations of cholesterol along the trafficking route in eukaryotic cells control the asymmetrical distribution of sphingolipids during vesicular traffic by modulating their spontaneous transbilayer movement.

However, and finally, data obtained with SL-Cer and SL-dihyCer in a l_o phase show that simple intuition can be misleading since we did not evidence any significant effect of cholesterol on the flip-flop of such molecules.

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