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Membrane bilayer properties of sphingomyelins with amide-linked 2- or 3-hydroxylated fatty acids

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ABSTRACT

The bilayer properties and interactions with cholesterol of *N*-acyl hydroxylated sphingomyelins (SM) were examined, and results were compared to nonhydroxylated chain-matched SM. The natural OH(D)-enantiomer of hydroxylated SM (with 16:0 or 22:0 acyl chain lengths) analogs was synthesized. Measuring steady-state diphenylhexatriene anisotropy, we observed that pure 2OH-SM bilayers always showed higher (5–10 °C) gel–liquid transition temperatures (T_m) compared to their nonhydroxylated chain-matched analogs. Bilayers made from 3OH (D)-palmitoyl SM, however, had lower T_m (5 °C) than palmitoyl SM. These data show that hydroxylation in a position-dependent manner directly affected SM interactions and gel state stability. From the c-laurdan emission spectra, we could observe that 2OH-palmitoyl SM bilayers showed a redshift in the emission compared to nonhydroxylated palmitoyl SM bilayers, whereas the opposite was true for c-laurdan emission in 3OH-palmitoyl SM bilayers. All hydroxylated SM analogs were able to form sterol-enriched ordered domains in a fluid phospholipid bilayer. 2-Hydroxylation appeared to increase domain thermostability compared to nonhydroxylated SM, whereas 3-hydroxylation appeared to decrease domain stability. When sterol affinity to bilayers containing SM analogs was determined (cholestatrienol partitioning), the affinity for hydroxylated SM analog bilayers was clearly reduced compared to the nonhydroxylated SM bilayers. Our results with hydroxylated SM analogs clearly show that hydroxylation affects interlipid interactions in a position-dependent manner.

1. Introduction

Hydroxylation is a fairly common modification in different sphingolipids. The sphingosine long-chain base has a hydroxyl group at 3 position, but additional hydroxylation is common (i.e., phytosphingosine has a hydroxyl group at 4 position [1–3]). In addition, hydroxylation in the *N*-linked acyl chain is common in brain cerebrosides, where up to 50% of myelin cerebroside can be 2-hydroxylated (for a recent review of 2-hydroxylated sphingolipids, see reference 4). The hydroxyl group at 2 position of the *N*-linked acyl chain has the D configuration in mammalian brain cerebroside and in honey bee sphingomyelin ([5]). The D configuration corresponds to the R configuration in the R/S system.

Hydroxylated sphingolipids have been shown to have important biological effects in cells and tissues. It is known that hydroxylated cerebrosides are important for the stability of the myelin sheet in neural tissue [4]. Furthermore, the association of the Alzheimer's beta-amyloid peptide with galactosylceramide membranes was recently reported to be stabilized by the presence of 2-hydroxylated acyl chains in the cerebrosides [6]. Hydroxylated ceramides exist abundantly in the skin where they, together with sterols and fatty acids, help to maintain the barrier function of skin [7]. The presence of 2-hydroxylation in cerebroside sulfates is known to stabilize their mutual interactions and to increase their melting temperature in hydrated model membrane systems [8,9]. A similar membrane stabilization by a 2OH(D)-fatty acid in galactosylceramide has been reported [10]. The stabilization induced by 2-hydroxylation is most likely due to increased possibilities for intermolecular hydrogen bonding. Hydroxylated ceramides are also known to be more condensed in monolayer membranes compared to their nonhydroxylated counterparts [11].

Hydroxylated sphingomyelins are not generally abundant but have been detected in testes and spermatozoa [12], in kidney and intestinal mucosa [13], and in neural tissue of knockout mice lacking UDP-galactose ceramide galactosyltransferase [14]. In these tissues and cells, the acyl chains of the hydroxylated SMs are often very long, and in the case of kidney and intestinal mucosa, the 2OH fatty acids were coupled to phytosphingosylphosphorylcholine [13]. The guinea pig Harderian gland has also been reported to contain a fairly high percentage of hydroxylated SM species [15], but the biological function or importance of hydroxylated SM species remains unknown. In an early study from our laboratory, racemic palmitoyl SM (PSM) with a hydroxyl group in the 2 position was shown to be condensed by cholesterol in binary monolayer membranes [16], but otherwise, there are few or no studies

Abbreviations: 22:0-SM, *N*-behenoyl-D-*erythro*-sphingomyelin; 2OH-22:0-SM, *N*-2OH (D)-behenoyl-D-*erythro*-sphingomyelin; 2OH-PSM, *N*-2OH(D)-palmitoyl-D-*erythro*-sphingomyelin; 3OH-PSM, *N*-3OH(D)-palmitoyl-D-*erythro*-sphingomyelin; 7SLPC, 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine; CTL, cholesta-5,7(11)-trien-3-beta-ol; DPH, 1,6-diphenyl-1,3,5-hexatriene; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PSM, *N*-palmitoyl-D-*erythro*-sphingomyelin

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on proper biophysical or functional characterization of hydroxylated SM analogs.

SMs are abundant phospholipids in the plasma membrane of cells [17,18] and appear to have major impact on the lateral structure of biological membranes [19–21] and thereby also on their function. Mammalian SMs normally have sphingosine as the long-chain base [17] and long or very long acyl chains linked to the primary amine at carbon 2 of the long-chain base [17]. The acyl chains or SMs are predominantly saturated (e.g., palmitic (16:0) and stearic (18:0) acid) or monounsaturated (nervonic acid, $24{:}1^{\Delta 15c}$). SMs are known to increase acyl chain order in fluid bilayer membranes [22], and they interact favorably with cholesterol and other sterols in the bilayer, forming sterol and SM-rich lateral ordered domains [22–27]. Since hydroxylated SMs occur in some tissues and cells, it is of interest to better understand how the hydroxylation in the *N*-linked acyl chain affects the biophysical properties of SMs in bilayer membranes. We have in this study prepared SMs with hydroxylated fatty acids in the *N*-linked position and studied how this modification changes the membrane properties of the analogs compared to nonhydroxylated palmitoyl SM. All hydroxy fatty acids had the biologically relevant D configuration. We used 2OH and 3OH palmitic acid to allow us to compare molecular properties to PSM, although the 2OH-palmitoyl SM (2OH-PSM) is only a minor species among hydroxylated sphingomyelins. 3OH-palmitoyl SM (3OH-PSM) is not biologically relevant but was included to allow us to compare the effect of the position of the OH on SM properties. 2OH-22:0-SM was prepared to represent a biologically relevant hydroxy-SM species. We find that hydroxylation at 2 position stabilized intermolecular interactions (increased T_m compared to nonhydroxylated SM), whereas hydroxylation at the 3 position destabilized molecular interactions (decreased T_m compared to 2OH-PSM and PSM). Sterol affinity to bilayers containing hydroxylated SM analogs was markedly attenuated as compared to the affinity for bilayers containing nonhydroxylated SM species.

2. Materials and methods

2.1. Materials

Highly pure POPC and D-*erythro*-sphingosyl phosphorylcholine were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. Racemic 2- or 3-hydroxylated palmitic or stearic acid was obtained from Larodan Fine Chemicals (Malmö, Sweden). PSM was isolated to 99% purity from egg SM (Avanti Polar Lipids) using preparative HPLC [28]. Stock solutions of lipids were prepared in methanol or hexane/2-propanol (3/2, by vol.). All phospholipid solutions were taken to ambient temperature before use. The concentration of all phospholipid solutions was determined by phosphate assay [29] subsequent to total digestion by perchloric acid. Stock solutions of the phospholipids were stored in the dark at −20 °C.

Highly pure cholesterol was from Sigma Chemicals (St. Louis, MO, USA). (7-Doxyl)-stearic acid was obtained from TCI (TCI Europe N.V., Belgium) and was used for the synthesis of 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine (7SLPC) [28]. CTL was synthesized from 7-dehydrocholesterol (Sigma Chemicals) and purified as described [30,31]. The identity of CTL was positively verified by mass spectrometry. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Molecular Probes (Leiden, The Netherlands). c-Laurdan was kindly provided by professor Bong Rae Cho (Department of Chemistry and Center for Electro- and Photo-Responsive Molecules, Korea University 1-Anamdong, Seoul 136-701, Korea) and synthesized as described in reference 32. Probes were stored under argon in the dark at −87 °C until dissolved in argon-purged ethanol (CTL, c-laurdan) or methanol (DPH). The concentration of stock solutions of CTL, DPH, and c-laurdan was determined spectrophotometrically using their molar absorption coefficient (ε) values: 11,250 $M^{-1}\,cm^{-1}$ at 324 nm for CTL, 88,000 $M^{-1}\,cm^{-1}$ at 350 nm for DPH, and 20,000 $M^{-1}\,cm^{-1}$ at 365 nm for c-laurdan. Stock solutions of fluorescent reporter molecules were stored in the dark at −20 °C and used within a week. Phosphate buffered saline (PBS; 50 mM, pH 7.4 with 140 mM NaCl) was used as aqueous solvent in all studies. All other inorganic and organic chemicals used were of the highest purity available. The solvents used were of spectroscopic grade. Water was purified by reverse osmosis followed by passage through a Millipore UF Plus water purification system having a final resistivity of 18.2 MΩcm.

2.2. Synthesis and identification of hydroxylated SM

The hydroxylated SM analogs were prepared from sphingosyl phosphorylcholine and *N*-hydroxysuccinimide esters of the respective racemic hydroxy fatty acids. *N*-hydroxysuccinimide esters were prepared as described in [33]. Coupling of the activated fatty acid and sphingosyl phosphorylcholine was performed as described previously [34,35]. Products were isolated using preparative HPLC (Supelco Discovery C18 column; dimensions 250×21, 2 mm, 5 micron particle size, with 100% methanol as the mobile phase). The 2OH(D) analog of the PSM eluted later than the corresponding 2OH(L) analog (22 min vs. 18.5 min, respectively). Since the 2OH(D) form is the naturally occurring form in sphingolipids, we used this form in our experiments. Identification of products was done using ESI-MS and by comparing the retention times of hydroxylated ceramides (derived from the hydroxylated SM analogs using sphingomyelinase) with hydroxylated ceramide standards provided by Avanti Polar Lipids.

2.3. Preparation of vesicles

Lipid vesicles used in the study were prepared to a lipid concentration of 50 μM for fluorimetric studies. Required amounts of the lipids and probes were mixed and the solvents were evaporated under a constant flow of N_2 at 37 °C. When preparing liposomes with mixed lipid compositions, the lipids were redissolved in chloroform to assure a homogeneous mixing of the lipids. Once the lipids were thoroughly mixed, the solvent was redried to yield a lipid film. After further drying under high vacuum for at least 1 h at room temperature, the lipid mixtures were hydrated by adding PBS buffer. The temperature of the PBS buffer was kept above the gel–liquid crystalline phase transition temperature of the lipid with the highest melting temperature before addition to the dry lipid film. The lipid suspension was maintained above T_m during the hydration period of 20 min. Samples were then vortexed to disperse the lipids in the buffer. For fluorescence measurements, multilamellar vesicles were prepared by probe sonication (sonicated for 2 min with 20% duty cycle and 15 W power output) using a Branson probe sonifier (W-450; Branson Ultrasonics).

2.4. Fluorescence quenching measurements

In order to follow the formation and melting of ordered domains containing sterol, the steady-state quenching of CTL by the quencher 7SLPC was measured on a PTI QuantaMaster spectrofluorimeter (Photon Technology International, Lawrenceville, NJ, USA) [30]. The excitation and the emission slits were set to 5 nm and 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 70 °C at a rate of 5 °C/min. The measurements were done in quartz cuvettes with a light path length of 1 cm and the sample solutions were kept at a constant stirring (350 rpm/min) throughout the fluorescence measurement. Fluorescence intensity of CTL was detected with excitation and emission wavelengths at 324 nm and 374 nm. The fluorescent probe was protected from light during all the steps of experiments. Fluorescence emission intensity was measured in the *F* sample (quenched) consisting of POPC/7SLPC/SM analog/cholesterol, (30:30:30:10, molar ratio) and in

the F_0 sample (non-quenched), in which 7SLPC had been replaced with POPC. The fluorescence intensity in the F sample was divided by the fluorescence intensity of the F_0 sample giving the fraction of non-quenched CTL fluorescence plotted vs. the temperature. CTL replaced 1 mol% of cholesterol in the mixed bilayer.

2.5. Determination of steady-state fluorescence anisotropy

The steady-state fluorescence anisotropy of DPH was measured on a PTI QuantaMaster spectrofluorimeter operating in the T-format, essentially following the procedure described previously [36]. The wavelengths of excitation and emission were 360 nm and 430 nm, respectively. The steady-state anisotropy was calculated as described in reference 37.

2.6. Determination of c-laurdan fluorescence

The emission spectra of c-laurdan were determined in pure SM analog bilayers. The temperature for analysis was selected to be −5 °C or +5 °C from the T_m for each SM analog. Vesicles were prepared as described above. Each experimental temperature is indicated in the figure legend. The total lipid concentration in the assay was 50 μM and the c-laurdan concentration was 1 mol%. Excitation was at 365 nm and emission spectra were recorded between 390 and 550 nm.

2.7. CTL equilibrium partitioning between unilamellar vesicles and cyclodextrin

The distribution of CTL between methyl-β-cyclodextrin (Sigma Chemicals) and extruded large unilamellar phospholipid vesicles was determined as described in reference 38, using a method significantly modified from the procedure reported by Niu and Litman [39]. The assay yields the molar fraction partition coefficient, K_x, for CTL. A high K_x indicates a higher affinity of CTL for the bilayer as compared with methyl-β-cyclodextrin.

3. Results

3.1. DPH steady-state anisotropy

2-Hydroxylated cerebrosides have been reported to have increased T_m as compared with nonhydroxylated chain-matched species [10], suggesting that hydroxylation at the 2 position stabilizes intermolecular interactions. Similar effects of 2-hydroxylation was also seen in ceramide monolayers [11]. To examine whether 2-hydroxylation also stabilized SM interactions in bilayer membranes, we formed multilamellar vesicles from pure hydroxy-SM analogs and determined steady-state DPH anisotropy as a function of temperature (Fig. 1). PSM is known to undergo a gel–liquid transition around 41 °C [40,41], and this transition was reported by DPH (T_m at 40 °C). Bilayers formed from 2OH-PSM displayed a significantly higher gel–liquid transition temperature, and the transition appeared to be biphasic in contrast to the transition seen for PSM (Fig. 1). A similar stabilization of the gel phase by 2OH was seen in 2OH-22:0-SM bilayers when compared to the acyl chain-matched nonhydroxylated SM bilayer. When the hydroxyl group was on the third carbon of the *N*-linked palmitic acid (3OH-PSM), the gel–liquid transition occurred at a lower temperature (T_m, ~34 °C) compared to PSM. These DPH anisotropy results suggest that hydroxylation in the *N*-linked acyl chain of SM can both stabilize (2 position) and destabilize (3 position) intermolecular interactions, in a position-dependent manner. Since the hydroxyl has the D configuration in both analogs, different orientation is not the likely explanation for the observed difference but rather the position of the hydroxyl group relative to the interface.

3.2. c-Laurdan emission

Since both of the examined hydroxylated SM analogs have their hydroxyl groups close to the interface, it is expected that they contribute to interfacial hydration. The emission spectra of c-laurdan are sensitive to changes in interfacial hydration and lateral packing [42]. We therefore determined c-laurdan emission both in the gel state and in the liquid state of the two hydroxylated SM analogs. We selected to measure c-laurdan emission at temperatures that were 5 °C below (gel) or above (liquid) the measured T_m of the gel–liquid transition, and the spectra for the two hydroxylated SM analogs and PSM are shown in Fig. 2. In the gel phase, the c-laurdan emission spectra were very different for all three SMs. The spectra of c-laurdan in 3OH-PSM bilayers were blueshifted relative to PSM bilayers, whereas the spectra were redshifted for 2OH-PSM bilayers (Fig. 2, upper panel). In the liquid state, all SM analogs gave a c-laurdan emission maxima close to 470 nm, but the emission from 3OH-PSM around 410 nm differed somewhat from both PSM and 2OH-PSM bilayers.

3.3. Formation of sterol-enriched lateral domains

In complex bilayer membranes, one measure of sterol/SM interaction is the formation of lateral ordered domains, which are enriched in sterol and SM and depleted in the fluid matrix phospholipid. The presence of such sterol-enriched domains can be determined using a fluorescence quenching assay by which the lateral distribution of CTL is determined in ternary bilayer membranes containing both disordered (POPC) and ordered (SM) phospholipids together with cholesterol [30]. Quenching of CTL fluorescence is accomplished by 7SLPC which behaves like a fluid phospholipid and partitions preferentially with POPC in a ternary bilayer membrane. When cholesterol and CTL are forming ordered domains with SM, CTL is more protected against collision-induced quenching by 7SLPC (in the fluid phase). If quenching is determined as a function of temperature, sterol-enriched ordered domains can be seen to "melt" at temperatures which indicate the relative stability of such domains. Fig. 3 (panel A) shows three quenching curves for CTL in complex bilayer membranes containing either PSM, 2OH-PSM, or 3OH-PSM as the ordered lipid. Panel B of Fig. 3 shows corresponding melting curves for long-chain SM species. Cholesterol and CTL are known to form ordered domains with PSM in which CTL is protected against quenching by 7SLPC, and these domains have melted at temperatures around or slightly above 40 °C, with the midpoint of melting at 32–

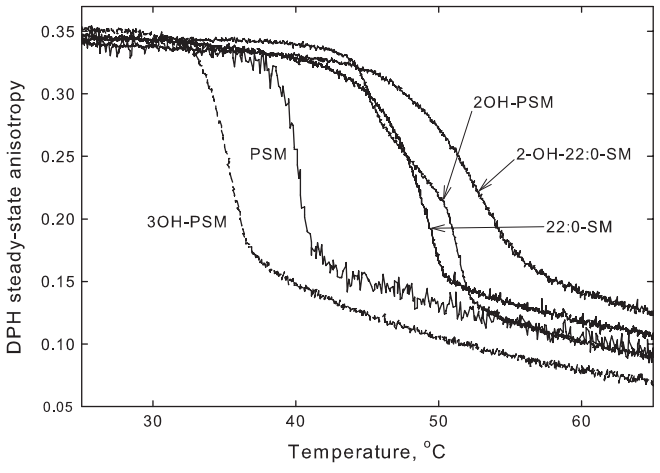

Fig. 1. Steady-state DPH anisotropy in multilamellar vesicles as a function of temperature. Vesicles were prepared from hydroxylated or nonhydroxylated SM species to a lipid concentration of 50 μM with 1 mol% DPH. Temperature was ramped at a rate of 2 °C/min. The curves are representative heating scans of several reproducible runs.

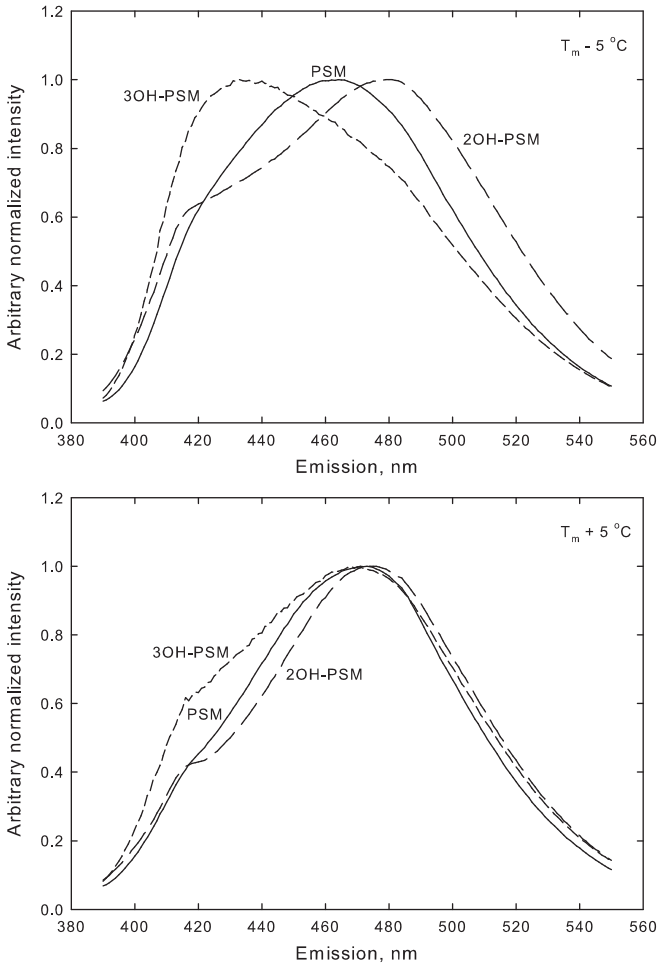

Fig. 2. c-Laurdan emission spectra from multilamellar vesicles containing PSM, 2OH-PSM, or 3OH-PSM. The total lipid concentration in the assay was 50 μM and the c-laurdan concentration was 0.1 mol%. Excitation was at 365 nm and emission spectra were recorded between 390 and 550 nm. The upper panel shows data obtained from the gel phase at T_m minus 5 °C, which corresponds to 36 °C for PSM, 42 °C for 2OH-PSM, and 30 °C for 3OH-PSM. The lower panels show data obtained from the fluid phase at T_m plus 5 °C, which corresponds to 46 °C for PSM, 52 °C for 2OH-PSM, and 40 °C for 3OH-PSM.

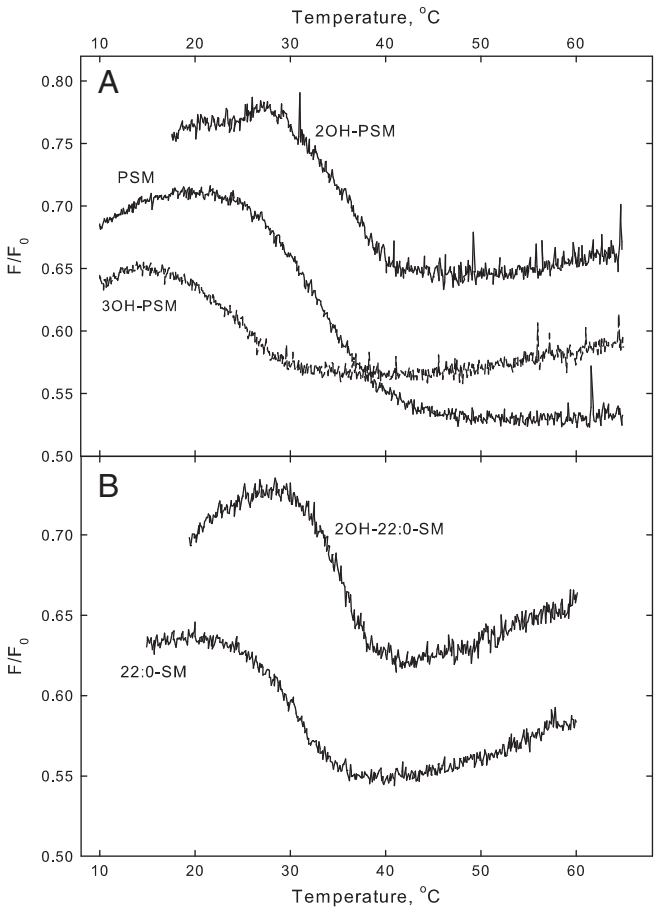

Fig. 3. Melting of ordered domains containing sterol observed from the quenching of CTL fluorescence. The melting profile is shown as the F (quenched)/F_0 (non-quenched) ratio plotted vs. temperature. The F sample consisted of POPC/7SLPC/SM/cholesterol/CTL (30:30:30:9:1 mol%), and in the F_0 sample, 7SLPC was replaced with POPC. The total lipid concentration was 50 μM and samples were heated at 5 °C/min. Panel A shows results for PSM and its hydroxylated analogs, whereas panel B shows results with 22:0-SM and 2OH-22:0-SM. The graphs show representative data from reproducible experiments.

33 °C (Fig. 3) [30]. 2OH-PSM formed CTL-enriched domains which were slightly more stable compared to the ordered domains seen for PSM; midpoint of melting, 35 °C (Fig. 3). 3OH-PSM also formed CTL-enriched domains in a similar manner as seen with both PSM and 2OH-PSM, but their thermostability was markedly diminished (midpoint of melting, 23 °C; Fig. 3A). For long-chain SM analogs, the CTL-enriched ordered domains with 2OH-22:0-SM were more stable than the domains formed together with 22:0-SM (Fig. 3B). The delta F/F_0 (maximum F/F_0 minus post-melting F/F_0) was smaller for the hydroxylated SM analog domains (and also the 22:0-SM domains) as compared to the PSM-containing domains, suggesting that the CTL (and sterol) contents in these were lower than in PSM-rich domains. These results indicate that, whereas cholesterol/CTL were able to interact with hydroxylated PSM analogs because they form ordered domains in which CTL was partially protected against quenching by 7SLPC, their affinity towards hydroxylated SM analogs appeared to be less than for nonhydroxylated SM species.

3.4. Sterol partitioning into bilayers containing SM analogs

A direct measure of sterol/SM interaction is to determine the affinity of the sterol for an SM-containing bilayer membrane. Relative sterol/SM affinity can be determined using an equilibrium partitioning assay in which a sterol is allowed to partition between SM-containing bilayer membranes and cyclodextrins [38,39,43]. We have examined the partitioning of 2 mol% CTL between methyl-β-cyclodextrin and acceptor vesicles (unilamellar) containing SM analogs and determined the molar fraction partitioning coefficient (K_x). Acceptor bilayers were prepared to contain 80 mol% POPC and 20 mol% SM analog, and equilibrium partitioning was followed at 37 °C (Table 1). As shown in the table, the K_x for CTL was markedly higher for PSM-containing bilayers (about 12.2 mM) as compared to pure POPC bilayers (about 5.9 mM). This difference demonstrates the high affinity that CTL has for PSM in a POPC matrix. This observation is consistent with similar data for comparable systems [38]. When PSM was replaced with 2OH-PSM or 3OH-PSM, the measured K_x was about 9.3 for 2OH-PSM and 7.2 for 3OH-PSM-containing bilayers. The CTL affinity for POPC bilayers containing 20 mol% 22:0-SM was about 9.4 mM, whereas the affinity to bilayers containing the 2OH-hydroxylated 22:0-SM was decreased to 7.4 mM (Table 1).The sterol partitioning assay indicates clearly that CTL has a reduced affinity to bilayer membranes containing hydroxylated SM analogs when compared to nonhydroxylated acyl-matched SM bilayers.

4. Discussion

Hydroxylations in sphingolipids is fairly common, both in the long-chain base and in the *N*-linked acyl chains (see references

3 and 4 and references therein). The hydroxyl groups are often located in the interfacial region (2OH in acyl chains, 3OH and 4OH in the long-chain base) and have been shown to stabilize interlipid interactions in model membrane systems [8–10]. The stabilization induced by the interfacial hydroxyl groups is most likely a result of increased possibilities for hydrogen bonding in the interfacial region within the molecules, between neighboring molecules, and to interfacial water molecules. For ceramides, Pasher [11] showed that the L-oriented 2OH appeared to prefer intramolecular hydrogen bonding, whereas the natural D-form of the 2OH appeared to prefer intermolecular hydrogen bonding. Such a difference in hydrogen bonding could also explain why the L- and D-form of hydroxy-SM analogs separated so efficiently on reverse-phase C18 HPLC and allowed for efficient isolation of the D-form of both 2OH-PSM and 3OH-PSM. Although separation of reverse-phase columns is mostly due to changes in solute hydrophobicity, differences on how OH groups can hydrogen bond intra- or intermolecularly can be envisioned to affect the overall balance of hydrophobicity and hydrophilicity in complex SM molecules and thus also affect their retention on C18 columns.

The hydroxyl group in the *N*-linked palmitic chain of SM dramatically affected intermolecular packing in a position-dependent manner, as evidenced by DPH anisotropy measurements of pure SM analog bilayers as a temperature function. The 2OH-SM bilayers had a markedly stabilized gel phase (against thermally induced melting) when compared to nonhydroxylated SM bilayers (Fig. 1). The shift in T_m was larger for hydroxylation in PSM than it was for hydroxylation in 22:0-SM, suggesting that chain length mismatch (and interdigitation) in 22:0-SM bilayers attenuated the stabilization induced by the 2OH function. Bilayers made from the 3OH(D)-PSM analog were clearly destabilized as compared to PSM, indicating that an OH on carbon 3 of the acyl chain caused intermolecular repulsion in the gel phase. This repulsion could have a steric origin or be caused by the polar hydroxyl group being located in a more hydrophobic environment away from the interfacial region.

In addition to affecting intermolecular interactions, the OH function in the SM palmitic chain also appear to affect interface hydration as probed by c-laurdan. Laurdan emission is sensitive to the polarity of its environment, and it displays a large redshift of its emission in polar solvents when compared to nonpolar solvents [42]. c-Laurdan is a modified laurdan probe with an additional carboxylic function on the laurdan amine, anchoring it efficiently to the membrane interface [32]. Going from the gel to the fluid phase, 3OH-PSM displayed the largest redshift of c-laurdan emission (~40 nm), whereas the redshift for PSM bilayers was much more modest (~10 nm). The emission maxima of c-laurdan in gel and fluid 2OH-PSM bilayers were almost unaltered. In these bilayers, the c-laurdan emission was very redshifted already in the gel phase, suggesting that the 2OH increased the hydration in the interface and around the probe despite the gel phase nature of the bilayer. This finding contrasts greatly with 3OH-PSM bilayers in their gel phase, in which c-laurdan emission was very blueshifted. The c-laurdan emission profile in the gel and fluid phases of 3OH-PSM was very similar to what has been reported for the respective phases of DPPC bilayers using laurdan as a probe [44]. It remains unclear whether 3OH-PSM bilayers really were less hydrated in their gel state compared to either PSM or 2OH-PSM or whether the 3 position of the OH interfered with the depth location of the c-laurdan probe.

To study how the hydroxylated PSM analogs were able to form sterol-enriched ordered domains in ternary bilayer systems also containing a fluid glycerophospholipid (POPC), quenching studies with CTL were performed. CTL and cholesterol form sterol-enriched lateral domains with PSM when admixed to a POPC bilayer [30]. CTL in ordered domains is more protected from collision-induced quenching by 7SLPC (which preferentially partitions into the fluid and disordered POPC-rich phase), but when temperature is increased, the ordered domains "melt" and CTL becomes more susceptible to quenching by 7SLPC [30]. This assay has been used by us to demonstrate whether or not an ordered lipid can form sterol-enriched domains in fluid POPC matrix bilayers [28,36,45–47]. Here, we were able to show that all SM analogs tested could form sterol-enriched ordered domains in a POPC bilayer (Fig. 3). The 2OH-PSM domains were slightly more stable as compared to PSM domains, whereas 3OH-PSM domains were markedly less thermostable compared to PSM (Fig. 3A). For long-chain SM analogs, the 2OH analog also formed more thermostable domains as compared to the nonhydroxylated analog (Fig. 3B). The stability of the ordered domains formed by the SM analogs in the ternary bilayers correlated with the T_m:s determined for pure SM analog bilayers using DPH (Fig. 1). The fraction of CTL fluorescence that was protected against quenching by 7SLPC was in general less for hydroxylated SM analogs as compared to chain-matched nonhydroxylated analogs This was deduced from the smaller difference in delta F/F_0 before (low temperature) and after (higher temperature) melting (Fig. 3) for CTL domains containing hydroxylated SM analogs. This suggests that the sterol content of the sterol-enriched domains varies between the three analogs so that it is highest for PSM and lower for all the other SM analogs. This could mean that the affinity of sterol (and CTL) to the ordered SM analogs differs with regard to chain length, the degree of hydroxylation and, possibly, to the OH position.

This possibility was explored by measuring CTL partitioning between bilayer membranes and cyclodextrins [38]. This assay yields the molar fraction partitioning coefficient for CTL and allows for the comparison of CTL affinities to different types of bilayer membranes. We found that while K_x for CTL was 5.9 for POPC bilayers at 37 °C, it was around 12 for POPC bilayers also containing 20 mol% PSM (Table 1). A higher K_x indicates higher affinity of the CTL for a bilayer as compared to cyclodextrin. When PSM was replaced with 2OH-PSM, the K_x of CTL decreased to about 9 and decreased further to about 7 when 3OH-PSM was used as the ordered lipid (Table 1). These results show that CTL (and sterol) affinity was relatively speaking highest for PSM, lower for 2OH-PSM, and lower still for 3OH-PSM. Its affinity to pure POPC bilayers was the lowest of the phospholipids tested. Interestingly, the CTL affinity to POPC bilayers containing 20 mol% saturated 22:0-SM was lower than it was for PSM-containing bilayers, suggesting that interdigitation and/or chain length mismatch affected sterol/SM interaction. Even with the long-chain SM analog, 2-hydroxylation appeared to slightly stabilize the sterol-containing ordered domain.

Taken together, our results demonstrate clearly that acyl chain hydroxylation in the interfacial region has dramatic effects on SM interlipid interaction and markedly attenuates SM/sterol interaction. This finding is interesting in light of an older study in which the 3OH group in the long-chain base of SM was shown not to markedly influence cholesterol exchange rates from bilayer membranes [48]. This difference further emphasizes the importance of hydroxyl group location on SM properties and interactions with sterols in membranes.

Table 1
Sterol partitioning between unilamellar vesicles and cyclodextrin. CTL partitioning at 37 °C was determined as described under Materials and methods. Values are averages from 3–5 separate experiments at each condition ± SD.

Vesicle composition (mol%)		K_x at 37 °C
POPC	100	5.9 ± 0.9
POPC:PSM	80:20	12.2 ± 1.8
POPC:2OH-PSM	80:20	9.3 ± 0.3
POPC:3OH-PSM	80:20	7.2 ± 0.3
POPC:22:0-SM	80:20	9.5 ± 0.2
POPC:2OH-22:0-SM	80:20	7.0 ± 0.6

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