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ORIGINAL PAPER

Ceramide acyl chain length markedly influences miscibility with palmitoyl sphingomyelin in bilayer membranes

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Abstract Ceramides are precursors of major sphingolipids and can be important cellular effectors. The biological effects of ceramides have been suggested to stem from their biophysical effects on membrane structure affecting the lateral and transbilayer organization of other membrane components. In this study we investigated the effect of acyl chain composition in ceramides (C4-C24:1) on their miscibility with *N*-palmitoyl-sphingomyelin (PSM) using differential scanning calorimetry. We found that short-chain (C4 and C8) ceramides induced phase separation and lowered the T_m and enthalpy of the PSM endotherm. We conclude that short-chain ceramides were more miscible in the fluid-phase than in the gel-phase PSM bilayers. Long-chain ceramides induced apparent heterogeneity in the bilayers. The main PSM endotherm decreased in cooperativity and enthalpy with increasing ceramide concentration. New ceramide-enriched components could be seen in the thermograms at all ceramide concentrations above $X_{Cer} = 0.05$. These broad components had higher T_m values than pure PSM. C24:1 ceramide exhibited complex behavior in the PSM bilayers. The miscibility of C24:1 ceramide with PSM at low ($X_{Cer} = 0.05$–0.10) concentrations was exceptionally good according to the cooperativity of the transition. At higher concentrations, multiple components were detected, which might have arisen from interdigitated gel-phases formed by this very asymmetric ceramide. The results of this study indicate that short-chain and long-chain ceramides have very different effects on the sphingomyelin bilayers. There also seems to be a correlation between their miscibility in binary systems and the effect of ceramides of different hydrophobic length on sphingomyelin-rich domains in multicomponent membranes.

Keywords Differential scanning calorimetry · Melting temperature · Molecular structure · Membrane structure

Introduction

Ceramides are simple sphingolipids that normally are only minor constituents of cellular membranes. Ceramides have attracted substantial interest because of their involvement in biological processes such as the apoptotic pathway, cellular proliferation, and senescence (Taha et al. 2006; Hannun and Obeid 2002; Kolesnick 2002). Ceramides can be generated through de novo synthesis or through hydrolysis of more complex sphingolipids (for a recent review see Zhang et al. 2009). The physical effects of ceramides on the membrane bilayer might contribute significantly to their biological effects (Cremesti et al. 2002).

Sphingomyelin is the most common sphingolipid in mammalian plasma membranes. In these membranes sphingomyelin readily associates with cholesterol and together they form liquid-ordered domains (i.e., analogous with lipid rafts). Within these sphingomyelin/cholesterol domains other sphingolipids and some raft-associated proteins may be found. When sphingomyelin is degraded through the action of sphingomyelinase, ceramide is generated. Sphingomyelinase-induced ceramide generation within rafts substantially alters the structure of these

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domains and the lateral partitioning of membrane components (Zhang et al. 2009). Ceramides are known to displace cholesterol from rafts, thereby increasing the chemical activity of cholesterol in the cell (Lange et al. 2005). Ceramide molecules have also been shown to associate with each other in biological membranes, forming small ceramide-enriched gel-phase domains (Grassme et al. 2002, 2003; Kolesnick et al. 2000). The ceramide-enriched domains have a tendency to fuse into larger ceramide-enriched membrane platforms, which may be important for the lateral clustering of certain proteins (Zhang et al. 2009). A recent study showed that the proteins GPI-PLAP and cholera toxin B-subunit (which binds to ganglioside GM1) can be found immobilized in ceramide-rich domains in model bilayer membranes (Chiantia et al. 2008).

The formation of ceramide-enriched domains and platforms is largely dependent on ceramide structure, which regulates their biophysical properties. Ceramides are *N*-acylated sphingosines. The small polar head group together with their predominantly saturated acyl chains and extensive hydrogen-bonding capacity enables ceramides to pack tightly together in membranes. Because of their small polar head group ceramides have a conical molecular shape which at higher membrane concentrations induces a negative curvature in membranes and gives a propensity to promote formation of non-lamellar phases (Veiga et al. 1999). Ceramides in membranes have also been shown to induce membrane fusion and vesiculation processes (Ruiz-Arguello et al. 1996, 1998; Holopainen et al. 2000a; Montes et al. 2002, 2004), which suggests that ceramides might also be centrally involved in vesicular transport and in endo-/exocytosis in cells. As in most biological sphingolipids, the acyl chains of ceramides mainly vary from 14 to 24 carbons. It has recently been shown that the synthesis of ceramides of different hydrophobic lengths is tightly regulated through the presence of a large set of ceramide synthases (Pewzner-Jung et al. 2006). Although the sphingolipids present in biological membranes mostly contain long and saturated acyl chains, many studies focusing on the effects of ceramides have utilized short-chain analogs, as these are more easily administered to cells because of higher water solubility (Sot et al. 2005b). However, it has been realized that acyl chain length is likely to influence the biological effects of ceramides (Chiantia et al. 2006; Di Paola et al. 2000; Ghidoni et al. 1999; Kolesnick et al. 2000).

Ceramides are at least partly miscible with sphingomyelin and have been shown to partition favorably into sphingomyelin-rich domains (Björkqvist et al. 2005; Wang and Silvius 2003; Sot et al. 2006; Holopainen et al. 1998). Recent findings have revealed more information on domain structures formed with ceramides (Ira and Johnston 2008; Ira et al. 2009). Ceramides were, for example, shown by atomic force microscopy (AFM) and time-of-flight secondary ion mass spectrometry (TOF-SIMS) to be included in ordered domains with sphingomyelin and cholesterol, and to create a heterogeneous environment therein. It was observed that the ceramides segregated to some extent to form "islands" within the ordered domains in phase-separated monolayers (Popov et al. 2008). The same kind of behavior has been reported in bilayer membranes where AFM and fluorescence correlation spectroscopy (FCS) showed that C16 and C18 ceramides recruit sphingomyelin and segregate into ceramide-enriched gel-phase domains in bilayers containing sphingomyelin, cholesterol, and a fluid phosphatidylcholine (Chiantia et al. 2007; Silva et al. 2006b). It was concluded that this is a chain length-dependent property and that only C16 and longer ceramides would have the ability to induce formation of such laterally segregated ceramide-enriched domains in the presence of C18:0 sphingomyelin (Goni and Alonso 2009; Chiantia et al. 2007).

Biophysical characterizations of very long-chain ceramides are fairly scarce in the literature (Carrer and Maggio 1999; Carrer et al. 2003; Holopainen et al. 1997, 2001; Pinto et al. 2008). C24:1 ceramide has recently been shown to form gel-phase domains in binary bilayers with a monounsaturated PC, which indicates the possibility this ceramide participates in the formation of ordered domains also in more complex lipid bilayers (Pinto et al. 2008). Short-chain ceramides in binary lipid bilayers have, on the other hand, been shown to order the fluid phase, rather than phase separating into a segregated gel-phase, at least with dimyristoyl-phosphatidylcholine (DMPC) (Carrer et al. 2006).

The effect of ceramides in membranes containing sphingomyelins have mainly been studied using natural ceramide mixtures. A fluorimetric study has shown that bovine brain ceramides increase the order in gel-phase sphingomyelin bilayers (Massey 2001). The same study also showed that ceramide generation in the sphingomyelin bilayer through the action of sphingomyelinase converted a fluid sphingomyelin bilayer at physiological temperature into a gel-phase sphingomyelin/ceramide bilayer. A differential scanning calorimetry (DSC) study has shown the effects of egg-ceramide on egg-sphingomyelin in bilayer membranes (Sot et al. 2006). It was shown that ceramide already at 5 mol% concentration broadened the phase transition and shifted it to higher temperatures. The transition was also shown to consist of several components indicating the presence of coexisting domains of varying compositions. It seems that the reason for limited miscibility of ceramides and sphingomyelin in two-component liposomes will be influenced by ceramide chain length. In this study we used DSC to investigate the effect of acyl chain length in ceramides (C4-C24:1) on their miscibility

with the biologically common and structurally symmetric sphingolipid D-*erythro*-*N*-palmitoyl-sphingomyelin (PSM).

Materials and methods

Material

PSM was purified from egg sphingomyelin (Avanti Polar Lipids, Alabaster, AL, USA) by reversed-phase HPLC (Supelco Discovery C18 column, dimensions 250 × 21.2 mm, 5 μm particle size) using 100% methanol as eluent. This method separates the PSM very effectively from sphingomyelins of other chain lengths, which can be seen as separate components in the chromatogram. Also, dihydro-PSM is effectively removed by the procedure. The acyl-chain-defined ceramides were from Avanti Polar Lipids. The purity and identity of all lipids were verified by ESI-MS (HCT-Ultra ion trap mass spectrometer; Bruker Daltonics, Bremen, Germany). Sphingomyelin and ceramide stock solutions were prepared in hexane/isopropanol (3:2, *v*/*v*). Solutions were stored in the dark at −20°C, and warmed to ambient temperature before use. The water used for all experiments was purified by reverse osmosis, followed by passage through a Millipore (Billerica, MA, USA) UF Plus water-purification system, to yield a product of resistivity 18.2 MΩ cm.

Sample preparation

The lipids were mixed in hexane/isopropanol to yield the indicated lipid ratio after which the lipids were dried under a constant flow of nitrogen. The dry film was redissolved in chloroform, dried again under nitrogen and any residual solvent was removed under vacuum overnight. The samples used for the DSC experiments were prepared by dispersing the dry lipids in 1 ml water at 80°C. The lipids were allowed to swell for 20 min in water in sealed vials at 80°C, then vortex mixed vigorously followed by ten freeze–thaw cycles, with the water-bath used for thawing kept at 80°C. The final total lipid concentration in the samples was 1.4 mM. After the freeze–thaw cycles the samples containing multilamellar vesicles (MLVs) were cooled to room temperature, degassed under vacuum, transferred to the DSC, and slowly cooled to 10°C.

After hydration, the pure, long-chain (C16-C24:1) ceramide samples went through an additional step of bath sonication at 90°C with a Branson bath sonifier 2510 (Branson Ultrasonics, Danbury, CT, USA). The pure ceramide suspensions were however, not quite homogeneous and the exact concentration of the material finally transferred to the DSC was hard to determine.

Differential scanning calorimetry

DSC heating and cooling thermograms were recorded between 10 and 60°C for short-chain ceramides (C4–C12) and 10–100°C for longer-chain ceramides (C16–C24), at a rate of 1°/min with a high-sensitivity Microcal (Northampton, MA, USA) VP-DSC. The data were analyzed, including peak-fitting and deconvolution of multicomponent endotherms, and plotted with Origin software (OriginLab, Northampton, MA, USA).

Results

The ceramides used in this study are presented as molecular models in Fig. 1. The working hypothesis of this study was that molecules with such different molecular structures must have different effects on the membranes they are incorporated in. In the absence of ceramide the PSM heating scan showed one highly cooperative transition

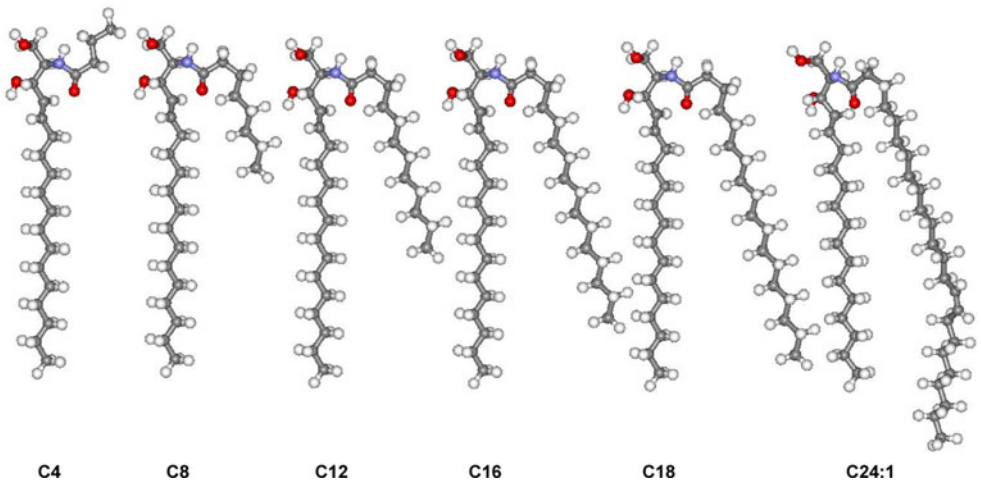

Fig. 1 Molecular models of the ceramides used in this study (C4-C24:1)

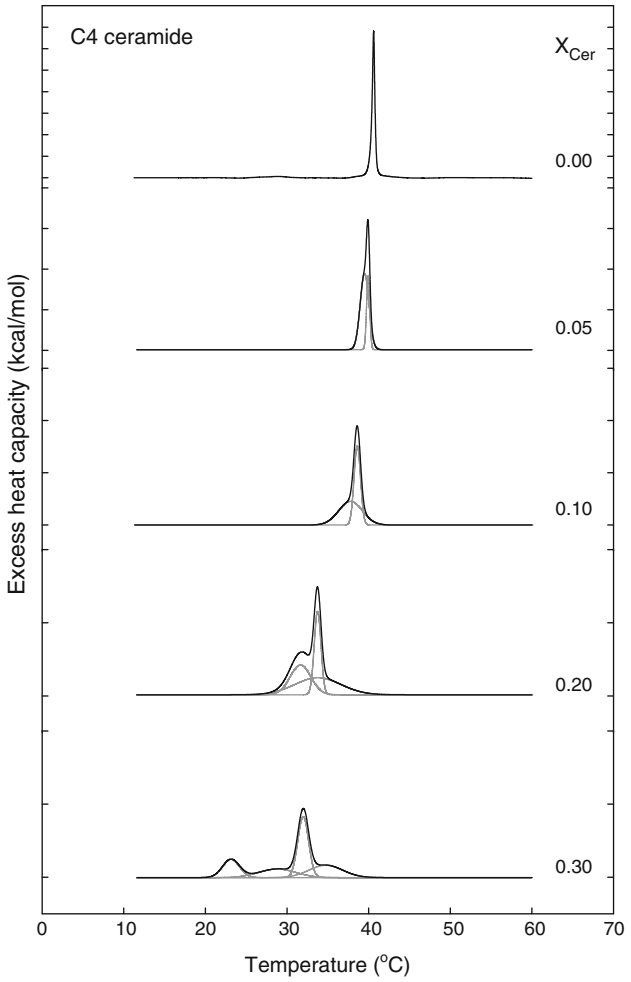

Fig. 2 Representative heating thermograms of PSM/C4 ceramide binary mixtures. DSC heating scans from 10 to 60°C were run at a rate of 1°/min. The ceramide concentration is indicated to the *right*. The *y*-scales were adjusted so that the ticks on the right and the left occur every 2 kcal/mole

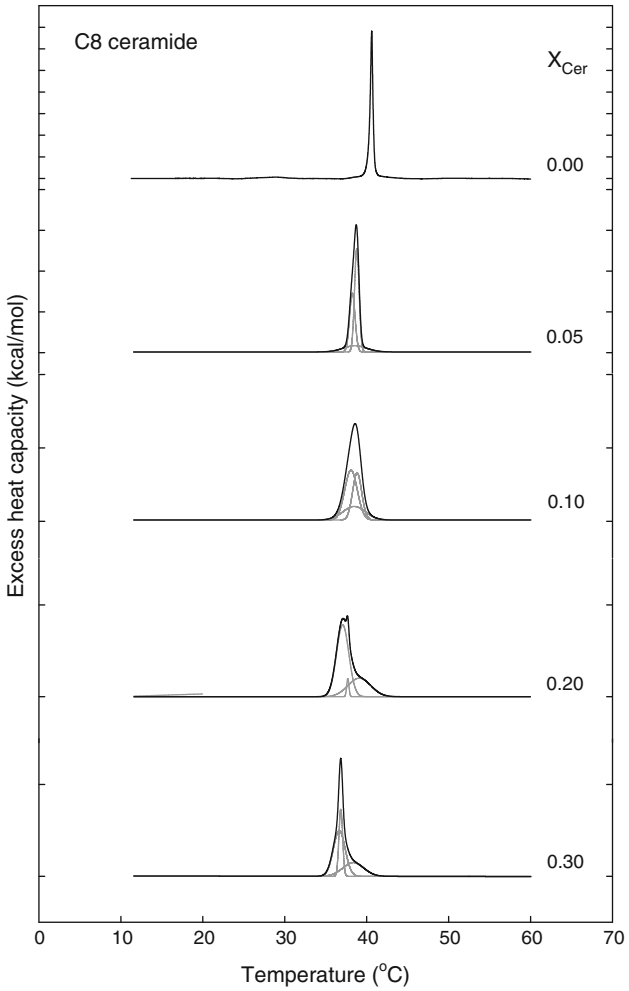

Fig. 3 Representative heating thermograms of PSM/C8 ceramide binary mixtures. Other conditions as defined for Fig. 2

centered at 40.6°C, ΔH ~7.4 kcal/mole (shown for comparison as the first line of Figs. 2, 3, 4, 5, 6, 7). There was also a barely visible pretransition at 28.9°C (ΔH ~0.5 kcal/mole) in good agreement with previous studies (Bar et al. 1997; Kuikka et al. 2001; Nyholm et al. 2003; Ramstedt and Slotte 1999). In bilayers containing PSM and one of the ceramides, deconvolution analysis of the main transitions revealed, in most cases, two or more overlapping endotherms. The different endotherm patterns seen are presented below for all the individual ceramides studied and compared with each other for deeper understanding of the complex behavior of these acyl chain-defined ceramides in mixtures with PSM. The highest ceramide content used in this study was $X_{\mathrm{Cer}} = 0.30$. Above this concentration some short-chain ceramides have been reported to have detergent like effects, forming micelles with phospholipids (Sot et al. 2005a) and longer-chain ceramides might cause extensive aggregation and restructuring of the MLVs (Holopainen et al. 2000b). Such effects would render the results incomparable and we therefore chose to keep X_{Cer} lower.

Melting temperatures for the pure ceramides

Because the melting temperatures of all the ceramides used in this study have not been reported previously, we determined the main transition temperature of fully hydrated pure ceramide dispersions. The aggregates formed by the ceramides in water were large and oil-like at higher temperatures. At lower temperatures the ceramides formed crystals. Metastable crystalline phases were also formed by some of the ceramides; exotherms in the heating scans indicated these changed spontaneously into more stable gel-phases before melting. However, the chain-melting transitions, taken as the highest reproducible endotherm, for all the ceramides could be detected and the T_{m} values for these are listed in Table 1. All samples were treated

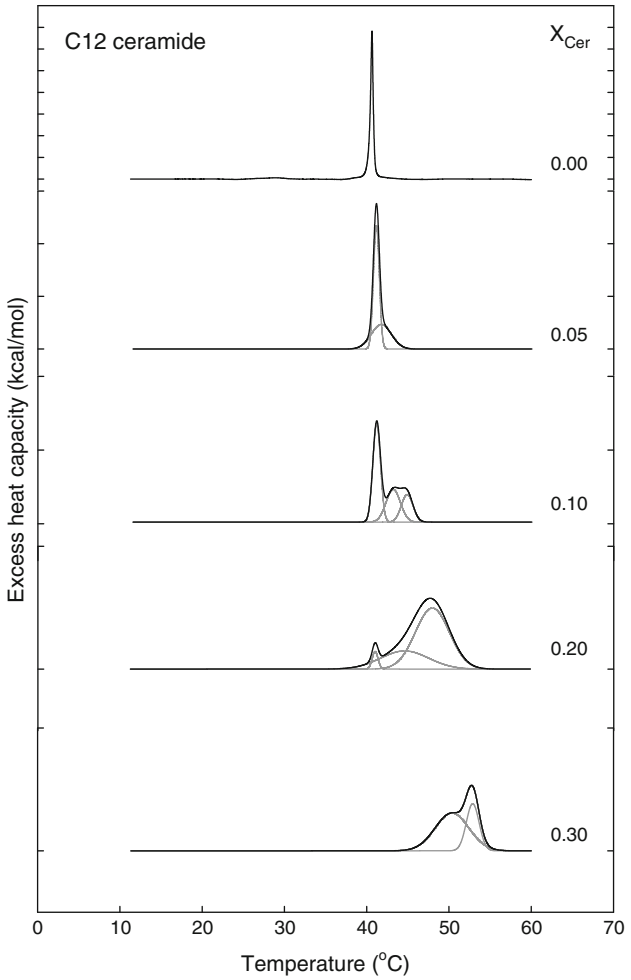

Fig. 4 Representative heating thermograms of PSM/C12 ceramide binary mixtures. Other conditions as defined for Fig. 2

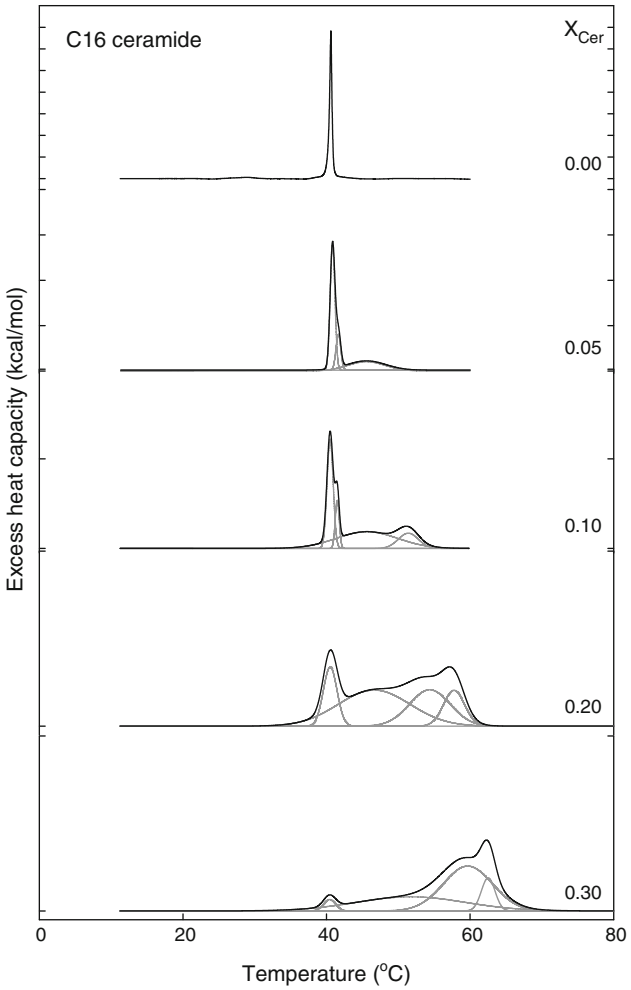

Fig. 5 Representative heating thermograms of PSM/C16 ceramide binary mixtures. DSC heating scans from 10 to 100°C were run at a rate of 1°/min. The results shown have been truncated at 60°C when no peaks were detected at higher temperatures. The ceramide concentration is indicated to the *right*. The scale for the *y*-axis was adjusted so that the ticks occur at every 2 kcal/mole

similarly for this comparison. We are, however, well aware that ceramide thermotropic behavior, with metastable crystalline phases present, is dependent on the scan rate and incubation time at different temperatures. The T_m values reported in Table 1 did not vary much after repeated heating/cooling cycles. An earlier very thorough investigation of the thermotropic behavior of pure C16 ceramide has shown that the behavior is largely dependent on the thermal history of the sample (Shah et al. 1995). The main melting transition for this ceramide was found at about 90°C, in accordance with our results for fully hydrated samples (Shah et al. 1995). These investigators were able to find some exotherms not seen by us at about 60°C when the heating and cooling speed was faster than ours. A recent study by Pinto et al. (2008) reported calorimetric data for pure C24:1 ceramide. They reported a broad endotherm with two maxima, one at 52°C and the other at about 65°C, for samples prepared in phosphate buffer. Our results for C24:1 ceramide at a higher concentration in water consistently showed the main transition at about 65°C.

Effect of short-chain ceramides on miscibility with PSM

Representative DSC up-scans for PSM bilayer dispersions containing different amounts of C4 ceramide are shown in Fig. 2. When C4 ceramide was added at low concentrations the enthalpy of the main endotherm was reduced and the T_m was shifted towards lower temperature. The shift of the heat capacity profiles towards lower temperatures clearly indicated better miscibility of C4 ceramide in the fluid than in the gel-phase of PSM. Also, already at $X_{Cer} = 0.05$ another component appeared in the thermogram that

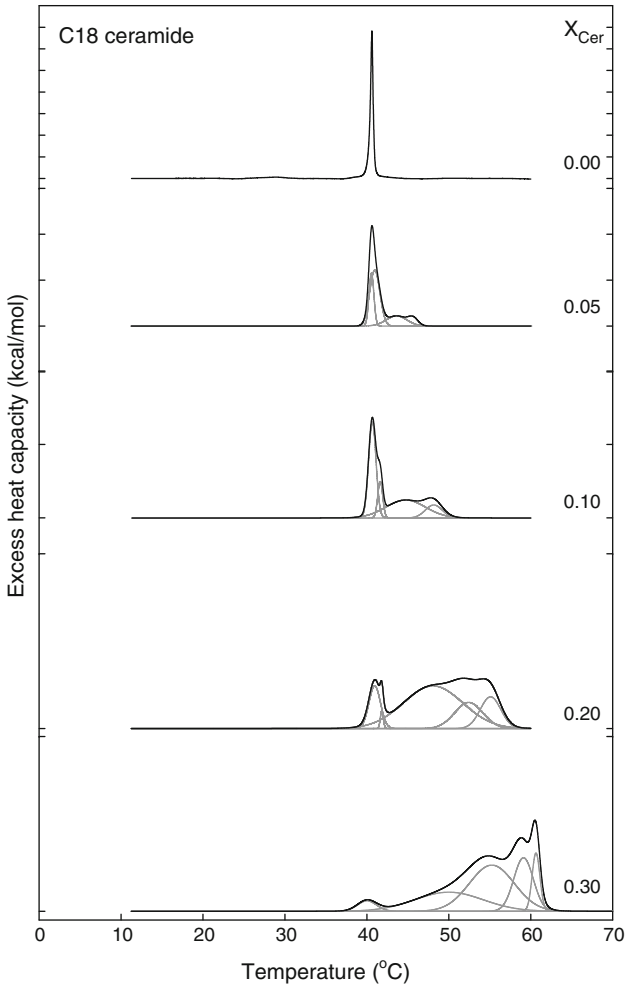

Fig. 6 Representative heating thermograms of PSM/C18 ceramide binary mixtures. Other conditions as defined for Fig. 5

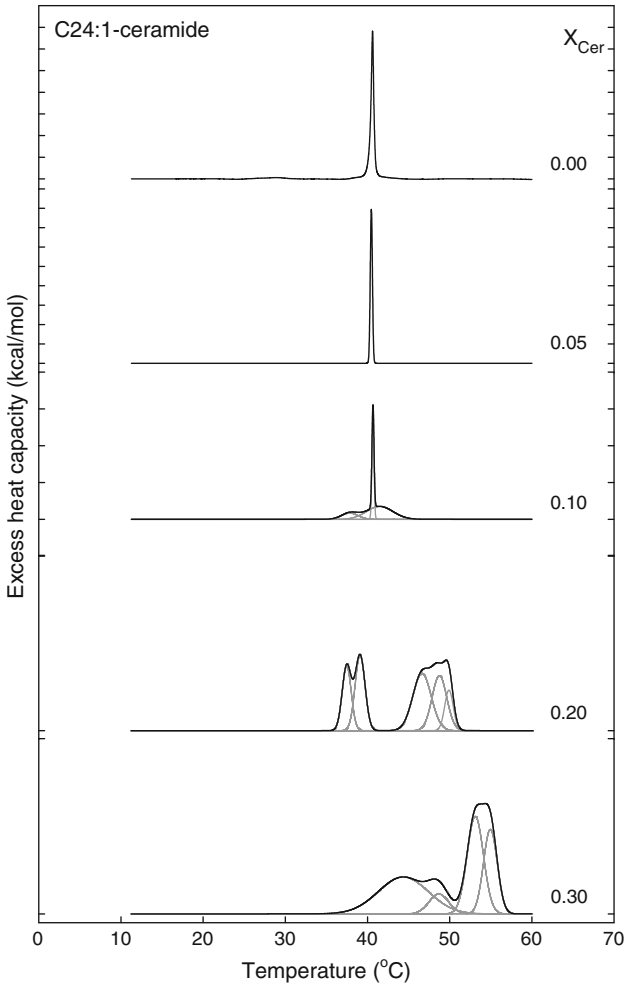

Fig. 7 Representative heating thermograms of PSM/C24:1 ceramide binary mixtures. Other conditions as defined for Fig. 5

melted at even lower temperature. The sphingomyelin-enriched phase seemed to persist up to $X_{Cer} = 0.10$ although the height and enthalpy of the endotherm reporting this transition was greatly reduced. At $X_{Cer} = 0.20$ peak-fitting deconvolution analysis gave the best fit for a sum of three components. At $X_{Cer} = 0.30$ of C4 ceramide a fourth component appeared at lower temperature in the thermogram.

As shown in the representative DSC up-scans in Fig. 3 for C8 ceramide/PSM mixtures, the peak-widths are substantially narrower than for PSM mixtures with the other ceramides, indicating fairly cooperative thermal behavior. The melting temperatures for all the mixtures that contained C8 ceramide were lower than for pure PSM. When ceramide was added, already at $X_{Cer} = 0.05$ the deconvolution procedure gave the best fit for three superimposed thermal events. These three components of the endotherm shifted in enthalpy, cooperativity, and melting temperature with increasing ceramide concentration. It is hard to say from our data what the different co-existing phases might consist of, but it is evident that no pure ceramide or PSM phases were present under these conditions. This short-chain ceramide also seemed to have better miscibility in the fluid phase of PSM than in the gel phase.

Table 1 Melting temperatures (T_m) for ceramides

Ceramide	Melting temperature (°C)
C4 ceramide	56.5
C8 ceramide	64.4
C12 ceramide	85.7
C16 ceramide	91.5
C18 ceramide	91.1
C24:1 ceramide	65.5

Melting temperatures for the ceramides used in this study were determined by DSC as the highest melting endotherm in aqueous dispersions of the pure ceramides. The values given are mean values from several consecutive heating scans

Because short-chain ceramides at high concentrations have sometimes been shown to function as detergents and to form mixed micelles with phospholipids with increasing temperature (Sot et al. 2005b), the possibility cannot be excluded that some of the endotherms seen by us at higher ceramide concentrations with the short-chain ceramides were due to such structures being formed. However, because repeated heating and cooling scans of our samples were reproducible, we conclude that no irreversible ceramide-induced micellization of the PSM membranes occurred in our samples during heating.

Effect of intermediate-chain ceramides on miscibility with PSM

The effect of the C12 ceramide on the melting of PSM was different from that of the shorter-chain ceramides (Fig. 4). The sharp peak of the PSM endotherm was present but decreased in enthalpy with increasing ceramide concentration, and another component with rather low cooperativity emerged at $X_{Cer} = 0.05$ at higher temperature. The sharp component was completely abolished at $X_{Cer} = 0.30$ for C12 ceramide, whereas deconvolution and best-fit analysis showed the broad component to consist of two superimposed thermal events.

Effect of long-chain ceramides on miscibility with PSM

C16 and C18 ceramides behave very similarly in the PSM bilayers studied here (Figs. 5 and 6, respectively). Already at $X_{Cer} = 0.05$ a broad higher-temperature component appeared in the PSM mixtures with the C16 ceramide. At $X_{Cer} = 0.10$ deconvolution of the peaks gave the best fit with four components present in the endotherm. The sharp component of the PSM-rich phase decreased in enthalpy and cooperativity with increasing ceramide concentration, and was almost abolished at $X_{Cer} = 0.30$. Essentially the same pattern was seen for C18 ceramide, although at $X_{Cer} \geq 0.20$ five components seemed to be present.

At ceramide concentrations above $X_{Cer} = 0.20$ in 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC):C16 ceramide bilayers, it has been reported that vesicle aggregation and tubule formation can occur (Silva et al. 2006a). The formation of such structures could possibly explain the large number of endothermal components exhibited by the C16 and C18 ceramide/PSM mixtures at these higher ceramide mole fractions.

The C24:1 ceramide exhibited the most complex thermotropic behavior of all the ceramides included in this study (Fig. 7). The miscibility of C24:1 ceramide with PSM was very good at low concentrations as indicated by the single highly cooperative endotherm at $X_{Cer} = 0.05$. Although some broader components started to emerge, the sharp and highly cooperative melting still dominated at $X_{Cer} = 0.10$. The C24:1 ceramide was recently characterized by Pinto and co-workers (Pinto et al. 2008). They studied the effect of this ceramide on mixing behavior with POPC. Their results indicated that C24:1 ceramide can form different interdigitated gel phases. Interestingly, at $X_{Cer} = 0.20$ in our mixtures with PSM and C24:1 ceramide, the endotherm was split into two broad baseline-separated peaks, both composed of two or more overlapping thermal events. One of the peaks occurred below the melting temperature of the pure PSM and the other was shifted towards substantially higher temperatures close to that of the pure ceramide. At $X_{Cer} = 0.30$ these two peaks again merged into one multicomponent endotherm.

Partial binary phase diagrams for ceramide/PSM mixtures

Figure 8 shows partial phase diagrams for the ceramide/PSM mixed bilayers as determined by DSC. The phase co-existence regions were defined by tangent construction from the heat-capacity profiles of the mixtures. Below the temperature of the lower phase boundary (filled circles), gel and crystalline phases or more than one gel-phase may co-exist. In the "phase co-existence region" (between the two lines) fluid and gel or crystalline phases co-exist, and above the temperature of the upper phase boundary (indicated by the open circles) only fluid phases are present. As seen in Fig. 8 both C4 and C8 ceramides destabilized the PSM gel-phase. Simultaneously, the fluid phase was formed at lower temperatures with increasing concentration of these ceramides. Ceramides with chains longer than C12 stabilized the gel-phase or induced more stable crystalline phases in the bilayers. The most temperature-stable phases were formed with C16 ceramide, which also induced the thermally broadest region of phase co-existence. The phase diagram for PSM/C24:1 ceramide included two regions of phase co-existence at ceramide fractions above $X_{Cer} = 0.20$.

Discussion

Chain length dependence for the ceramide-induced effects on the thermotropic behavior of PSM

In mixtures with PSM the C4 and C8 ceramides behaved differently from those with longer acyl chains. The C4 and C8 ceramides shifted the T_m for the main endotherm of PSM to lower temperatures with increasing amounts of ceramide, indicating that these short-chain compounds were more miscible in the fluid than in the gel-phase PSM bilayer. Huang et al. (1998) showed that short-chain

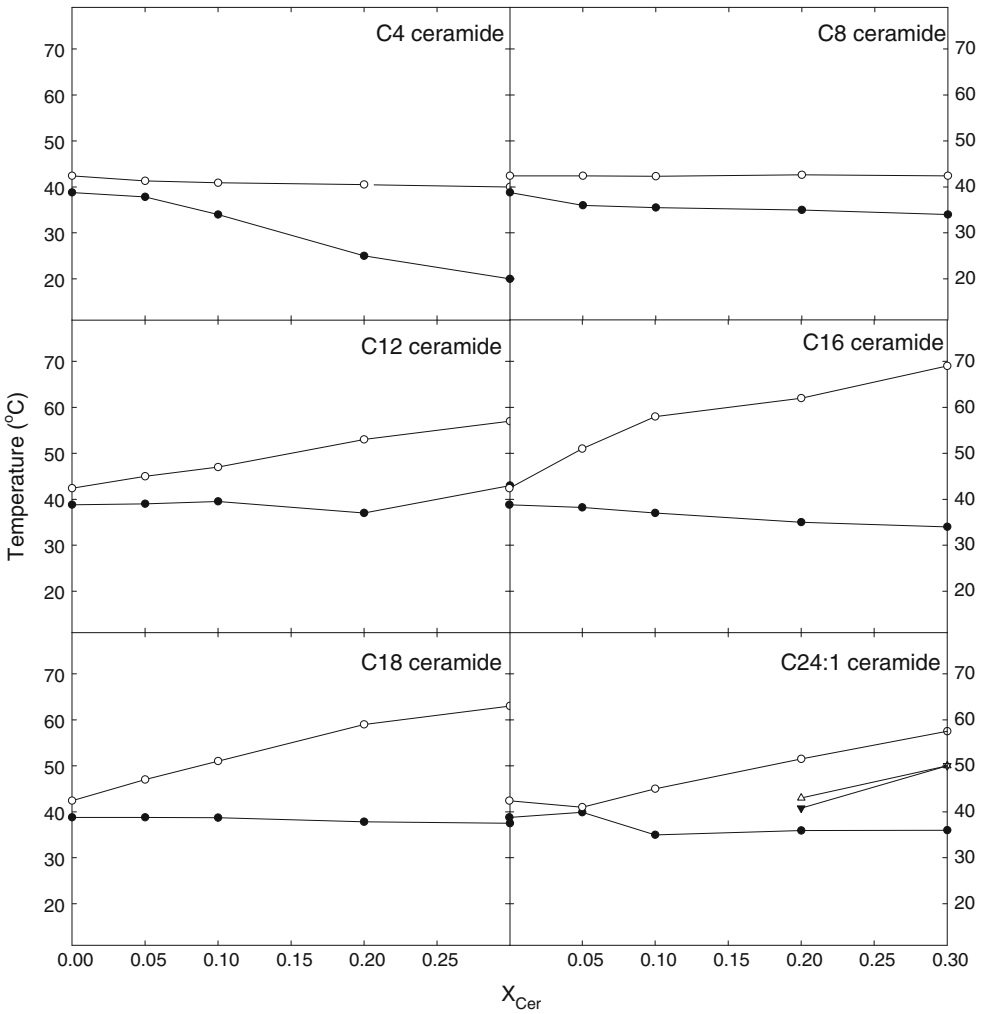

Fig. 8 Partial phase-diagrams of PSM/ceramide mixtures derived from calorimetric data. The temperature intervals for phase co-existence (between the two lines) were defined by tangent construction from the heat capacity profiles for the mixtures (Figs. 2, 3, 4, 5, 6, 7). The start and end temperatures from different experiments vary by less than 5%. The lower phase boundary is indicated by *filled circles* and the upper phase boundary by *open circles*. The *triangles* in the *last panel* show where the first phase co-existence region ends (*filled*) and where the second starts (*open*). The *ceramide* represented is indicated in each *panel*

ceramides were incorporated into, and ordered, fluid-phase di-palmitoyl-phosphatidylcholine (DPPC) bilayers. Carrer et al. (2006) have studied the mixing of C8 ceramide with DMPC and published a partial phase diagram for these two compounds. Already at low ceramide concentrations the cooperativity and enthalpy of the DMPC phase transition were greatly reduced. There was an apparent phase-coexistence region between about 3 and 30 mol% ceramide where two liquid phases seemed to be present over a certain temperature interval. Electron paramagnetic resonance (EPR) data also indicated that the ceramide-DMPC mixture is more ordered than the pure DMPC at 30 mol% ceramide at all positions along the acyl chain (Carrer et al. 2006). As noted in the above mentioned studies for ceramide/phosphatidylcholine mixtures, we can also conclude that short-chain ceramides in PSM bilayers probably order the fluid phase while perturbing the gel-phase. This is in agreement with the phase diagrams presented in this study for the C4 and C8 ceramides, for which the upper boundary of the gel/fluid co-existence region is a horizontal line indicating the presence of two fluid phases above this line.

With the longer-chain ceramides (C12–C18) the calorimetric data showed an endotherm at the T_m of PSM which was not shifted much in temperature with addition of more ceramide. Instead the enthalpy and cooperativity of the peak was diminished and with C12 ceramide the transition was completely abolished at 30 mol% ceramide. New transitions at a higher temperature appeared with increasing amounts of long-chain ceramides in the PSM bilayer. The sharp components probably arose from fairly cooperative hydrocarbon chain melting of domains enriched in PSM and poor in ceramide. Reduction of the sharp

components could mean that the ceramide perturbed the gel-phase PSM organization and/or that it reduced domain sizes. The broader components displayed less cooperative melting of gel-phase domains enriched in ceramide and PSM. The interactions of long-chain ceramides with phosphatidylcholines and sphingomyelins have been investigated previously (Carrer and Maggio 1999; Huang et al. 1998; Massey 2001). C16 ceramide has been shown to induce phase-separation in DPPC bilayers (Huang et al. 1998) and in giant unilamellar vesicles composed of egg-sphingomyelin and egg-ceramide gel-phase domain formation has been visualized by fluorescence microscopy (Sot et al. 2006). The effect of bovine brain ceramide on DPPC was actually very similar, as reported by DSC (Carrer and Maggio 1999) to what we see here for C16 and C18 ceramides with PSM. The results from DPH fluorescence polarization showed an increase in melting temperature for the phospholipid after addition of long-chain ceramides. The same study showed that the effect of C16, C18, and C24:1 ceramides on the melting temperature of bovine brain sphingomyelin was quite similar (Massey 2001). The effect of ceramide on sphingomyelin was much larger than the effect on phosphatidylcholine.

The C24:1 ceramide was the only unsaturated ceramide included in our study and also the one with the longest acyl chain. It was chosen because it is a naturally occurring molecular species, and because its behavior in fluid phospholipid bilayers was recently characterized by Pinto et al. (2008). They found that the behavior was very complex because of the large structural dissimilarity between these two lipids. C24:1 ceramide had relatively low solubility in fluid POPC bilayers, although it was higher than for C16 ceramide (Pinto et al. 2008). In our study C24:1 ceramide had surprisingly good miscibility with PSM at low concentrations ($X_{Cer} = 0.05$), as indicated by the highly cooperative melting endotherms for these mixtures. Phase separation was evident at $X_{Cer} \geq 0.1$, with two broad regions of phase co-existence present in the $X_{Cer} = 0.2$ sample, probably resulting from the complex interdigitated gel-phases that can be formed by this very long-chain ceramide (Pinto et al. 2008). Of the ceramides studied by us, C24:1 ceramide had totally unique behavior in the sphingomyelin bilayers, not quite resembling either short or other long-chain derivatives. In biological membranes C24:1 sphingolipids are fairly abundant and, therefore, C24:1 ceramide can also be generated through their hydrolysis. De-novo synthesis of C24:1 ceramide also contributes to its abundance, especially in liver, kidney, and brain (Laviad et al. 2008).

There are significant differences between the concentration effects on the thermotropic behavior of ceramide/PSM mixtures which markedly depend on ceramide chain length. The number of broad components, obtained through best-fit analysis of the thermograms, varies for different ceramides. All ceramides induced at least two broad components, indicating the presence of distinct populations of independently melting, ceramide-enriched structures. At this point, it is also worth mentioning that the acyl chain-dependent effects of ceramides might be dependent on the acyl chain composition of the sphingomyelin also. For example, C18 sphingomyelin interactions are perturbed by C12 and shorter ceramides (Chiantia et al. 2007), whereas we show here that the PSM gel-phase is already slightly stabilized by the presence of C12 ceramide.

Ceramide effects on PSM thermotropic behavior correlate with domain formation in complex lipid bilayers

Work with model membrane systems (Megha and London 2004), lipoproteins (Björkqvist et al. 2005; Guarino et al. 2005), and caveolin-rich lipid rafts (Yu et al. 2005) have revealed that ceramides are able to displace cholesterol from sphingomyelin-rich (Yu et al. 2005; Guarino et al. 2005; Björkqvist et al. 2005) or saturated phosphatidylcholine-rich (Megha and London 2004) domains. It was also shown that the partitioning of ceramides into the sphingomyelin-rich domains (from which cholesterol was displaced) resulted in marked stabilization of the ceramide/sphingomyelin domains against temperature-induced melting (Björkqvist et al. 2005). These findings may imply that ceramides have a more favored interaction or miscibility with sphingomyelin compared with cholesterol. We have previously studied the effects of ceramide acyl chain lengths on their ability to affect sterol/PSM domains in multicomponent bilayers (Nybond et al. 2005). The ceramides tested ranged in length from C2 to C14 (Nybond et al. 2005). Fluorescence quenching of *trans*-parinaric acid-labeled sphingomyelin revealed that domains enriched in PSM were slightly destabilized against temperature-induced melting by C4 ceramide when added in equimolar amounts to PSM. C8 ceramide completely disrupted domain formation by PSM and sterol. Short-chain ceramides have been shown (by fluorescence correlation spectroscopy and atomic force microscopy) to mix with the liquid-ordered phase and fluidize it, whereas longer-chain ceramides formed and stabilized more ordered phases (Chiantia et al. 2007). Strong stabilization of PSM domains against temperature-induced melting was also observed by us with C12 and C14 ceramides (Nybond et al. 2005). Another study of ours showed that C16 ceramide also was included in PSM-rich domains and stabilized them, as indicated by fluorescence quenching of *trans*-parinaric acid-labeled ceramide (Björkqvist et al. 2005). We also noted that C16 ceramide was fairly miscible with PSM in an equimolar mixture (Björkqvist et al. 2005). C16

ceramide in three-component membranes (with POPC and PSM) was later shown to induce highly ordered gel-phase structures and formed three-phase co-existence regions with several gel phases (Castro et al. 2007). Also, Megha et al. (2007) have shown that pure porcine brain sphingomyelin (mainly C18:0 and C24:1 acyl chains) domains in a di-oleoyl-phosphatidylcholine (DOPC)-based bilayer were destabilized by ceramides having chain lengths shorter than or equal to C8. On the other hand, C12 and longer-chain ceramides appeared to stabilize the domains against temperature. Supporting these findings, it has been shown in multicomponent membranes mimicking the situation in the plasma membrane of cells that C16 and C18 ceramides recruit sphingomyelin and segregate into gel-phase domains (Chiantia et al. 2007). The diffusion of components, for example proteins or fluorescent probes, present in the liquid-disordered and liquid-ordered phases were not much affected by the presence of C18 ceramide in similar bilayers (Chiantia et al. 2008). Acyl-chain length also affected the propensity of ceramides to displace cholesterol from liquid-ordered domains in such complex membranes. It was observed that short-chain ceramides did not displace cholesterol, whereas C12 and longer ceramides seemed to stabilize sphingomyelin/cholesterol domains and displace the sterol from the domains, as reported for the fluorescent cholesterol analog cholestatrienol (Nybond et al. 2005).

The results of this study show a clear correlation with the findings discussed above. The advantage of studying binary mixtures here was that we could exclude the effect of the fluid lipid and yet were able to confirm the trends we had previously seen in more complex lipid bilayers. The interaction with PSM changed with increasing ceramide chain length at a chain length of approximately 8–10 carbon atoms. Shorter-chain ceramides were miscible in the fluid phase PSM bilayer and destabilized the gel-phase. Comparing this result with our previous findings indicates that these ceramides can co-exist with cholesterol and sphingomyelin in a liquid-ordered phase domain. The ceramides with *N*-linked chains longer than 12 carbons displaced sterol from sphingomyelin-rich domains and induced gel-phase domain formation in multicomponent membranes. In this study these long-chain ceramides also induced highly ordered phases with PSM in two-component membranes.

Biological implications

Ceramide levels have been shown to be elevated in cells during signaling events and during cell stress situations, apoptosis, senescence, and cell-cycle arrest. The generation of ceramides during such responses has been suggested to occur in rafts through the action of sphingomyelinase, thus simultaneously reducing the amount of sphingomyelin (Cremesti et al. 2002; Zhang et al. 2009). The acyl chains of ceramides generated in this way would be predominantly long and saturated. The generation of such ceramides would induce formation of large ceramide-enriched platforms (Cremesti et al. 2002; Zhang et al. 2009). These platforms could then take part in receptor clustering, thereby mediating the ceramide signaling effects (Grassme et al. 2002, 2003, 2007). However, some studies indicate that, for example in neutrophil spontaneous apoptosis, sphingomyelinases are not involved in ceramide generation, but de-novo synthesis of ceramides would govern their production (Seumois et al. 2007). Such synthesis of ceramides of different hydrophobic lengths is regulated through the action of a large set of ceramide synthases (Pewzner-Jung et al. 2006). It seems increasingly evident that the cellular processes involving ceramides are regulated in different ways in different situations and cell lines, which makes the concept of studying ceramide as a second messenger very complicated. Many cell studies on the effects generated by ceramides have employed short-chain ceramides because of their easy administration to cells, others have generated long-chain ceramides through sphingomyelinase treatment. The conclusions regarding ceramide chain-length effects are often contradictory and might be caused by artifacts, because of the difficulty of delivering long-chain ceramides to cells. The induction of apoptosis in cancer cells has, for example, been reported to be dependent on ceramide chain length, i.e. short-chain analogs seemed to be more cytotoxic than long-chain ceramides (Shabbits and Mayer 2003). On the other hand the formation of pores in the cellular plasma membrane induced by ceramides has been implicated in apoptosis as an acyl chain-independent effect of ceramides (Siskind and Colombini 2000).

On the basis of recent biophysical studies, including the results presented in this article, we can conclude that ceramides of different chain lengths will have very different effects on membrane lateral organization (Chiantia et al. 2007; Megha et al. 2007; Nybond et al. 2005; Sot et al. 2005a, b). The results of both our previous study on membrane domains (Nybond et al. 2005) and this study indicate that ceramides of different hydrophobic length will have substantially different effects on PSM. Sphingomyelinase-induced generation of ceramide in rafts in biological membranes will create at least a transient situation in which the generated ceramide and sphingomyelin co-exist as near neighbors. One effect of the generated ceramide would be, as shown by many previous studies, that it will compete with cholesterol for sphingomyelin and displace cholesterol from rafts (Alanko et al. 2005; Lange et al. 2005; Megha and London 2004). Such a situation would alter the chemical activity of cholesterol in cells (Lange et al. 2005) and induce the formation of both liquid-ordered

and gel-phase regions in the membrane. Staneva and coworkers have shown that sphingomyelin in bilayer membranes inhibits the hydrolytic action of phospholipase A_2 (PLA_2) (Koumanov et al. 1997, 1998). However, if the SM is hydrolyzed to ceramide by sphingomyelinase, the activity of PLA_2 will increase (Koumanov et al. 2002). The authors concluded in their recent study on giant unilamellar vesicles that the defects induced in the membrane by the ceramides at the gel-phase domain boundary would be the site for enzyme action (Staneva et al. 2009). Another extensive model membrane study by the same authors was recently reported. They used synchrotron X-ray powder patterns to compare four-component membrane systems containing phosphatidylcholine, sphingomyelin, ceramide, and cholesterol (Staneva et al. 2008) and concluded that sphingomyelin acts to mediate a transition between gel phase structures enriched in ceramide and liquid-ordered phases enriched in cholesterol. With increasing temperature they report that the liquid-ordered phase in the multicomponent membranes was increasing as SM was mobilized from the ceramide-rich gel-phase. The authors also note that the coexisting phase structures were very complex. In the light of such new findings it is easy to conclude that a short-chain ceramide which would destabilize sphingomyelin-rich domains without cholesterol displacement (this study and those of Megha et al. (2007) and Nybond et al. (2005)) would have a very different effect on enzyme activity also. It is clear that the differences seen in the effect of ceramide on sphingomyelin thermotropic behavior will reflect the effect ceramides of different acyl chain lengths will have on biological membranes also.

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