RAPID TRANSBILAYER MOVEMENT OF CERAMIDES IN PHOSPHOLIPID VESICLES AND IN HUMAN ERYTHROCYTES*

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The transbilayer diffusion of unlabeled ceramides with different acyl chains (C₆-Cer, C₁₀-Cer and C₁₆-Cer) was investigated in giant unilamellar vesicles (GUVs) and in human erythrocytes. Incorporation of a very small percentage of ceramides (approximately 0.1% of total lipids) to the external leaflet of egg phosphatidylcholine GUVs suffices to trigger a shape change from prolate to pear shape vesicle. By observing the reversibility of this shape change the transmembrane diffusion of lipids was inferred. We found a half-time for unlabeled ceramide flip-flop below 1 minute at 37°C. The rapid diffusion of ceramides in a phosphatidylcholine bilayer was confirmed by flip-flop experiments with a spin-labeled ceramide analogue incorporated into large unilamellar vesicles. Shape change experiments were also carried out with human erythrocytes in order to determine the transmembrane diffusion of unlabeled ceramides into a biological membrane. Addition of exogenous ceramides to the external leaflet of human erythrocytes did not trigger echinocyte formation immediately as one would anticipate from an asymmetrical accumulation of new amphiphiles in the outer leaflet but only after approximately 15 minutes incubation at 20°C in the presence of an excess of ceramide. We interpret these data as indicative of a rapid ceramide equilibration between both erythrocyte leaflets as indicated also by ESR spectroscopy with a spin labeled ceramide. The late appearance of echinocytes could reveal a progressive trapping of a fraction of the ceramide molecules in the outer erythrocytes leaflet. Thus, we cannot exclude the trapping of a ceramides into plasma membrane domains.

Ceramide is the backbone and an intermediate molecule in the metabolism of sphingolipids. It is a minor lipid component of the plasma membrane of eukaryotic cells and can be generated in vivo by the degradation of sphingomyelin or of gangliosides. Free ceramide was shown to play a role as second messenger for many cellular functions. Its potent biological activity has been extensively reviewed (1, 2). Because enzymes involved in ceramide generation and metabolism are localized in different subcellular compartments, a challenging question remains unanswered: how do such water insoluble molecules overcome the successive solubility barriers found during traffic between the membranes of different organelles? In addition, the transmembrane orientation of ceramides must be controlled at each step. Ceramide is synthesized on the cytosolic surface of the ER and is converted to GalCer in the lumenal leaflet of the ER. It is not known if this reorientation within the membrane takes place spontaneously or is catalyzed by a protein. The Golgi system also requires regulated ceramide distribution between leaflets as synthesis of GlcCer occurs on the cytosolic side and sphingomyelin (SM) on lumenal side (3). The transverse diffusion of several sphingolipid derivatives has been measured with spin-labeled and fluorescent analogues in LUVs and in different biological membranes (erythrocytes, hepatocytes, Golgi). SM with a nitroxide on a short acyl chain has almost no capacity to flip in the...
PM of erythrocytes and diffuses very slowly in Golgi membranes from rat liver. GaLCer and GlcCer experience a slow flip rate in LUVs with a half time of several hours (4). There are conflicting reports in the literature concerning spontaneous flip-flop of natural ceramide. For Blitterswijk et al. (5) “natural ceramide may be restricted for quite some time on the side of the bilayer where it is generated”. On the contrary, for van Helvoort and van Meer, “ceramide flips in seconds” (6). Bai and Pagano have measured the transmembrane diffusion of a ceramide analogue possessing a fluorescent BODIPY group on a short acyl chain in POPC-LUVs (7). A half time of diffusion of approximately 22 minutes at 22°C was reported for this molecule. Their result suggests that the spontaneous diffusion of ceramide is much faster than that of lipids with a zwitterionic head group like SM or PC. It is important to measure the transverse diffusion of unlabeled long chain ceramides to determine the extent of the label influence on the flip-flop rates previously measured. Because it has been suggested that ceramide interacts with SM to form segregated domains (8) it is also important to measure the transverse diffusion in a SM containing membrane to establish if the presence of domains affects the transmembrane diffusion of ceramides. Goñi’s group reported recently that the in situ formation of ceramides by the action of SMase on LUVs or erythrocyte ghosts accelerates the transmembrane diffusion of other lipids such as NBD-PE. The authors attributed the latter phenomenon to the formation of non-lamellar lipid phases (9).

In the present article, we first measured ceramide transverse diffusion in lipid vesicles that did not contain proteins. An originality of our approach is that we can measure flip-flop rates in a single GUV without having to label the lipids. To this end, a small amount of non-labeled ceramides was incorporated into the outer leaflet of EPC GUVs and the transmembrane diffusion of ceramides was measured by monitoring the GUV shape changes which can be triggered by a very small excess of lipid in one monolayer (10-12). The half-time of ceramide flip-flop in EPC GUVs was found to be 2 minutes at 20°C and about 30 seconds at 37°C, hence about one order of magnitude below the value reported by Bai and Pagano (7).

Independent experiments were carried out with spin-labeled analogues of ceramide or of phosphatidylcholine incorporated into LUVs that confirmed the rapid transmembrane diffusion of ceramides in EPC and the slow diffusion of the PC analogue.

In order to investigate ceramide flip-flop in a biological membrane, which contains sphingomyelin and cholesterol, we carried out also series of experiments with human erythrocytes to which small amounts of unlabeled ceramides were added. Following the strategy employed successfully several years ago for the investigation of phospholipid translocation in human erythrocytes (13, 14), shape change of erythrocytes was used to detect the transmembrane diffusion of unlabeled ceramides. These experiments as well as ESR experiments with spin-labeled ceramides suggested a rapid ceramide transmembrane diffusion in erythrocytes excluding at least a complete trapping of such molecules in immobilized domains of the outer monolayer.

**EXPERIMENTAL PROCEDURES**

**Chemicals.** Egg Phosphatidylcholine (EPC) was purified according to (15). Brain sphingomyelin (SM), cholesterol and NBD-C6-PC were purchased from Avanti-Polar Lipids (USA). RTPE was acquired from Molecular Probes (USA). Lyso-PC was obtained by hydrolysis of EPC with phospholipase A2. C6-PS and C6-PC were synthesized as described in (16). Sucrose, glucose, fatty acid free BSA and all other chemicals were purchased from Sigma-Aldrich (USA). Dimethyldichlorosilane solution was bought from BDH (England).

**Synthesis of sphingosine.** Sphingosine used for the synthesis of ceramides was the reduction product of a selectively protected precursor. The sphingosine precursor, (2S, 3R, 4E)-2-azido-4-octadecen-1, 3-diol, was obtained according to the method described in (17). D-galactose was used as starting material and as a source of chiral center. Carbons 4 and 5 proper asymmetry respectively for carbon 2 and 3 of sphingosine precursor were obtained in
several steps. The objective of the synthesis was to obtain only one isomer with the good olefin geometry (4E), which was made possible by a Wittig reaction performed in presence of lithium bromide and phenyl-lithium between n-tetradecyltriphenylphosphonium and an aldehyde intermediate obtained in two steps from D-Galactose.

**Synthesis of paramagnetic fatty acid.** 4-doxylpentanoic acid was obtained in three steps according to the method described in (16). The first step was the condensation of a amino-alcohol (2-amino-2-methyl-propan-1-ol) on the butyl levulinate followed by an oxidation of the obtained oxazolidine. The saponification of the paramagnetic ester yielded the expected paramagnetic acid.

**Synthesis of ceramides.** Unlabeled ceramides (C₆-Cer, C₁₀-Cer, C₁₆-Cer) and the spin-labeled ceramide, SL-Cer (Fig. 1a), were obtained by grafting a fatty acid respectively n-caproic acid, n-capric acid, palmitic acid, 4-doxylpentanoic acid on the amine function of a synthetic sphingosine in presence of EEDQ in ethanol according to the method described in (18).

**Ceramide purification and characterization.** C₆-Cer, C₁₀-Cer, C₁₆-Cer and SL-Cer were purified on silica gel column with a gradient elution (chloroform : methanol 100:0; 99:1; 98:2; 97:3; 95:5) yielding respectively 65 %; 70 %; 87 % and 80 % of the pure expected product. Unlabeled ceramide (C₆-Cer, C₁₀-Cer, C₁₆-Cer) were characterized by NMR (¹H and ¹³C) analysis in deuterated chloroform and by TLC on silica plate (eluant: chloroform : methanol : water 80:20:2,7) giving respectively rf = 0.74; rf = 0.76; rf = 0.75. The spin-labeled ceramide was characterized by ESR spectroscopy in absolute ethanol and by TLC on silica plate (eluant: chloroform : methanol : water 80:20:2,7) giving rf = 0.8.

**Synthesis of spin-labeled phosphatidylcholine.** Synthesis of a spin-labeled phospha-tidylcholine (Fig. 1b) was performed by acylation of lyso phosphatidylcholine free alcohol function with 4-doxylpentanoic acid in presence of DCC and DMAP according to (19) as explained in (16).

**Erythrocytes.** Venous blood samples were obtained from voluntary healthy people. Sodium citrate 0.1M was added (9:1 v/v) in order to avoid coagulation. Erythrocytes were isolated by resuspension of 500 μl of blood in 4500μl of an isonic PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4) and centrifugated at 1000 g for 10 min. The supernatant was removed by aspiration. This washing procedure was repeated three times.

**Giant unilamellar vesicles preparation.** Giant Unilamellar Vesicles (GUVs) made of EPC or EPC:SM:cholesterol:RTPE (1:1:1:0.03 % mol) were prepared following the electroformation method (20, 21). The fabrication chamber is composed by two conductor cover slides coated with a thin transparent film of indium tin oxide and separated by a Teflon spacer of 0.5 mm. 15 μl of lipid solution in chloroform at 0.25 mg/ml concentration were deposited on both conductor cover slides. After organic solvent evaporation, the chamber was placed in vacuum for 1 hour. The film was re-hydrated with a sucrose solution (200 mOsm). The chamber was rapidly connected to an A.C. power supply, generating a low frequency voltage (8 Hz) that was progressively increased from 0 to 1.1 V in 40 minutes. After one night, the voltage was increased to 1.3 V while decreasing the frequency to 4 Hz for 1 hour. The obtained vesicles had a spherical shape.

**Shape changes experiments with GUVs at 20°C.** A few μl of the solution containing spherical GUVs were transferred with a micropipette from the fabrication chamber to the observation chamber containing 500 μl of glucose solution with an osmolarity slightly above 200 mOsm. The density difference between outside and inside media allowed the vesicles to sediment in the observation chamber and provided a better contrast for light microscopy. In order to obtain prolate shape vesicles, the glucose solution was allowed to evaporate for more than 1 hour at room temperature. Due to the difference between inside and outside osmotic pressure, 10-20% of the vesicles deflated and adopted a prolate shape. Once a vesicle was targeted a flux of the desired lipids or protein was injected at
a sufficiently large distance (>1 mm) from the selected vesicle in order to avoid undesirable concentration gradients, turbulence due to flow of water or organic solvent at the vicinity of the GUVs. Typically 10 µl of 0.1mM lipid in glucose solution was injected for water-soluble lipids (C6-Cer, Lyso-PC, C6-PS). For the less soluble long chain ceramides (C10-Cer, C16-Cer) a mixture of ethanol:dodecane (98:2, v:v) was used as solvent for the injection (2.5 µl of 0.8mM for C10-Cer and 2.5 µl of 2mM for C16-Cer). The final concentration of organic solvent was ~0.5% in water (22). Shape changes induced by the redistribution of lipids and the recovery of initial shape were monitored by observation with an optical microscope (inverted microscope Zeiss IM35 or Nikon Eclipse E600FN) equipped with a CCD camera (Micro Max 5MHz system Princeton Instruments, Inc. or Nikon DS-L1).

Temperature dependence of ceramide transmembrane diffusion in GUVs. In order to measure the temperature dependence of the transmembrane diffusion, microscope observation chamber was placed on a Peltier effect module (Linkam PE94 in an inverted microscope Leica DMIREZ and equipped with a CCD camera Photometrics Coolsnap CF), allowing observations to be done at temperatures varying from 9° to 30°C. The temperature of the external solution could be controlled and monitored at any time by a thermocouple thermometer (Fluke 51K/J thermometer) inserted into observation chamber solution. Shape changes experiments were carried out as described before.

Shape changes experiments with erythrocytes. 5µl of pelleted erythrocyte were resuspended in 5 ml of an isotonic PBS buffer. The mixture was homogenized by shaking. Finally, 500µl were loaded in observation chamber. Few minutes were necessary to allow erythrocytes to sediment. In order to avoid the “glass effect” (23) cover slides of observation chambers were passivated with dimethyl-dichlorosilane. For that purpose covers slides were incubated 10 minutes in a dimethylchlorosilane solution (2% in 1-1-1 trichloromethane) rinsed with abundant methanol, ethanol and water and allowed to dry for at least 30 minutes. Once an erythrocyte group was targeted, injection of molecules was proceeded as in shape changes experiments with GUVs. Briefly, 30 µl of 0.1mM lipid in PBS solution was injected for water soluble lipids (C6-Cer, C6-PC, C6-PS) and 3µl of 10mM of C16-Cer in ethanol:dodecane (98:2, v:v). Shape change observations were performed as in GUVs experiments.

ESR experiments with LUVs. ESR experiments were carried out with a 9 GHz ESR Bruker spectrometer (Bruker ER 200D SRC) connected to a PC-computer for data accumulation and treatment (Stelar: DS EPR, Mede, Italy). ESR has to be carried out with LUVs because GUVs are too dilute and fragile, hence not suitable for spectroscopic techniques. 200 nm LUVs made of EPC were obtained by extrusion and by freezing and thawing method (23, 24). The spin-labels (0.25 % mol of total lipid composition) were added to the dry lipids before vesicles formation in order to obtain a symmetrical distribution of probes in the two leaflets. To measure the transmembrane diffusion of spin-labeled molecules initially incorporated in both leaflets of LUVs (8mM of lipids), sodium ascorbate (final concentration 10mM) and bovine serum albumin (4.4 % in weight) were added to the solution in which the LUVs were suspended. The nitroxides exposed on the outer leaflet were subsequently chemically reduced in less than a minute (16). The spectral intensity of the low field line was recorded at 20°C immediately after ascorbate addition. The decrease of signal intensity, indicating the probe diffusion from the inner to the outer monolayer (flop), was plotted as function of time. Half-life times were calculated by the fit to a single exponential.

ESR experiments with erythrocytes. Erythrocytes were washed 3 times in buffer (145mM NaCl, 5mM KCl, 1mM MgSO4, 10mM Glucose, 20mM Hepes, pH 7.4). The buffy coat and supernatant were carefully removed by aspiration and the cells resuspended in 500 µl at a hematokrit of 50%. 440µl of SL-Cer in buffer (10µM final concentration) were added to cell suspension. The labeled erythrocytes were incubated at 37°C for 15 minutes in order to
allow the spin-labels to equilibrate between both leaflets of membranes. Sodium ascorbate (5mM final concentration) added to the cell suspension reduced instantaneously the labels on the outer leaflet. Further evolution of the line intensities corresponded to the outward lipid translocation.

KINETIC MODEL

In order to estimate the translocation rate of unlabeled ceramide in a GUV, we used a model (Fig. 2), which is a simplified version of the model developed by Needham and Zhelev for the incorporation of lyso-PC into lipid vesicles (26). Data analysis has to take into account the non-negligible time of ceramide incorporation compared to the time of lipid flip-flop. The slow ceramide incorporation is due to 2 factors: i) ceramide injection with a micropipette is done deliberately far from a selected GUV. Molecules, either monomers or micelles, have to diffuse in the water before reaching a selected GUV; ii) the intake or penetration of a ceramide molecule within the outer GUV’s leaflet can also be slow. Finally, the volume of water compared to the volume occupied by the phospholipid membranes is very high, therefore whatever the partition coefficient, only a small fraction of the ceramide injected will be localized eventually in the membrane of a lipid vesicle. It is hard to know at which stage the mixture of ethanol:dodecane plays a role to accelerate the intake except to say that in the absence of small amount of organic solvent the incorporation of long chain ceramides (C<sub>10</sub>-Cer and C<sub>16</sub>-Cer) is almost null. Because a detailed model would require too many unknown parameters (that would be very difficult or impossible to evaluate independently in particular because we used unlabeled molecules) we chose to account for the ceramide incorporation rather empirically by using for the rate of uptake an exponentially decreasing function of time that was validated by independent measurements carried out with a fluorescent NBD-PC molecule injected in a similar fashion into EPC GUVs. Briefly, 10 µl of a 0.1 mM NBD-PC solution in glucose (200 mM) were injected at the vicinity of an EPC giant vesicle and the fluorescence intensity compared to the fluorescence of a control vesicle made of EPC:NBD–PC (99:1 mol %). These experiments showed that the concentration of NBD-PC incorporated in GUVs can be fitted by an equation of the form:

\[ c(t) = A(1 - e^{-\frac{t}{t_i}}) \]

indicating that the incorporation rate is an exponentially decreasing function of time of the form:

\[ f(t) = \frac{A}{t_i} e^{-\frac{t}{t_i}} \]

\( t_i \) is the characteristic time of incorporation, which is about 5 minutes for NBD-PC under those experimental conditions. \( A \) is the final concentration of amphiphilic molecules incorporated in the outer monolayer and is expressed in % of total lipids in the GUV. \( A \) depends upon the concentration and chain lengths of the molecules injected.

For the ceramide experiments we can write the following differential equation system:

\[
\frac{dc_{\text{out}}}{dt} = f(t) - K_{\text{out}} c_{\text{out}} + K_{\text{in}} c_{\text{in}}
\]

\[
\frac{dc_{\text{in}}}{dt} = K_{\text{out}} c_{\text{out}} - K_{\text{in}} c_{\text{in}}
\]

where: \( c_{\text{out}}(t) \) and \( c_{\text{in}}(t) \) are the ceramide concentrations (in % of total lipids) in the outer and the inner monolayer respectively. \( K_{\text{out}} \) and \( K_{\text{in}} \) are the translocation rates from the outer to the inner monolayer and from the inner to the outer monolayer respectively. We have assumed that the fraction of ceramides in the inner monolayer, which spontaneously partitions into the internal volume of vesicle, is negligible because the internal volume of the vesicles is very small in comparison to the external volume. Furthermore one can assume that for a GUV both leaflets are equivalent (in terms of curvature and tension). Therefore: \( K_{\text{out}} \approx K_{\text{in}} = K \). The equations can be written:
\[
\frac{dc_{out}}{dt} = f(t) - Kc_{out} + Kc_{in}
\]
\[
\frac{dc_{in}}{dt} = Kc_{out} - Kc_{in}
\]

The GUV transition associated with a shape change depends upon the difference of lipid concentration \(\Delta c = c_{out}(t) - c_{in}(t)\) between both monolayers. \(\Delta c\) is the lipid asymmetry. By taking into account the expression of \(f(t)\) the following analytical solution is obtained:

\[
\Delta c(t) = \frac{Ae^{-1}}{2K-t_i} \left( e^{\frac{t}{t_i}} - e^{-2Kt} \right)
\]

In case of GUVs, the minimum asymmetry needed to induce a shape change is of the order of 0.1% of the total area of a vesicle (10-12). This means that budding transition would occur when \(\Delta S/S > 0.1\), or \(\Delta c > 0.1 \frac{a_{EPC}}{a_{Cer}}\) where \(a_{EPC}\) is the area occupied by an EPC molecule and \(a_{Cer}\) that of a ceramide molecule.

Shape recovery happens when \(\Delta S/S = 0.1\), i.e. when the area of the two leaflets becomes almost identical. The area occupied by a ceramide molecule is probably slightly lower than the area of a PC molecule. However because of the uncertainty in the threshold for the budding transition, we think that the actual difference in area between EPC and Cer is not an important source of error, so we may assume that \(\Delta c > 0.1\). Furthermore our objective was not to determine an accurate value for \(A\) and \(t_i\) but essentially to deduce reliable values for \(K\).

Representative theoretical curves are shown in Fig. 3. Fig. 3-A shows \(\Delta c\) for \(t_i\) fixed and \(K\) variable. Fig. 3-B shows \(\Delta c\) for \(K\) fixed and \(t_i\) variable. Each curve intercepts twice the threshold line: \(\Delta c = 0.1\) which enables one to deduce two values \(t_i\) and \(t_2\) corresponding respectively to the budding transition and to the reversible shape transition. Times \(t_1\) and \(t_2\) were measured for every experiment. \(K\) and \(t_i\) were then deduced by using equation (1).

To determine if \(K\) and \(t_i\) values obtained are very sensitive to \(A\) and to the threshold \(\Delta c\), we allowed \(A\) and \(\Delta c\) to vary when using equation (1). \(A\) was varied from 1 to 5 (in percent of total lipids) with 10 steps, and \(\Delta c\) from 0.05 to 0.15 percent of membrane lipids with 5 steps. A matrix of 66 possible pairs \((K, t_i)\) was then obtained after using equation (1) and finally an averaged value of \(K\) and \(t_i\) were calculated for each experiment and for each ceramide. The uncertainties appearing in Table 1 were deduced from this averaging.

**Erythrocytes.** Ceramide transmembrane diffusion in erythrocytes was analyzed in a more qualitative fashion than in the case of ceramide diffusion in GUVs. The percentage of lipid asymmetry necessary to trigger a shape change in erythrocytes is known to be significantly higher than in a GUV. Typically the lipid asymmetry \(\Delta c\) must be of the order of 1% to trigger a shape change from discocytes to echinocytes (27). All experiments with human erythrocytes were repeated from three to six times and different observation areas were always inspected. Thus the figures shown in the Results section are representative of many observations.

**RESULTS**

GUVs shape changes triggered by lipids that do not flip. Fig. 4 shows the sequence of events observed after addition of lyso-PC to an EPC GUV. The vesiculation (or budding transition) that takes place progressively (Fig. 4, a to h) is completed after approximately 1 minute and remains stable for more than 90 minutes. It indicates a stable asymmetry between both leaflets. This stability demonstrates the very slow lyso-PC flip-flop in a lipid bilayer as reported previously (28, 29 and references therein). When fatty acid free BSA was added to such vesicles to remove lyso-PC the original shape was recovered confirming that lyso-PC had not flipped to the inner monolayer. It is difficult to work out the actual concentration of lyso-PC in the membranes. The average concentration of phospholipids due to the GUVs is itself difficult to determine with precision because there is a large scatter of GUV’s size and their distribution in volume is not homogeneous. However the shape change by itself is an indication of the percentage
of lyso-PC incorporated into the lipid vesicles. Other molecules with a charged or zwitterionic head group gave the same stable shape change. For example, a phosphatidylserine with a long sn-1 chain and short sn-2 acyl chain (C₆) was added in the environment of a prolate GUV. A budding transition took place that was stable for more than one hour (data not shown).

**Shape changes triggered by unlabeled ceramides at 20°C.** We have added various unlabeled ceramide molecules to preformed EPC GUVs. C₆-Cer was dissolved in water, but C₁₀-Cer and C₁₆-Cer were incorporated with the help of a mixture of ethanol:dodecane (98:2, v:v). Figs. 5 and 6 are representatives shape change pathways for C₆-Cer and C₁₆-Cer respectively. Similar figures were obtained with C₁₀-Cer (not shown). These data indicate that unlabeled ceramides trigger a budding transition that is reversible in a short period of time. Control experiments (not shown) indicated that the amount of organic solvent used for C₁₀-Cer and C₁₆-Cer does not trigger any budding in the absence of ceramide. Furthermore experiments with C₆-Cer in the presence of ethanol:dodecane (98:2, v:v) did not allow us to measure a significant acceleration of C₆-Cer transmembrane diffusion in GUVs at 20°C. We found that the time (t₁) required for the formation of a bud varied from one ceramide to another. t₁ was slightly longer for longer acyl chains. The lag time probably reflects the time of insertion within the outer monolayer of the various molecules. For C₆-Cer the incorporation took place approximately 1 minute after injection of molecules. For C₁₀-Cer and C₁₆-Cer, t₁ was longer. But the difference may not be very meaningful because it probably depends upon the amount of organic solvent in the latter cases. The flip time (or τ₁/2) can be estimated from the re-opening of the neck formed when a budding vesicle is apparent. The reopening process was always rather sudden and took place approximately 9 to 15 minutes after t₁. The discontinuity in the shape change evolution confirmed that one can consider the shape change as a thermodynamic phase transition (30). However the vesicle shapes in the steady state (approximately 30 minutes after injection) presented some variability, especially when the long chain ceramides were injected. Even if seventy five per cent of vesicles studied were able to recover prolate shape, sometimes a second budding transition arose in the following minutes after the disappearance of the first bud. We have observed a few double budded vesicles, i.e., three vesicles interconnected by two little necks, or invaginated vesicles. Such variability is expected when doing a series of experiments with individual lipid vesicles.

**Quantitative evaluation of ceramides translocation rate in EPC GUVs.** A rigorous quantitative evaluation of τ₁/2 must take into account simultaneously the superposition of the kinetics of ceramides insertion in the outer monolayer and ceramide diffusion towards the inner monolayer. The model used to explicitly introduce the two rate constants for insertion and flip-flop has been explained in the *Kinetic Model* section. Fig. 7 shows the evolution with time of the asymmetry function Δc deduced from the experiments with the unlabeled ceramide tested at 20°C. The curves are drawn with the most probable values of the various kinetic parameters, which are indicated in Table 1. The intercepts of these curves with the line corresponding to Δc = 0.1% allows one to determine t₁ and t₂ and to infer values of K and τ₁/2. t₁ and t₂ were measured for various temperatures comprised between 9°C and 30°C for C₆-Cer, and C₁₆-Cer. Ceramide translocation rates K for different temperatures are plotted in Fig. 8, which shows Arrhenius plots of K in GUVs. At 20°C we show that the flip time constant for each kind of ceramide is closed to 1min and increases with the chain length. The temperature dependence is consistent with an Arrhenius law. We deduced the activation energy barrier for C₆-Cer and C₁₆-Cer. Direct measurement of the ceramide diffusion rate at 37°C by the shape change method is in practice impossible because of the very small τ₁/2 value. Experiments attempted at 37°C showed that budding was not observed after ceramide injection at 37°C, suggesting that the molecule equilibrates too rapidly.
between the two leaflets at high temperature. Reliable $\tau_{1/2}$ values at 37°C could be nevertheless obtained rigorously by extrapolation of $\tau_{1/2}$ values measured at lower temperature by using the activation energy deduced from Arrhenius plots (See Fig. 8 and Table 1), knowing that EPC undergoes no thermal transitions in the temperature range covering 9-37°C.

**Shape change of human erythrocytes.** When experiments were carried out with vesicles containing an equimolar amount of (EPC:SM:cholesterol) and a trace of fluorescent lipid (RTPE) lateral domains were formed and can be seen by fluorescence microscopy (31). However, the line tension existing between two phases, by maintaining the membrane under lateral stress (32), precluded the use of such vesicles for measurement of ceramide flip-flop by shape transformation. Because of the difficulty to measure ceramide transmembrane diffusion by shape change in GUVs containing EPC/SM/cholesterol with physiological proportions, we have investigated instead a biological plasma membrane. Since the pioneer work of Sheetz & Singer (27) it is well known that erythrocytes undergo shape changes after the insertion of amphiphiles that accumulate in one leaflet of the plasma membrane. The kinetic of such shape change from discocyte to echinocyte, or from discocyte to stomatocyte has been used to measure the selective transport of phosphatidylserine in red cells (13, 14).

Thus we have followed shape changes in human erythrocytes to which ceramides with various acyl chains were added. Fig. 9-row I shows a control with no addition of lipid on the outer leaflet. Row II shows that the addition of phosphatidylcholine molecules with a short sn-2 chain (C6-PC) to an erythrocyte suspension triggers in a few seconds the formation of stable echinocytes in red cells (13, 14). When C6-Cer or C16-Cer (rows IV and V) were added to erythrocytes instead of phospholipids, echinocytes were formed in about 20 minutes. Thin layer chromatography of the erythrocyte lipids after extraction carried out at time $t = 15$ minutes and $t = 45$ minutes (not shown) indicated that a high percentage of ceramides injected had been incorporated into the erythrocyte membrane ruling out the possibility that ceramide molecules were not incorporated. This amount should be able to trigger echinocyte to spherocyte transformation if all molecules remained in the outer monolayer as in case of C6-PC. We conclude that randomisation between both leaflets was very fast. However a slow asymmetrical distribution of ceramides seemed to build up and to cause the formation of spicules after 10-20 minutes as if the life-time of ceramides in both monolayers were different due to the presence of liquid-ordered domains in the outer monolayer where ceramide would be trapped.

**ESR experiments with a spin-label analogue of ceramide.** The transmembrane diffusion of a spin-label ceramide with a short sn-2 chain was measured in liposomes and in human erythrocytes. The transmembrane diffusion of spin-label ceramide and of a spin-labeled analogue of PC were measured first in EPC LUVs. The large excess of ascorbate used in these experiments reduced instantaneously nitroxides radicals exposed initially on the outer monolayer. With both probes, 50% of signal was destroyed from ascorbate reduction a few seconds after ascorbate addition. Fig. 10-A shows the subsequent reduction to zero of the ESR signal due to inside-outside spontaneous transbilayer diffusion (flop) at 20°C. For the spin-label ceramide, the curve has a $\tau_{1/2}$ of 1.1 minutes, which characterizes the “flop rate” of SL-Cer in EPC-LUVs. The plateau in Fig. 10-A obtained with spin-label PC after addition of ascorbate and BSA indicates that 50% of PC analogue remain in the inner leaflet. The stability of the latter signal indicates that PC does not diffuse significantly from the inner to the outer monolayer during at least 30 minutes nor does ascorbate penetrate the bilayer. In addition, we have verified that the presence of 1% of unlabeled C16-Cer had no significant effect on the diffusion of spin-
As in case of GUVs, the activation energy and the $\tau_{1/2}$ value at 37°C were calculated from experiments which were carried out at different temperatures for SL-Cer in EPC LUVs (See Fig. 8 and Table 1). Experiments were also carried out with LUVs of different lipid composition. Various proportions of SM (up to 25%) and/or cholesterol (33%) were mixed with EPC. The spontaneous transmembrane diffusion of spin-label ceramide was determined in these vesicles. Exponential fits of the inside-outside movements of the spin-label ceramide in PC:SM vesicles, containing various proportion of SM, are shown in Fig. 10-B. Even 25 % of SM did not modify significantly the transverse diffusion of the ceramide analogue ($\tau_{1/2} = 1.3$ minutes).

Furthermore with a lipid composition SM/PC/Cholesterol (1:1:1 % mol) the transmembrane diffusion rate of the spin-labeled ceramide was identical (data not shown).

Finally, experiments were made to measure the transmembrane diffusion of spin-labeled ceramide in the human erythrocyte membrane. A rapid spontaneous transmembrane diffusion was also found ($\tau_{1/2} = 1.1$ minutes at 20°C, Fig. 10-C).

DISCUSSION

Lipid traffic investigation in cells has often been undertaken with fluorescent lipids and also, though at a lesser extent, with spin-label derivatives. These approaches have been extremely powerful and have led to many important findings on the routes followed by the main lipids in eukaryotic cells. The biological relevance of measurements done with modified lipids is an on going discussion, which is certainly justified when absolute rates (for example of transmembrane diffusion) are measured. Mimicking ceramide traffic within a cell with ceramide analogues possessing a fluorescent moiety on a short acyl chain is one approach that has been used (7). The partial solubility of such molecules provides a technical advantage but also has a drawback because these molecules can diffuse spontaneously from one organelle to another unlike long chain lipids. In addition the probe may (or may not) perturb the determination of the transmembrane diffusion rates (or flip-flop) (33).

The present results provide the first flip-flop measurement of natural ceramide with different acyl chain lengths. It is compared with the transbilayer diffusion of a spin-labeled ceramide. We confirm the tendency of Bai and Pagano’s experiments (7), which indicated that ceramide flip-flop was much faster than that of most of phospholipids (for which $\tau_{1/2}$ of flip-flop is of several hours). However here we measured a flip-flop rate about an order of magnitude faster than the reported value with a BODIPY ceramide. The rapid translocation of ceramide that we have observed (less than 1 minute at 37°C) can have important consequences in all processes in which ceramides are involved in vivo.

It is generally believed that ceramides in the plasma membrane are segregated into rigid domains or rafts that would trap them in one monolayer and prevent or at least slow down their transmembrane diffusion (34). Shape change experiments with lateral domains could not be used for lipid transmembrane diffusion measurements for reasons indicated briefly above. GUVs can be made with SM/cholesterol mixtures forming a $l_o$ phase (35). It is possible to induce shape change in such vesicles by addition of lyso-PC. However lyso-PC was expelled from the rigid phase after injection in the experiments of Tanaka et al. (35). Furthermore when a high a level of lyso-PC was used, it induced fission of the bud, a phenomenon which does not happen with the $l_d$ phase vesicles. Finally the bending modulus of lipids in the $l_o$ phase must be higher than in the fluid $l_d$ phase, thus the threshold for a budding transition must require a much larger lipid asymmetry than in the $l_d$ phase. If the threshold was slightly above 0.1 %, Fig. 7 shows that no shape change would be seen.

Spin-label experiments in LUVs containing a high proportion of SM and cholesterol indicated a rapid transmembrane diffusion of the spin-labeled ceramides. In biological membranes, the same lipid composition, somehow because of the presence of proteins, forms probably small size domains that do not inhibit shape changes that take place at the scale of
several micrometers. Yet insertion of ceramides in erythrocytes did not induce shape changes as rapidly as it does in GUVs. A combination of slow insertion of the ceramide molecules in the viscous membrane of erythrocytes and a rapid flip-flop of ceramide can explain why we did not observe immediately the formation of echinocytes as in the case of C<sub>6</sub>-PC (Fig. 9-II). It should be emphasize also that in many eukaryotic cells the shape is controlled by the cytoskeleton. In red cells, the shape is to a large extent dependent upon the transmembrane distribution of the lipids (27). However, probably because of the cytoskeleton, a lipid asymmetry of about 1% is necessary to trigger a shape change in erythrocytes, which is about 10 times higher than the lipid asymmetry that triggers a shape change in an EPC-GUV.

We have systematically observed echinocytes being formed approximately 15 minutes after ceramide addition to fresh red cells. This observation cannot be explained by the well known “glass effect” on erythrocyte shape, which does not take place if glass has been treated with a silane derivative or it happens eventually after a much longer incubation as verified by several control experiments carried out without ceramide addition. We hypothesize that the late appearance of echinocytes reveals a progressive trapping of a fraction of ceramides in the outer erythrocyte leaflet that causes an expansion of the outer surface. Such trapping could be explained by a displacement of cholesterol by the ceramide injected near the membrane (36) or by the formation of ordered domains due to the increasing concentration of ceramide trapped in the outer leaflet (37). Fig. 11 is a schematic representation of the outer monolayer expansion of an erythrocyte membrane following the progressive ceramide trapping in the outer monolayer, where rafts are more likely to exist than in the inner monolayer (38). The full line arrows indicate that the flip-flop of ceramides is likely to follow an indirect route (ABCD) rather than AD, which is probably a slower process. Hence rapid lateral diffusion in the I, phase of the outer monolayer (39) permit ceramides to short-cut the transmembrane barrier in the I<sub>0</sub> phase.

As already mentioned in the introduction, ceramide is a precursor in sphingolipids synthesis. To overcome topological problems associated with the cascade of transfer from one membrane to another, either an ATP dependent transport or a rapid spontaneous flip-flop is required at different steps (3, 6, 40). Our measurements highlight and favour the latter possibility: rapid transmembrane diffusion can be sufficient to reach the luminal monolayer of organelles.

Ceramide has been implicated as modulator in endocytosis (41, 42). Budded and endocytosed vesicle formation has been explained by ceramide-rich domain formation and negative spontaneous curvature of ceramide through experiments of asymmetrical enzymatic formation of ceramide in GUVs (43). The authors rejected the possibility of a rapid translocation rate of ceramides.

Although we have not been able to measure with unlabeled ceramide the diffusion of ceramide in giant vesicles containing SM and cholesterol, the data obtained in LUVs containing a spin-label analogue of ceramide indicated that the presence of ordered domains does not significantly slow down ceramide flip rate. Similar conclusion was reached with erythrocyte. A plausible explanation of these observations is that ceramide even though it may accumulate in the ordered domains (or rafts) probably partitions into the fluid phase where it can flip rapidly from the outer monolayer to the inner monolayer.

Experiments carried out with EPC LUVs containing 1% of natural ceramide in addition to 0.25% of SL-PC show that this amount of ceramide is not sufficient to promote SL-PC translocation. This result allows us to rule out that the PC translocation coupled to ceramide production reported by Goñi’s group (9) takes place in the vesicles of the lipid composition we are using. We can therefore assure that in former experiments shape changes triggered in GUVs by the small amount of ceramide incorporated were only due to the high transmembrane diffusion of ceramide.
Contrary to Montes et al. (44) and to Siskind and Colombini (45), we did not observe any decrease of contrast of vesicles by efflux of their content when ceramide was injected. However in our experiments, only a low percentage of ceramides was incorporated (less than 5%), a quantity much lower than in the experiments of Montes et al. or Sisiskind and Colombini, where ceramide represented 20% and 10% of total lipid respectively.

In conclusion, we have shown that natural ceramide possesses a rapid translocation rate in the order of a few tens of seconds at 37°C. This very rapid flip-flop must be taken into account in ulterior interpretations when experiments are carried out with ceramides.

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**FOOTNOTES**

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1 The abbreviations used are: C₆-Cer, C₆-Ceramide; C₁₀-Cer, C₁₀-Ceramide; C₁₆-Cer, C₁₆-Ceramide; C₆-PC, C₆-Phosphatidylcholine; C₆-PS, C₆-phosphatidylserine; DCC, Dicyclohexylcarbodiimide; DMAP, 4-N-Dimethylaminopyridine; EPC, Egg phosphatidylcholine; ER, Endoplasmic reticulum; ESR, Electron Spin Resonance; GalCer, Galactosylceramide; GluCer, Glucosylceramide; GUV, Giant Unilamellar Vesicle; LUV, Large Unilamellar Vesicle; Lyso-PC, Lysophosphatidylcholine; NBD-PE, 1-acyl-2-[6-(7-nitro-2,1,3-benzoxadiazol-4-yl) amino] caproyl]-sn-glycero-3-phosphoethanolamine; NBD-PC, 1-acyl-2-[6-(7-nitro-2,1,3-benzoxadiazol-4-yl) amino] caproyl]-sn-glycero-3-phosphocholine; PM, plasma membrane; POPC, 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine; RTPE, Red Texas Phosphatidylethanolamine; SL-Cer, Spin-label ceramide; SL-PC, Spin-label phosphatidylcholine; SM, Sphingomyelin.
FIGURE LEGENDS

Fig. 1. Scheme of the Spin-labeled molecules. a) Spin-labeled analogue of ceramide with a short sn-2 chain (SL-Cer). b) Spin-labeled analogue of phosphatidylcholine with a short sn-2 chain (SL-PC).

Fig. 2. Schematic representation of the incorporation and translocation of ceramides in giant EPC vesicles. \( f(t) \) represents the time dependent rate of incorporation of ceramides in the external monolayer. \( K \) is the time constant of lipid flip-flop within the membrane. \( c_{in}(t) \) and \( c_{out}(t) \) are the concentration of ceramides inserted in the inner monolayer and the outer monolayer respectively. Superscripts refer to the direction of translocation.

Fig. 3. Theoretical curves for the asymmetry of ceramide distribution between the two monolayers (\( \Delta c \)) as a function of time for various values of the parameters \( t_i \) and \( K \). \( K \) is the rate constant of ceramide flip-flop and \( t_i \) is the characteristic time of ceramide insertion in the outer monolayer. Panel A: \( t_i \) fixed (25 min) and \( K \) variable: ( ), \( K = 0.2 \) min\(^{-1}\); (----), \( K = 0.3 \) min\(^{-1}\); (....), \( K = 0.4 \) min\(^{-1}\); (-- • -- ), \( K = 0.5 \) min\(^{-1}\); (-----), \( K = 0.6 \) min\(^{-1}\). Panel B: \( K \) fixed (0.5 min\(^{-1}\)) and \( t_i \) variable: ( ), \( t_i = 5 \) min.; (----), \( t_i = 10 \) min.; (....), \( t_i = 20 \) min.; (-- • -- ), \( t_i = 40 \) min. \( A \) = 3 %. The horizontal line corresponds to the threshold of lipid asymmetry above which the budding transition occurs. Similar families of curves were generated for different \( t_i \) or \( K \) values.

Fig. 4. Budding transition after injection of Lyso-PC into an EPC GUV. 90 min. after lyso-PC injection, the initial shape was not recovered indicating the absence (or the very slow) lyso-PC flip-flop. Experiments were carried out at 20°C. Time after injection: a) \( t = 0 \); b) \( t = 50 \) s; c) \( t = 51 \) s; d) \( t = 52 \) s; e) \( t = 53 \) s; f) \( t = 54 \) s; g) \( t = 55 \) s; h) \( t = 90 \) min. The scale bar corresponds to 10 µm. The same sequence was observed after the injection of phosphatidylserine with a short sn-2 chain.

Fig. 5. Shape recovery after injection of C\(_6\)-Cer into an EPC GUV. Time after injection: a) \( t = 0 \); b) \( t = 15 \) s; c) \( t = 30 \) s; d) \( t = 45 \) s; e) \( t = 1 \) min; f) \( t = 9 \) min; g) \( t = 9 \) min 4 s; h) \( t = 9 \) min 5 s; i) \( t = 9 \) min 6 s; j) \( t = 9 \) min 30 s; k) \( t = 12 \) min s; l) \( t = 15 \) min. Scale bar corresponds to 10 µm. Temperature 20°C.

Fig. 6. Shape recovery after injection of C\(_{16}\)-Cer in an EPC vesicle. Time after injection: a) \( t = 0 \); b) \( t = 2 \) min 10 s; c) \( t = 2 \) min 36 s; d) \( t = 2 \) min 46 s; e) \( t = 2 \) min 50 s; f) \( t = 15 \) min 22 s; g) \( t = 15 \) min 57 s; h) \( t = 16 \) min 27 s; i) \( t = 16 \) min 37 s; j) \( t = 16 \) min 47 s; k) \( t = 17 \) min 32 s; l) \( t = 22 \) min 53 s. Scale bar is 10 µm. Temperature 20°C.

Fig. 7. Calculated evolution of ceramide asymmetrical distribution \( \Delta c = c_{out}(t) - c_{in}(t) \) plotted as a function of time for most probable values of parameters \( K \) and \( t_i \). \( K \) is the time constant of ceramide flip-flop and \( t_i \) is the characteristic time of ceramide penetration: ( ), C\(_6\)-Cer; (----), C\(_{10}\)-Cer; (....), C\(_{16}\)-Cer. See text for more details.

Fig. 8. Rate constants of transmembrane diffusion for different ceramides in EPC vesicles. ■ SL-Cer in LUVs ( ), ● C\(_6\)-Cer in GUVs (----), ▲ C\(_{16}\)-Cer in GUVs (....). Activation energies and \( \tau_{1/2} \) at 37°C (O), given in Table 1, were deduced from the linear fits for each molecule.
Fig. 9. Shapes of human erythrocytes after injection of various lipids near the cells. Temperature 20°C. I. Control (no lipids injected): a) t = 0, b) t = 10 min, c) t = 20 min, d) t = 30 min. II. C₆-PC. a) t = 0, b) t = 10 sec, c) t = 1 min 45 sec, d) t = 20 min. III. C₆-PS. a) t = 0, b) t = 8 min 20 sec, c) t = 21 min 40 sec, d) t = 40 min 15 sec. IV. C₆-Cer. a) t = 0, b) t = 10 min, c) t = 20 min, d) t = 29 min. V. C₁₆-Cer. a) t = 0, b) t = 10 min, c) t = 23 min 30 sec, d) t = 30 min. Scale bar is 10 µm.

Fig. 10. Kinetic of inside-outside translocation of spin-labels lipids. A) ■ SL-PC in EPC LUVs, □ SL-PC in EPC LUVs in presence of 1 % of C₁₆-Cer, ● SL-Cer in EPC LUVs. B) SL-Cer translocation in EPC:SM LUVs, semi-logarithmic scale: ■ 5% SM, □ 15 % SM, ● 25% SM. C) SL-Cer in human erythrocytes. Temperature 20°C.

Fig 11. Schematic model showing how an asymmetrical distribution of ceramides can be generated because of the formation of domains enriched in ceramides in the outer monolayer. a) In case of coexistence between l₀ and lₘ phases, a lipid in the lₘ phase limited to a small area, can diffuse out of the l₀ domain and reach the inner monolayer at the rate specific for flip-flop in a lₘ fluid phase. The transmembrane diffusion from the outer to the inner monolayer is then determined by the rate of lateral diffusion in- and out of the lₘ phase and not by the true transmembrane diffusion within the liquid ordered phase l₀. A rough calculation shows that for a 100 nm domain the time scale of diffusion out of this domain is of the order of 50 ms at 20°C (39). Consequently even if the route AD is slow, ABCD can be rapid and enable the 2 sides of a lₘ phase to exchange lipids. b) Ceramides tend to cluster in rafts in the outer monolayer or to form ceramide lateral domains that should induce a lateral pressure in the outer monolayer (indicated by white horizontal arrows). Eventually in spite of the rapid exchange of the ceramides between raft and non-raft PM domains and between outer and inner monolayers through the route BC, ceramides in the outer monolayer outnumber ceramides in the inner monolayer and induce a membrane bending.

### Table 1

| Ceramide | Number of experiments at 20°C | K (min⁻¹) at 20°C | τ₁/2 (min) at 20°C | tᵢ (min) at 20°C | Activation energy (Kcal/mol) b | τ₁/2 (sec) at 37°C c |
|----------|-----------------------------|------------------|-------------------|-----------------|-------------------------------|---------------------|
| SL-Cer a | 3                           | 0.7± 0.1         | 1.1± 0.2          | -               | 11.4±1.5                     | 18.6± 2.4           |
| C₆-Cer   | 5                           | 0.6 ±0.3         | 1.2 ±0.6          | 18.6 ±8.8       | 11.7± 4.1                    | 18.1± 6.3           |
| C₁₀-Cer *| 6                           | 0.4 ± 0.2        | 1.6 ± 0.7         | 25.8 ±12.2      | -                             | -                   |
| C₁₆-Cer *| 9                           | 0.3±0.1          | 2.4 ±1.0          | 38.9 ±18.4      | 8.4± 3.3                     | 57± 22              |

| a : experiments carried out in LUVs; b and c: values deduced from Arrhenius plots. |
| * Indicates that ceramides were incorporated with the help of ethanol:dodecane (see Materials and Methods for details). |
FIGURE II

\[ f(t) \text{ (DIFFUSION, PARTITION)} \]

\[ C_{out} \quad K_{out} \quad K_{in} \quad C_{in} \]
FIGURE IV
FIGURE VI
FIGURE VII

![Graph showing % of total lipid over time](image-url)
FIGURE VIII
Rapid transbilayer movement of ceramides in phospholipid vesicles and in human erythrocytes

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