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Acyl chain length affects ceramide action on sterol/sphingomyelin-rich domains

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Abstract

The effects of ceramides with varying saturated *N*-linked acyl chains (C2–C14) on cholesterol displacement from sphingomyelin-rich domains and on the stability of ordered domains were studied. The bilayers examined were made from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), D-*erythro*-*N*-palmitoyl-sphingomyelin (PSM), D-*erythro*-*N*-*acyl*-sphingosine, and cholesterol (60:15:15:10 mol%, respectively). Cholestatrienol (CTL) or D-*erythro*-*N*-*trans*-parinoyl-sphingomyelin (tParSM) were used as reporter molecules (at 1 mol%) for the ordered domains, and 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine (7SLPC) as a fluorescence quencher (30 mol%, replacing POPC) in the liquid-disordered phase. The results indicate that the ceramide had to have an *N*-linked acyl chain with at least 8 methylene units in order for it to displace cholesterol from the sphingomyelin-rich domains at the concentration used. The melting of the sphingomyelin-rich domain shifted to higher temperatures (compared to the ceramide-free control) with C2, C12 and longer chain ceramides, whereas C4–C10 ceramides led to domain melting at lower temperatures than control. This study shows that short-chain ceramides do not have the same effects on sterol- and sphingomyelin-rich domains as naturally occurring longer-chain ceramides have.

Keywords: Sterol partitioning; Cholestatrienol; Trans-parinaric acid; Quenching; 7-doxyl phosphatidylcholine

1. Introduction

Sphingomyelins are major solubilizers of cholesterol in the plasma membrane compartment in most cell types [1,2]. Sphingomyelins are well suited to interact with cholesterol, since their acyl chains are long and mostly saturated [3,4], and since they have more possibilities for hydrogen bonding as compared with phosphatidylcholines [5,6]. It has been argued that most of the cellular cholesterol is confined to plasma membranes [7,8], where a substantial fraction of it is found in sphingomyelin-rich domains [9,10]. However, conditions exist where cells appear to enrich plasma membranes with cholesterol even if their sphingomyelin-level is markedly reduced [11]. When plasma membrane sphingomyelin is enzymatically degraded, the cellular homeostasis of cholesterol is dramatically changed and cholesterol is translocated from the cell surface to intracellular membranes [12]. Ceramide is a much less effective solubilizer of cholesterol than sphingomyelin, in part because ceramide has no protecting head group to shield the exposed hydrophobic portions of the molecules [13]. The miscibility of ceramide with sphingomyelin is, on the other hand, good based on, e.g., DSC analysis of equimolar mixtures of PSM and C16-ceramide [14]. On a macroscopic scale, ceramide has also been shown to partition favorably into sphingomyelin-rich domains [5,12,15]. Very recent data from both model membrane systems [14,16], lipoproteins [17], and caveolin-rich lipid rafts [18] show that ceramide is able to displace cholesterol from sphingomyelin-rich [14,17,18] or saturated phosphatidylcholine-rich [16] domains. It was also shown that the partitioning of ceramide into the sphingomyelin-rich domain (from which cholesterol was displaced) resulted in a marked stabilization of the ceramide/sphingomyelin domain against temperature-induced melting [14].

Abbreviations: 7-SLPC, 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine; CTL, Cholestatrienol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PSM, D-*erythro*-*N*-palmitoyl-sphingomyelin; tParSM, D-*erythro*-*N*-*trans* parinoyl-sphingosylphosphorylcholine

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Since ceramides can have such dramatic effects on the sterol content of sphingomyelin-rich domains, it would be of interest to evaluate the effect of ceramide structure on the displacement process. This would be important in light of the common use of short-chain and medium-chain ceramides as tools to induce or inhibit apoptosis in cells [19,20]. It is possible that some of the effects of ceramides reported in the literature stems from their effects on cholesterol/sphingomyelin domains rather than from direct effects on target proteins [21,22]. In this study, we have used cholestatrienol (CTL) and *N-trans* parinoyl-sphingomyelin (tParSM) as fluorescent probes which partition into sterol- and sphingomyelin-rich domains, respectively, and 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine (7SLPC) as a quencher lipid mainly located in the liquid-disordered phase. We have determined the effect of the ceramide *N*-linked chain length (C2–C14) on sterol displacement from and the temperature-dependent stability of sphingomyelin-rich domains in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles containing 10 mol% sterol.

2. Material and methods

2.1. Materials

D-*erythro*-*N*-palmitoyl-sphingomyelin (PSM) was purified from egg yolk sphingomyelin (Avanti Polar Lipids, Alabaster, AL, USA) by reverse-phase HPLC (Supelco Discovery C18-column, dimensions 250 × 21.2 mm, 5 μm particle size) using 100% methanol as eluent. The purity and identity of the products were verified on a Micromass Quattro II mass spectrometer (Manchester, UK). POPC was obtained from Avanti Polar Lipids. (7-Doxyl)-stearic acid was obtained from TCI (TCI Europe N.V., Belgium) and was used for the synthesis of 7SLPC [23]. D-*erythro*-*N*-*acyl*-sphingosines were obtained from Larodan Fine Chemicals (Malmö, Sweden) or synthesized from D-*erythro*-sphingosine and fatty acid [24]. All products were characterized by mass spectrometry. Stock solutions of lipids were prepared in hexane/2-propanol (3:2 by vol), stored in the dark at −20 °C, and warmed to ambient temperature before use CTL (cholesta-5,7,9(11)-trien-3-beta-ol) was synthesized and purified using the method published by Fisher and coworkers [25]. tParSM was synthesized from *trans*-parinaric acid (Molecular Probes, Eugene, OR, USA) and D-*erythro*-sphingosylphosphorylcholine (Matreya LLC, Pleasant Gap, PA, USA) according to Cohen and co-workers [24]. The fluorescent probes were purified by reverse-phase HPLC on a RP-18 column with methanol/acetonitrile (70:30, by vol) as eluent for CTL and 100% methanol for tParSM. All compounds were positively identified by mass spectrometry. CTL and tParSM were stored dry under argon in the dark at −87 °C until solubilized in argon-purged ethanol (CTL) or methanol (tParSM). Stock solutions of fluorescent lipids were stored in the dark at −20 °C and used within a week.

2.2. Fluorescence quenching method

The F samples contained quencher (7SLPC) and a complex lipid mixture as described above, while POPC replaced 7SLPC in F_o samples. The fluorescence intensity in the F samples was compared to the fluorescence intensity in F_o samples giving the fraction of quenched fluorescence. In lipid vesicles in which ordered and disordered domains co-exist, POPC together with the nitroxide labeled quencher (7SLPC) formed the disordered phase in the vesicles [26]. Fluorophores residing in the quencher-rich disordered domains give a weaker fluorescence than fluorophores in the ordered domains, which are quencher-poor. CTL is a fluorescent cholesterol analogue that has been shown to mimic the membrane behavior of cholesterol quite well [25,27–30]. In complex lipid bilayer vesicles, in which lateral domain formation is expected, CTL can be used as a fluorescent sterol analogue that associates with sterol-rich domains [14]. The amount of CTL exposed to quenching by 7SLPC gives a measure of CTL distribution between ordered and disordered membrane domains. The stability of the ordered domains containing CTL is measured by looking at the quenching susceptibility as a function of temperature. This quenching method can be used with other fluorescent reporter molecules as well. In this study, we used CTL and tParSM as fluorescent probes which partition predominantly into domains enriched in sterol and PSM, respectively.

2.3. Preparation of vesicles

Vesicles used in steady-state fluorescence measurements were prepared at a lipid concentration of 50 μM. The lipid mixtures were dried under nitrogen, dispersed in argon-purged water and heated above the gel- to liquid-crystalline phase-transition temperature. The samples were vortexed and then sonicated for 2 min (20% duty cycle, power output 15 W) with a Branson probe sonifier W-250 (Branson Ultrasonics, CT, USA). This procedure gives liposomes with a rather large size distribution with a mean diameter of 200 nm (determined using a Malvern 4700 multiangle laser spectrometer at an angle of 90°). The water used in the experiments was purified by reverse osmosis followed by passage through a Millipore UF Plus water purification system, to yield a product with a resistivity of 18.2 MΩ cm.

In fluorescence quenching studies, F samples consisted of POPC: 7SLPC: PSM: ceramide: cholesterol (30:30:15:15:10 molar ratio) and in F_o samples POPC replaced 7SLPC. The samples were studied with CTL or tParSM as the fluorescent probe, which replaced cholesterol or PSM, respectively, to give a final probe concentration of 1 mol%. The fluorescent probes were protected from light during all steps. Solvents were saturated with argon before use in order to minimize the risk of oxidation. All experiments were performed in duplicate or triplicate and curves shown are representative of reproducible experiments.

2.4. Steady-state fluorescence measurements

Fluorescence measurements were performed on a PTI QuantaMaster-1 spectrofluorimeter (Photon Technology International, Lawrenceville, NJ, USA). The excitation and emission slits were set to 1–4 nm. The temperature was controlled by a Peltier element, with a temperature probe immersed in the sample solution. The samples were heated from 8 °C to 90 °C at a rate of 5 °C/min during the measurements. The measurements were performed in quartz cuvettes and the sample solutions were kept at constant stirring (260 rpm) throughout the measurements. Fluorescence intensity of CTL was detected with excitation and emission wavelengths at 324 nm and 374 nm, respectively. Fluorescence emission of tParSM was detected at 410 nm, while excitation occurred at 305 nm.

3. Results

The basic membrane system examined in this study contained POPC, PSM and cholesterol (75:15:10). According to the phase diagram of de Almeida and coworkers [31] the only phases present in such membranes at room temperature are the liquid disordered phase (L_d) containing mostly POPC, and the liquid ordered phase (L_o) containing PSM and most of the cholesterol. The "melting" of the L_o domains with increasing temperature can be detected using DSC [32] or CTL quenching [32] (see also Fig. 1). The amount of CTL exposed to quenching by 7SLPC gives a measure of CTL distribution between ordered and disordered membrane domains. The bottom trace in Fig. 1 shows the change in CTL quenching susceptibility as the temperature of the sample is increased. The $\Delta(F/F_o)$ change is not very

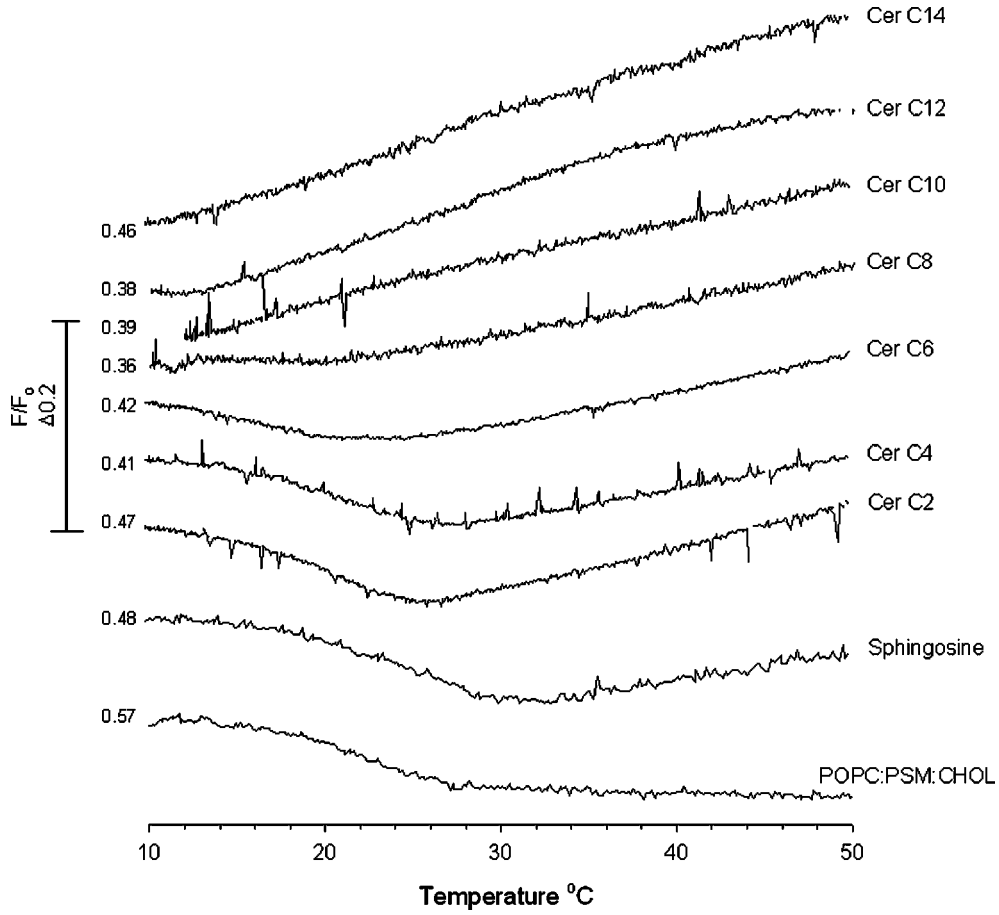

Fig. 1. Melting of sterol/PSM-rich domains in a fluid bilayer as examined by fluorescence quenching of CTL. For fluorescence quenching experiments, the F_o-samples were prepared to contain POPC/PSM/sterol (75:15:10 mol%) or POPC/PSM/ceramide(or sphingosine)/sterol (60:15:15:10 mol%). The sterol fraction contained cholesterol and CTL (9:1, mol/mol). 7SLPC was used as a quencher in F-samples at 30 mol% (replacing some of the POPC). The lipid concentration was 50 μM. The temperature gradient was 5 °C/min.

dramatic, but the melting of the L_o domains can clearly be seen between 10 and 28 °C. The observed $\Delta(F/F_o)$ is typical for 15 mol% PSM and 10 mol% sterol, but is larger when the PSM content in the membrane is higher [14]. To test the effects of ceramides with varying *N*-linked acyl chain lengths on the sterol-rich domains, 15 mol% ceramide was included in the bilayers during preparation. Both the C2 and the C4 ceramide induced only minor effects on the $\Delta(F/F_o)$ of CTL fluorescence, whereas C6 ceramide shifted the melting of the sterol-rich domain to lower temperature (Fig. 1). In bilayers containing C10, C12, C14 (Fig. 1) or C16 ceramide [32], the CTL-quenching method did not report any sterol-rich domain melting, suggesting that these ceramides were able to completely displace sterol from the PSM domains. Sphingosine was previously shown to slightly stabilize sterol/PSM domains using CTL as the probe [32], so sphingosine is included for the convenience of the reader again in Fig. 1.

In order to determine the effects of the ceramides on the stability of the PSM-rich domains, tParSM was used as the fluorescent probe (Fig. 2). The bottom trace in Fig. 2 is for a bilayer containing 15 mol% PSM and 10 mol% cholesterol, but no ceramide. The $\Delta(F/F_o)$ for the quenching of CTL and tParSM in these membranes differ depending on the probe used (bottom traces in Figs. 1 and 2). The temperature interval for domain melting was however the same (as expected if PSM and CTL/cholesterol are in the same domain). Sphingosine and C2 ceramide appeared to stabilize the PSM domain slightly, with a similar change in $\Delta(F/F_o)$.

Both C4 and C6 ceramides destabilized the PSM-rich domains and attenuated the $\Delta(F/F_o)$, indicating that less ordered domains existed under these conditions. With C8 ceramide, no domain melting could be observed in the temperature interval analyzed. With longer chain ceramides, PSM-domain stabilization became again apparent, and this stabilization was clearly chain-length dependent. C16 ceramide stabilized the PSM-rich domain even further as compared to the C14 ceramide [32].

4. Discussion

Although we have not determined the membrane concentration of the ceramides, we argue that the actual concentrations are indeed very close to the prepared concentrations. The calculated log*P* (octanol/water partition coefficient) for the C2 ceramide is 6.14 and higher than that

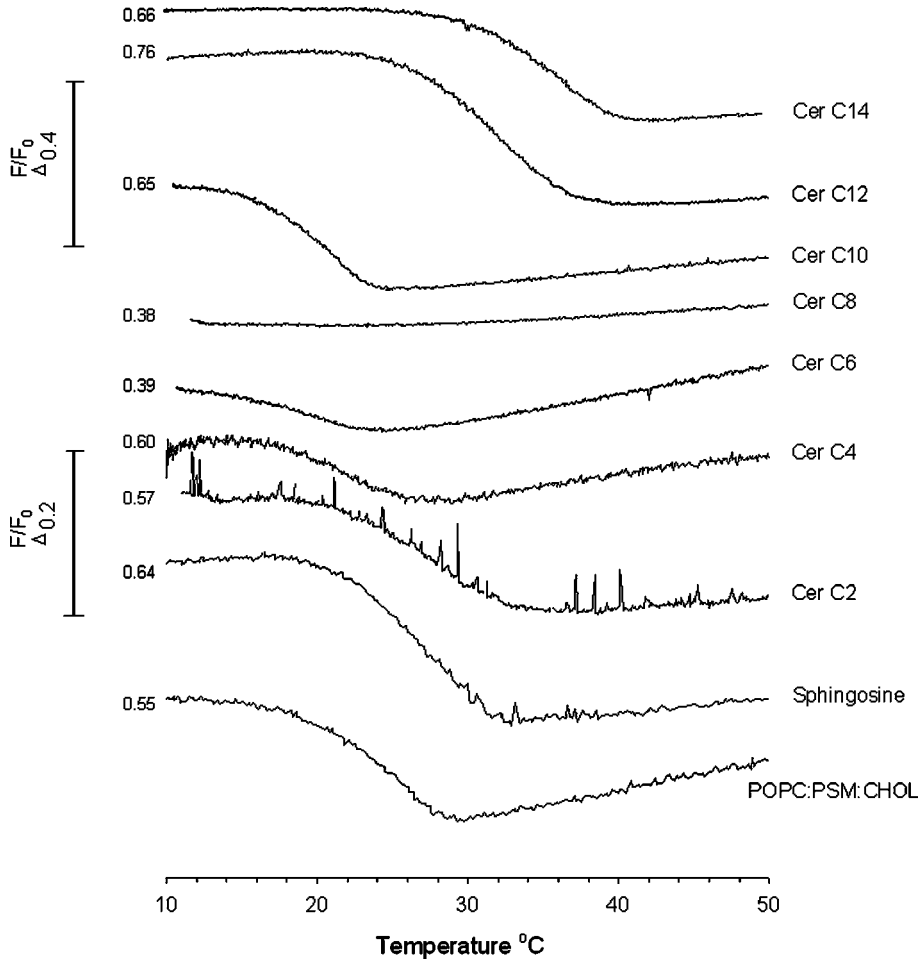

Fig. 2. Melting of sterol/PSM-rich domains in a fluid bilayer as examined by fluorescence quenching of tParSM. For fluorescence quenching experiments, the F_o-samples were prepared to contain POPC/PSM/cholesterol (75:15:10 mol%) or POPC/PSM/ceramide (or sphingosine)/cholesterol (60:15:15:10 mol%). The PSM fraction contained PSM and tParSM (14:1, mol/mol). 7SLPC was used as a quencher in F-samples at 30 mol% (replacing some of the POPC). The lipid concentration was 50 μM. The temperature gradient was 5 °C/min. The scale bar $\Delta(F/F_o)$ 0.2 applies to the five lower curves, whereas the scale bar $\Delta(F/F_o)$ 0.4 applies to the four upper curves.

for sphingosine (5.94; data not shown). The calculated log$P$ values varied linearly between 6.14 and 12.48 for ceramide chain lengths C2 to C14 (data not shown). Sphingosine was previously shown by us to stabilize PSM domains under similar conditions used in this study (this study and [32]), indicating that its membrane concentration was significant. The critical micelle concentration of sphingosine has been reported to be around 112 μM [33]. Also the most "water-soluble" of our ceramides (C2 ceramide) clearly stabilized PSM domains (Fig. 2) suggesting it had a significant membrane concentration. Although the short-chain ceramides are considered "water-soluble", their cmc is only 5–6 μM [34], which is three orders of magnitude less than the critical micelle concentration of deoxycholate, which was shown by us to have a fairly high membrane partition coefficient (2200 M^{-1} with egg yolk sphingomyelin vesicles) [35]. All these things considered, and since these short-chain ceramide sphingolipids in general have an affinity for other sphingolipids (in this case PSM [15]), we are confident that their membrane concentration is close to the prepared concentration.

The C2 ceramide resembles sphingosine more than it does a natural ceramide, with the N-acetyl chain at the water/lipid interface (Fig. 3). The effects of the C2 ceramide in this study were very similar to the effects observed with sphingosine in a similar membrane system [32], suggesting that it co-existed with the sterol in the PSM-rich domain. It is interesting to note that both sphingosine and C2-ceramide can be pro-apoptotic in cells [36,37], whereas C6 and longer ceramides can be potent inhibitors of apoptosis.

The acyl chain of the C4 ceramide will most likely influence the interfacial lipid packing in its vicinity, since the C4 chain probably wobbles between the aqueous phase and the more hydrophobic interface (Fig. 3). In our system C4 ceramide disturbed the lipid packing in the sterol/PSM-rich domain, partially destabilizing them, (Figs. 1 and 2) but was not capable to displace a lot of sterol from the domains (Fig. 1). The C6 ceramide behaved similarly, although the melting temperature of the domains was further decreased (Figs. 1 and 2). It may be argued that short-chain ceramides may form micelles in membranes which at higher temperatures actually may be expelled from the bilayers. However,

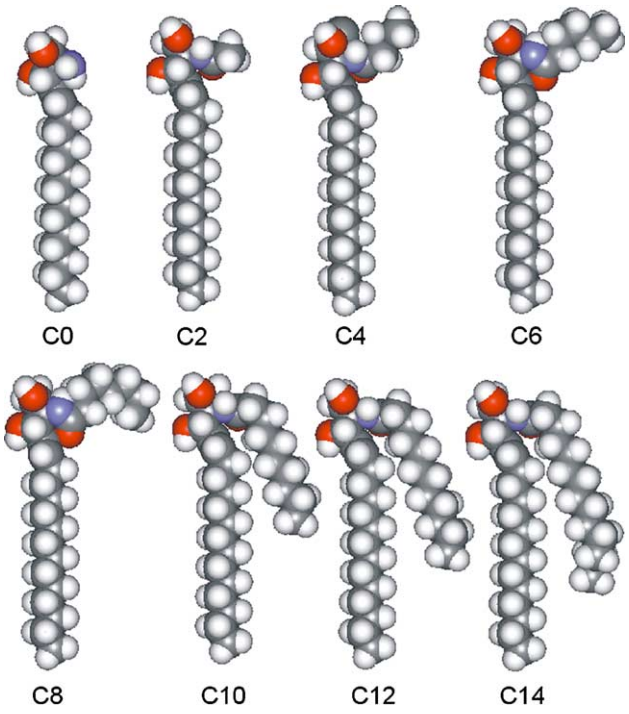

Fig. 3. CPK models of sphingosine (C0) and the different ceramides (C2–C14) used in the study. Energy-minimized models were prepared in Chem3D (Cambridge Software, MA, USA) and combined and visualized using DS ViewerPro 5.0 (Accelrys Inc.).

using the C4 and C6 ceramide systems (both with CTL and tParSM as probes), the quenching curves were almost identical during temperature up-scan and down-scan (data not shown), implying that it is very unlikely that these short-chain ceramides induced irreversible micellization during the heating scans.

It is interesting here to note that while C2 ceramide destabilized the lamellar-to-hexagonal phase transition of dielaidoylphosphatidylethanolamine bilayers [38], the C6 ceramide actually stabilized the same transition [38], possibly implying that the C6 acyl chain in fact is partially embedded in the phosphatidylethanolamine bilayer giving the ceramide molecule an inverted cone shape. The membrane packing of the C6 chain in the ceramide may not, however, be identical in a sphingomyelin-rich environment which has a different interfacial hydration. The acyl chain of the C8 ceramide is probably long enough to be partially embedded within the bilayer interface. C8 ceramide displaced sterol from the PSM-domains and destabilized them (no melting with either probe can be detected above 10 °C). Ceramides with C10 to C14 displaced all sterol from the PSM-domains and a clear ceramide-induced stabilization of the PSM-domains was evident (Fig. 2). When the acyl chain of the ceramides are C10 or longer they are very likely to be completely in the bilayer, adjacent to the neighboring long-chain bases or acyl chains (Fig. 3). In a study of the membrane properties of *N*-acyl phosphatidylethanolamines, it was observed that the *N*-linked acyl chain had to have at least 10 methylene units before it was embedded in the bilayer [39,40]. Although the *N*-linked acyl chain must have a slightly different membrane affinity depending on its attachment to a phosphorylethanolamine head group or to a long chain base, our results agree reasonably well with the studies reported in [39,40].

Taken together, our results show that although all tested ceramides appeared to partition into PSM-rich domains, their effect on the sterol composition of the PSM-domains varied markedly with the *N*-linked acyl chain length. Only medium and long-chain ceramides could displace sterol from PSM-rich domains at the selected concentration. The effect of the ceramides on the stability of the PSM-rich domains was also markedly influenced by the ceramide *N*-linked chain length. Strong stabilization of the PSM-rich domains was seen only with C12 and longer ceramides, whereas the short-chain ceramides mostly destabilized the PSM-rich domains. Since ceramides generated in situ using sphingomyelinase have been shown to induce membrane leakage [41], ceramide aggregation into domains [42], and transmembrane scrambling of lipids [43], it would be of some interest to correlate those ceramide effects with the length of the *N*-linked acyl chain in order to better understand how they are influenced by the molecular shape of the ceramides.

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