

Vectorial Budding of Vesicles by Asymmetrical Enzymatic Formation of Ceramide in Giant Liposomes

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ABSTRACT Sphingomyelin is an abundant component of eukaryotic membranes. A specific enzyme, sphingomyelinase can convert this lipid to ceramide, a central second messenger in cellular signaling for apoptosis (programmed cell death), differentiation, and senescence. We used microinjection and either Hoffman modulation contrast or fluorescence microscopy of giant liposomes composed of 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC), *N*-palmitoyl-sphingomyelin (C16:0-SM), and Bodipy-sphingomyelin as a fluorescent tracer (molar ratio 0.75:0.20:0.05, respectively) to observe changes in lipid lateral distribution and membrane morphology upon formation of ceramide. Notably, in addition to rapid domain formation (capping), vectorial budding of vesicles, i.e., endocytosis and shedding, can be induced by the asymmetrical sphingomyelinase-catalyzed generation of ceramide in either the outer or the inner leaflet, respectively, of giant phosphatidylcholine/sphingomyelin liposomes. These results are readily explained by 1) the lateral phase separation of ceramide enriched domains, 2) the area difference between the adjacent monolayers, 3) the negative spontaneous curvature, and 4) the augmented bending rigidity of the ceramide-containing domains, leading to membrane invagination and vesiculation of the bilayer.

INTRODUCTION

Ceramide has recently been confirmed to function as a second messenger in several cellular processes, including apoptosis (programmed cell death), growth suppression, differentiation, and cell senescence (for a review, see Hannun, 1996). Studies on ceramide-mediated cell signaling cascades are focused on the search for its protein effectors; however, only protein kinase C- ξ , phospholipase A₂, and CAPP (ceramide-activated protein phosphatase) have been demonstrated to be activated by this lipid *in vitro* (for a review, see Hannun, 1996). In addition to its role in cell signaling, ceramide has been suggested to enhance the formation of atherosclerotic plaques (Kruth, 1997) and to be responsible for retaining normal skin functions such as its impermeability (Elias and Menon, 1991). As an amphipathic molecule ceramide strongly favors partitioning into bilayers, thus implying the possibility that changes in the physical state of the membrane might be involved in ceramide-mediated cell functions. Therefore, an understanding of the properties of ceramide-containing membranes is needed to elucidate the roles of lipid-lipid and lipid-protein interactions in determining the biological activities of this lipid. To this end, it has been suggested that ceramide causes packing defects in dipalmitoylphosphatidylcholine (DPPC), resulting in an activation of phospholipase A₂ (Huang et al., 1996).

In cellular membranes ceramide is formed upon the hydrolytic removal of the phosphocholine moiety of sphingomyelin by sphingomyelinase, and at least five sphingomyelinase species serving distinct functions have been identified in eukaryotic cells (Liu et al., 1997). These enzymes are activated subsequent to the binding of extracellular ligands to their plasma membrane receptors, as well as by ionizing radiation, serum deprivation, and dexamethasone (Liu et al., 1997). At an early stage in apoptosis activation of sphingomyelinase is observed (Jarvis et al., 1996), and subjecting cells to the action of externally added sphingomyelinase has been shown to cause apoptosis (Hannun and Obeid, 1995).

Sphingomyelin (SM) is a ubiquitous and abundant phospholipid of eukaryote cells and in plasma membrane is mainly present in the outer monolayer. Although the physicochemical properties and thermal phase behavior of SM are well known (Koynova and Caffrey, 1995), the significance of this lipid to the structure and function of membranes remains unidentified. SMs possess a high degree of saturation, leading to considerably higher transition temperatures and higher microviscosity of the membranes compared to phosphatidylcholines (Kolesnick, 1991). Interestingly, the contents of phosphatidylcholines (PCs) and SMs seem to be reciprocally controlled in cells, thus compensating the rigidifying effect caused by increased SM (Kolesnick, 1991). Besides a structural role, SM also seems to be involved in several cellular processes, such as viral fusion, attachment of cells to the substrate, and, e.g., disorders such as Niemann-Pick disease (Jwa Hidari et al., 1997; Nieva et al., 1994; Merrill and Jones, 1990).

In contrast to SM, only a few studies have been made that have been able to relate the physicochemical properties of ceramide to its cellular effects. The thermotropic behavior

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of non-hydroxy- and α -hydroxy fatty acid ceramides has been resolved by differential scanning calorimetry and x-ray diffraction (Shah et al., 1995a,b). The contents of ceramide in the membranes of cells undergoing apoptosis may reach 10 mol% of the total phospholipid (Hannun, 1996). A similar content of natural ceramides was required for the formation of microdomains enriched in this lipid in vitro in both the gel state and fluid large unilamellar vesicles (LUVs) (Holopainen et al., 1997, 1998). Ceramide-enriched microdomains also form upon generation of ceramide by sphingomyelinase in vitro in PC/SM LUVs (Holopainen et al., 1998). Accordingly, after the conversion of sphingomyelin to ceramide, the organization of the membrane no longer corresponds to thermodynamic equilibrium, but the membrane is in a metastable state. Subsequently, the latter decays to a new steady state; the rate of this reorganization process is diffusion controlled. As hydrophobic mismatch (Holopainen et al., 1997) could be excluded as the cause of lateral segregation of this lipid, this seems likely to arise from the properties of the ceramide headgroup. Although the exact mechanism remains somewhat uncertain, a feasible explanation is intermolecular hydrogen bonding, when the chemical structure of ceramide is taken into account. Compared to glycerophospholipids, which can act only as acceptors of hydrogen bonds, sphingolipids such as sphingomyelin and ceramide can act as both acceptors and donors because of their hydroxyl and amino groups, respectively, and because of the phosphate moiety of the former lipid (Pascher, 1976). To this end, recent Fourier transform infrared (FTIR) spectroscopy provided detailed information on acyl chain and headgroup inter- and intramolecular interactions in non-hydroxy (NFA) and α -hydroxy (HFA) fatty acid ceramides and revealed strong intermolecular hydrogen bonds, particularly for the former type (Moore et al., 1997).

Alterations in plasma membrane morphology with shedding of vesicles into the extracellular space is one of the hallmarks of apoptosis (Majno and Joris, 1995). Yet the possible relationship between changes in lipid composition (formation of ceramide) and changes in membrane morphology has not been investigated. Because of the remarkable similarity of the characteristic morphological changes in all cell lines studied, similar mechanisms have been suggested to be responsible. The mechanism of budding of the fluid-filled, structureless, and membrane-enclosed vesicles in apoptosis is not understood but is thought to arise from the disconnection of the cell membrane and cytoskeleton (Majno and Joris, 1995). Investigation of processes such as undulation, healing, and budding has become possible with the use of the so-called giant unilamellar vesicles ($\varnothing \approx 50\text{--}500 \mu\text{m}$) (Menger and Keiper, 1998) and has provided unprecedented insight into the mechanistic basis of changes in vesicle morphology (Lipowsky, 1991; Seifert and Lipowsky, 1996). In this study we used giant liposomes and microinjection to explore the possibility that formation

of ceramide in membranes could also be involved in the underlying mechanisms controlling cell morphology.

MATERIALS AND METHODS

Materials

C16:0-sphingomyelin was from Northern Lipids (Vancouver, BC, Canada), and SOPC was from Avanti Polar Lipids (Alabaster, AL). The concentration of Bodipy-sphingomyelin (Molecular Probes, Eugene, OR) was determined spectrophotometrically, using $91,000 \text{ cm}^{-1}$ at 505 nm as its molar extinction coefficient. Concentrations of the other lipids were determined gravimetrically with a high-precision electrobalance (Cahn, Cerritos, CA). The purity of the lipids was checked by thin-layer chromatography on silicic acid-coated plates (Merck, Darmstadt, Germany) developed with chloroform/methanol/water (65:25:4, v/v/v). Examination of the plates after iodine staining or, when appropriate, by fluorescence illumination revealed no impurities. Pro analysis grade solvents were from Merck, and other chemicals were from standard sources.

Liposome preparation

Lipid stock solutions ($\sim 10 \text{ mM}$ and $\sim 2.5 \text{ mM}$ for SOPC and C16:0-sphingomyelin, respectively) were mixed in chloroform to obtain the desired composition, with Bodipy-sphingomyelin included as a fluorescent probe (final molar ratio 0.75:0.20:0.05 for SOPC, C16:0-sphingomyelin, and Bodipy-sphingomyelin, respectively). The solvent was evaporated under a stream of nitrogen, and subsequent evacuation under reduced pressure for at least 12 h. The dry residue was dissolved in diethylether:methanol (9:1, v/v) to yield a final total lipid concentration of 1 mM. Approximately one microliter of this solution was applied onto the surface of each Pt electrode (Angelova and Dimitrov, 1986), which was then dried with a stream of nitrogen and evacuation in vacuum for 1 h. A glass chamber with the attached electrodes and a quartz window bottom was placed on the stage of a Zeiss IM-35 inverted fluorescence microscope (see instrument setup from Wick et al., 1996). An AC voltage of 0.2 V ($f = 4 \text{ Hz}$) was applied before 1.3 ml of 0.5 mM HEPES buffer (pH 7.4) was added. During the first minute of hydration the voltage was raised to 1 V. After 2 h the AC field was turned off, and giant liposomes were observed with Hoffman modulation contrast optics with a ($10\times$) objective or by epifluorescence with a Nikon ELWD ($20\times$) objective. The excitation and emission wavelengths were selected with filters (Melles-Griot) transmitting in the range of 420–480 nm and $>500 \text{ nm}$, respectively. Images were viewed with a Peltier-cooled digital camera (C4742-95; Hamamatsu, Japan) connected to a computer.

Microinjection of sphingomyelinase

Micropipettes with inner tip diameters of $>0.5 \mu\text{m}$ (Schnorf et al., 1994) were made from borosilicate capillaries (1.2 mm outer diameter) by a microprocessor-controlled horizontal puller (P-87; Sutter Instrument Co., Novato, CA). Picoliter aliquots of sphingomyelinase (sphingomyelin phosphodiesterase, EC 3.1.4.12, *Bacillus cereus*, from Sigma, 1 unit/ml, specific activity 100–300 units/mg protein in 9 mM CaCl_2 , 1.8 mM MgCl_2 aqueous solution) were applied either to the outer surface or to the interior of individual giant vesicles with a pneumatic microinjector (PLI-100; Medical Systems Corp., Greenvale, NY). For easier handling only vesicles attached to the electrode surface were used.

All experiments were repeated at least 15 times with excellent reproducibility. Yet the lag time in the formation of domains after external application of SMase varied to some extent (from 10 to 150 s). This

variation is likely to be due to the varying distance of the micropipette tip from the vesicle surface, allowing for the diffusion and uncontrolled dilution of the enzyme into the medium.

RESULTS

We investigated the possibility that the apoptotic lipidic messenger, ceramide, could be involved in causing changes in the membrane morphology of cells undergoing apoptosis. Giant liposomes composed of 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) and *N*-palmitoyl-sphingomyelin (C16:0-SM) readily formed in an AC field and were

visualized by fluorescence microscopy of the included fluorescent lipid tracer (molar ratio 0.75:0.20:0.05 for SOPC, C16:0-SM, and Bodipy-sphingomyelin, respectively) (Fig. 1 A). Uniform distribution of the fluorescent lipid Bodipy-SM is evident at the resolution of the optical microscope, in keeping with the miscibility of sphingomyelin in PC membranes (Calhoun and Shipley, 1979; McKeone et al., 1986).

We have recently demonstrated by using large unilamellar liposomes that in contrast to sphingomyelin, ceramide forms microdomains in both fluid as well as in gel-state PC

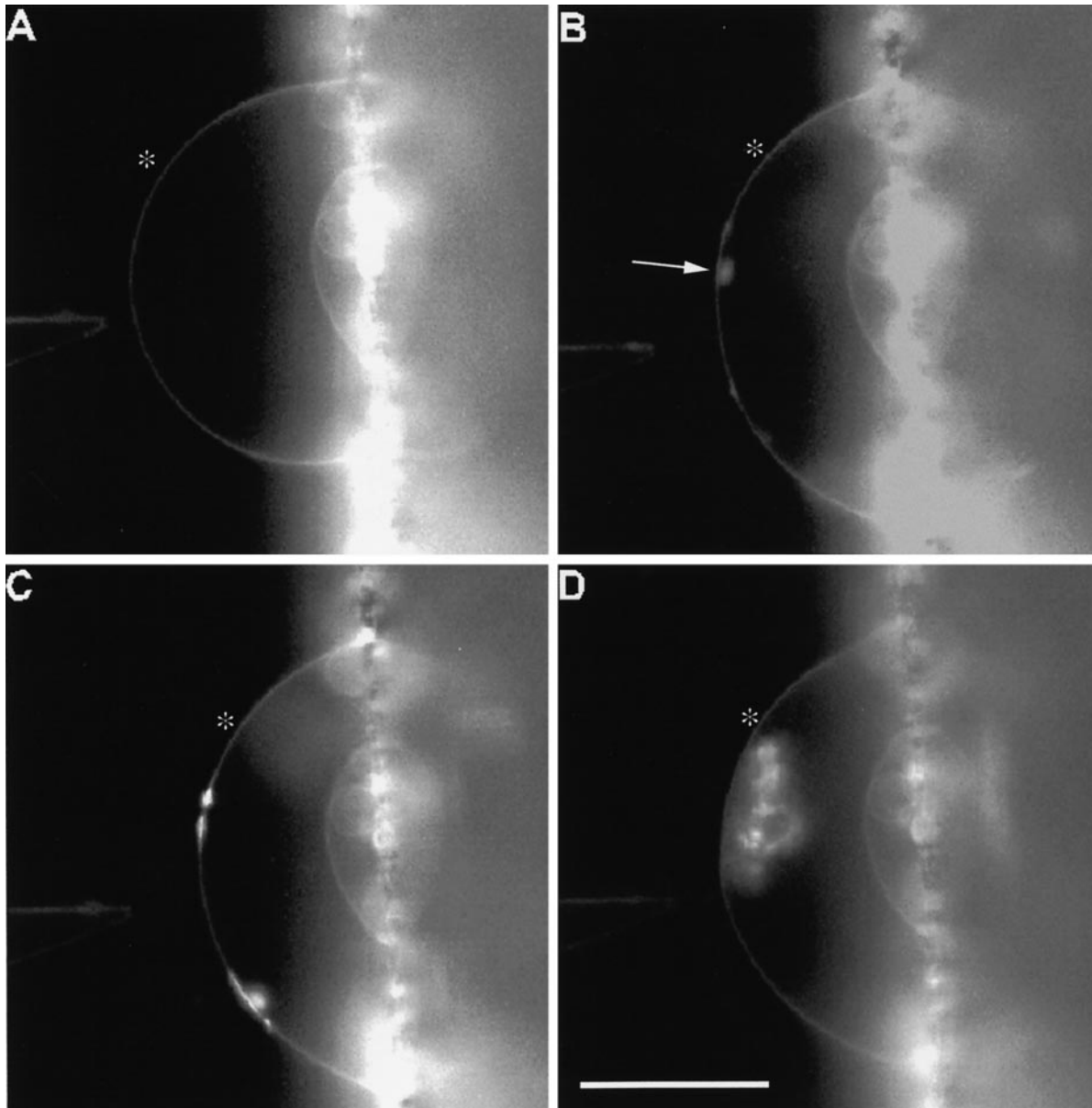


FIGURE 1 Transformations of a single SOPC/C16:0-sphingomyelin/Bodipy-sphingomyelin (molar ratio 0.75:0.20:0.05) giant vesicle, induced by the addition of *Bacillus cereus* sphingomyelinase in the vicinity of the vesicle. Still fluorescence images were taken (A) before enzyme administration and after (B) 20 s, (C) 40 s, and (D) 2 min. The length of the scale bar in D corresponds to 100 μm .

membranes (Holopainen et al., 1997, 1998). To investigate the consequences of enzymatic conversion of sphingomyelin to ceramide in a fluid lipid membrane, we treated the above giant phosphatidylcholine/sphingomyelin vesicles by sphingomyelinase. Within ~ 30 s after the external application of this enzyme to the vicinity of the outer membrane surface, domains with increased fluorescence intensity become observable (Fig. 1 *B*, arrow). The reason for their diffuse appearance is likely to be their 3-D nature, so that each domain is only partly in the focal plane. Likewise, fairly long exposure times (0.5–2 s) had to be used in fluorescence microscopy, thus resulting in Brownian motion, causing haziness of the domain appearance. Notably, we did use a rather high probe concentration ($X = 0.05$) and did not want to increase this, so as to limit the membrane perturbing effect of Bdp-sphingomyelin. The resolution further suffers from the 3-D nature of the system. Subsequent to their emergence, these membrane regions increase in brightness (Fig. 1, *C*), and in ~ 1 –2 min the domains are “endocytosed” as smaller vesicles into the interior of the giant liposome (Fig. 1 *D*). The phase separation process was quantitated by measurement of the loss of fluorescence intensity of the probe at a distal part of the GUV (marked by * in Fig. 1); this clearly shows the emission intensity to be reduced in the membrane outside the fluorescence domains (Fig. 2). A possible explanation for this is photobleaching of the probe; however, on the time scale of our experiments this was negligible. Moreover, the intensity is reduced by $\sim 30\%$, thus indicating that half of the Bodipy-sphingomyelin in the outer leaflet is contained in the formed microdo-

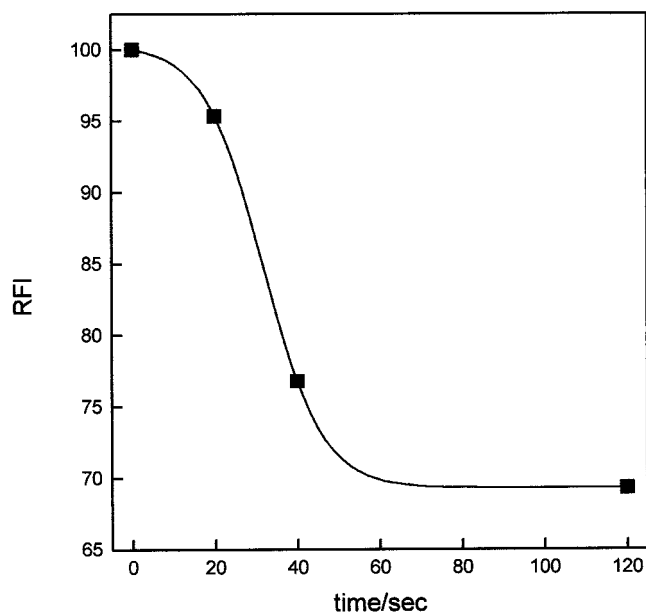


FIGURE 2 Time course for the decrease in the fluorescence intensity in the bilayer after application of *Bacillus cereus* sphingomyelinase. The intensity changes were measured at a place distal to the enzyme addition (marked with * in Fig. 1).

main (either as such or as the corresponding ceramide). As a further progression, when this process is viewed in real time, the microdomains then increase in thickness toward the interior. Subsequently, we see the appearance of small fluorescent vesicles within the GUV, together with disappearance of the domains on the GUV surface. To verify our results indicating vesicle formation inside GUVs, we used Hoffman modulation contrast optics, allowing for the use of short exposure times (< 1 ms) (Fig. 3 *A*). In keeping with the above fluorescence images, within 60 s a number of small vesicles became observable (Fig. 3 *B*, arrow). A further increase in time results in progressive accumulation of small vesicles in the GUV interior (Fig. 3, *C* and *D*). The diameter of these vesicles is quite constant ($2.7 \pm 0.08 \mu\text{m}$). In this case the newly formed small vesicles stay apart from each other, whereas in Fig. 1 they are clustered. The reason for this remains unknown but could be related to the amount of ceramide in the vesicles. Interestingly, Alonso and co-workers have shown that hydrolysis of SM/PE/cholesterol vesicles by sphingomyelinase causes their aggregation/fusion (Ruiz-Argüello et al., 1996; Basáñez et al., 1997).

Notably, in the above experiment the formation of ceramide probably takes place only in the outer leaflet of the giant liposome. The formation of ceramide in both leaflets of the bilayers would require either 1) permeation of *B. cereus* SMase through the bilayer and/or 2) rapid transbilayer diffusion of ceramide. We do not consider the first mechanism to be feasible for an enzyme with a molecular mass of 37 kDa. The second mechanism would require the flip-flop rate of ceramide to be very fast. This is in contrast to the halftime of ~ 22 min for the spontaneous transbilayer movement rates measured for the fluorescently labeled ceramide (Bai and Pagano, 1997). However, our previous work showed that extensive degradation of sphingomyelin in LUVs (average diameter of 100 nm) did exceed 50% (Holopainen et al., 1998). Taken together, it is possible that ceramide could also transfer into the inner surface of GUVs.

It is very likely that external application of the enzyme results in an asymmetry in bilayer lipid composition. It was therefore of interest to investigate the consequences of formation of ceramide in the *inner* leaflet of the giant vesicle. For this purpose, sphingomyelinase was microinjected into the interior of the giant liposome (Fig. 4, *A*). As in the formation of ceramide by externally applied sphingomyelinase, within ~ 30 s we observed the appearance of diffuse fluorescent domains in the membrane (Fig. 4, *B*). After ~ 1 –2 min small ($\varnothing \approx 5.0 \pm 0.17 \mu\text{m}$) liposomes emerged on the outer surface of the giant liposome (Fig. 4, *C*); their number continued to increase for several minutes (Fig. 4, *D*). These vesicles eventually formed a layer on the giant liposome surface and had a remarkably homogeneous size distribution. Compared to the external addition of the enzyme, the reaction seems to proceed for a longer time. This difference could be explained by the externally added enzyme becoming diluted into the bulk aqueous phase,

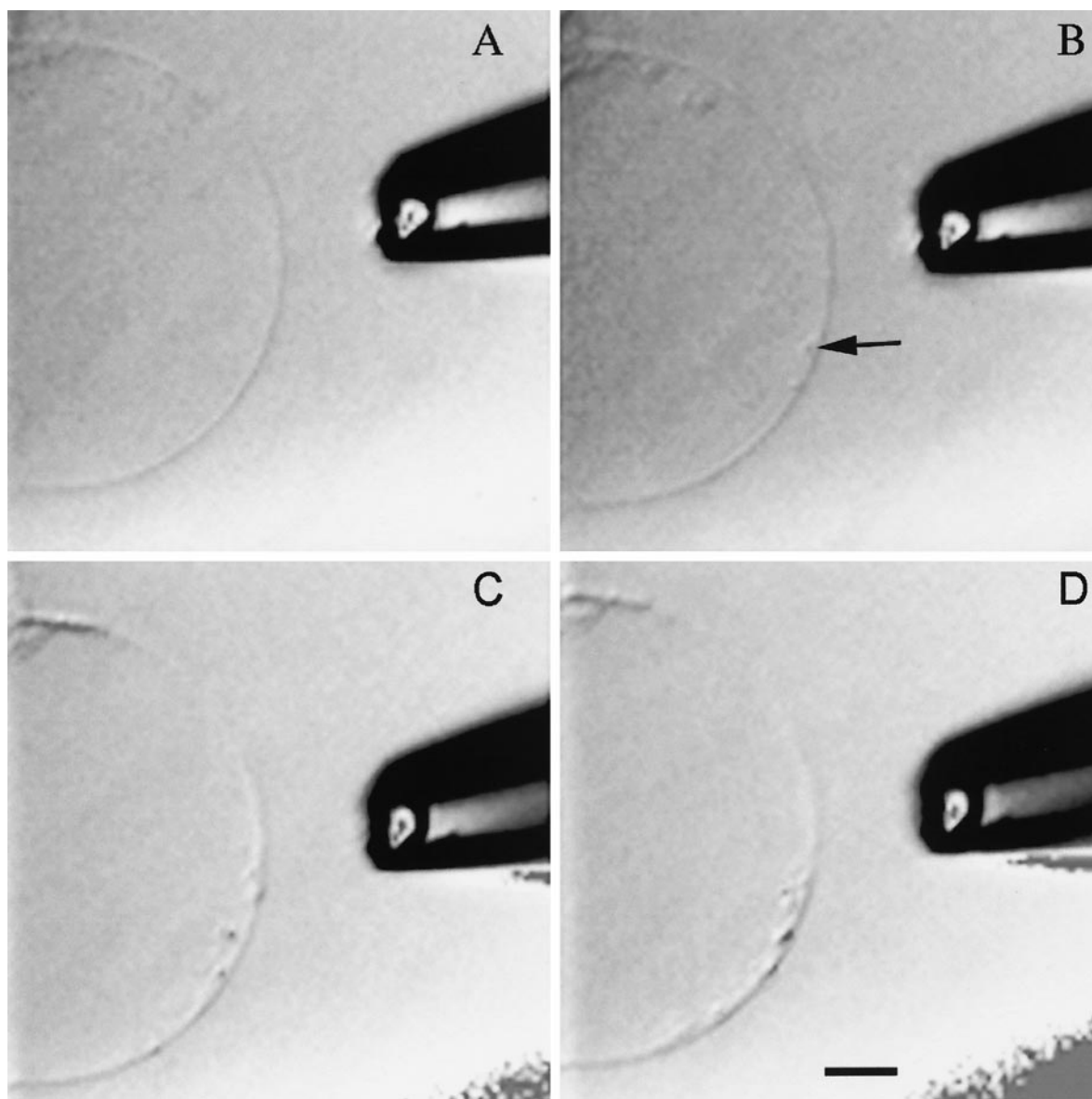


FIGURE 3 Changes in the vesicle topology induced by the addition of sphingomyelinase in the vicinity of SOPC/C16:0-sphingomyelin/Bodipy-sphingomyelin (molar ratio 0.75:0.20:0.05) giant vesicle. Still Hoffman modulation contrast microscopy images were taken (A) before enzyme administration and after (B) 60 s, (C) 120 s, and (D) 180 s. The length of the scale bar in D corresponds to 20 μm .

resulting in the attenuation of the local rate of ceramide formation.

Perhaps, somewhat unexpectedly, the vesicle diameter does not decrease, although vesiculation retracts lipids from the GUV surface into the interior of the vesicle. For technical reasons vesicles that were not attached to the platinum wire could not be used. Accordingly, the connection of the vesicle to the lipid on the electrode is likely to provide the source for the new lipid that is transferred into the giant vesicle. The reasons for the lack of vesicle shrinkage are likely to be the following. First, the phosphocholine head-group released into the interior of the vesicle acts as an osmolyte, causing water influx from outside of the GUV.

Second, it is possible that blebbing of vesicles is accompanied by transient connection of the aqueous compartments (Zhang et al., 1998).

DISCUSSION

The present study provides the first demonstration of vectorial formation of vesicles, i.e., endocytosis and budding, to be induced by the asymmetrical formation of ceramide by sphingomyelinase in either the outer or inner leaflet, respectively, of giant liposomes. This is distinct from the previously reported effects of another lipolytic enzyme, phos-

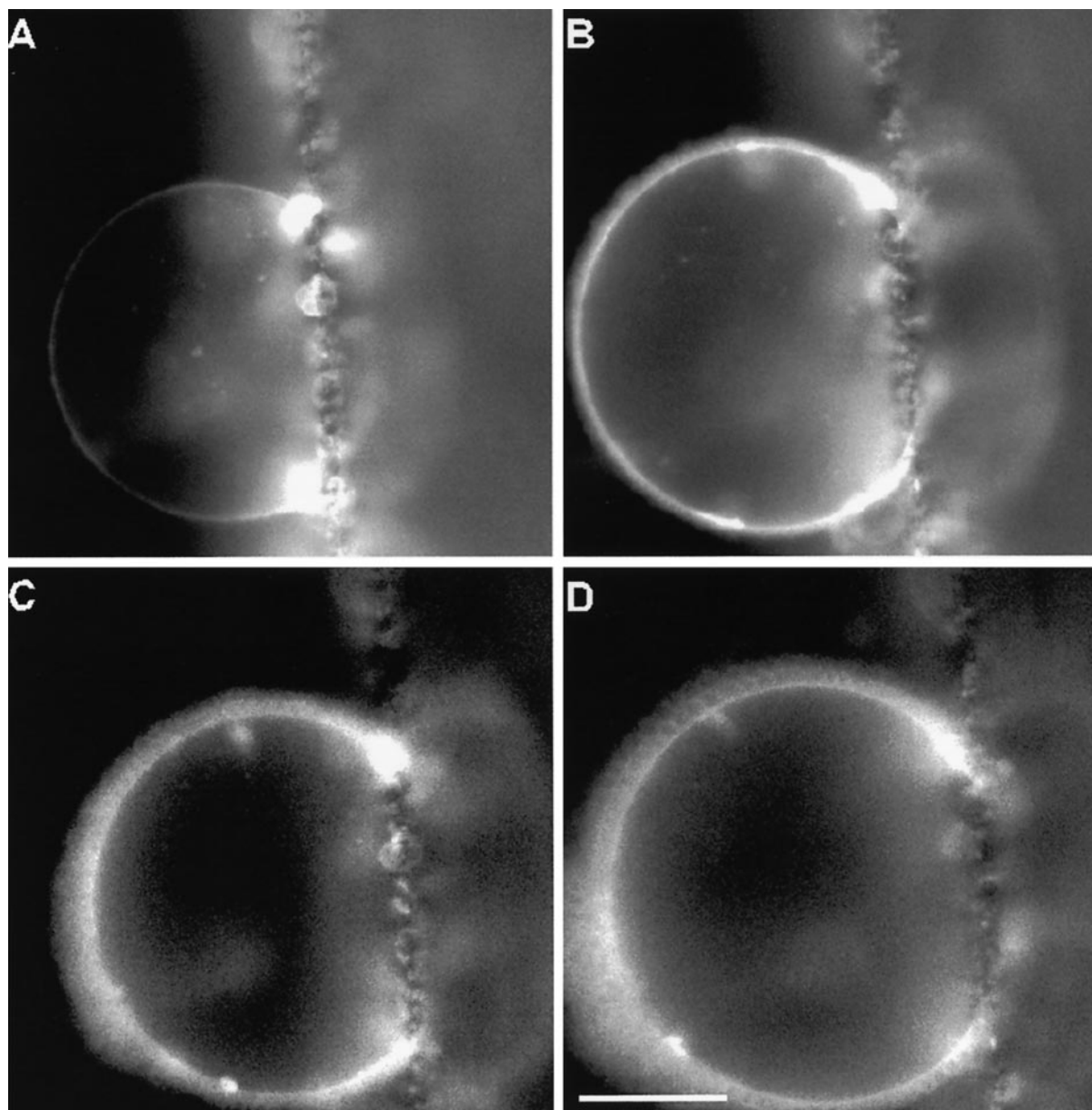


FIGURE 4 Microinjection of sphingomyelinase into a single SOPC/C16:0-sphingomyelin/Bodipy-sphingomyelin (molar ratio 0.75:0.20:0.05) giant vesicle. Still fluorescence images were recorded (A) before the enzyme addition and (B) 1 min, (C), 3 min, and (D) 10 min after the microinjection. The length of the scale bar in *D* corresponds to 100 μm .

pholipase A_2 . In brief, subjecting giant 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) liposomes to either the external or internal action of phospholipase A_2 causes these vesicles to burst and shrink, respectively (Wick et al., 1996). Importantly, these authors showed that vesicle morphology is not altered by lysozyme, because protein has no enzymatic activity toward the bilayer. The formation of vesicles from GUV is preceded by the rapid emergence of laterally segregated domains enriched in the fluorescent marker.

The physical foundations of the determinants of vesicle shapes are reasonably well established and have been laid in

terms of excess area in one of the leaflets of a bilayer (Farge and Devaux, 1993), area-to-volume ratio (Döbereiner et al., 1993), bending elasticity, and spontaneous curvature (Evans, 1974; Gruner, 1989; Helfrich, 1973; Lipowsky, 1991, 1992; Sackmann, 1994; Seifert and Lipowsky, 1996). Osmotically induced shape changes and fission of small vesicles have been shown previously (for a review, see Sackmann, 1994). Also lateral domain formation establishing domain boundaries contributes to vesicle budding (Jülicher and Lipowsky, 1993; Lipowsky, 1992; Seifert and Lipowsky, 1996). The equilibrium shape for a multicompo-

ment system is achieved at the free energy minimum for the alloy, the relevant forces also including bending energy, causing either mixing or lateral phase separation of the individual components. Because of the coupling of curvature and local enrichment of lipids, introducing two or more components into the membrane results in a nonuniform membrane and leads to nonzero spontaneous curvature. Perhaps the simplest two-component system is represented by the coexistence region in a membrane undergoing a phase transition between fluid and gel phases. To this end, upon increasing the temperature one can observe the pinching off of small vesicles ($\varnothing \approx 1 \mu\text{m}$) from bovine brain sphingomyelin giant vesicles (Döbereiner et al., 1993). An important conclusion from these data is that the dynamics required for these processes to occur in living cells could be inherent to the shape phase diagram of the lipid membranes, thus making proteins unnecessary as the driving force (Döbereiner et al., 1993).

A model schematically delineating the present results is illustrated in Fig. 5. In Fig. 5 A the lipids in the SOPC/SM

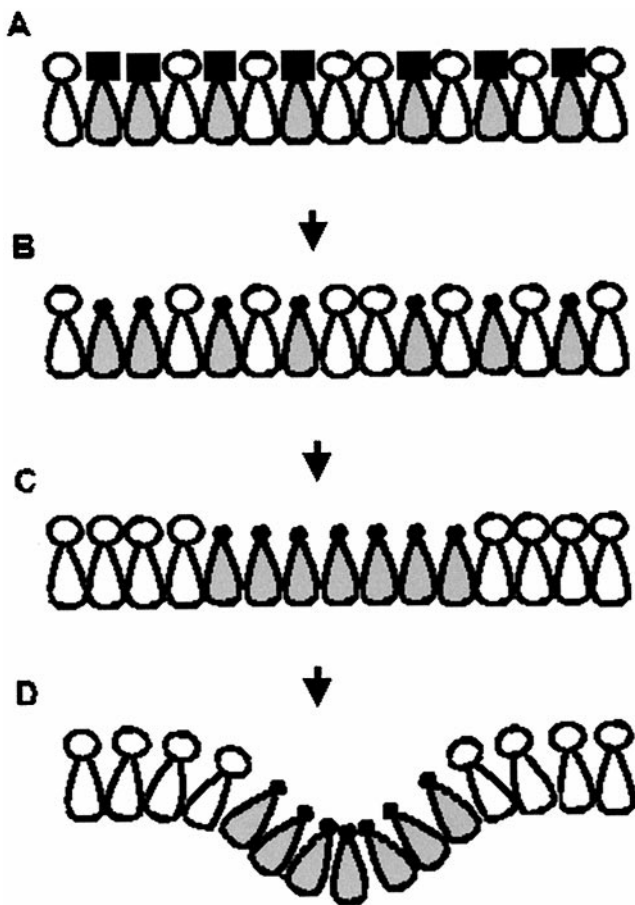


FIGURE 5 A schematic model for the mechanism of ceramide domain formation and invagination in a leaflet of a PC/sphingomyelin liposome, induced by the asymmetrical hydrolytic action of sphingomyelinase. For the sake of clarity only the monolayer subject to the action of sphingomyelinase is illustrated. \circ , SOPC; \blacksquare , sphingomyelin; \bullet , ceramide.

monolayer are randomly distributed. Hydrolytic cleavage of the phosphocholine headgroup from SM by sphingomyelinase generates ceramide, a lipid with a small, less hydrated headgroup (Fig. 5 B). Compared to a typical phosphatidylcholine such as POPC with a mean molecular area of 70 \AA^2 (Feng and MacDonald, 1995), the area for ceramide in the monolayer is small, $\sim 40 \text{ \AA}^2$ (Löfgren and Pascher, 1977). Accordingly, the leaflet containing ceramide condenses, thus causing an area difference with respect to the adjacent leaflet of the bilayer. Subsequently (Fig. 5 C), the formed ceramide segregates into a domain; a likely driving force is intermolecular hydrogen bonding (Pascher, 1976). Because of the tendency of ceramide to form inverted nonlamellar phases (Ruiz-Argüello et al., 1996), an invagination is formed by the ceramide-enriched domain (Fig. 5 D). The driving force is thus provided by 1) the area difference between the adjacent monolayers (Farge and Devaux, 1993), and 2) the negative spontaneous curvature for the ceramide-containing domain (Ruiz-Argüello et al., 1996; Veiga et al., 1998). In addition, the bending rigidity of the ceramide-enriched domain must exceed that of the adjacent leaflet of the bilayer. The domain then grows in size and finally forms an enclosed cavity (not shown). It should be emphasized that although illustrated above as separate events, the formation of ceramide, its local enrichment, and membrane bending should all occur simultaneously.

In light of the present results demonstrating budding of vesicles from giant liposomes after the microinjection of sphingomyelinase into the vesicle interior, it seems reasonable to suggest that the enzymatic formation of ceramide may well cause the observed membrane blebbing in apoptosis, in a manner not requiring metabolic energy. Our results thus indicate a possible central role for sphingomyelinase in controlling 3-D ordering of cellular membranes. Regarding apoptosis, the contents of ceramide in such cells have been reported to be very high, 10% of the total lipid at most (Hannun, 1996). As the precursor sphingomyelin is not evenly distributed in different cellular membranes, it seems reasonable to assume that the local concentration of ceramide could well exceed 25 mol%. Bearing this in mind, we note that one possibility is that not only does ceramide function as a traditional "lipid second messenger," but the effects of this bioactive lipid could also involve changes in the physical state of the plasma membrane.

In addition to cellular signaling mechanisms, vesicle budding and endocytosis are crucial in the membrane trafficking and internalization of plasma low-density lipoprotein (LDL), for instance. In the plasma membrane endocytosis involves caveolae, 50–60-nm diameter invaginations (Parton et al., 1994). These ceramide-enriched membrane domains contain G-protein-coupled receptors, and a role in signal transduction has also been suggested (Lisanti et al., 1994). There is evidence that interleukin- 1β binds to a sphingomyelin-rich plasma membrane domain with the characteristics of caveolae. Hormone binding was accom-

panied by the hydrolysis of sphingomyelin to yield ceramide, and ceramide production was concluded to be highly compartmentalized in the cell surface (Liu and Anderson, 1995). Very recently it was shown that exogenous sphingomyelinase treatment of ATP-depleted macrophages and fibroblasts results within 10 min in the budding of numerous vesicles from the plasma membrane into the inside of these cells (Zha et al., 1998). These vesicles have a diameter of $\sim 0.4 \mu\text{m}$ and lack any observable protein coating. Very recently, it was demonstrated that exogenously incorporated *N*-hexanoyl-sphingosine (C_6 -ceramide) induced time- and dose-dependent formation of vesicles (diameter 2–10 μm) in the interior of fibroblasts (Li et al., 1999). Our data show that except for sphingomyelinase, no proteins are necessary for endocytosis but can readily occur in giant liposomes. Evidence for the involvement of sphingomyelinase and phosphatidylcholine-specific phospholipase C in the entry of *Neisseria gonorrhoeae*, *Staphylococcus aureus*, and species of mycobacteria into human cells has recently been demonstrated (Grassmé et al., 1997; Johansen et al., 1996; Walev et al., 1996). This mode of endocytosis could be mediated by the concerted hydrolytic action of sphingomyelinase/phospholipase C of the plasma membrane of the infected cells in a manner similar to that described here. Experiments assessing these issues are currently in progress in our laboratory.

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