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Biochimica et Biophysica Acta 1715 (2005) 111 – 121
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Displacement of sterols from sterol/sphingomyelin domains in fluid bilayer membranes by competing molecules

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Received 3 June 2005; received in revised form 5 August 2005; accepted 5 August 2005
Available online 19 August 2005

Abstract

The formation of sterol and palmitoyl sphingomyelin enriched ordered domains in a fluid bilayer was examined using domain selective fluorescent reporter molecules (cholestatrienol and *trans*-parinaric acid containing lipids) together with a quencher molecule in the fluid phase. The aim of the study was to explore how stable the ordered domains were and how different, biologically interesting, membrane intercalators could affect domain stability and sterol distribution between domains. We show that sterols easily can be displaced from ordered domains by a variety of saturated, single- and double-chain membrane intercalators with a small polar group as a common denominator. Of the two-chain intercalators examined, both palmitoyl ceramide and palmitoyl dihydroceramide were effective in displacing sterols from ordered domains. Of the single-chain intercalators, hexadecanol and hexadecyl amide displaced the sterol from sterol/sphingomyelin domains, whereas palmitic acid, sphingosine and sphinganine failed to do so. All molecules examined stabilized the sphingomyelin-rich domains, as reported by *trans*-parinaric-sphingomyelin and by scanning calorimetry. Parallels between the displacement of sterol from ordered domains in our model membrane system and the ability of the above mentioned molecules to alter the chemical activity and distribution of sterols in biological membranes are discussed.

Keywords: Cholesterol; Cholestatrienol; Sterol displacement; Quenching; Spin-labeled phosphatidylcholine; Ceramide

1. Introduction

In biological membranes, the lipids and proteins are organized into domains of different lipid lateral diffusion. Such domains can be formed due to lipid–lipid interactions, in which case domain formation is dependent on the structure and biophysical properties of the lipid components. The formation of ordered domains, also called rafts, in biological membranes is favored by the presence of long-chain saturated sphingolipids as well as by the presence of cholesterol [1–3]. The high content of sphingolipids and cholesterol in these membrane domains has consequently directed the focus of research in this field to elucidate the rules governing sterol and sphingolipid-induced domain formation and stability, as well as intermolecular interaction.

Cholesterol has important effects on the properties of lipids in membranes. Cholesterol causes phase-separation into cholesterol-poor and cholesterol-rich domains in bilayer membranes containing both low and high T_m lipids [4–8]. Cholesterol and sphingomyelin have long been implicated to exhibit a preferential interaction in model and biological membranes. They co-localize in the plasma membrane of mammalian cells and on the surface of lipoprotein particles [9,10]. Cholesterol homeostasis in cells is affected by both the depletion and enrichment of plasma membrane sphingomyelin [11–15]. In model membrane studies, the interaction between cholesterol and sphingomyelin and the formation of domains in bilayers by these two lipids has been given a lot of attention in recent years [16–20]. Cholesterol (or a related sterol) promotes phase separation of saturated sphingomyelins and is essential for the formation of the liquid-ordered phase at higher cholesterol

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0005-2736/$ - see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.bbamem.2005.08.002

concentrations [21–23]. However, cholesterol is also able to form ordered domains with saturated phosphatidylcholines in model membranes [4,6,24]. The interaction with cholesterol is very sensitive to the length and saturation of the acyl-chains of the phosphatidylcholines [25,26]. Cholesterol interaction with sphingomyelin seems to be less dependent on the chain length [27]. However, the tendency of sphingomyelin and cholesterol to form domains is greatly reduced if the acyl chain in sphingomyelin is unsaturated [26–28]. The molecular packing in cholesterol/sphingomyelin bilayers is known to differ from that of cholesterol/phosphatidylcholine membranes as evidenced by bilayer compressibility and water permeability studies [29,30].

In recent studies, it has been shown that ceramide is able to compete with cholesterol for association with ordered domains in bilayer membranes [31,32]. These findings may imply that ceramides have a more favored interaction or miscibility with sphingomyelin as compared with cholesterol. Megha and London speculate in their recent study that the displacement can be due to cholesterol and ceramide having structures that allow tight packing with saturated phospho- and sphingolipids [32]. In another recent study, Lange and co-workers showed that cultured cells which were exposed to millimolar concentrations of octanol displayed an increased mobilization of plasma membrane cholesterol into the endoplasmic reticulum [33], in analogy with what has been shown for sphingomyelinase-treated cells [34]. It seemed likely that simple single-chain alcohols (e.g., octanol) may indeed interfere with cholesterol-miscibility in sphingolipid-rich domains in a similar manner as shown for ceramides. In this model membrane study, we have therefore studied how different simple membrane intercalators with a small polar function (analogously to ceramide and cholesterol) affect the lateral distribution of sterols in complex bilayer membranes containing fluid phospholipids (POPC) and saturated sphingomyelins.

2. Materials and methods

2.1. Materials

D-*erythro*-*N*-palmitoyl-sphingomyelin (PSM) and D-*erythro*-*N*-palmitoyl-dihydro-sphingomyelin (DHPSM) were 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). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-stearoyl-(12-doxyl)-*sn*-glycero-3-phosphocholine (12SLPC) were 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 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine (7SLPC)[2]. β-Cyclodextrin (β-CyD), cholesterol, D-sphingosine and palmitic acid (16:0-COOH) were from Sigma Chemicals (St. Louis, MO, USA) and 1-hexadecanol (16:0-OL), D-*erythro*-dihydrosphingosine (sphinganine) and D-*erythro*-*N*-palmitoyl-sphingosine (PCer) were from Larodan Fine Chemicals (Malmö, Sweden).

D-*erythro*-*N*-palmitoyl dihydrosphingosine (DHCer) was prepared from PCer by hydrogenation using palladium oxide (Aldrich Chemical Co., Milwaukee, WI, USA) as catalyst [35], and purified as described for PSM. Palmitic amide (16:0-NH_2) was produced by reacting palmitic chloride with ammonia. Stock solutions of lipids were prepared in hexane/2-propanol (3:2 vol/vol), stored in the dark at −20 °C, and warmed to ambient temperature before use.

Cholestatrienol (cholesta-5,7,9(11)-trien-3-beta-ol, CTL) was synthesized and purified using the method published by Fisher and coworkers [36]. D-*erythro*-*N*-*trans*-parinoyl-sphingomyelin (tParSM) was synthesized from *trans*-parinaric acid (Molecular Probes, Eugene, OR, USA) and D-*erythro*-sphingosylphosphorylcholine (lyso-SM, Matreya LLC, Pleasant Gap, PA, USA) according to Cohen and co-workers [37]. D-*erythro*-*N*-*trans*-parinoyl-sphingosine (tParCer) was synthesized from *trans*-parinaric acid and D-sphingosine using the method described for D-*erythro*-*N*-*trans*-parinoyl-sphingomyelin synthesis. The fluorescent probes were purified by reverse-phase HPLC on a RP-18 column with methanol/acetonitrile (70:30, vol/vol) as eluent for CTL and tParCer and 100% methanol for tParSM. All compounds were positively identified by mass spectrometry. CTL, tParSM and tParCer were stored dry under argon in the dark at −87 °C until solubilised in argon-purged ethanol (CTL) or methanol (tParSM and tParCer). Stock solutions of fluorescent lipids were stored in the dark at −20 °C and used within a week.

2.2. 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). The water used in the experiments was purified by reverse osmosis followed by passage

[2] During the course of our experiment, both 12SLPC and (12-doxyl)stearic acid became unavailable from commercial suppliers. We were therefore forced to start using 7SLPC which we made from 1-palmitoyl-2-OH-*sn*-glycero-3-phosphocholine and (7-doxyl)stearic acid, which was available and which we believed was a better choice than (5-doxyl- or 16-doxyl)stearic acid. Both 7SLPC and 12SLPC have similar (albeit not identical) quenching properties in our systems.

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: SLPC: PSM: variable lipid: cholesterol (30:30:15:15:10 molar ratio) or POPC: SLPC: PSM: PCer: 16:0-OL (25:30:15:15:15) and in F_0 samples POPC replaced SLPC. The samples were studied with CTL, tParCer or tParSM as the fluorescent probe, which replaced 1 mol% of cholesterol, PCer or PSM, respectively. 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.

2.3. Steady-state fluorescence measurements and quenching of steady-state fluorescence

Fluorescence measurements were performed on a PTI QuantaMaster-1 spectrofluorimeter (Photon Technology International, Lawrenceville, NJ, USA) operating in the T-format. The excitation and emission slits were set to 5 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 tParCer and tParSM was detected at 410 nm, while excitation occurred at 305 nm.

The F samples contained quencher (SLPC) and a complex lipid mixture as described above, while POPC replaced SLPC in F_0 samples. The fluorescence intensity in the F samples was compared to the fluorescence intensity in F_0 samples giving the fraction of quenched fluorescence.

2.4. Differential scanning calorimetry

DSC measurements were performed in a Calorimetry Sciences Corporation Nano II DSC (Provo, UT). Multilamellar vesicles containing lipids in different proportions (details are given in the figure legends) were co-mixed from organic solvent (hexane/2-propanol 3:2 vol), dried under a flow of nitrogen, and then dissolved in a small volume of benzene. The benzene was evaporated under a stream of nitrogen, and the samples kept in high vacuum for 2 h. Water at 55 °C was added to the dry lipid samples to give a final lipid concentration of a few mM. The tubes were vortexed and freeze/thawed 15 times in liquid nitrogen and a water bath at 55 °C. Samples that did not form a homogenous suspension after the freeze/thaw procedure were heated to 80 °C and bath sonicated (Branson 2510E-MT, Branson Ultrasonics, Danbury, LT, USA). The samples were loaded into the calorimeter and subjected to two consecutive heating and cooling scans at a rate of 0.5 °C/min between 0 and 100 °C.

2.5. Cholesterol desorption from mixed monolayers to cyclodextrin in the subphase

Monolayers of cholesterol, PSM and PCer were spread on a clean water surface, and after 5 min (to let the solvent evaporate) the monolayer was compressed to 20 mN/m and kept at that pressure during the entire experiment (at 22 °C). To initiate cholesterol desorption, 1.4 mM βCyD (final concentration) was injected into the subphase and the rate of cholesterol desorption was calculated as described previously [38]. The ratio of cholesterol to PSM in the monolayer was equimolar, and the amount of PCer was varied. The rate of desorption was corrected for the amount of cholesterol that was available in the monolayer, calculated as the observed rate divided by the mole fraction of cholesterol. Average values from three determinations for each composition were determined. Control experiments showed that β-CyD could not remove PSM or PCer from monolayers containing only PSM, PCer or an equimolar mixture of the two.

3. Results

3.1. Fluorescence quenching assay for sterol association with membrane domains

Domain formation and sterol association with specific domains were studied by fluorescence quenching. Lipid vesicles in which ordered and disordered domains co-exist were used. PSM was used as the main saturated lipid forming the ordered domains. POPC together with a nitroxide labeled quencher (7SLPC or 12SLPC) formed the disordered phase in the vesicles [4]. Fluorophores residing in the quencher-rich disordered domains will give a weaker fluorescence in these vesicles 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 [36,39–42]. 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 [31]. The amount of CTL exposed to quenching by SLPC gives a measure of CTL distribution between ordered and disordered membrane domains. The stability of the 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 tPar-labeled sphingolipids as fluorescent probes which partition predominantly into sterol- and sphingolipid-rich domains, respectively, and examined how domain stability and probe partition is affected by competing molecules.

To illustrate the usefulness of domain-selective fluorescent reporter molecules and quenchers (see also [31,32,43]) we prepared complex bilayers containing POPC, 12SLPC,

PSM, cholesterol and either CTL or tParSM (at 1 mol%) as fluorescent probes (Fig. 1). In this system, the fluorescent probe will be shielded from quenching by SLPC if it partitions into ordered membrane domains. The melting of ordered domains containing a certain probe will be seen as a decrease in F/F_0 with temperature due to increased quenching when the bilayer becomes more homogenous after domain melting. In a disordered POPC/SLPC environment, the quenching susceptibility of CTL is high at all temperatures studied here, as shown by Björkqvist and coworkers [31]. In Fig. 1A, we show the quenching of CTL or tParSM in PSM-rich domains as a function of temperature (the bilayer composition was POPC 60 mol% (or POPC and 12SLPC at 30 mol% each), 30 mol% PSM and 10 mol% sterol with CTL or tParSM, replacing the corresponding bulk lipid giving a final probe concentration of 1 mol%. The ratio of the intensity for the F and F_0 samples varied somewhat between the two probes, but the temperature-interval for domain melting reported by the probes was fairly similar. A DSC thermogram for a corresponding bilayer system (POPC/PSM/cholesterol 60:30:10 mol%) is shown in Fig. 1B. The melting of the PSM-rich phase occurring between 10 and 40 °C was observed to be in reasonable agreement with the domain melting reported by either CTL or tParSM. However, the melting of sphingomyelin/sterol-rich domains seems to start earlier as reported by CTL than by tParSM (Fig. 1A). This effect might be due to CTL having slightly lower affinity for these domains than tParSM. It might also be that the sterol is released at the onset of the gradual domain melting process in a slightly different manner than the sphingomyelins.

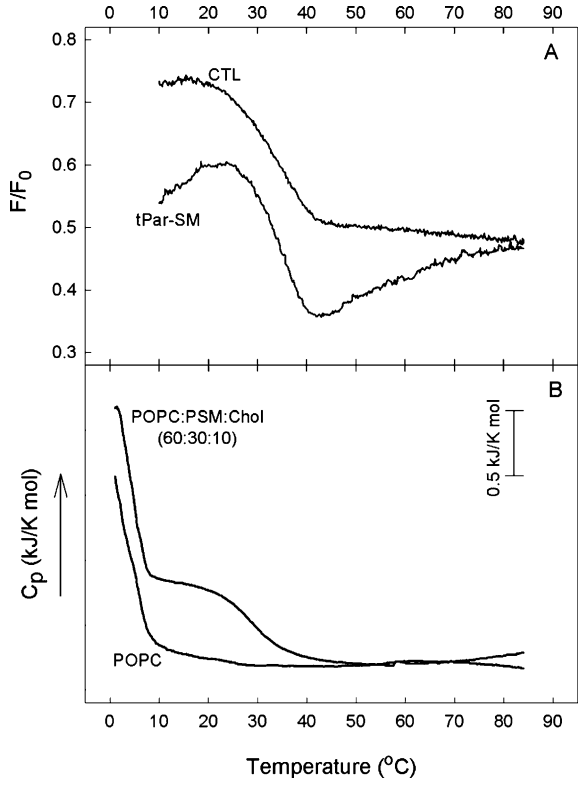

Fig. 1. Melting of sterol/PSM-rich domains in a fluid bilayer as examined by fluorescence quenching and differential scanning calorimetry. For fluorescence quenching experiments (panel A), the bilayers were prepared to contain POPC/PSM/sterol (60:30:10 mol%). The sterol fraction contained 9 mol% cholesterol and 1 mol% CTL. When tParSM was used as the fluorophore, cholesterol was used at 10 mol% and 1 mol% of PSM was replaced by tParSM. 12SLPC was used in F samples at 30 mol% (replaced some of the POPC). In panel B, thermograms of bilayers containing either POPC or POPC/PSM/cholesterol (60:30:10 mol%) were obtained (the second heating scan is shown). The final phospholipid concentration was 4–5 mM. The temperature gradient was 5 °C/min and 0.5 °C/min for quenching and calorimetry experiments, respectively.

3.2. Ceramide and dihydroceramide affect sterol/PSM domains

It was recently demonstrated that ceramide can displace cholesterol from sterol/PSM or sterol/DPPC domains [31,32]. Here, displacement is taken to mean that a large fraction of the sterol no longer was associated with the ordered domains in the presence of ceramide. Since dihydroceramides reportedly behave differently from ceramides in cell systems [44–46] and also have different molecular properties in monolayer systems [47], we wanted to see whether DHCer was able to replace sterol from sterol/PSM domains. To do so, we used the quenching assay described above with CTL as the fluorescent probe for sterol partitioning into ordered domains and tParSM as the reporter molecule in sphingolipid-rich domains (Fig. 2). As shown by our CTL quenching assay, the PSM/sterol domains formed by 10 mol% sterol and 15 mol% PSM (Fig. 2A) in a POPC matrix melted as a function of increasing temperature (between 10 and 30 °C) and were somewhat less stable than those formed by 30 mol% PSM and sterol (Fig. 1A). When PCer or DHCer was introduced to the bilayer systems (at 15 mol%), CTL no longer reported domain melting (Fig. 2A). This finding could be due to the ceramides disrupting the ordered domain formation or to the displacement of sterol from the domains. To verify which explanation was more likely, we studied the same complex lipid systems probed with tParSM (Fig. 2C). This probe reported domain melting in the presence of both PCer and DHCer and it was thereby confirmed that ordered domains were present in the bilayers. From Fig. 2C, we could further see that both ceramides substantially stabilized the domains formed by PSM. These results, together with the results reported in our previous paper [31], in which tParCer was used in a similar system to verify the formation of ceramide-rich domains, were found by us to clearly indicate displacement of sterols from ordered domains by both PCer and DHCer. From a miscibility point of view, these results are not surprising, since both PCer [31] and DHCer (Ramstedt and Slotte, unpublished observations) show good miscibility with PSM in bilayers. We observed further that DHCer was able to shift the melting of the ordered domains to a significantly higher temperature than PCer (Fig. 2C).

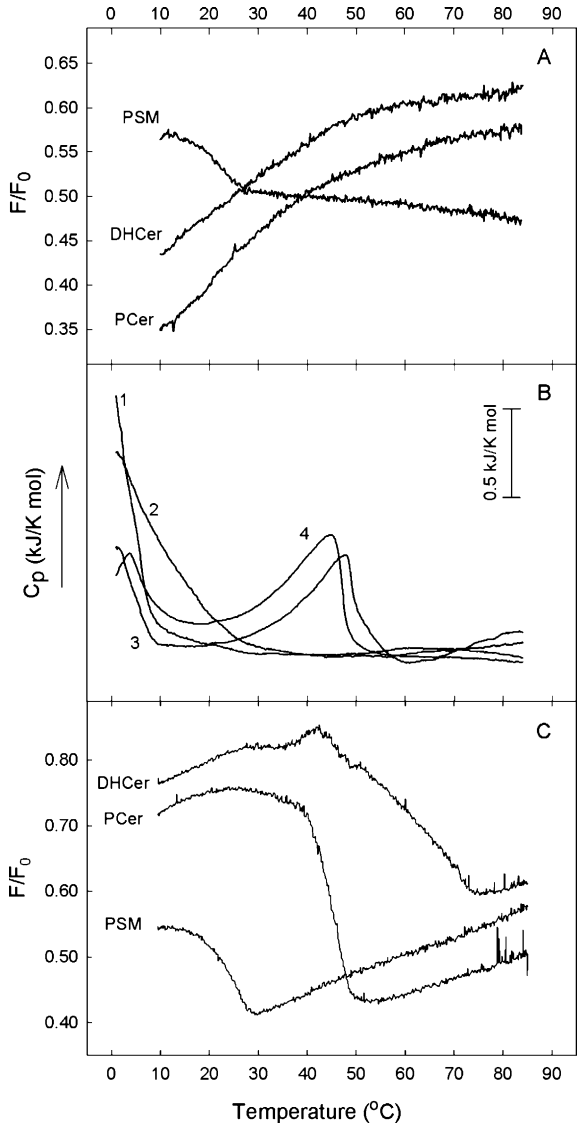

Fig. 2. Properties of fluid bilayers containing sterol, PSM, and ceramide. For fluorescence quenching experiments (panels A and C), the bilayers were prepared to contain POPC/PSM/sterol (75:15:10 mol%) or POPC/PSM/ceramide/sterol (60:15:15:10 mol%). In panel A CTL was used as fluorescent reporter molecule replacing some of the cholesterol to yield a final probe concentration of 1 mol%. 12SLPC was used in *F* samples at 30 mol% (replaced some of the POPC). In panel C, tParSM was used as fluorescent probe replacing sphingomyelin to yield 1 mol% final concentration and 7SLPC was used as a quencher in the *F* samples (see Materials and methods). The ceramide was either PCer or DHCer. In panel B, thermograms of bilayers containing either pure POPC (line 1), POPC/PSM/cholesterol (line 2, 75:15:10 mol%), POPC/PSM/PCer (line 3, 70:15:15 mol%), or POPC/PSM/PCer/cholesterol (line 4, 60:15:15:10 mol%) are shown (the second heating scan is displayed). The final phospholipid concentration was 4–5 mM. The temperature gradient was 5 °C/min and 0.5 °C/min for quenching and calorimetry experiments, respectively.

The DSC thermograms obtained from the complex bilayer systems with and without ceramides suggest that whereas cholesterol/PSM domains appeared to melt between 10 and 40 °C (Fig. 2B, line 2), inclusion of PCer (at 15 mol%) clearly led to the phase-separation of a ceramide-rich component which most likely also contained PSM but not much cholesterol, since the presence or absence of 10 mol% cholesterol in the system did not markedly shift the melting of the ceramide-rich component (Fig. 2B, lines 3 and 4). These results are in good agreement with the quenching data of CTL, showing displacement of sterol from PSM domains by ceramides. The melting temperature of the ceramide-rich component as reported by DSC (Fig. 2B, line 4) is also in very good agreement with the melting data reported here by tParSM (Fig. 2C) and in our previous study by tParCer [31].

Cholesterol desorption from a mixed monolayer containing equimolar amounts of cholesterol and PSM to βCyD in the subphase was also markedly enhanced by the presence of increasing amounts of ceramide (Fig. 3). These results together with those obtained from the quenching studies show that ceramide, compared to cholesterol, displayed a better miscibility with PSM, and hence gave lateral displacement of cholesterol from PSM-rich regions in the membrane.

3.3. Displacement of sterols by long-chain molecules

Already in 1984 Lange and her co-workers showed that millimolar amounts of octanol increased the cholesterol oxidase susceptibility of cholesterol in red cell membranes [48], indicating that the alcohol could somehow affect the lateral distribution of cholesterol in red cell membranes. In a more recent paper, Lange and colleagues showed that octanol (below 1 mM) increased cholesterol flow from the cell surface to the endoplasmic reticulum in cultured fibroblasts, again suggesting that octanol somehow affects the chemical activity of cholesterol in cell membranes [33].

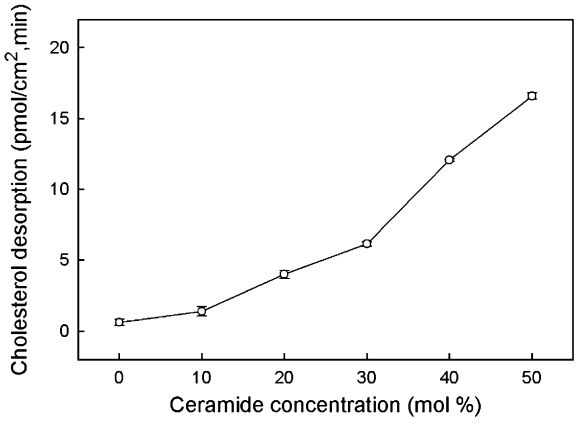

Fig. 3. Desorption of cholesterol from mixed monolayers containing cholesterol, PSM and PCer. Monolayers with the indicated lipids were spread on a clean water surface, and after 5 min (to let the solvent evaporate) the monolayer was compressed to 20 mN/m and kept at that pressure during the entire experiment (at 22 °C). To initiate cholesterol desorption, 1.4 mM βCyD (final concentration) was injected into the subphase and the rate of cholesterol desorption was calculated as described by Ohvo and Slotte [38]. Average values from three determinations for each composition is indicated (± S.D.). The ratio or cholesterol to PSM was equimolar, and the increased amount of PCer in the monolayer is indicated as mol% on the *x*-axis scale.

Because of these findings, we wanted to test the effects of various membrane intercalators, with a single long acyl chain, on the distribution of sterol between ordered and disordered domains in model bilayer systems. Using the CTL quenching assay, we could show that both hexadecanol and hexadecyl amide (at 15 mol%) could displace CTL from PSM domains, since the CTL quenching efficiency in these membranes was high at low temperatures where domains of PSM and hexadecanol exist (see Fig. 4). The effect of hexadecanol on CTL displacement was concentration-dependent in the range 5–15 mol%, with maximal quenching of CTL fluorescence being achieved at 15 mol% (Fig. 5). Palmitic acid could not displace the sterol from ordered domains with PSM, rather palmitic acid had an ordering effect on the sterol-rich domains, shifting the melting temperature of these to higher temperatures (Fig. 4A).

When the quenching susceptibility of tParSM was determined instead of using CTL as a probe (Fig. 4B), the inclusion of 15 mol% hexadecyl amide, hexadecanol or palmitic acid in the bilayers appeared to markedly stabilize the ordered PSM domains as determined from the higher

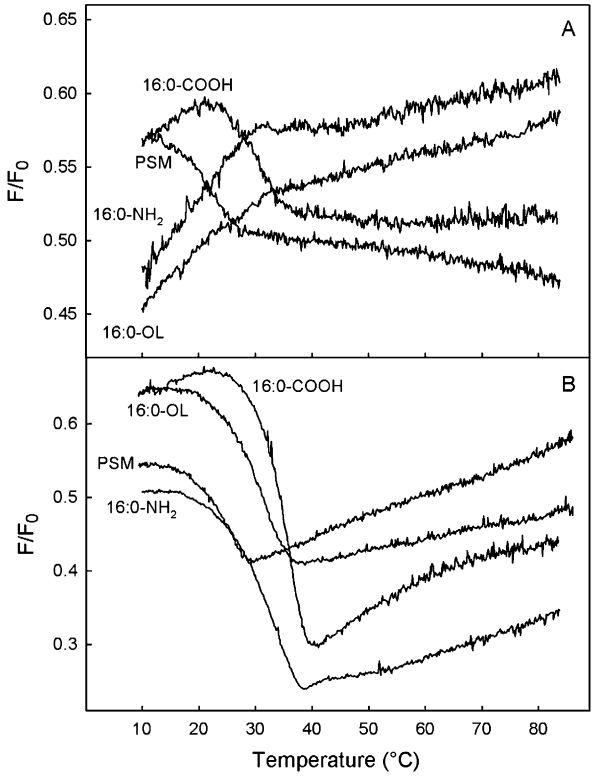

Fig. 4. Displacement of sterols from PSM domains by saturated long-chain molecules. The association of sterols with PSM domains was detected by 12SLPC quenching of CTL emission. Emission intensities were measured in F and F_0 samples, which consisted of POPC: (12SLPC or POPC): PSM: X: cholesterol (30:30:15:15:10, mol%) where X was 16:0-OL, 16:0-NH_2 or 16:0-COOH. The temperature was increased by 5 °C/min. The domains were probed with either CTL (panel A) or tParSM (panel B), which replaced 1 mol% of cholesterol or PSM, respectively. For the system with pure PSM and PSM/16:0-COOH with tParSM (panel B), 7SLPC was used as quencher (see Materials and methods).

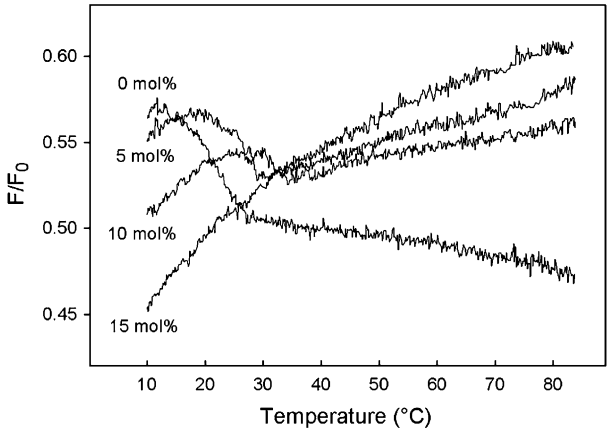

Fig. 5. Concentration dependence of sterol displacement from PSM domains by hexadecanol. The association of sterols with PSM domains was detected by 12SLPC quenching of CTL. Emission intensities were measured in F and F_0 samples, which consisted of POPC: (12SLPC or POPC): PSM: X: cholesterol: CTL (45:30:15:X:9:1, mol%) where X was 0, 5, 10 or 15 mol% 16:0-OL. As the bilayer concentration of 16:0-OL increased, the amount of POPC was decreased correspondingly, with PSM and sterol concentrations kept unchanged. The samples were heated by 5 °C/min.

melting temperatures for these domains. The partitioning of the tParSM probe into the PSM-rich domains also appeared to increase in the presence of the intercalators, as compared to the intercalator-free system, since the amplitude of the quenching difference was bigger when the intercalators were present. From DSC analysis, it was observed that hexadecanol was miscible with PSM (at equimolar amounts in the bilayer) and markedly increased the melting temperature of the binary mixture (about 68 °C, Fig. 6) as compared to PSM alone (which melts around 41 °C). The results from the thermal analysis agree well with the quenching data showing that the PSM domains were stabilized by the long-chain alcohol.

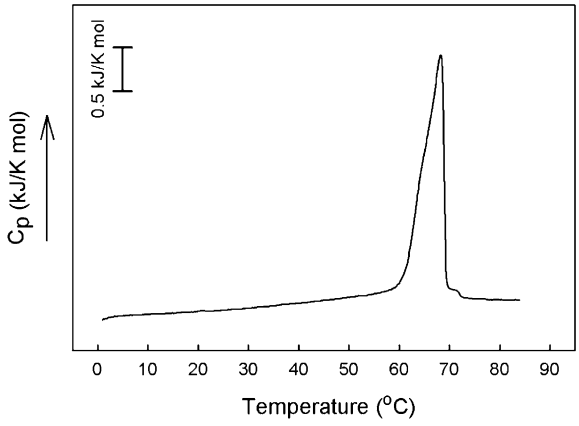

Fig. 6. Miscibility of hexadecanol with PSM. Bilayers containing 50 mol% each of PSM and 16:0-OL were prepared by sonication (see Materials and methods) and analyzed by DSC. The final PSM concentration was 4.5 mM, and the temperature gradient was 0.5 °C/min. The second heating scan is shown.

3.4. Competition between ceramide and hexadecanol

Since both PCer and hexadecanol independently displaced sterols from sterol/PSM domains, it was of interest to compare how these intercalators would behave when added together. Would either of them displace the other in a ternary system? To address this question, we prepared bilayers consisting of POPC: PSM: PCer (70:15:15 nmol) and compared them to bilayers consisting of POPC: PSM: PCer: hexadecanol (55:15:15:15 nmol). The probes used were tParSM (Fig. 7A) or tParCer (Fig. 7B) replacing the respective bulk lipid to give a total probe concentration of 1 mol%. The quencher 12SLPC was present at 30 nmol in *F* samples (with a corresponding reduction in the amount of POPC). The results show that hexadecanol stabilized the domains formed by PSM and PCer, and hence was miscible with these domain-forming lipids. Both fluorescent probes showed qualitatively similar results (Fig. 7 panels A and B for tParSM and tParCer, respectively), although tParCer reported a slightly lower temperature for the onset of domain melting in the system containing PSM, PCer and hexadecanol. This result can be seen as an indication that ceramide is released somewhat more easily from the domains during melting than sphingomyelin is.

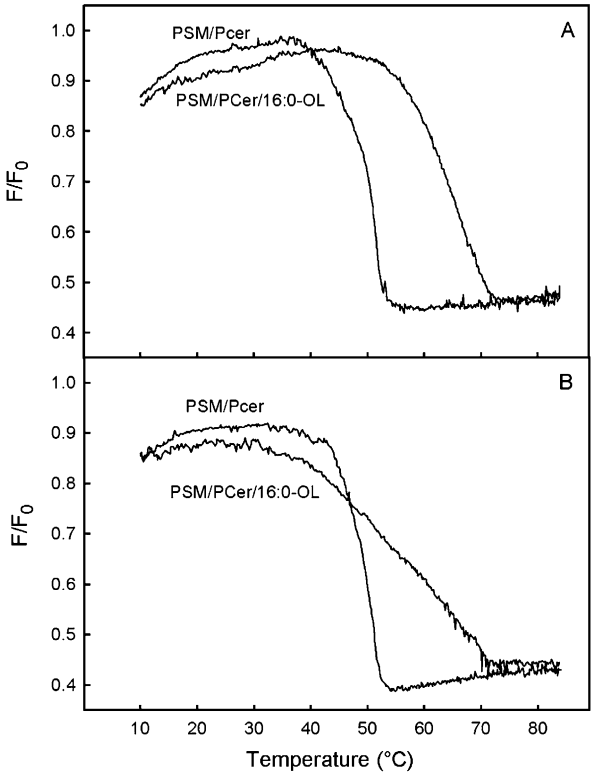

Fig. 7. Properties of fluid bilayers containing PSM/PCer and hexadecanol without sterol. Bilayer membranes were prepared to contain either POPC: (12SLPC or POPC): PSM: PCer (40:30:15:15, mol%) or POPC: (12SLPC or POPC): PSM: PCer: 16:0-OL (25:30:15:15:15, molar ratio). The probes used were either tParSM (panel A) or tParCer (panel B). The probes replaced 1 mol% of the respective bulk lipid. The temperature was increased by 5 °C/min.

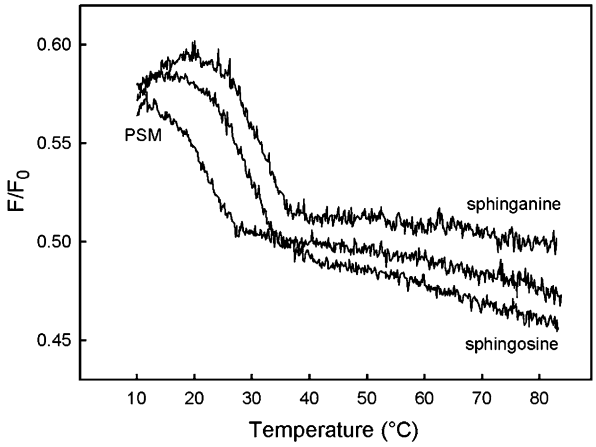

Fig. 8. Effects of sphingosine or sphinganine on PSM/sterol domain melting as detected by 12SLPC quenching of CTL. *F* and F_0 samples consisted of POPC: (12SLPC or POPC): PSM: *X*: cholesterol: CTL (30:30:15:15:9:1, molar ratio) where *X* was either sphingosine or sphinganine. The samples were heated by 5 °C/min.

3.5. Effects of sphingosine and sphinganine on sterol/PSM domains

Since single-chain membrane intercalators and ceramides were shown to displace sterols from ordered domains, we also found interest in looking at the sphingolipid long-chain bases, sphingosine and sphinganine. These molecules contain both hydroxyls and an amine group, a single long hydrocarbon chain and, like ceramides, they are sphingolipid precursors and potent signaling molecules. It was therefore of interest to investigate whether these lipids were able to disturb the packing in and displace sterol from sterol/PSM domains. This was tested in bilayers containing POPC, PSM, sphingosine (or sphinganine), and sterol using CTL as the fluorescent reporter molecule in the quenching assay. As seen from Fig. 8, neither sphingosine nor sphinganine was able to displace sterol from PSM-rich domains, but rather appeared to slightly stabilize the sterol/PSM domains (i.e. melting occurred at an elevated temperature compared to the control system).

4. Discussion

4.1. Fluorescence quenching as a method to study membrane domains

We have in this study used a fluorescence quenching method, based on domain selective fluorescent probes and quenchers, recently described [31,32,43]. As quenchers, we have used 7SLPC or 12SLPC which prefer to be in the fluid phase [4,49]. The probe used for sterol partitioning was CTL, which has been shown to mimic the behavior of cholesterol well [36,39–41]. We have found that CTL in mixed lipid bilayers reports the melting of PSM/sterol domains in good agreement with the melting reported by

DSC results of similar mixtures ([31] and Fig. 1). In this study, we also used lipids labeled with *trans*-parinaric acid (that is tParCer and tParSM) to study domains formed by these together with a corresponding unlabeled lipid. These probes effectively reported on domain melting in the quenching assay at temperatures expected for the corresponding host lipids in the ordered domains. However, we have not determined the partitioning of these probes between the fluid and ordered phases explicitly. Calculating partition coefficients for these probes from fluorescence data would be very complicated in these multi-component lipid mixtures, since the quantum yield of the fluorophores varies somewhat in different environments. The quenching method also has the drawback that it does not distinguish between domains in the liquid-ordered and the gel-phase, nor does it report on domain size and morphology. As pointed out by Fastenberg and co-workers, the susceptibility to quenching of fluorophores in a domain might be higher if domains are very small or have domain shapes with a high edge-to-core ratio [50]. There is a possibility that domain size might be altered by the membrane intercalators used in this study, since inclusion of intercalators in the bilayers will affect the ratio of the other domain-influencing lipids as well.

In Figs. 2A, 4A and 5, there is an apparent increase in F/F_0 values with increasing temperature that needs further clarification. For example, in Fig. 2, when ceramide is present, the increase cannot be explained by CTL associating with ceramide-rich domains, since the melting of these is not detected by this probe. The increase seems to be a consequence of the way these results are calculated. When CTL is displaced from domains the fluorescence in the quenched samples (F values) at low temperatures is already much lower than in the unquenched samples (F_0 values). Due to temperature effects on the probe the CTL fluorescence will decrease as a function of temperature as shown by Björkqvist and co-workers [31]. This decrease will always be a certain fraction of the initial fluorescence. This will lead to a smaller change in absolute intensity values for the quenched than for the unquenched samples due to temperature effect on CTL. Indirectly, the F/F_0 values will go up with temperature, since the difference in absolute fluorescence (F and F_0 values) will be bigger at low than at higher temperatures. The quenching efficiency of 12SLPC in a DOPC or POPC environment has been shown not to be affected by temperature to any significant degree, which excludes that as an explanation for the F/F_0 increase [31,51].

However, having pointed out the above mentioned limitations of the method, we find this method very useful for determining the presence of sterol in ordered domains, since CTL effectively reports this. Second, the stability of ordered domains, as reported by CTL or tPar-labeled lipids, can easily be studied by looking at the quenching as a function of temperature. The melting temperatures for domains in several of our lipid mixtures have been verified by DSC and we have concluded that they are fairly reliable. However, the melting of ordered domains is a complex event in these multi-component bilayer systems, as evidenced by for example the broad shoulder in the DSC thermogram presented in Fig. 1B. The gradual melting indicates that the lipid composition of the domains is temperature dependent. This is further strengthened by the small but reproducible variations in melting temperatures reported by different fluorescent probes in the same lipid mixture (for example, Fig. 4 A vs. B or Fig. 7 A vs. B). The third parameter of interest reported by this method is the amplitude of the quenching difference, i.e., the difference in F/F_0 below and above the melting temperature of the domains. This amplitude cannot be compared between probes, but can be used as a measure of the relative amount of the same probe partitioning into ordered domains in similar bilayer systems. The amplitude is, however, a feature that might be affected by domain morphology and size as discussed above.

4.2. Sterol distribution is affected by neutral membrane intercalators

In this study, we have examined the ability of single and double chained membrane intercalators with small polar head groups to displace sterols from ordered domains in a fluid lipid matrix. We were able to draw apparent parallels to the previously reported ability of these molecules to redistribute cellular cholesterol [33,48,52–56].

It was recently shown that saturated ceramide and diacylglycerol are able to displace sterols from ordered domains [32]. Sphingomyelinase treatment of cells leading to higher ceramide content of the plasma membrane has long been known to affect cholesterol homeostasis and lead to re-organization of cellular cholesterol [54–58]. Megha and London conclude in their paper that these effects are a direct consequence of the ceramide induced displacement of cholesterol from rafts in the plasma membrane [32]. We have also seen, from the inability of CTL to report a discontinuity in quenching efficiency in PCer containing membranes, that PCer displaces CTL from sterol-rich domains in membranes with DHPSM, PSM or DPPC [31]. In this study, we show that DHCer also is able to displace sterol from ordered domains with PSM in mixed lipid bilayers.

Both PCer and DHCer stabilized ordered domains with PSM as reported by tParSM and, for PCer, also by DSC. DHCer stabilized the ordered domains to a much greater extent than PCer did, which indicates an apparent effect of the saturation of the *trans*-double bond. There are large differences in the biological activity between dihydroceramide and ceramide [44–46,59]. These biologically active molecules often exhibit opposite functions, in for example enzyme activation [45]. According to our results, this behavior does not seem to be a consequence of their biophysical domain forming properties or their ability to redistribute sterols between lateral domains.

The results described above indicate that the structural features of diacylglycerol and ceramides, small head group and saturated acyl chains, give them the ability to displace sterols from ordered domains. We therefore wanted to study other structurally related molecules to see if these had the same effect on sterol-rich domains. Our interest in long-chain alcohols for this purpose came from the studies by Lange and co-workers, who showed that octanol had an effect on the chemical activity of cholesterol in cells, increasing its susceptibility for cholesterol oxidase and cyclodextrin extraction in red cell membranes and increasing the level of ER cholesterol in fibroblasts [33,48]. It has also been shown by others that the effect of long-chain alcohols on membrane proteins, as, for example, G-protein-coupled receptors, is a lipid-mediated mechanism [60]. Hexadecanol has been shown to intercalate between the acyl chains of saturated phospholipids and to induce tighter lateral packing in phospholipid membranes [61]. In this study, we show that hexadecanol mixes with PSM and stabilizes domains formed by PSM in a fluid lipid matrix. Hexadecanol also displaced sterol from ordered domains in our mixed lipid bilayers. It seems that hexadecanol substitutes for cholesterol as a spacer between the acyl chains of PSM, which leads to an increase in order and probably to a more solid phase in the bilayers. However, hexadecanol was not able to displace ceramide from ordered domains formed by PSM and ceramide, rather it seemed to stabilize these domains to some extent. Hexadecyl amide always gave similar results as hexadecanol when included in our studies.

Palmitic acid was not able to displace sterol in the same way as hexadecanol or hexadecyl amide. However, palmitic acid stabilized domains formed by PSM to much the same extent as hexadecanol did, which correlates well with the reported ordering effect of palmitic acid and hexadecanol on DPPC in monolayers [62]. The fatty acid is most likely ionized at the conditions used by us whereas hexadecanol and hexadecyl amide are neutral molecules, which could explain the difference in ability to displace cholesterol. Since pH was not monitored in our experiments and the pK of the fatty acid varies, being different in membrane environment than in aqueous solution [63] we have no direct evidence for this assumption.

The long chain sphingoid bases, sphingosine and sphinganine, were not able to displace sterols from ordered domains with sphingomyelin. Instead, the long-chain bases seemed to stabilize the sterol-rich domains. This is in good agreement with previous studies that showed no effect of sphingosine on the distribution of cellular cholesterol [52,53]. One study even shows that incubation with sphingosine increases the total cholesterol level in human B lymphocytes, leading to more rigid membranes as measured by DPH polarization [64]. A recent monolayer and cell biological study also suggests that sphingosine favors interaction with cholesterol, which could explain why they co-localize in the same domains [65].

4.3. Conclusions

The aim of this work was to study ordered domains formed by PSM and cholesterol and how different membrane intercalators could affect domain stability and sterol distribution in the bilayers. The formation of ordered cholesterol/PSM domains is thought to be driven by the favorable packing of the cholesterol ring structure with the saturated acyl chains of the sphingomyelin and might also involve specific interactions, like hydrogen bonding [66–68]. The ability of neutral membrane intercalators to disrupt these interactions and displace cholesterol from ordered domains has to be driven by thermodynamically more favorable interactions. The displacement process might for example involve favorable rearrangements at the interface as described for alcohols by Rowe and coworkers [69]. As discussed above, the displacement of sterols from ordered domains in our mixed model membranes correlate well with results from previous biological studies of the chemical activity of cholesterol in cells [33,48,52]. Our results show that sterols easily can be displaced from their interaction with PSM by a variety of single and double-chain (saturated) membrane intercalators which all have a small polar head-group as a common denominator. The effects of such molecules on the raft-association of sterols in biological membranes might be of key importance for their biological functions.

Acknowledgements

This study was supported by the Academy of Finland, the Sigrid Juselius Foundation, the Svenska Kulturfonden Foundation, the Borg Foundation and the Medicinska Understödsföreningen Liv och Hälsa.

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