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Surface tension induced by sphingomyelin to ceramide conversion in lipid membranes

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Abstract

We have investigated the effect of sphingomyelin (SM) to ceramide enzymatic conversion on lipid bilayers using Giant Unilamellar Vesicles (GUVs). Sphingomyelinase was added externally to GUVs containing various proportions of SM. In situ asymmetrical SM conversion to ceramide reduced the area of one leaflet. In the absence of equilibration of all the lipids between the two leaflets, a mismatch between the two monolayers was generated. The tension generated by this mismatch was sufficient to trigger the formation of membrane defects and total vesicle collapse at relatively low percentage of SM (≈5% mol). The formation of nanometric size defects was visualised by AFM in supported bilayers. Vesicle rupture was prevented in two circumstances: (a) in GUVs containing a mixture of l_d and l_o domains and (b) in GUVs containing 5% lysophosphatidylcholine. In both cases, the accumulation of enough ceramide (at initial SM concentration of 10%) allowed the formation of ceramide-rich domains. The coupling between the two asymmetrical monolayers and the condensing effect produced by the newly formed ceramide generated a tension that could underlie the mechanism through which ceramide formation induces membrane modifications observed during the late stages of apoptosis.

Keywords: Ceramide; Sphingomyelinase; Membrane surface tension; Lipid flip-flop; Lipid asymmetry; Lipid scrambling

1. Introduction

Ceramide is produced in cell membranes from its precursor sphingomyelin (SM) through the enzymatic activity of endogenous sphingomyelinase [1–3]. Ceramide function in the plasma membrane during different cell processes as apoptosis, senescence, immune response, bacterial pathogenesis and cell-cycle arrest [4], is supposed to be a second messenger [5,6]. However, its mode of action is still unclear. It is not known if its action is exerted through binding to specific intracellular protein targets or if, on the contrary, it is its strong impact on membrane structure (microdomain function, membrane vesiculation, fusion, fission and vesicular trafficking) that plays a role in cellular signalling [7].

The presence of ceramide has important effects on the physical properties of the membrane. The small size of ceramide polar headgroup affects the overall membrane curvature and stability, possibly by promoting the lamellar–hexagonal phase transition [8,9], pore formation [10], and endocytosis stimulation [11–13]. Ceramide also shows a strong tendency to segregate from other lipids forming highly ordered ceramide-enriched microdomains. Ceramide phase diagrams for different PC fluid matrix have been extensively studied [14–18]. In addition, the presence of SM and cholesterol augments ceramide partitioning into gel or liquid ordered domains [15,16,19]. It has been also reported that ceramide competes with cholesterol in forming ordered lipid domains [20]. Finally, ceramide, compared with other lipids, has an unusual characteristic, namely its rapid transmembrane diffusion (flip-flop). Bai and Pagano measured a half-time of 22 min at 37 °C for a fluorescent analogue of ceramide in lipid vesicles [21] and more recently a half-time of less than 1 min at 37° was reported for unlabeled-C_{16} ceramide in giant vesicles and in the erythrocyte plasma membrane [22].

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Most of the results described above were obtained by adding ceramide to a lipid mixture that is used to form bilayers. An interesting and complementary approach to study the influence of ceramide in lipid bilayers is to increase in situ its concentration through SM enzymatic digestion by SMase [8,23–28]. This asymmetric increase in ceramide concentration closely resembles the mechanism of ceramide generation which takes place in vivo before triggering other cellular responses. The newly formed ceramides are also capable of inducing visible membrane transformations: membrane fusion [8,23,24], vesicle budding [25], and also to facilitate the transbilayer translocation of other lipids (scrambling), possibly through the formation of lamellar to inverted hexagonal phase transition in membranes [26]. Scrambling in apoptosis and ceramide generation seems to be closely linked [29]. However, not much attention has been paid to the fact that when the ceramide content in the membrane is increased due the SMase hydrolysing activity, physical changes occur besides overall increase in ceramide concentration due to the very slow transmembrane diffusion of SM, hence to the very asymmetrical structure of the bilayer that is formed.

GUVs, with a diameter in the range 10–100 μm i.e. visible by light microscopy, are useful model systems to investigate membrane domains [30]. They have allowed the observation of vesicle deformations by membrane asymmetry [22,31]. Here, we used GUVs to explore the effect of the lateral stress generated by the SMase when hydrolysing SM in situ. We have analysed the effect on relaxed vesicles with an excess area, on quasi-spherical lipid vesicles in the fluid phase, and in vesicles where l_o–l_d domains coexist. The overall morphological changes suggest that SMase induces a lateral tension caused both by the asymmetrical SM distribution following SMase treatment and by the condensing effect of the newly formed ceramide. Observations on supported bilayers with AFM confirmed the ceramide production, its segregation in gel domains after its redistribution membrane and the generation of small holes in the membrane induced by the lateral tension.

2. Materials and methods

2.1. Chemicals

Egg phosphatidylcholine, brain sphingomyelin, egg phosphatidylethanolamine, lyso-phosphatidylcholine, rhodamine phosphatidylethanolamine and cholesterol were purchased from Avanti-Polar Lipids (USA). Red Texas phosphatidylethanolamine was acquired from Molecular Probes (USA). Sphingomyelinase from *Bacillus cereus*, sucrose, glucose, and all other chemicals were purchased from Sigma-Aldrich (USA).

2.2. Giant unilamellar vesicles preparation

Giant Unilamellar Vesicles (GUVs) made of EPC, EPC:SM (98:2% mol) or EPC:EPE:SM:Lyso-PC:cholesterol:RTPE:RhoPE (at different mol ratios, indicated when necessary) were prepared following the electroformation method [32,33]. 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 conducting cover slides. After evaporation, the chamber was placed in vacuum for 1 h to eliminate residual solvent. 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 min. After one night, the voltage was increased to 1.3 V while decreasing the frequency to 4 Hz for 1 h in order to detach the vesicles from the cover slides. The vesicles obtained had a spherical shape. Domains could be seen by fluorescence microscopy for particular lipid mixtures: EPC:EPE:SM:chol (0:1:2:1), (1:1:1:1), (1.5:1:0.5:1) molar ratios due to the presence 1% mol fluorescent lipids (RTPE or RhoPE) in the lipid composition. SM proportion was progressively decreased (down to 5%) and replaced with EPC. We used EPC, because of EPE does not form lipid vesicles at high molar ratios. Molar ratios tested were EPC:EPE:SM:chol (0:1:2:1), (1:1:1:1), (1.5:1:0.5:1), (1.6:1:0.4:1) and (1.8:1:0.2:1). EPC was substituted in experiments carried out to study Lyso-PC influence. Lipid molar ratios were EPC:EPE:SM:Chol:Lyso-PC (1.6:1:0.2:1:0.2) and (1.2:1:0.4:1:0.4).

In order to obtain prolate shape vesicles, i.e. to have a large surface to volume ratio necessary for shape changes, the glucose solution was allowed to evaporate for more than 1 h at room temperature.

2.3. Supported lipid bilayers preparation

EPC:EPE:SM:Chol (1:1:1:1 mol) Large Unilamellar Vesicles (LUVs) of 200 nm size were fabricated at 1 mg/ml by extrusion in buffer (20 mM HEPES, 150 mM KCl, pH 7.5) at 42 °C. A drop of the LUVs solution was incubated on freshly cleaved circular pieces of mica glued onto a Teflon surface in presence of 10 mM $CaCl_2$. After several minutes (down to 30 min), the samples were extensively washed with buffer and used for AFM imaging. AFM images were taken with an Atomic Force Microscope (Nonatec Electrónica, Madrid, Spain) operated in the jump mode [34]. The scanning piezo measurement was calibrated using silicon calibrating gratings. Silicon nitride tips (DI instruments) with constant force of 0.73–0.38 N/m were used.

2.4. Sphingomyelinase assay in GUVs

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 difference in density between outside and inside mediums permitted vesicle sedimentation in the observation chamber and provided a better contrast for light microscopy. Once a vesicle was targeted, 10 μl of 1 unit/ml protein concentration (in 9 mM $CaCl_2$ and 1.5 mM $MgCl_2$ aqueous solution) was added. Shape changes induced by sphingomyelin to ceramide transformation were monitored by observation with a fluorescent 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).

2.5. Sphingomyelinase assay in supported bilayers

As in case of GUVs, once a lipid bilayer region was targeted, up to 30 μl of 1 unit/ml protein concentration (in 9 mM $CaCl_2$ and 1.5 mM $MgCl_2$ aqueous solution) was added locally while continuously observing the same region.

3. Results

3.1. Vesicles with sphingomyelin that lack lipid ordered domains were unstable upon SMase addition

Incubation with SMase of spherical vesicles containing SM triggered membrane rupture after a few minutes. The membrane collapse was observed by optical microscopy even when SM content in vesicles was low (~5% mol). Vesicle rupture was preceded by opening (lasting up to seconds), enabling the observation of small vesicles efflux, when giant vesicles had encapsulated smaller vesicles, as shown in Fig. 1. When small vesicles were outside the giant "mother" vesicle, they became accessible to SMase and eventually collapsed themselves.

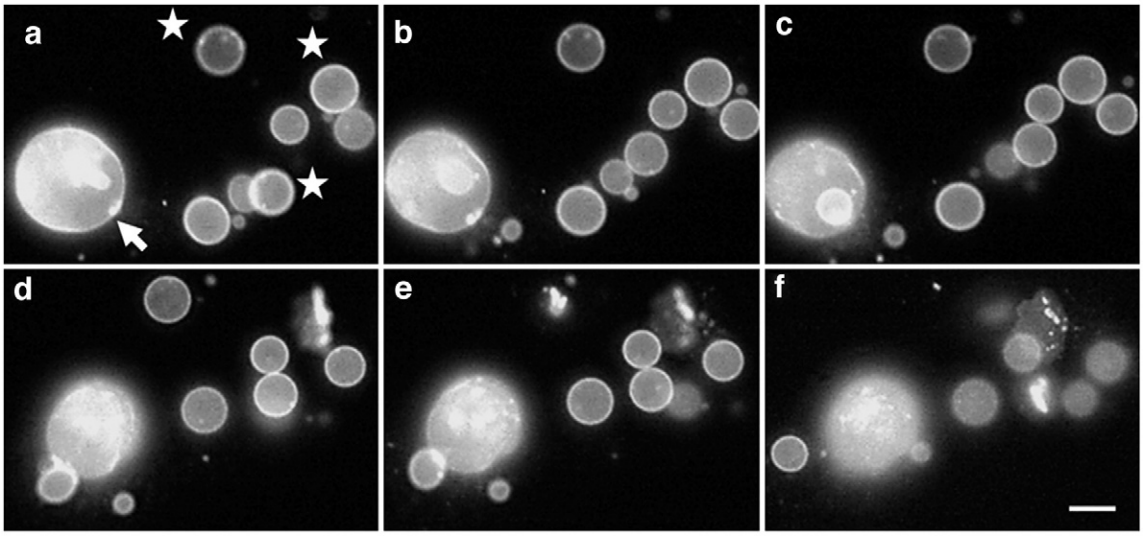


Fig. 1. Transient opening in an EPE:SM:chol (1:2:1) vesicle after addition of SMase. Time after injection: (a) 30 s, (b) 50 s, (c) 1 min, (d) 1 min 30 s, (e) 1 min 45 s, (f) 2 min 5 s. The white arrow points towards a transient opening through which small vesicles efflux from inside to outside. White stars indicate vesicles which undergo total membrane rupture when in contact with SMase. Scale bar: 10 μm.

Membrane rupture was taken as an indication of the release of the tension induced by the asymmetric membrane generated by the enzyme. Several control experiments were carried out with vesicles formed with EPC, in lack of SM. Alternatively SMase was added to vesicles containing SM in the absence of Ca^{2+} and Mg^{+2} cations, which are known to be essential for SMase activity [35]. In both cases vesicles did not undergo any transformation. If the aqueous solution containing Ca^{2+} and Mg^{+2} was injected in the absence of SMase, no transformation took place either. Finally, if EDTA (25 mM) was present in the external medium the enzyme had no activity and the vesicles did not undergo shape change (not shown).

3.2. Deflated vesicles formed invaginations upon SMase addition

Spherical vesicles that were deflated by osmotic pressure took a prolate shape, and possessed a surface excess, which allowed a smooth relaxation of membrane tension after SMase addition. When deflated EPC GUVs containing 2% of SM were incubated with SMase at 20 °C, a prolate-oblate transition took place usually in the first 5 min and an invagination appeared 1–2 min later (Fig. 2). We always observed a single stable invagination at the pole of the giant vesicle. The dark appearance indicated that the medium inside the invagination was that of the outer environment (glucose) and not that of the inner one (sucrose) (see Materials and methods). The invagination therefore constituted a lighter pocket within the GUV. This explains why it was always found at the centre and on the top of the GUV. The presence of an invagination rather than a budd was confirmed by changing the focus of the image. The shape change observed was in accordance with vesicles shape changes theory and indicated a surface asymmetry, with the inner monolayer area larger than the outer one [36].

3.3. Vesicles containing domains can be used to estimate the lateral strain induced by SM hydrolysis

Some of the vesicles prepared with lipid composition EPC: EPE:SM:Chol (X:1:2–X:1 mol) presented coexisting liquid ordered and liquid disordered phases. If the bilayers equilibration was allowed, smaller domains of identical composition coalesced until total phase separation occurred, minimizing the contact region between phases, and maintaining the membranes under tension [30] (Fig. 3a). When vesicles were deflated due to solvent evaporation the initial spherical vesicles adopted an eight shape with two spherical caps with different radii connected by an interfacial region. The line tension between domains precluded their mixing within the GUV. Membranes of both spherical caps were under lateral tension as revealed by the absence of membrane undulations.

Subsequent SMase treatment of vesicles containing several lateral domains induced further shape transformation. The membrane tension due to SM hydrolysis was relaxed by adjusting the reduced vesicle surface to volume ratio. The higher surface tension (Figs. 3b and c) increased the contact area between different domains. Thus, the interfacial area relaxed the surface tension and initially prevented membrane rupture. In a second step, mixing of domains occurred and domain segregation disappeared (Figs. 3d to f). Afterwards, ceramide rich-domains were observed, with a domain typology similar to that observed when lyso-PC was present (see below, Fig. 5c). Further exposure of these vesicles to enzymatic activity produced vesicle rupture.

Micrographs observations were used to estimate the area loss induced by ceramide formation. Taking into account that fluorescent images represent slices of three-dimensional objects with spherical symmetry, simple geometrical calculations allows one to estimate the area decrease associated with a transition from double budded vesicle to a sphere. We assumed

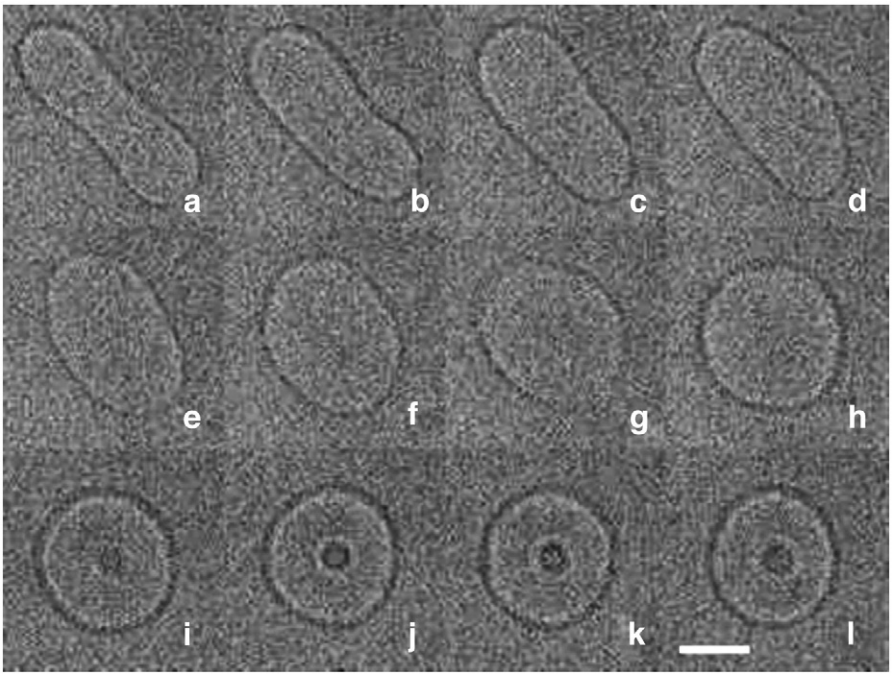


Fig. 2. Formation of an invagination in an EPC:SM (98:2 mol) vesicle after addition of SMase. Time after injection: (a) 0; (b) 7 min 10 s; (c) 7 min 20 s; (d) 7 min 30 s; (e) 7 min 40 s; (f) 7 min 50 s; (g) 8 min; (h) 8 min 10 s; (i) 8 min 49 s; (j) 8 min 51 s; (k) 8 min 53 s; (l) 9 min 10 s. Scale bar: 10 μm. See text for more information.

that the volume remained constant during shape transformation since no contrast modification was detected with vesicles observed under bright field illumination. As shown in Fig. 4, the initial shape corresponded to that of two spherical caps and the final shape corresponded to a single sphere. R_1, R_2, h_1, h_2 and R_f were measured on micrographs for different vesicles and SM ratios deduced from the lipid composition (Fig. 4). The percentage of area reduction is: $100\cdot(S_f - S_0)/S_0$. The measured area decrease for such transformation was on the order of 10%. This percentage, which characterises the shape change, was independent of the initial SM concentration in the lipid mixture (see Discussion and Fig. 4). When vesicles reached the spherical conformation, continuous SM hydrolysis produced the formation of domains which are likely to be ceramide-enriched domains. For a SM concentration higher than 12.5% mol the liposomes rupture occurred (not shown).

3.4. Lyso-PC stabilised membrane integrity and allowed ceramide rich domains formation

The presence of Lyso-PC in the membrane composition (see Materials and methods) protected the vesicles from

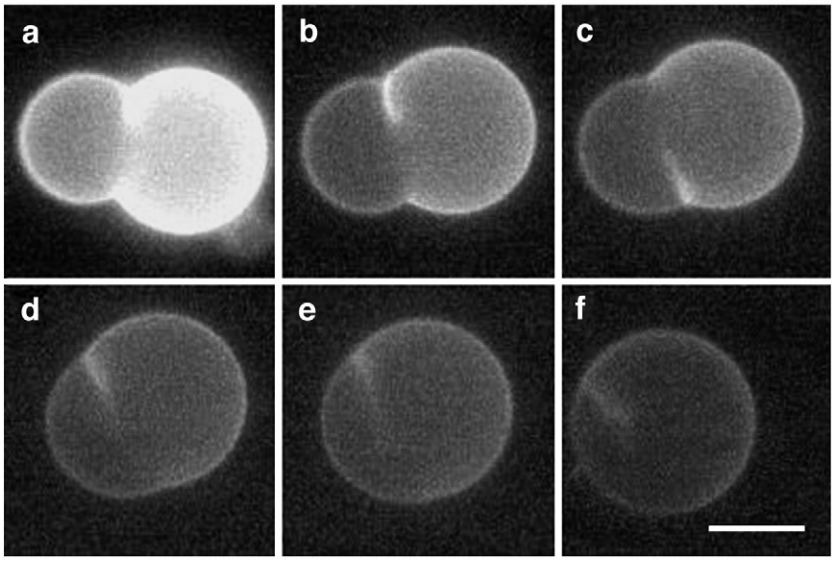
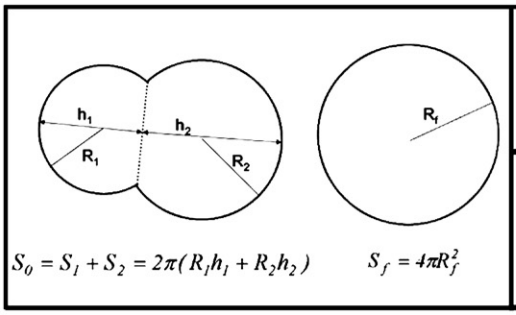


Fig. 3. Shape change produced after addition of SMase in an EPE:SM:chol (2.5:0.5:1 mol) vesicle containing lipid phase separation (l_o+l_d domains). Time after injection: (a) 0, (b) 7 min 17 s, (c) 7 min 50 s, (d) 8 min 28 s, (e) 8 min 40 s, (f) 9 min. Scale bar: 10 μm.



Vesicle	$[SM]_0$ (% mol)	Monolayer area reduction [a] (% of total monolayer area)	Area reduction from condensing effect [b] (% of total area)	SM asymmetry [c] (% of total area)	100 x $(S_0-S_f)/S_0$ (% of total area)
1	50	10	~30	25	*13,2 ± 2,8*
2	25	5	~15	12,5	*9,4 ± 2,8*
3	12.5	2,5	~2	6,25	*10,2 ± 4,3*
4	12.5	2,5	~2	6,25	*11,1 ± 2,4*

Fig. 4. Area decrease measurements after addition of SMase to vesicles presenting lipid phase separation (l_o+l_d domains). Both areas at initial state and final state are parameterised by assuming that images which are taken in equatorial plane possess spherical symmetry. h_1, h_2, R_1, R_2 and R_f are measured from micrographs. Three contributions to the surface decrease are estimated theoretically and shown in the same table: (a) The outer monolayer area decrease given by the enzymatic conversion to ceramide of the outer SM. Data represent the area reduction by a 20% of the SM concentration in the outer monolayer according to the mean molecular areas of ceramide and SM obtained in monolayers at 30 mN/m from [37]. This contribution is mitigated by the rapid redistribution of created ceramide. (b) The total area reduction given by the condensing effect of the new synthesized ceramides on the fluid lipid matrix. Data represent the variation in the mean molecular area of the overall membrane depending on proportion of synthesized ceramide, according to [42]. Because of heterogeneity of acyl chains in brain SM, the area decreased used here was an average value from $C_{16:0}$ ceramide and $C_{24:1}$ ceramide described in [42]. (c) The SM asymmetry defined as the area difference between both monolayers over the total lipid area, after the total redistribution of the newly formed ceramide. The shape change is characterised by an experimental area reduction on the order of 10% (last column), and is independent of SM content in the initial lipid composition.

collapse induced by SMase treatment. SMase addition to a vesicle suspension containing Lyso-PC produced an average decrease in volume of 23±12% (for 8 independent vesicles, the high standard deviation value takes into account 2 vesicles which presented volume decrease below 7%) after a few minutes (Figs. 5a and b). This phenomenon was accompanied by the formation of small lipid vesicles and/or filaments inside the GUVs. The stabilising effect of lyso-PC can also be inferred from the observation that vesicles with 10% of SM, when treated with SMase, showed the presence of ceramide-enriched domains (Fig. 5c), indicating that in situ enzymatically produced ceramide reached a concentration high enough to allow domains formation before any appreciable irreversible membrane destabilisation occurred.

3.5. Direct observation of nanoscale defects in supported lipids bilayers with AFM

Supported bilayers containing EPC:EPE:SM:Chol (1:1:1:1 mol ratio) were prepared for AFM observation. These bilayers always exhibited two types of domains differing in height by 0.6 nm. The thicker domains corresponded to liquid ordered (l_o) domains, whereas the thinner ones corresponded to liquid disordered (l_d) domains [27]. Heterogeneity in lipid vesicle adhesion on the mica surface originated the diversity in relative ratio and connectivity of the two types of domains observed in different regions on the mica. Addition of SMase to the aqueous solution induced three different surface rearrangements that were apparent to a different extent depending on the ratio of l_o/l_d present at the surface analyzed: lo domain reduction, appearance of higher domains corresponding to ceramide rich areas, and the formation of "holes". Regions presenting a discontinuous l_o phases as depicted in Fig. 6 showed a clear decrease in l_o areas during the first minutes of enzymatic exposure, as reported before [27]. Figs. 6a and b show this shrinkage in size of l_o domains. The second type of enzymatic induced rearrangement observed is shown in Fig. 6c. New patches appear that differ in height, shape and position from the original l_o regions observed before enzymatic treatment. The height difference of these newly appeared patches was now 1 nm (Fig. 6c), with respect to the l_d phase, is compatible with ceramide domains constituted after accumulation of enzymatic generated

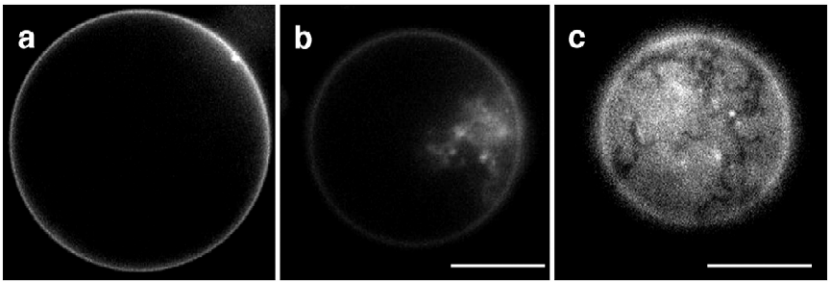


Fig. 5. Membrane stabilisation by the presence of lyso-PC in lipid composition and visualisation of rich-ceramide domains in an EPC:EPE:SM:Chol:Lyso-PC (1.2:1:0.4:1:0.4 mol) vesicle after incubation with SMase. (a) GUV before addition of SMase, (b) The same vesicle 7 min after SMase addition. Both decrease of volume and invaginated small vesicles and/or filaments can be observed. (c) Rich-ceramide domains (visualised as dark "channels") were observed when focusing at the top of a vesicle (here a different vesicle from (a) and (b) and 13 min after Smase addition). Scale bars: 10 μm.

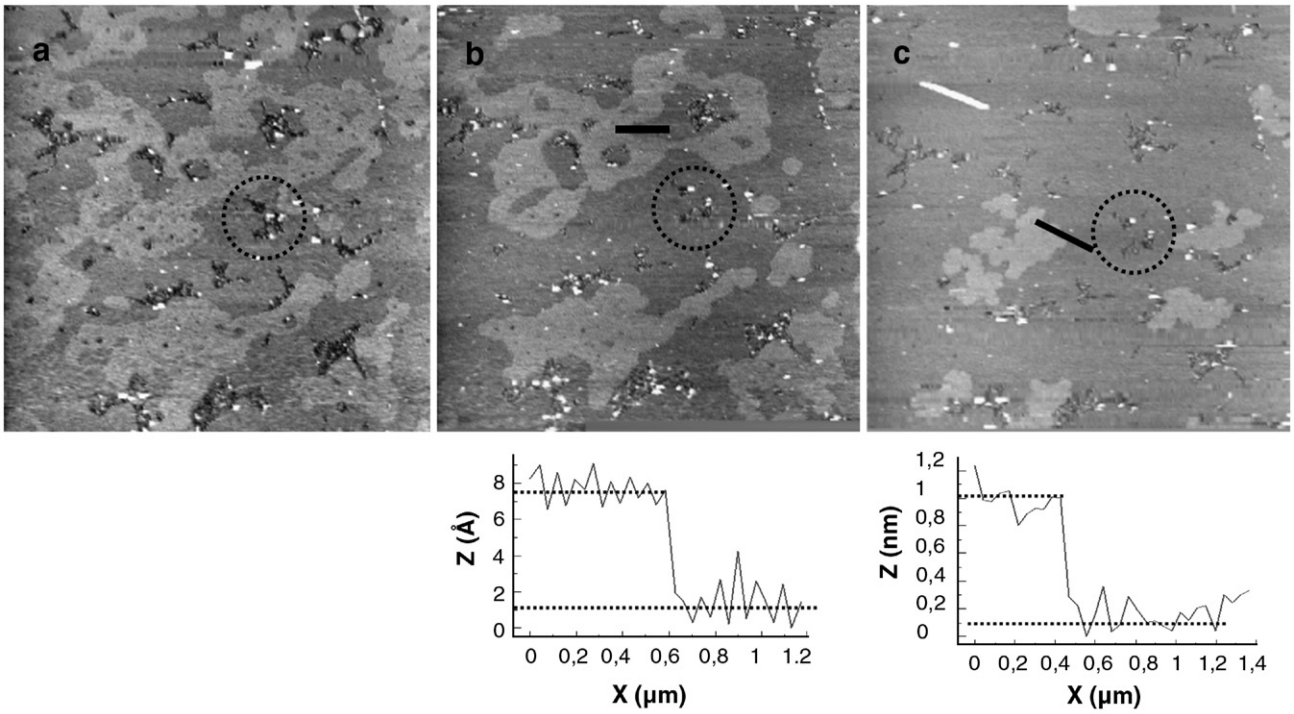


Fig. 6. Domain redistribution in supported lipid bilayers containing l_o domains (PC:SM:PE:Chol 1:1:1:1) after addition of SMase. Time after injection: (a) 19 min; (b) 60 min; (c) 75 min. l_o phase area is decreased from (a) to (b) by SM to ceramide conversion. Finally, in (c), l_o phase disappears and the new thicker ceramide patches appear. The height profiles (indicated with a black bar on AFM images) show the height difference between the l_d–l_o phase (0,6 nm) and between ld-ceramide domains (1 nm). Defects in lipid coating (dashed circle) serve as a reference to indicate that the images represent the time evolution of the same region.

ceramides [27]. In the region depicted in Fig. 6 the shape deformation can be interpreted as being indicative of tension generated by the enzymatic activity, this tension is evidenced through the appearance of holes formed in the membrane after enzymatic treatment in regions where the domain prevalence was reversed and the l_o phase constituted the continuous phase surrounding discontinues l_d phase regions (Fig. 7). The appearance of small holes on an enzymatic treated surface is shown in Fig. 7b. Previous reports had also described enzymatic generated holes [27]. Thicker regions similar to the ones observed in Fig. 6 can also be seen in Fig. 7c, reinforcing the interpretation that they correspond to domains formed by the accumulation of ceramides.

4. Discussion

The work presented here shows that in situ enzymatic conversion of sphingomyelin into ceramide on only one side of a lipid vesicle generates a surface tension that in turn can induce shape changes and membrane rupture. SM to ceramide conversion reduces the mean molecular area occupied by this lipid by approximately 20% (measured in monolayers at 30 mN/m) [37]. Since only SM in the outer monolayer was accessible to SMase injected in the medium, the enzyme activity generated a surface asymmetry in the bilayer. The area asymmetry can be relaxed by bending the membrane if vesicles possess a surface excess. It is well documented that an area difference as low as 0.1% is sufficient to induce a couple of forces associated with the difference of lateral tension and can trigger budding or invagination in a deflated GUV [31,38]. Together with the inverted cone shape of ceramide molecules, a negative curvature should be induced to the overall membrane and invaginations must be observed [25]. On the other hand, the newly synthesized ceramide is expected to eventually equilibrate between both monolayers [22]. This effect could partially inhibit the induction of negative curvature in membranes by ceramide. However the very slow SM diffusion from the inner to the outer monolayer should maintain a bilayer asymmetry preventing the vesicle from reversing to a prolate shape. Our experiments indicated that a stable membrane invagination was produced by exogenous SMase treatment in prolate vesicles containing 2% of SM.

SM asymmetry explains why spherical vesicles (i.e. unable to bend) with as little as 5% of SM, and no visible lipid ordered domains, became unstable upon SMase addition. In the hypothetical case where ceramide did not redistribute between both monolayers, the maximum expected molecular area reduction from SM to ceramide that could be reached after full SM hydrolysis in the outer leaflet is about 1% (20% reduction of 5% of the area, assuming that all lipids contribute equally to the total area). This estimated area reduction is smaller than a priori necessary for vesicle rupture (3–5% of total lipid surface, [39]). However, ceramides formed on the outer monolayer are expected to redistribute between both monolayers within a few minutes [22]. The flow of new molecules towards the inner monolayer can decrease the area difference between leaflets. However a large initial asymmetry in our hands can generate transitory defects and/

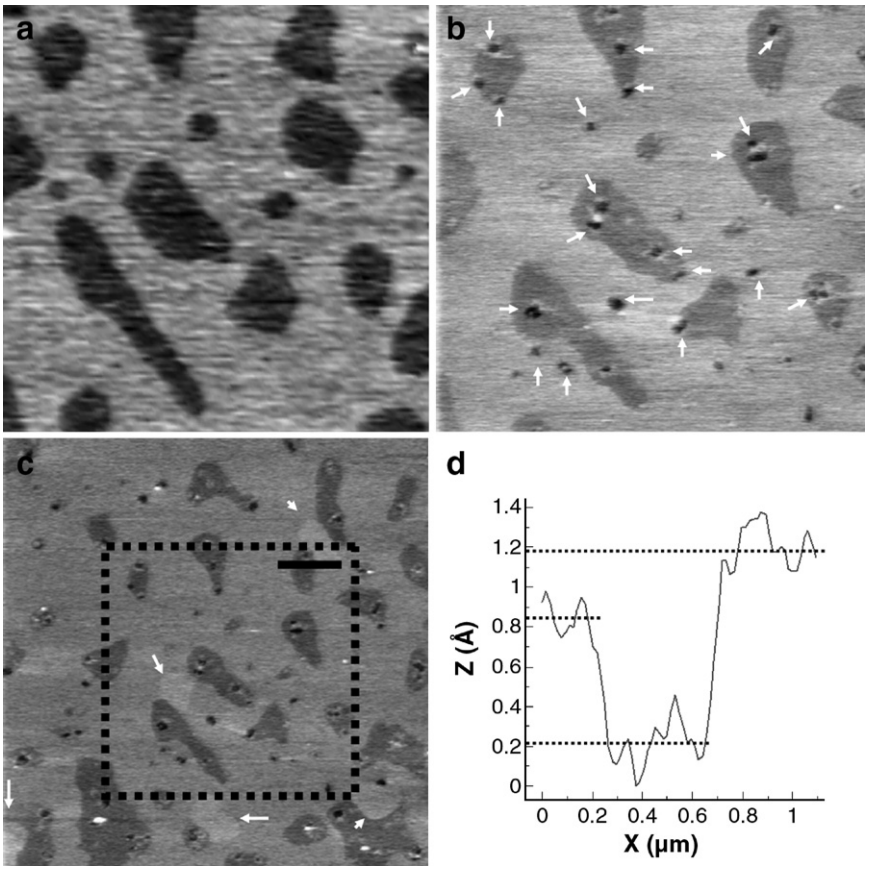


Fig. 7. Direct observation of nanoscale defects produced by SM to ceramide conversion. Time after injection of Smase: (a) 0 min, (b) 60 min and (c) 65 min. The scanned region in (a) and (b) is the same as that delimited by a dashed square in (c). Dimensions of the dashed square are 5 μm × 5 μm. The nanometre-sized holes that appear upon enzymatic treatment are pointed out by arrow heads (b). The ceramide rich domains indicated by white arrows are also observables in (c). The profile from the black bar on AFM images indicating the relative heights of different kinds of domains is shown in (d).

or ruptures in spherical GUVs by stretching the outer monolayer [40]. In any case the high tension asymmetry provokes instabilities in the lipid bilayer [41,42].

Another contribution to the surface tension is given by the condensing effect of new ceramides on the molecular packing of PC matrix [43]. In the latter case, and because of the ceramide redistribution, the membrane tension acts in both monolayers and becomes a symmetric tension. Decreases of ~1 to ~22% on mean molecular areas have been measured depending on ceramide concentrations (from 5 to 50% mol) and on acyl chains composition in a DMPC matrix [43]. Apart from, or in conjunction with the SM asymmetrical distribution, the condensing effect of the newly formed ceramides should be an important contribution to the surface tension for high SM ratios (>15% mol) because of the high heterogeneity of acyl chains of the brain SM used here.

Recently the group of Ohki showed that SMase incubation of vesicles containing DOPC, C_{16}–Cer, C_{16}–SM and Cholesterol transform the liquid-ordered and disordered phases into gel and liquid phase [28]. No mention was made of the overall apparent membrane area reduction in their results. Our observations indicated that the SMase activity produced observable shape changes that reflect an overall vesicle area decrease. At constant volume the measured area reduction was ~10%, (see Fig. 4). This quantity is larger than the estimated area reduction coming from the SM asymmetry and the condensing effect contributions for vesicles with a small SM content. Therefore, the area reduction cannot be explained by these two contributions in this case. In fact, deflated vesicles with domains posses an area excess. Thus, this measured area reduction of 10% corresponds to a geometrical accommodation to the volume of the new decreased surface, and consequently independent of SM content. The surface tension associated to such high lateral stress is never reached because the interfacial tension between domains relaxes the stretched vesicle thereby preventing rupture in a first moment. Once the spherical shape was reached, vesicles underwent the formation of ceramide-enriched domains and ruptured, according to the critical area strains (2–3%) for vesicle containing high amounts of cholesterol or SM [39]. Vesicles containing EPE:EPC:SM:chol, and 5% or 10% of both of SM and lyso-PC did not rupture under SMase treatment. They decreased in size and were able to accumulate enough ceramide on their surface to form visible ceramide rich domains. Change in vesicle volume without apparent rupture suggests the leakage of the inner content by transient defects as a response to the tension created by the enzyme activity but healed after enough inner volume was expelled and reduced vesicle size and membrane tension. The origin of defects can be diverse. One hypothesis is the potential of newly formed ceramide molecules to induce lamellar-inverted hexagonal phase transitions on PE

and cholesterol matrix [9]. This possibility is however unlikely within the range of temperatures (20 °C) and for the lower concentrations of initial SM, i.e. 5% mol, present in our experiments [9]. In addition, the presence of Lyso-PC should mitigate the ceramide HII-phase induction [23]. Another hypothesis that we favour is the formation of hydrophilic transient pores stabilised by the high positive spontaneous curvature of Lyso-PC. The positive curvature of lyso-PC can contribute to the membrane stabilisation by reducing the hydrophobic region in contact with water and by connecting the two opposing monolayers. These transient pores must be of nanometres size since they are not visible by optical microscopy. The latter hypothesis was confirmed by AFM visualisation of planar lipid domains possessing an average size of several nanometres (see Fig. 7). Several reasons can explain why we do not detect transient pores with a longer life-time. First, the presence of cholesterol increases the line tension of pores and reduces their lifetimes. Second, the low viscosity of aqueous solution prevents the visualisation of macroscopic transitory pores [44].

A direct consequence of transient pore formation is the transfer of lipids from the inner monolayer to the outer monolayer along the pores' edges [45]. Furthermore, the surface mismatch produced by ceramide formation could be rapidly relaxed by this mechanism. Defect lifetimes of 10^{-3} to 10^{-1} s involve a net transbilayer flux of 10^3–10^5 lipids trough a defect with radius of several nanometres [46,47]. This mechanism could be the way by which SM and ceramide production allow lipid scrambling, an effect that has been documented in different biological and reconstituted systems [26,48]. In agreement with the interpretation that it is the ceramide asymmetric generation that promotes lipid scrambling [48,49], one could extrapolate this observation to a biological situation and postulate that endogenous SMase could be a lipid scramblase.

5. Conclusion

In this article SM to ceramide conversion was produced by hydrolysis of SM polar head group in a single leaflet. Several phenomena then contributed to the formation of an area difference between the two monolayers: (1) the disappearance of SM head group of the outer monolayer shrinks the outer monolayer area; (2) the absence of transmembrane distribution of SM between the two monolayers caused by the very slow SM transmembrane diffusion does not enable equilibration between the two monolayers; (3) the symmetrical area decrease coming from the condensing effect of synthetised and redistributed ceramides.

All these processes fully explain in a quantitative way membrane bending, transitory pore formation, and/or membrane collapse observed in experiments with SM containing GUVs when incubated with SMase. Pore formation is possibly helped by the presence of lyso-PC in the lipid composition of the membranes. The high positive curvature of Lyso-PC, should favour transient pores, enabling lipids to flip from one monolayer to the other. These observations by optical microscopy and confirmed in AFM experiments, are responses of the vesicles that attempt to relax the surface tension generated by the lipid asymmetry. This process results in lipid scrambling, thus SM located in the inner monolayer can become also accessible to SMase.

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