Biochimica et Biophysica Acta 1828 (2013) 785–791

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Cholesterol's interactions with serine phospholipids — A comparison of *N*-palmitoyl ceramide phosphoserine with dipalmitoyl phosphatidylserine

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ARTICLE INFO

Article history:
Received 31 August 2012
Received in revised form 2 November 2012
Accepted 5 November 2012
Available online 13 November 2012

Keywords:
Sterol domain
Lateral structure
Lipid interaction
Laurdan
Cholestatrienol
trans-Parinaric acid

ABSTRACT

In this study we have prepared ceramide phosphoserine (CerPS) and examined its sterol-interacting properties. CerPS is a hydrogen-bonding sphingolipid, but its head group differs from that found in sphingomyelin (SM). Based on diphenylhexatriene steady-state anisotropy measurements, we observed that fully hydrated *N*-palmitoyl CerPS had a gel-to-liquid crystalline phase transition temperature of about 51 °C in 50 mM sodium phosphate buffer (pH 7.4). This was close to the T_m measured for 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine (DPPS) bilayers (T_m 50.5 °C). Based on cholestatrienol (CTL) quenching experiments in liquid disordered ternary bilayers (containing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphcholine; POPC), cholesterol/CTL formed sterol-enriched ordered domains with CerPS. These had similar thermostability as the sterol domains formed with *N*-palmitoyl SM. Cholesterol failed to form sterol-enriched ordered domains with DPPS under comparable conditions. Based on the equilibrium partitioning of CTL, we observed that the affinity of sterol for bilayers containing POPC/CerPS/cholesterol (6:3:1 by mol) was much higher than the affinity measured for control fluid POPC/cholesterol (9:1 by mol) bilayers, but slightly less than seen for comparable PSM-containing bilayers. We conclude that the phosphoserine head group was less efficient than the phosphocholine head group in stabilizing sterol/sphingolipid interaction. However, hydrogen bonding apparently can overcome some of the negative effects of the phosphoserine head group, since CerPS interacted more favorably with cholesterol compared to DPPS.

1. Introduction

The core structure of biological membranes is the phospholipid bilayer. While e.g., a monounsaturated phosphatidylcholine could make up a physically stable fluid bilayer, biological membranes are much more complex and contain many different lipid classes and thousands of different molecular species [1,2]. Clearly, lipid diversity is important for providing biological membranes with varying lateral and transmembrane structures, and for optimizing membrane protein function and solute permeability [3–7]. The transmembrane distribution of phospholipids in cell membranes is highly asymmetric [8–10], with all the aminophospholipids being in the endoleaflet of the membrane, while the outer leaflet contains most of the sphingolipids. The transbilayer distribution of cholesterol in cell membranes is not yet firmly established. While cholesterol must exist to some extent in the exoplasmic leaflet, because of the presence of sphingomyelin (SM) and other sphingolipids in that leaflet which it interacts with [11], some experiments suggest that most of cell membrane sterols would actually reside in the endoplasmic leaflet [12]. If that is the case, cholesterol must be able to interact with e.g., aminophospholipids which reside in that compartment.

Only a limited number of studies exist in which cholesterol/aminophospholipid interaction has been examined in more detail [13]. The maximum solubility of cholesterol in phosphatidylethanolamine bilayers has been shown to be markedly lower (50%) as compared to the solubility in phosphatidylcholine bilayers (66%) [14,15]. It was suggested that the phosphoethanolamine head group is not big enough to shield cholesterol from unfavorable water interactions at the membrane interface when adjacent to a small head group phospholipid. When the head group of SM was replaced with a phosphoethanolamine head group, the sphingolipid also lost its capacity to interact favorably with cholesterol [16,17], supporting the view that head group properties markedly influence phospholipid/cholesterol interaction. In cholesterol exchange studies, it has been established that both phosphatidylethanolamine and

Abbreviations: 7SLPC, 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine; CerPS, *N*-palmitoyl ceramide phosphoserine; CTL, cholesta-5,7 (11)-trien-3-beta-ol; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPS, dipalmitoyl-*sn*-glycero-3-phosphoserine; mβCD, methylβcyclodextrin; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PSM, *N*-palmitoyl-D-*erythro*-sphingomyelin; SM, sphingomyelin; tPA, *trans*-parinaric acid

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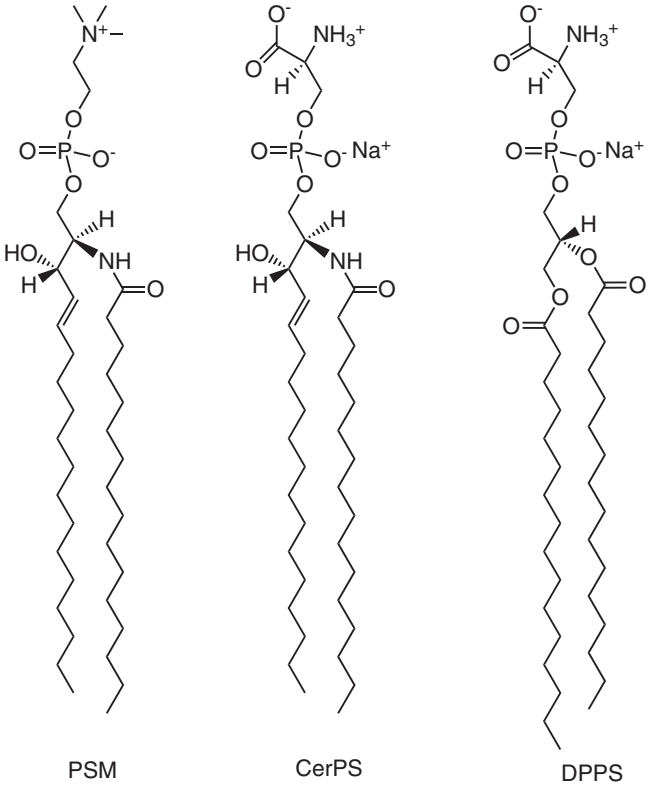
http://dx.doi.org/10.1016/j.bbamem.2012.11.009

phosphatidylserine are poor acceptors of cholesterol, when compared to phosphatidylcholines or sphingomyelins [18]. However, one equilibrium partitioning study has indicated that cholesterol can have a higher affinity to POPS bilayers as compared to POPC bilayers [19]. Data obtained from cholesterol desorption studies in monolayer membranes, on the other hand, suggest that cholesterol's interactions with aminophospholipids are less favorable than cholesterol's interactions with phosphocholine-containing phospholipids. The desorption rate of monolayer cholesterol to sub-phase cyclodextrin was much higher (i.e., less favorable interaction between cholesterol and phospholipid) from POPE and POPS monolayers (with 50 mol% cholesterol) when compared to a similar POPC monolayer (Fig. 7 in [2]). The slowest rate was seen with *N*-oleoyl SM, when mono-unsaturated phospholipids were compared.

The aim of the present study was to examine the interaction of cholesterol with saturated phospholipids which had phosphoserine as their head group function (Scheme 1). To allow for variation of hydrogen bonding properties in the interfacial region of the phospholipid studies, one phospholipid selected was *N*-palmitoyl ceramide phosphoserine (CerPS), and the other was 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine (DPPS). Although the sphingolipid long-chain base is synthesized from L-serine and palmitic acid, L-serine has not, to the best of our knowledge, been reported as a head group of biologically relevant sphingolipids. We show that cholesterol was able to form sterol-enriched ordered domains with CerPS in fluid bilayers, but failed to do so when DPPS was the ordered lipid. Both phospholipids displayed a similar gel-to-liquid phase transition temperature (T_m 50–51 °C), so their phase behavior was not the likely explanation for the difference in sterol interaction. We conclude that while the phosphoserine head group did not contribute to favorable cholesterol/phospholipid interaction, increased interfacial hydrogen bonding (in CerPS) could compensate and still make cholesterol/CerPS interactions possible.



Scheme 1. Molecular structures of the saturated phospholipids used in the study.

2. Material and methods

2.1. Materials

High purity POPC and egg SM (99%+) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. PSM was isolated to 99% purity from egg SM as described [20]. Cholesterol (99% pure) and mβCD were 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-(7-doxyl) stearoyl-*sn*-glycero-3-phosphocholine (7SLPC) [21]. Stock solutions of lipids were prepared in methanol/hexane (9:1 by vol), and kept at −20 °C. All lipid solutions were taken to ambient temperature before use. The concentration of all phospholipid solutions was determined by phosphate assay [22], subsequent to total digestion by perchloric acid.

Cholesta-5,7,9(11)-triene-3-beta-ol (CTL) was synthesized and purified as described [23,24]. DPH and laurdan were purchased from Molecular Probes (Leiden, the Netherlands). Fluorescent probes were stored under argon at −87 °C until dissolved in argon-purged ethanol (for CTL) or methanol (for diphenylhexatriene (DPH), laurdan). The concentration of stock solutions of the fluorophores was determined with spectroscopy using their molar absorption coefficients (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 laurdan. Stock solutions of fluorescent reporter molecules were stored at −20 °C and used within a week. Water was purified by reverse osmosis followed by passage through a Millipore UF Plus water purification system having final resistivity of 18.2 MΩcm.

N-palmitoyl-D-*erythro*-ceramide phosphoserine was prepared via a 15 step synthesis approach in 4.5% overall yield. Details of the synthesis can be found in the Supplemental information.

2.2. Preparation of vesicles

Lipid vesicles used in the study were prepared as described previously [20,25], to a lipid concentration of 0.050 mM for fluorescence studies. The solvent was 50 mM sodium phosphate buffer (pH 7.4, with 140 mM NaCl). Vesicles were prepared by probe sonication (sonicated for 2 min 10% duty cycle 15 W power output with a Branson W-450 sonicator) (Branson Ultrasonics Corporation, CT, USA) unless otherwise indicated. The mild sonication yielded polydisperse and mostly multilamellar vesicles (200–800 nm diameter), as determined using a Malvern zeta-sizer instrument. Domain formation, as revealed by CTL or tPA quenching, is not sensitive to the size or lamellarity of vesicles, since multilamellar and extruded unilamellar (about 200 nm) yield very similar results for the compositions studied (unpublished observations).

2.3. Determination of steady-state DPH anisotropy

The steady-state fluorescence anisotropy of DPH was measured on a PTI Quanta-Master spectrofluorimeter (Photon Technology International, Inc. NJ, USA) operating in the T-format, essentially following the procedure described in [20]. Briefly, multilamellar vesicles were prepared from CerPS or DPPS, and contained 1 mol% DPH. The wavelengths of excitation and emission were 360 nm and 430 nm, respectively. The steady state anisotropy was calculated as described in [26]. The temperature gradient was 5 °C/min. T_m-values extracted from the DPH anisotropy experiments were taken as the temperature of the midpoint anisotropy change (between the start of the cooperative transition and the end of it).

2.4. Laurdan emission spectra

The multilamellar bilayer vesicles were prepared as described above (2.2.) with 1 mol% of laurdan. Laurdan was excited at 385 nm

and the emission spectra were detected at 5 °C below and above the melting temperature of PSM, CerPS or DPPS between 400 and 550 nm on a PTI QuantaMaster-3 spectrofluorimeter working in the T-format. The emission and excitation slits were set to 5 nm and the temperature was controlled by a Peltier element with a temperature probe submerged in the samples. The temperature resolution of the temperature probe was ±0.1 °C. The samples were constantly stirred during measurement.

2.5. Fluorescence quenching measurements

The fluorescence quenching of CTL or tPA by the quencher 7SLPC was measured on a PTI Quanta-Master spectrofluorimeter to observe the melting of ordered domains in the vesicles [27,28]. The excitation and emission slits were set to 5 nm and the temperature was ramped from 10 °C to 70 °C with a rate of 5 °C/min. Wavelengths for excitation and emission were 324/374 nm for CTL, and 305/405 nm for tPA, respectively. The F_0 (unquenched) samples were prepared with 60 nmol POPC, 10 nmol cholesterol (including 1 nmol CTL) and 30 nmol of PSM, CerPS or DPPS. The F (quenched) sample was identical to the F_0 sample with the exception of 30 nmol of POPC which was replaced by 7SLPC. 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 versus the temperature.

2.6. Life-time analysis of trans-parinaric acid

The life-time analysis of tPA in POPC/PSM/cholesterol (60:30:10 by mol) vesicles, or in vesicles in which PSM was replaced with either CerPS or DPPS, was determined using a PicoQuant Fluotime 200 instrument (PicoQuant GmbH, Berlin, Germany). A PLS300 led laser (298 nm) was used for excitation. Analysis was performed with sonicated multilamellar vesicles (100:0.5 lipid:tPA molar ratio) at 23 °C. Data analysis was performed using PicoQuant FluoFit software.

2.7. CTL partitioning assay

The distribution of CTL between mβCD and extruded large unilamellar phospholipid vesicles was determined as described in [29,30]. 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 to mβCD.

3. Results

3.1. Properties of pure phospholipid bilayers

Analysis of the steady-state DPH anisotropy in pure phospholipid bilayers reveals the T_m of the main transition, because the anisotropy of DPH changes dramatically when the gel phase is turned into a fluid disordered phase, with concomitant changes in the rotational freedom of the DPH molecule. Our analysis showed that the gel-to-liquid crystalline phase transition in CerPS bilayers occurred at 51 °C with the transition taking place over a temperature interval of about 5 degrees (Fig. 1). This T_m is significantly higher than observed for *N*-palmitoyl ceramide phosphocholine (or PSM, 41 °C, [31–33]), but lower than the temperature reported for *N*-palmitoyl ceramide phosphoethanolamine (65 °C, [16]). For pure DPPS bilayers, the observed T_m was ~50.5 °C (Fig. 1), i.e., close in temperature to that measured for CerPS. Previous literature values for the T_m of DPPS main phase transition are close to 53 °C [34–36]. It should be noted that the T_m of anionic phospholipids is markedly dependent on both pH and the presence of counter ions (the T_m of both DPPS and CerPS was close to 59 °C when vesicles were prepared in Dulbecco's phosphate-buffered saline — data not shown). The DPH anisotropy data also revealed that CerPS was more disordered (lower anisotropy) than DPPS at temperatures above the T_m (Fig. 1).

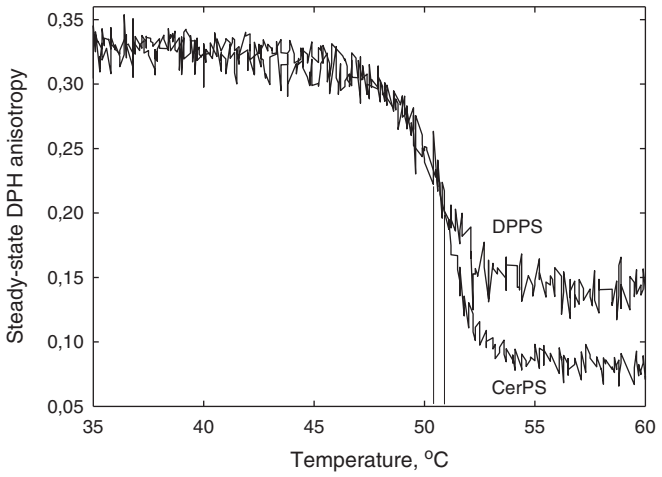


Fig. 1. Steady-state DPH anisotropy in pure phospholipid bilayers. Multilamellar vesicles containing CerPS or DPPS were prepared to a final concentration of 0.05 mM. DPH was added to 1 mol%. The temperature was ramped at 5 °C/min and the anisotropy plotted as a temperature function. Representative scans are shown. The left vertical line indicated T_m for DPPS phase transition, and the right vertical line T_m for CerPS phase transition.

The emission of laurdan is sensitive to changes in interfacial hydration [37], which in turn also is influenced by lateral packing. Therefore laurdan reports a very blue-shifted emission from gel phases compared to more red-shifted emission from fluid phase bilayers. In order to compare emission from different bilayer types, one has to assume that the bilayer depth of laurdan is unaffected by the type of phospholipid presented in the bilayer. The emission or laurdan fluorescence from gel-phase PSM bilayers has been shown to be more red-shifted than from e.g., dipalmitoyl phosphatidylcholine [38], suggesting that PSM bilayer interfaces are more hydrated (or more polar) possibly because of the more extensive hydrogen-bonding among PSM molecules. In this study, the PSM bilayers in the gel state gave more red-shifted emission as compared to both CerPS and DPPS (Fig. 2A), suggesting that laurdan experienced a more hydrated interface in PSM bilayers. In the liquid-crystalline phase, laurdan reported very similar emission spectra from both PSM and CerPS bilayers, whereas DPPS bilayers still gave very blue-shifted emission (Fig. 2B). Since the interfacial hydrogen bonding network is likely to be fairly similar for PSM and CerPS, the difference in gel phase emission is likely to derive from direct effects from the head group on interfacial hydration.

The life-time analysis of tPA can yield useful information about the nature of ordered phases, since it partitions preferentially into ordered phases and since its emission life-time is so markedly affected by the nature of the ordered phase [39,40]. We measured tPA life-times in ternary bilayer systems containing POPC/ordered lipid/cholesterol (60:30:10 by mol). The life-time analysis of tPA in all systems tested could best be described by three life-time components, a long one (>35 ns), one intermediate (~13–16 ns), and a shorter component (<6 ns) (Table 1). For the POPC/PSM/cholesterol bilayers, the long life-time component was about 38 ns, whereas the shorter life-time components were 16 and 5.7 ns. Very similar life-time behavior of tPA was observed in the POPC/CerPS/cholesterol bilayers (Table 1), suggesting that the phosphoserine head-group on ceramide did not change the ordered phase properties significantly when compared to PSM. However, if the phosphoserine was linked to a diacylglycerol (as in DPPS) in the POPC/ordered lipid/cholesterol bilayer, the long tPA life-time component increased to about 50 ns, with only small changes in the other life-time components (Table 1). The long life-time component of tPA in DPPS-containing bilayers suggests that the ordered phase was much more gel-like compared to the ordered domains formed by either PSM or CerPS under similar conditions. Such a gel-like state could be expected if cholesterol was not present in the DPPS-rich

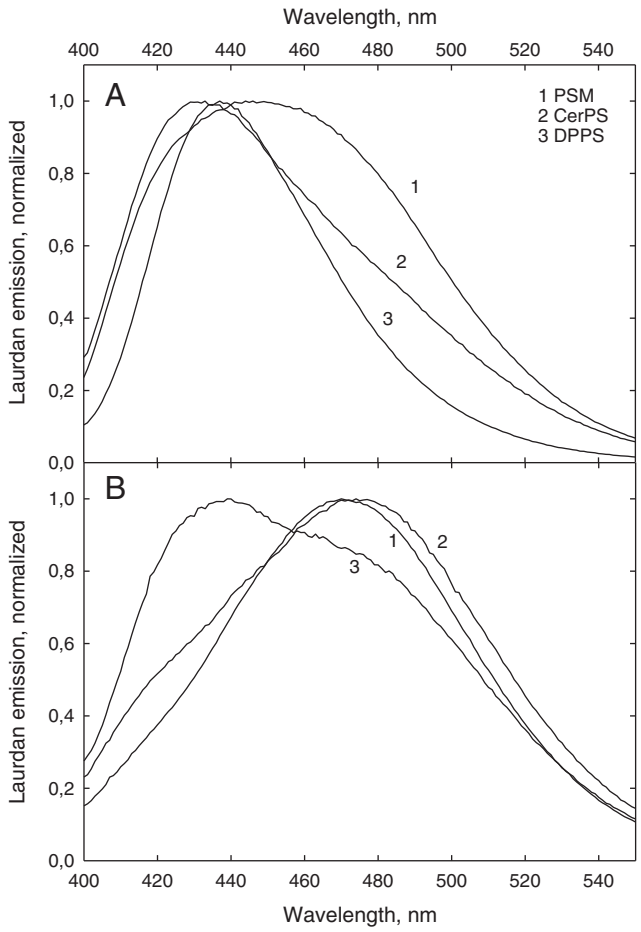


Fig. 2. Laurdan emission profile from bilayer membranes in their gel or fluid state. Multilamellar vesicles made from PSM, CerPS or DPPS were prepared to a final concentration of 0.05 mM. Laurdan was included at 1 mol%. The normalized laurdan emission was determined from vesicles in their gel state (panel A, the temperature was 36, 45 and 45 °C, for PSM, CerPS, and DPPS, respectively) or their fluid state (panel B, the temperature was 46, 55 and 55 °C, for PSM, CerPS, and DPPS, respectively).

domain, suggesting that no or very little l_o phase was formed. To further explore this possibility, we compared sterol interaction with the ordered phospholipids in similar ternary bilayers.

3.2. Interaction of sterol with CerPS and DPPS in complex bilayers

To study how the head group of CerPS affected interactions with cholesterol and CTL, we measured the formation of ordered sterol-enriched domains in ternary bilayers also containing POPC. With our CTL quenching assay [28], we are able to define whether possible ordered domains also contain sterol. Ordered domains which do not necessarily contain sterol can be detected using our tPA quenching assay [41]. As shown in Fig. 3A, CTL quenching as a temperature function reveals the presence of a PSM and sterol-enriched domain in the POPC bilayer. This PSM-domain melted with a T_m of about 30 °C and had completely melted around 40 °C. CerPS also appeared to form a sterol-enriched domain in the POPC-bilayer (Fig. 3A), and the ordered domain melted with an apparent T_m of about 28 °C and end-of-melting temperature of 35 °C. This CerPS-rich domain was thus less thermostable than the PSM domains, despite the fact that pure CerPS bilayers had a higher phase transition temperature than PSM bilayers. The delta F/F_0 (difference in F/F_0 before and after melting) also was less for CerPS domains as compared to PSM, domains, suggesting that the CTL (and thus cholesterol) concentration was less in these ordered domains. DPPS failed to form sterol-enriched ordered domains, as indicated by the monotonous F/F_0-temperature function (Fig. 3A). Based on tPA quenching (Fig. 3B), it was clear, however, that DPPS formed ordered domains in the POPC bilayer, they just did not contain significant amounts of sterol. Similarly, tPA quenching also detected ordered domains for PSM and CerPS in POPC bilayers. The tPA-reported end-of-melting for PSM domains was similar to the values obtained from CTL quenching. However, with CerPS domains, tPA reported a slightly higher end-of-melting than CTL did (Fig. 3A and B). The problem with the F/F_0 ratio exceeding unity is often seen with tPA as probe (but never with CTL). It may relate to a quencher dependent difference in phase partitioning of the fatty acid.

The determination of the affinity of CTL for a bilayer is another way to obtain information about sterol/phospholipid interaction in the bilayer. If the interaction is "unfavorable", the equilibrium partition coefficient of CTL is lower than for situations where sterol/phospholipid interaction is more favored. We carried out such measurements for bilayer systems which had similar compositions as in our quenching assays, and determined the K_x at two different temperatures (23 °C and 37 °C, Fig. 4). The K_x of CTL is fairly low for pure POPC bilayers, because sterols do not prefer to interact with disordered acyl chains [29]. The inclusion of 10 mol% cholesterol in the POPC bilayer increased the K_x significantly at 23 °C, reflecting a cholesterol-induced ordering of POPC acyl chains which also increased CTL affinity to the bilayers. This effect was much smaller at 37 °C. Inclusion of 30 mol% PSM in the POPC and cholesterol-containing bilayer increased the K_x of CTL dramatically (Fig. 4). PSM has previously been shown to markedly increase the partition coefficient of both CTL and cholesterol [19,29]. The effect on K_x is higher at 23 °C than at 37 °C, reflecting a higher disorder at elevated temperatures which leads to less favorable interactions. CerPS was also able to increase the affinity of CTL for the bilayer membranes, confirming that sterols were able to interact with this ordered lipid. The K_x for CerPS-containing bilayers was, however, slightly lower when compared to PSM-containing bilayers. At 23 °C, the presence of 30 mol% DPPS did not increase the K_x for CTL over the level found for POPC/cholesterol (9:1), confirming that cholesterol and CTL were not interacting favorably with this ordered phospholipid. At 37 °C, the K_x for DPPS-containing bilayers was similar to the value observed at 23 °C (Fig. 4), but still significantly less than measured for PSM or CerPS at 37 °C.

4. Discussion

The interaction of cholesterol with phospholipids in bilayer membranes is in part influenced by the acyl chain composition of the phospholipids, and by their polar head group properties. Cholesterol

Table 1
Life-time analysis of tPA in ternary bilayers containing POPC, saturated lipid, and cholesterol.

Composition	τ_1	f_1	α_1	τ_2	f_2	α_2	τ_3	f_3	α_3
POPC/PSM/Chol	37.7 ± 3.7	17.1 ± 0.9	5.9 ± 0.6	16.0 ± 0.7	59.3 ± 1.8	41.8 ± 1.1	5.7 ± 0.2	26.8 ± 1.7	53.2 ± 1.8
POPC/CerPS/Chol	39.7 ± 4.6	24.1 ± 1.6	6.7 ± 0.6	12.9 ± 0.8	61.3 ± 2.4	50.0 ± 2.1	4.8 ± 0.3	20.9 ± 2.4	44.2 ± 2.9
POPC/DPPS/Chol	49.5 ± 1.6	46.5 ± 7.2	15.2 ± 3.7	14.1 ± 1.6	44.4 ± 6.7	44.8 ± 4.8	5.0 ± 0.4	14.9 ± 1.5	42.5 ± 1.9

The molar ratio of lipids in the bilayers was 60:30:10. Measurements were performed at 23 °C. Values are averages from n = 4 ± SEM. Life-times (ns) τ; fractional intensities (%) f; fractional amplitudes (%) α.

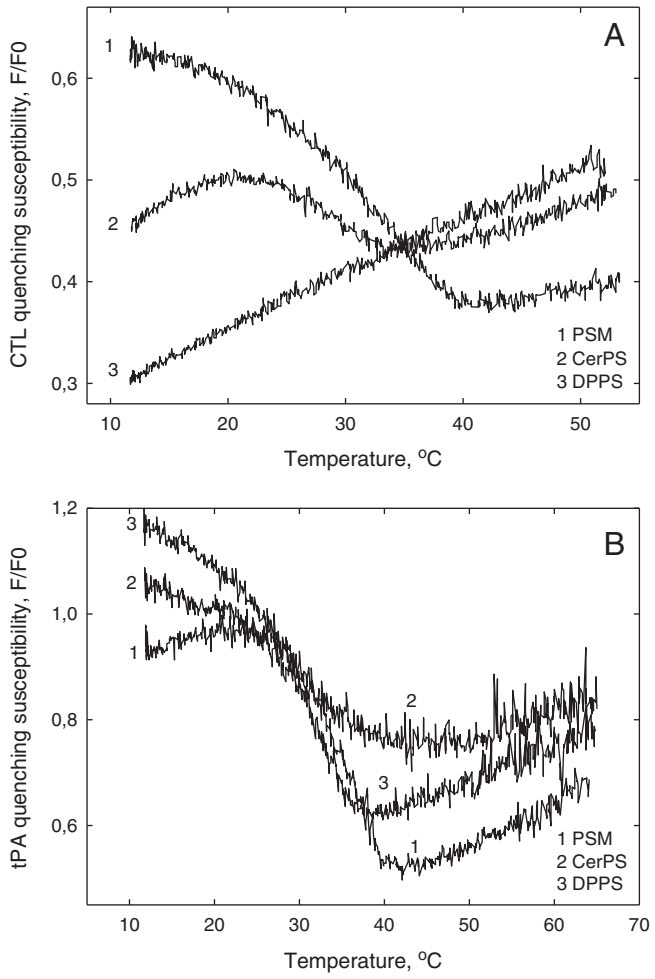


Fig. 3. Formation of ordered domains in ternary bilayer membranes. Multilamellar vesicles containing POPC/saturated phospholipid/cholesterol (60:30:10 by mol) were prepared (F_0-sample) to a final lipid concentration of 0.05 mM. 1 mol% of either CTL (panel A) or tPA (panel B) was included. Quenching of fluorescence was achieved by 7SLPC (50% of POPC was replaced by 7SLPC in F-samples). Samples were ramped at 5 °C/min. Representative scans are shown.

prefers to interact with phospholipids having more ordered acyl chains [20,29] when compared to more disordered phospholipids [42–44]. The interaction of cholesterol with a highly disordered acyl chain would impose a large entropy penalty compared to the situation when cholesterol can interact with an already more ordered acyl chain [45]. The head group size, hydrogen bonding properties, and charge are also important modulators of cholesterol's interactions with a phospholipid (or a glycosphingolipid for that matter) [15,16,46,47]. However, also differences in the interfacial properties of bilayer phospho- and sphingolipids will markedly affect cholesterol's ability to interact [48]. In this study we compared how the head group (CerPS versus PSM) affected interlipid interactions in bilayer membranes. We also examined how interfacial differences affected interlipid interactions, when the head group was phosphoserine (CerPS versus DPPS).

Pure CerPS bilayers had the gel-to-liquid crystalline phase transition centered at 51 °C, which is considerably higher than the T_m for pure PSM bilayers (41 °C, [31–33]). Hydrogen bonding and other electrostatic interactions among the head groups are likely to explain the observed gel phase stabilization in the CerPS bilayers [49]. A similar degree of gel phase stabilization has also been reported for DPPC and DPPS, whose main transition temperatures are 41 and 53 °C, respectively [34–36,50]. The importance of hydrogen bond interactions in the head group region of DPPS bilayers was clearly demonstrated by Hauser and collaborators, who showed that the T_m of *N*-methylated DPPS was 8 degree lower than the T_m of DPPS [36]. In the *N*-methylated DPPS molecule hydrogen bonding involving the head group NH_2 is abolished.

Fully hydrated DPPS bilayers, contrary to DPPC bilayers, do not show a pre-transition endotherm with DSC [36]. This may indicate that the orientation of the phosphoserine group is less parallel with the membrane bilayer plane as compared to the phosphocholine group [16,36,51]. At least for PSM bilayers, a phosphate oxygen in the head group is involved in hydrogen bonding with the 3OH of the long chain base [52,53]. This bond is likely to affect the orientation of the phosphocholine headgroup, and in this respect PSM and e.g., DPPC must differ, since similar hydrogen bonding is not possible in DPPC. It is unclear if the phosphoserine head group of CerPS is able to interact electrostatically with the 3OH of the long-chain base, but the high T_m of the gel phase melting suggests that such interaction would not be very likely.

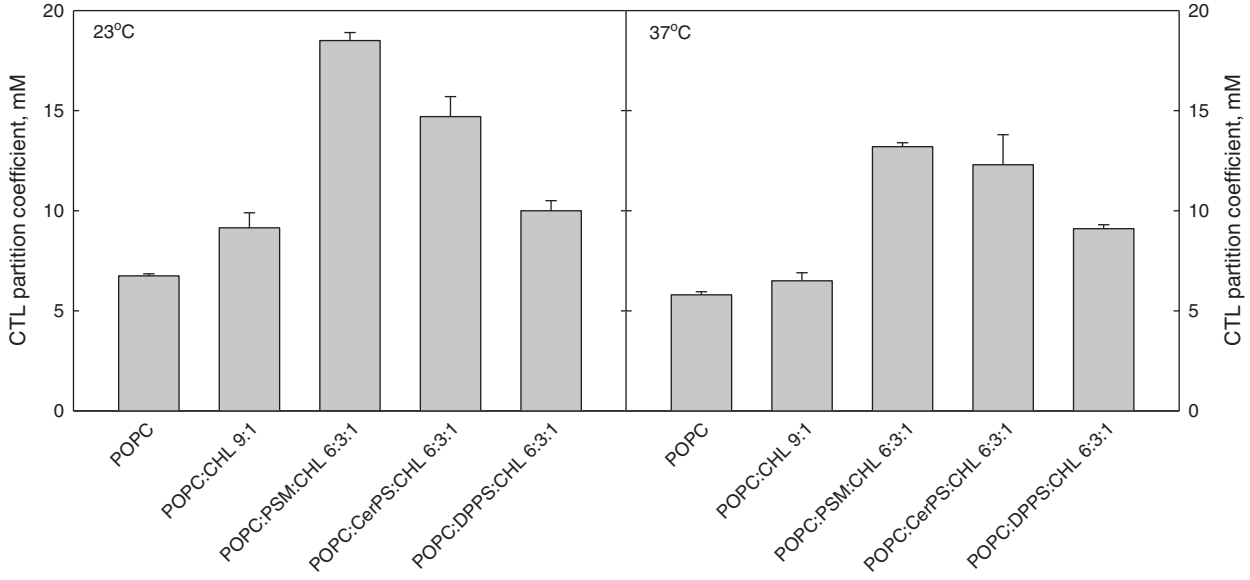


Fig. 4. Equilibrium partitioning of CTL between mβCD and unilamellar vesicles. The vesicle composition is indicated for each bar, with conditions for 23 °C shown to the left, and for 37 °C to the right. Values are averages from 3 different experiments.

Our laurdan emission spectra suggested that the gel phase CerPS bilayers were less hydrated compared to PSM, whereas the difference in apparent hydration became much smaller in the liquid-crystalline phase. The gel phase DPPS bilayer was even less hydrated than either CerPS or PSM bilayers, especially in the liquid crystalline phase (Fig. 2). This observation shows how the interfacial hydrogen bonding groups in both CerPS and PSM (2NH and 3OH) affected interfacial hydration. These functional groups are missing in DPPS. A comparison of laurdan emission profiles of DPPS and DPPC bilayers in the liquid crystalline phase reveals further that DPPS bilayers had much more blue-shifted emission compared to DPPC bilayers (Fig. 2, this study, and Fig. 2 in [38]). This marked difference in interfacial hydration also suggests that the phosphoserine and phosphocholine head groups directly affected interfacial hydration levels (differently), even in the more disordered and less densely packed liquid crystalline phase.

The interaction of cholesterol/CTL with the saturated phospholipids was examined in ternary bilayer membranes also containing the fluid POPC. This setup has the benefit of allowing the sterol component to choose its interaction partners. Based on a ternary phase diagram, the POPC/PSM/cholesterol (60:30:10 by mol) system at 23 °C should contain both liquid disordered (POPC rich), liquid ordered (PSM and sterol-enriched) and gel phase components (PSM) [54]. The POPC-rich component dominates, and most of the PSM is present as a gel phase. At 37 °C, the PSM gel phase has melted and the proportion of the liquid ordered phase has increased [55]. While we do not have access to similar data for the POPC/CerPS/cholesterol or POPC/DPPS/cholesterol systems, we can assume that the saturated phospholipids are dominant in the gel phase at 23 °C, and maybe also at 37 °C (because of their high T_m). With this background, we can now try to understand our data.

Our CTL quenching assay suggests that sterols (including cholesterol) formed a sterol-enriched phase with both CerPS and PSM, whereas they did not appear to form it with DPPS. The sterol concentration in the sterol-enriched CerPS domains appeared to be slightly less as compared to the sterol-enriched PSM domain (smaller F/F_0 amplitude), reflecting lesser affinity of CTL/cholesterol for CerPS compared to PSM (also shown in the K_x reported in Fig. 4). The sterol-enriched ordered domains observed with CTL quenching in the PSM- or CerPS-containing bilayers are most likely a sterol-containing gel phase [27], but the presence of a liquid-ordered phase cannot be ruled out for the CerPS bilayers. Direct comparison to published phase diagrams of e.g., POPC:PSM:cholesterol is difficult to do, since the presence of 7SLPC in some samples (F-samples) may obscure domain boundaries. On the other hand, quenching data (which involve 7SLPC) agree very nicely with DSC data for similar systems (see Fig. 1 in [27]), suggesting that 7SLPC does not markedly change the properties of the disordered phase.

The tPA life-time data lend support to the findings that sterols form both a liquid-ordered and a gel phase with PSM and apparently also with CerPS at 23 °C, since both systems gave very similar life-time components for tPA (Table 1). However, DPPS, which did not interact well with sterols in the POPC bilayer, appeared to be dominant in a gel phase at 23 °C even when cholesterol was present, as indicated by the very long life-time component of tPA (τ_1 was 49.5 ns for DPPS-containing bilayers, while it was below 40 ns for both PSM and CerPS-containing bilayers). The life-time of tPA is known to be very long in gel phase environments [39,40,56].

Our finding that cholesterol and CTL did not appear to interact favorably with DPPS is in line with old cholesterol exchange data, showing that phosphatidylserine/cholesterol interaction is much less favored than e.g., sphingomyelin/cholesterol interaction [2,18,57]. Cholesterol/sphingomyelin interaction is known to be stabilized by hydrogen bond-formation involving the interfacial functional groups (2NH and 3OH of PSM) [48]. Those same functional groups are of course present also in CerPS (while absent from DPPS), and are therefore likely to facilitate sterol/CerPS interactions. In DPPS-containing bilayers, it appeared that cholesterol preferred to interact with POPC over DPPS, at least when DPPS was in a gel state (i.e., at 23 °C). This was shown by the absence of sterol-enriched DPPS-domains at this temperature (Fig. 3), and also by the low CTL partitioning coefficient in POPC/DPPS/cholesterol bilayers (Fig. 4).

Sterols do not interact with all sphingolipids, irrespective of their head groups. We have shown previously that sterol/cerebroside interactions are not favored, probably because the small sugar head group does not efficiently protect sterols from unfavorable interactions with interfacial water [58]. Removal of a single methyl from the PSM choline moiety also makes sterol/sphingolipid interactions unfavorable [16]. The phosphoserine head group somehow made sterol/DPPS interactions unfavorable, but with CerPS the interfacial hydrogen bonding capability apparently compensated and made sterol/CerPS interactions possible (at least more favorable than sterol/POPC interaction). This finding strongly demonstrates the importance of interfacial hydrogen bonding in stabilizing sterol/sphingolipid interactions.

Acknowledgements

We thank Dr. Anders Björkbom for helpful comments on the manuscript. Funding was provided by Åbo Akademi University and the Sigrid Juselius foundation (JPS), and by the Ministry of Education, Culture, Sports, Science and Technology and Matching Fund Subsidy for a Private University, Japan (SK).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2012.11.009.

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