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Biochimica et Biophysica Acta 1778 (2008) 954–962
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbamem

N-palmitoyl-sulfatide participates in lateral domain formation in complex lipid bilayers

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Received 9 August 2007; received in revised form 11 December 2007; accepted 16 December 2007
Available online 16 January 2008

Abstract

Sulfatides (galactosylceramidesulfates) are negatively charged glycosphingolipids that are important constituents of brain myelin membranes. These membranes are also highly enriched in galactosylceramide and cholesterol. It has been implicated that sulfatides, together with other sphingolipids, take part in lateral domain formation in biological membranes. This study was conducted to characterize the lateral phase behavior of *N*-palmitoyl-sulfatide in mixed bilayer membranes. Going from simple lipid mixtures with sulfatide as the only sphingolipid in a fluid matrix of POPC, to more complex membranes including other sphingolipids, we have examined 1) ordered domain formation with sulfatide, 2) sterol enrichment in such domains and 3) stabilization of the domains against temperature by the addition of calcium. Using two distinct phase selective fluorescent probes, *trans*-parinaric acid and cholestatrienol, together with a quencher in the fluid phase, we were able to distinguish between ordered domains in general and ordered domains enriched in sterol. We found that *N*-palmitoyl-sulfatide formed ordered domains when present as the only sphingolipid in a fluid phospholipid bilayer, but these domains did not contain sterol and their stability was unaffected by calcium. However, at low, physiologically relevant concentrations, sulfatide partitioned favorably into domains enriched in other sphingolipids and cholesterol. These domains were stabilized against temperature in the presence of divalent cations. We conclude that sulfatides are likely to affect the lateral organization of biomembranes.

Keywords: Galactosylceramidesulfate; Calcium; Cholesterol; Cholestatrienol; *trans*-parinaric acid; Raft

1. Introduction

Sulfatides (galactosylceramidesulfates) are negatively charged glycosphingolipids, being minor constituents of most eukaryotic membranes, but are more abundant in the nervous system and especially important for brain myelin membranes [1]. In many cells sulfatides have been found to function as receptors for neurotransmitters, opiates, endorphins and a heat shock protein, Hsp70 [2–8]. In the myelin membranes sulfatides take part in contact formation between bilayers through interaction with galactosylceramide, a neutral glycosphingolipid [9–11]. This interaction is of crucial importance for the stability of the myelin in the central nervous system and the myelin membranes are also highly enriched in both galactosylceramide and cholesterol [9,11]. It is becoming increasingly evident that glycosphingolipids participate in cell–cell interactions, possibly through defined membrane regions called glycosignaling domains [10,12].

During the last decade it has been widely accepted that dynamic lateral domains, the so called "rafts" enriched in sphingolipids and cholesterol, are formed in biological membranes [13,14]. Many different kinds of lateral domains may however exist within the same membrane [13,14]. The enrichment of sterol in sphingolipid-rich domains in biological membranes is often taken for granted, whereas some studies suggest that these lipids do not always co-localize [15,16]. The use of the term "rafts" for lateral

Abbreviations: 7SLPC, 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine; AFM, atomic force microscopy; CTL, cholestatrienol; DPH, diphenylhexatriene; DSPC, di-stearoyl-*sn*-glycero-3-phosphocholine; FTIR, fourier transform infrared spectroscopy; PGalCer, *N*-palmitoyl-galactosylceramide; PGlcCer, *N*-palmitoyl-glucosylceramide; PLacCer, *N*-palmitoyl-lactosylceramide; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PSM, *N*-palmitoyl-sphingomyelin; tPA, *trans*-parinaric acid; tPar-SM, *N*-*trans* parinoyl-sphingomyelin; tPar-sulfatide, 3-*O*-sulfo-D-galactosyl-β1-1′-*N*-*trans* parinoyl-D-*erythro*-sphingosine

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doi:10.1016/j.bbamem.2007.12.016

domains has been widely debated and more clear definitions for different kinds of domains have recently been presented [17,18]. The domains studied within the context of this model membrane work are sphingolipid-rich, but we want to be careful with using the confusing term "rafts" when drawing parallels to the lateral domains of biological membranes.

The distribution of sulfatide within biological membranes is apparently heterogeneous but has not been widely studied. Sulfatides are found in the detergent insoluble fraction of the myelin membranes, which could suggest that they take part in lateral domain formation [19,20]. A very recent study has used time-of-flight secondary ion mass spectrometry to characterize the sub cellular distribution of sulfatide species in cerebellum [21]. It appeared that sulfatides with different acyl chain compositions were heterogeneously distributed within white matter and that the different regions contained different amounts of cholesterol [21].

Previous model membrane studies have shown that sulfatides are protected against antibody recognition in a sphingomyelin/cholesterol environment compared to a phosphatidylcholine/cholesterol environment [22], suggesting significant differences in the interaction with sphingolipid versus phospholipid rich membranes. A recent study used atomic force microscopy (AFM) on supported lipid bilayers to look at the partitioning of sulfoglycolipids in membranes compositionally resembling those of the sperm plasma membrane [23]. The results showed that sulfatide was localized in ordered domains in the supported bilayers. The above mentioned studies on biological as well as model membranes however leave it quite unclear whether sulfatide will actually co-localize with cholesterol in bilayer membranes.

Studies on the membrane properties of sulfatides have often been conducted on biological mixtures of sulfatides with a wide variety of acyl chains of different lengths and degree of hydroxylation [23,24]. Studies on the biophysical behavior of sulfatides are in general quite scarce. It is quite clear, however, that the ceramide part of the sulfatide molecule will influence their membrane properties [21,25]. The thermotropic behavior of pure acyl chain defined sulfatides has been extensively studied by Boggs and co-workers [25–28]. *N*-palmitoyl-sulfatide, the acyl chain species used in this paper, has a transition temperature significantly lower than that of the corresponding non-sulphated galactosylceramide [25,29]. *N*-palmitoyl-sulfatide was found to occur in two different gel phases of different stability and the complex thermotropic behavior was highly dependent on the hydration level [29].

The membrane properties of sulfatides have also been shown to be dependent on divalent cations, such as calcium [3,25,30]. The thermotropic behavior of sulfatide is dependent on the presence of counter ions in a complex and acyl chain dependent way [25]. In the presence of calcium a condensing effect on a pure sulfatide monolayer is detected, indicating a reduction in repulsive forces or an increase in attractive intermolecular interactions between sulfatide molecules within the plane of the membrane [31]. In bilayers composed of a saturated phosphatidylcholine mixed with sulfatide the phase transition temperature is increased in the presence of mM-concentrations of calcium as determined by DPH anisotropy [3]. Similarly to galactosylceramide, also sulfatide has been shown to form an intermolecular hydrogen bonding network in the head group region [32,33]. According to FTIR the amount of inter- and intramolecular hydrogen bonds of the head group are reduced within pure sulfatide bilayers when calcium binds to the sulphate [30,32].The binding causes molecular rearrangements in the head group region so that the bilayer properties of the molecules are significantly changed [30]. Calcium has also been found to stabilize the intermembrane carbohydrate–carbohydrate interactions between galactosylceramide and sulfatide [9,10,34]. It is clear from the studies mentioned above that calcium will affect the quite complex membrane properties of sulfatide in biological membranes as well as in mixed lipid bilayers.

In this study we wanted to explore whether sulfatides were able to participate in lateral domain formation with other sphingolipids in model bilayer membranes and if the domains formed contain sterol. We also wanted to study what effect calcium might have on the lateral domain formation by these lipids.

2. Materials and methods

2.1. Materials

3-*O*-sulfo-D-galactosyl-β1-1′-*N*-palmitoyl-D-*erythro*-sphingosine (sulfatide) was purchased from Larodan Fine Chemicals AB (Malmö, Sweden). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids (Alabaster, AL). D-*erythro*-*N*-palmitoyl-sphingomyelin (PSM) was purified from egg yolk sphingomyelin (Avanti Polar Lipids) by reverse-phase HPLC (Supelco Discovery C18-column, dimensions 250×21.2 mm, 5 μm particle size) using 100% methanol as eluent. *N*-palmitoyl-galactosylceramide (PGalCer) was synthesized from lyso-galactosylceramide (D-galactosyl-β1-1′-D-*erythro*-sphingosine, Avanti Polar Lipids) and fatty acid anhydride (Sigma Chemicals, St. Louis, MO). 7 μmol lyso-galactosylceramide (psychosine), 70 μmol of palmitic acid anhydride and 5 μmol of triethylamine were dissolved in 500 μl dry dichloromethane/methanol (4:1, by volume). The reaction was carried out at room temperature for 4 h. PGalCer was purified by reverse-phase HPLC on a preparative RP-18 column with 100% methanol as the eluent and UV-detection at 203 nm. (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) according to the method by Szolderitz et al. [35]. Cholesterol was from Sigma Chemicals. The purity and identity of all lipids were verified on a Micromass Quattro II mass spectrometer (Manchester, UK). Stock solutions of lipids were prepared in hexane/2-propanol (3:2, by volume) or in the case of glycosphingolipids in chloroform/methanol (2:1, by volume), 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 Fischer et al. [36]. The synthesized fluorescent probe was purified by reverse-phase HPLC on a RP-18 column with methanol/acetonitrile (7:3, by volume) as eluent. *trans*-parinaric acid (tPA) was synthesized from *cis*-parinaric acid according to the method by Kuklev and Smith [37]. *N*-acylation of 3-*O*-sulfo-D-galactosyl-β1-1′-*lyso*-D-*erythro*-sphingosine (Larodan Fine Chemicals) and D-*erythro*-sphingosylphosphorylcholine (Avanti Polar Lipids) with tPA to yield *trans*-parinaric-sulfatide (tPar-sulfatide) and *trans*-parinaric-sphingomyelin (tPar-SM), respectively, was done essentially as the synthesis of PGalCer described above, with the exception that DCC (dicyclohexylcarbodiimide) was used as a catalyst to activate the tPA during the reaction. All fluorescent compounds synthesized were positively identified by mass spectrometry. DPH was purchased from Molecular Probes (Leiden, the Netherlands). The probes were stored dry under argon in the dark at −87 °C until solubilised in argon-purged ethanol (CTL) or methanol (tPA and tPar-sulfatide). Stock solutions of fluorescent lipids were stored in the dark at −20 °C and used within a week.

2.2. Preparation of vesicles

Lipid mixtures containing fluorescent probes were prepared from the stock solutions mentioned above and dried under nitrogen. Since several different solvents were present, which in our experience sometimes lead to de-mixing of the lipids during evaporation, we re-dissolved the lipid mixture in one uniform solvent (either chloroform or benzene) and mixed thoroughly. The solvent was then evaporated under a stream of nitrogen and the samples finally kept in vacuum until all traces of solvent were removed. The same fluorescence quenching results were obtained with both solvents and the results were highly reproducible after re-dissolving. The dry lipids were dispersed in argon-purged buffer and heated to about 60 °C. The warm samples were vortexed and then sonicated for 2 min (25% duty cycle, power output 10 W) with a Branson probe sonifier W-450 (Branson Ultrasonics, Danbury, CT). This procedure gives liposomes with a rather large size distribution with a mean diameter of about 200 nm and the lipids are incorporated in the vesicles to the same extent independent of lipid species as verified by ESI-MS analysis. We have previously shown that sonication at ~60 °C gave similar results in fluorescence quenching studies and a similar vesicle size distribution as sonication at a temperature above the T_m of pure glycosphingolipids [15].

The buffer used contained 50 mM TRIS and 150 mM NaCl, pH 7.4. Calcium was added to the buffer as $CaCl_2$ at indicated concentrations prior to filtration. The water used for the buffer was purified by reverse osmosis followed by passage through a Millipore UF Plus water purification system, to yield a product with a resistivity of 18.2 MΩcm. The buffer was filtered through 0.45 μm nylon membrane filters (Gelman Sciences, Michigan) before vesicle preparation. In fluorescence quenching studies, vesicles were prepared in buffer at a lipid concentration of 50 μM. F samples consisted of POPC: 7SLPC: sterol (3:3:1, molar ratio) with sphingolipids added in indicated proportions and in F_0 samples POPC replaced 7SLPC. POPC and the nitroxide labeled quencher (7SLPC) mix well and together they make up the major part of the disordered phase in the vesicles at all temperatures studied (Halling, Ramstedt, Slotte 2006, unpublished data). The formation of ordered domains by the sphingolipids was studies with CTL as a selective marker for sterol-enriched domains and tPA as a marker for ordered domains in general. The probes were included in the bilayers to represent 1 mol% of the total lipid. The fluorescent probes were protected from light during all steps and solvents were saturated with argon before use in order to minimize the risk of oxidation.

For steady-state fluorescence intensity and anisotropy measurements lipid vesicles composed of PSM/POPC were prepared as follows. PSM and POPC were mixed at various ratios in a series of 11–30 samples. The solvent was evaporated under a stream of nitrogen and the dried samples were kept in vacuum to remove the last potential traces of solvent. The samples were hydrated with warm argon-purged buffer on a 60 °C water bath. Vesicles were produced by alternately vortexing and heating the samples a few times, before bath sonicating them for 20 min at 70 °C. X_{PSM} ranged from 0.3 to 0.72 with tPar-sulfatide and from 0 to 1 with DPH. tPar-sulfatide was added at a probe: lipid ratio of 1:400 with a final lipid concentration of 100 μM whereas DPH was used at a corresponding ratio of 1:200 with a final lipid concentration of 50 μM.

2.3. Quenching of steady-state fluorescence

Fluorescence measurements were performed on a PTI QuantaMaster-2 spectrofluorimeter (Photon Technology International, Lawrenceville, NJ) operating in the L-format. The temperature was controlled by a Peltier element, with a temperature probe immersed in the sample solution. The samples were heated from 10 to 60 °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 throughout the measurements. The excitation and emission slits were set to 5 nm and the wavelengths were adjusted with selectively transmitting filters. The wavelengths of the excitation and emission filters used for CTL were 321 nm (range ±5 nm) and >385 nm, respectively. Fluorescence emission of tPA was detected at >420 nm, while excitation occurred through a filter centered around 310 nm (range ±5 nm).

The fluorescence quenching method has been discussed in detail in previous publications [38,39]. Briefly, fluorescence intensity of quencher containing F samples is compared to the fluorescence intensity of quencher-free F_0 samples giving the fraction of unquenched fluorescence (F/F_0). In lipid vesicles in which ordered and disordered domains coexist, the amount of CTL or tPA (predominantly associated with ordered domains) exposed to quenching by 7SLPC gives a measure of the extent of ordered domains formed in the system. The stability of the sterol-enriched domains, for example, can be measured by looking at the quenching susceptibility of CTL as a function of temperature. The changes in quenching are reversible in bilayers containing PSM as the domain building lipid after temperature scans to at least 80 °C (results not shown) indicating probe and quencher-stability throughout the experiments.

2.4. Calculating the mean temperature of domain dissociation

The results from at least three different quenching experiments were used to calculate the temperature at the midpoint of domain dissociation as reported by a certain fluorescent probe. This temperature is defined as the point where half of the probe molecules that were initially shielded from quenching have been released during the domain dissociation process and thereby come in contact with the quencher in the fluid phase. The derivative of the quenching curve was taken by averaging the slopes of two adjacent data points as:

$$\frac{1}{2} = \left(\frac{y_{i+1} - y_i}{x_{i+1} - x_i} + \frac{y_i - y_{i-1}}{x_i - x_{i-1}}\right) \quad (1)$$

where x is the temperature and y the F/F_0 value. The "midpoint of domain dissociation" was defined as the point giving the steepest slope of the quenching curve. The results from three different curves were used to calculate the standard deviation in temperature and to evaluate the significance of the differences.

2.5. Steady-state fluorescence intensity and anisotropy measurements

Steady-state fluorescence measurements were performed on a PTI QuantaMaster-2 spectrofluorimeter (Photon Technology International, Lawrenceville, NJ) operating in the T-format. The temperature was controlled by a Peltier element, with a temperature probe immersed in the sample solution. The samples were kept at a constant temperature of 23 °C during the measurements. The measurements were performed in quartz cuvettes and the sample solutions were kept at constant stirring throughout the measurements. The excitation and emission slits were set to 5 nm and the wavelengths were adjusted by monochromators. The wavelengths of the excitation and emission used for DPH were 358 nm and 430 nm, respectively. Fluorescence emission of tPA was detected at 410 nm (for the intensity measurements) and 430 nm (to minimize the background in the anisotropy measurements), while excitation occurred at 305 nm.

2.6. Determination of the phase behavior of tPar-sulfatide

The partition coefficient of tPar-sulfatide between gel and fluid phase in POPC/PSM binary mixtures was determined from the fluorescence intensity and steady-state fluorescence anisotropy as described by Prieto and coworkers [40,41]. Briefly, the partition coefficient, K_p, was calculated from the fluorescence intensity (I) and steady-state anisotropy (r) according to the following equations:

$$I = \frac{K\left(\varepsilon_g \phi_g K_p X_g + \varepsilon_f \phi_f X_f\right)}{\left(K_p X_g + X_f\right)} \quad (2)$$

$$r = \frac{\varepsilon_g \phi_g r_g K_p X_g + \varepsilon_f \phi_f r_f X_f}{\varepsilon_g \phi_g K_p X_g + \varepsilon_f \phi_f X_f} \quad (3)$$

where the suffix g indicates results obtained in the gel phase and f indicates the fluid phase [41]. K is the factor used to normalize the fluorescence intensity values, ε is the molar extinction coefficient, ϕ is the quantum yield and X denotes the fraction of each phase present as determined from the binary phase-diagram. K_p was obtained by fitting the equations to the experimental data.

3. Results

3.1. Lateral domain formation by N-palmitoyl-sulfatide

The tendency of sulfatides to self-associate into ordered domains was studied by quenching of tPA and CTL fluorescence (Fig. 1). The bilayers studied had the composition POPC

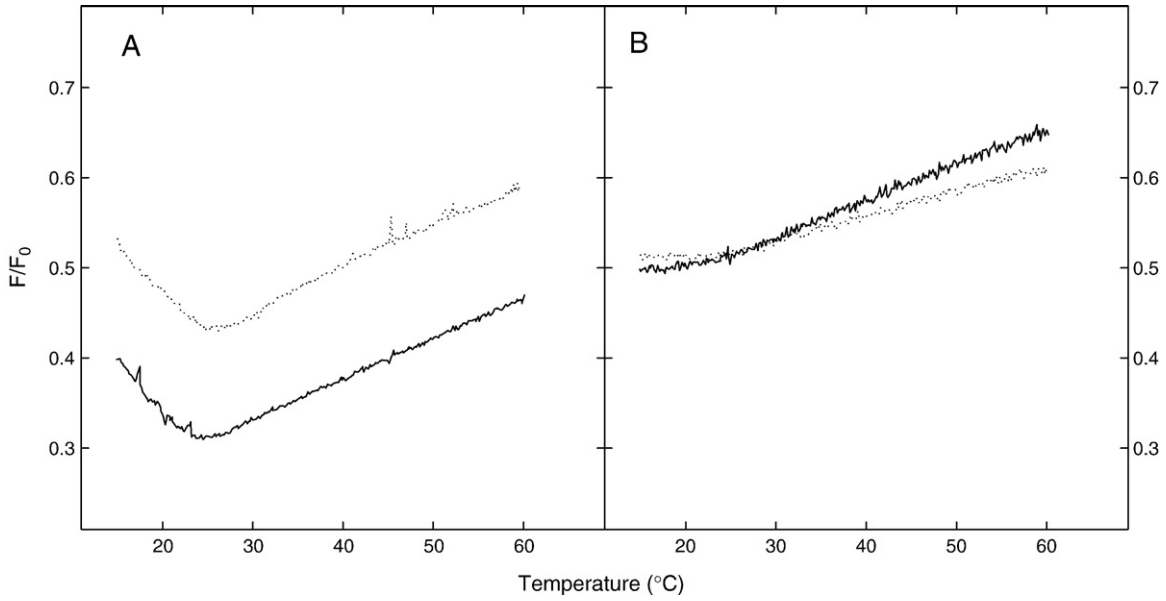

Fig. 1. Formation of ordered domains in complex lipid bilayers. The quenching of tPA fluorescence (A) was used as a measure of the amount of ordered (sterol-enriched or sterol-poor) domains formed in the membranes and the quenching of CTL (B) was used as a measure of the amount of sterol-enriched domains formed. The fraction of unquenched fluorescence (F/F_0) is plotted as a function of increasing temperature. The bilayers were prepared in TRIS–NaCl buffer to contain POPC (±7SLPC): sulfatide: cholesterol (60:30:10, molar ratio) with 1 mol% tPA or CTL, respectively. The quenching susceptibility of the probes in vesicles in a calcium free environment (dotted line) is compared to that in the presence of 10 mM $CaCl_2$ (solid line). The total lipid concentration was 50 μM and the temperature gradient 5 °C/min. The curves presented are representative of a set of at least three reproductions.

(±7SLPC): sulfatide: cholesterol (60:30:10, molar ratio) with 1 mol% probe added to the lipid mixture. As shown in Fig. 1A the sulfatides only formed lateral domains that included tPA at temperatures well below the physiological. The melting of these domains can be seen as an increase in the quenching efficiency with increasing temperature below 25 °C as the probe comes into contact with the quencher in the fluid phase. This occurred both in the presence (solid line) and absence (dotted line) of calcium. This finding indicates that the divalent counter ions were unable to facilitate self-aggregation of sulfatide molecules into clusters. Sterol did not seem to associate with the sulfatide-rich domains even at low temperatures since the quenching curves obtained with CTL were basically straight over the whole temperature range studied (Fig. 1B). In monolayers sulfatide was however miscible with cholesterol since cholesterol desorption to β-cyclodextrin in the subphase was much slower from a binary mixed monolayer than from a pure cholesterol monolayer (results not shown).

3.2. Partitioning of tPar-sulfatide between two phases in POPC/PSM binary mixtures

To study how well sulfatide partitions into ordered sphingolipid-rich domains we performed a partitioning assay for the *trans*-parinoyl-sulfatide probe in POPC:PSM mixtures along the tie-line in the binary phase-diagram at 23 °C. To verify the tie-line and the phase boundaries we determined the steady-state anisotropy for DPH. The phase boundaries agreed well with those reported previously [42]. We found that DPH partitioned equally between the fluid and gel phase domains in this two-phase system, hence the linear increase in anisotropy with the fraction of gel phase in the phase-coexistence region (Fig. 2). We also verified earlier results showing that free tPA had a preference for the gel phase in this system ($K_p = 1.95 \pm 0.09$) [40]. To determine the phase behavior of a tPar-sphingolipid we

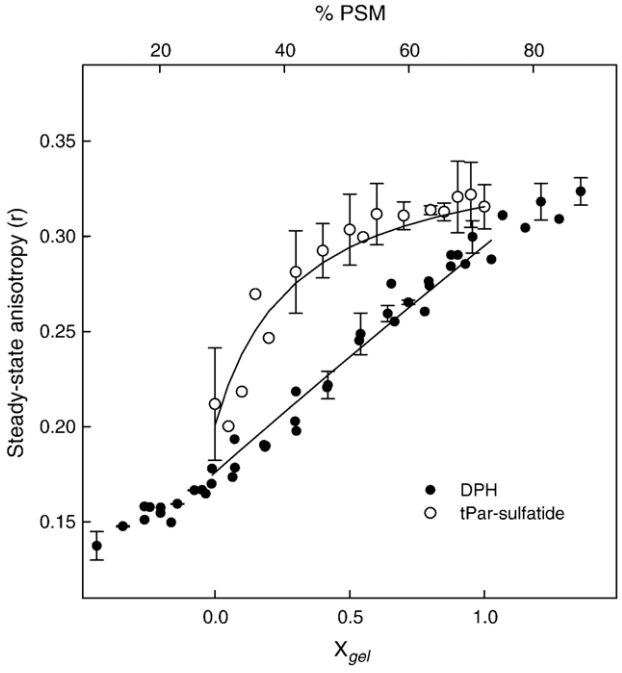

Fig. 2. Phase behavior of tPar-sulfatide in POPC:PSM binary mixtures. The steady-state anisotropy of DPH (filled circles) and tPar-sulfatide (open circles) are plotted against the fraction of gel phase (X_{gel}, at the lower *X*-axis) present along the tie-line in the binary phase-diagram at 23 °C [42]. The percentage of PSM in the bilayers (% PSM) is given by the upper *X*-axis. The data points show the anisotropy values given as the average from at least two different experiments with the variation shown by error bars.

started by comparing the partitioning of tPar-SM between gel and fluid phase in the binary PSM/POPC bilayer systems with that of PSM. We found that the partition coefficient for tPar-SM (K_p ~1.70) was only slightly lower than that for the saturated PSM (K_p ~2.0) as determined from the phase compositions according to the lever rule [42]. Based on this we assume that the partitioning of tPar-sulfatide also closely would resemble that of *N*-palmitoyl-sulfatide. The partition coefficient between PSM gel phase domains and POPC-rich fluid phase along the tie-line for tPar-sulfatide was 2.10±0.33, which indicates a clear preference of this probe for the ordered PSM-rich phase in these systems (Fig. 2). The lines in Fig. 2 are fits of the data points in the phase-coexistence region. Essentially the same partition coefficient (K_p=2.20±0.01) was obtained from the fluorescence intensity measurements for tPar-sulfatide.

3.3. Lateral domain formation by *N*-palmitoyl-sulfatide, sphingomyelin and sterol

According to the partition coefficient sulfatide associates preferentially with PSM-rich domains. The quenching of tPA fluorescence was used as a measure of the amount of ordered domains formed in bilayers consisting of POPC (±7SLPC): PSM: sulfatide: cholesterol (60:25:5:10, molar ratio) with 1 mol% tPA (Fig. 3A). These experiments showed very clearly that lateral domains were present in the bilayers as the quenching susceptibility of the probe increased substantially at the temperature of domain dissociation. The midpoint of this dissociation process was calculated using Eq. (1) and is indicated by the open circles with the standard deviation in temperature given by the error bars. The inset in panel A shows the corresponding curves for vesicles containing POPC (±7SLPC): PSM: cholesterol (60:25:10, molar ratio) with 1 mol% tPA as a control. The quenching susceptibility of the probe in vesicles in a calcium free environment (dotted line) is compared to that in the presence of 10 mM $CaCl_2$ (solid line). The domains containing sulfatide were stabilized against temperature by the addition of calcium to the surrounding buffer. A similar stabilization was seen with magnesium at corresponding concentration (results not shown), which leads us to believe that this is an effect that can be mediated by divalent cations in general. As shown in the inset in Fig. 3A without sulfatide no such stabilization of PSM-rich domains occurred with calcium. We could therefore conclude that sulfatide was present in the domains and that the increase in domain temperature stability was related to the presence of sulfatide in the domains. When calcium was not present no stabilization of PSM-rich domains was induced by the addition of sulfatide either (comparing the dashed line in Fig. 3A with that in the inset).

The quenching of CTL fluorescence was used as a measure of the amount of sterol-enriched domains formed in bilayers containing POPC (±7SLPC): PSM: sulfatide: cholesterol (60:25:5:10, molar ratio) with 1 mol% CTL. The fraction of unquenched fluorescence (F/F_0) at low temperatures indicated that sterol was included in the domains (Fig. 3B). The CTL probe was however released earlier, i.e. at lower temperature, during the domain dissociation process, than was tPA in corresponding bilayers (Fig. 3A).

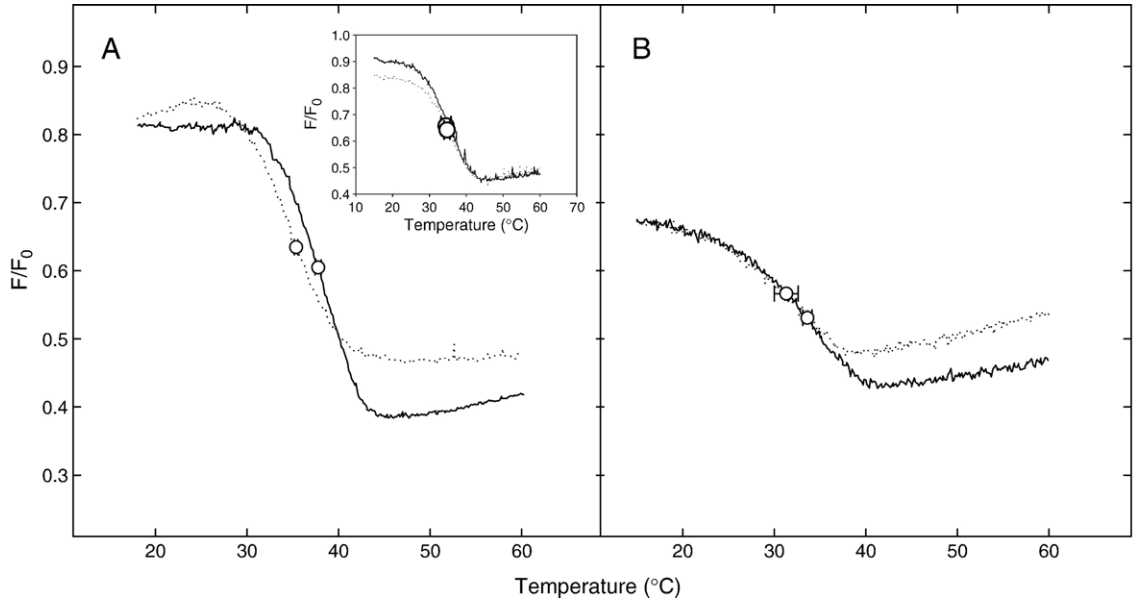

Fig. 3. Formation of ordered domains in complex lipid bilayers containing sphingomyelin and sulfatide. The quenching of tPA fluorescence (A) was used as a measure of the amount of ordered (sterol-enriched or sterol-poor) domains formed in the membranes and the quenching of CTL (B) was used as a measure of the amount of sterol-enriched domains formed. The fraction of unquenched fluorescence (F/F_0) is plotted as a function of increasing temperature. The bilayers were prepared in TRIS–NaCl buffer to contain POPC (±7SLPC): PSM: sulfatide: cholesterol (60:25:5:10, molar ratio) with 1 mol% tPA or CTL, respectively. The inset in panel A shows the corresponding curves for vesicles containing POPC (±7SLPC): PSM: cholesterol (60:25:10, molar ratio) with 1 mol% tPA, as a control. The quenching susceptibility of the probes in vesicles in a calcium free environment (dotted line) is compared to that in the presence of 10 mM $CaCl_2$ (solid line). The total lipid concentration was 50 μM and the temperature gradient 5 °C/min. The mean temperature of domain dissociation as reported by the release of the two probes was calculated as described in the Materials and methods section from at least three different experiments and is indicated by the open circles with the standard deviation indicated by horizontal error bars.

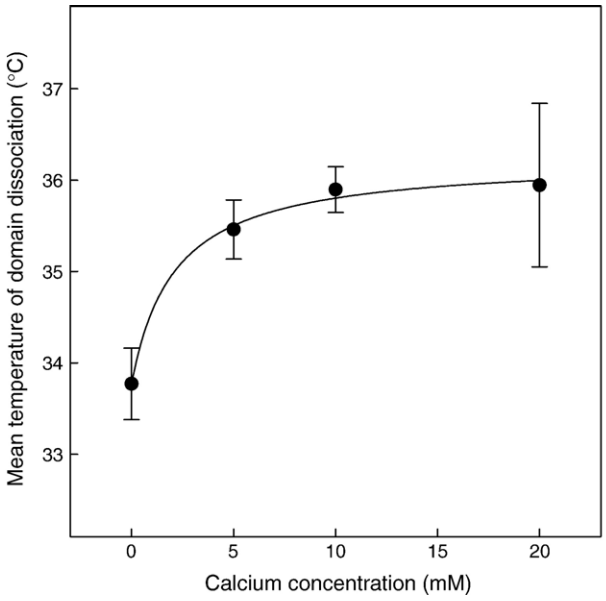

Fig. 4. Calcium concentrations versus domain dissociation temperatures as reported by tPA. The mean temperature of the domain dissociation as reported by the susceptibility of tPA for quenching was calculated as described in the Materials and methods section from at least three different experiments and plotted as a function of calcium concentration for vesicles containing POPC (±7SLPC): PSM: sulfatide: cholesterol (60:25:5:10, molar ratio) with 1 mol% tPA.

Fig. 4 shows the effect of different concentrations of calcium in the surrounding buffer on the mid temperature of tPA probe release during domain dissociation in bilayers containing POPC (±7SLPC): PSM: sulfatide: cholesterol (60:25:5:10, molar ratio) with 1 mol% tPA. The stabilization against temperature induced by calcium was concentration dependent and reached a plateau at a calcium concentration of about 10 mM which was also the concentration used in the experiments in this study. This calcium concentration is quite high compared to physiological concentrations although it is hard to know how high the local calcium concentrations in biological environments might get. Excess calcium has also previously been shown to be needed for detecting the calcium-induced effects on sulfatide-containing model membranes [25,43].

3.4. Formation of glycosphingolipid domains containing galactosylceramide and sulfatide.

Since sulfatide and galactosylceramide have been indicated to interact in biological membranes we also wanted to look at the ability of these lipids to form lateral domains together in a bilayer. Fig. 5 shows the quenching curves for mixtures containing POPC (±7SLPC): PGalCer: sulfatide: cholesterol (60:15:15:10, molar ratio) with 1 mol% tPA or CTL, respectively. The results show that the two lipids form ordered domains which are enriched in the tPA probe (Fig. 5A). These domains also seem to include some sterol according to the quenching of CTL fluorescence in Fig. 5B. However, the domains were not influenced to any larger extent by the presence of calcium ions, probably indicating that the domains could not be ordered to any further extent at such high glycolipid concentrations and the probes were therefore protected equally well in the absence as in the presence of calcium.

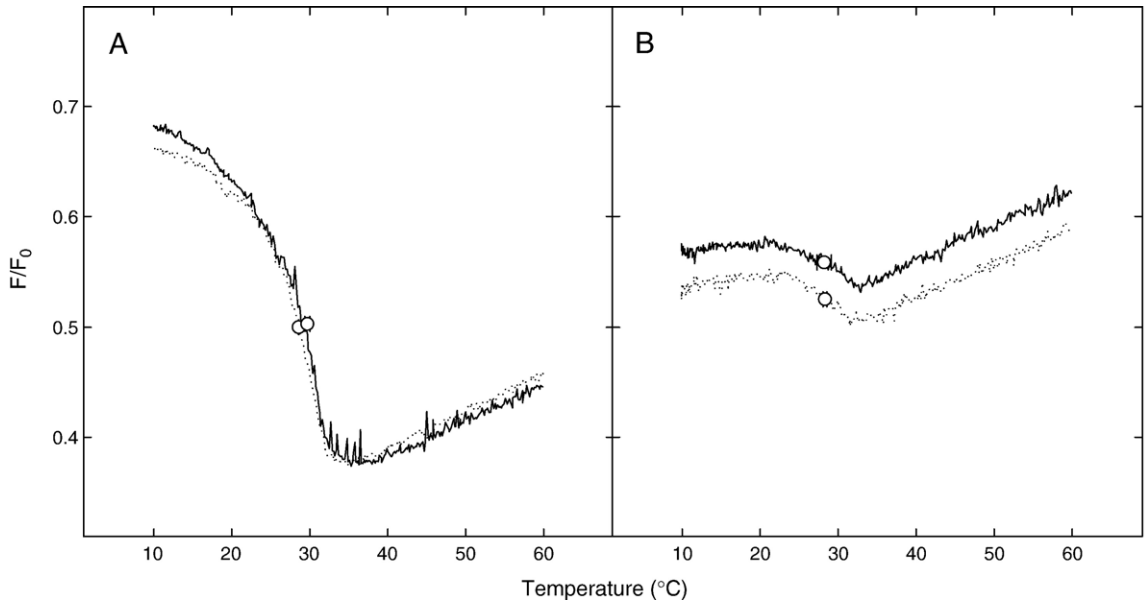

Fig. 5. Formation of ordered domains in complex lipid bilayers containing galactosylceramide and sulfatide. The quenching of tPA fluorescence (A) was used as a measure of the amount of ordered (sterol-enriched or sterol-poor) domains formed in the membranes and the quenching of CTL (B) was used as a measure of the amount of sterol-enriched domains formed. The fraction of unquenched fluorescence (F/F_0) is plotted as a function of increasing temperature. The bilayers were prepared in TRIS–NaCl buffer to contain POPC (±7SLPC): PGalCer: sulfatide: cholesterol (60:15:15:10, molar ratio) with 1 mol% tPA or CTL, respectively. The quenching susceptibility of the probes in vesicles in a calcium free environment (dotted line) is compared to that in the presence of 10 mM $CaCl_2$ (solid line). The total lipid concentration was 50 μM and the temperature gradient 5 °C/min. The mean temperature of domain dissociation as reported by the release of the two probes was calculated as described in the Materials and methods section from at least three different experiments and is indicated by the open circles with the standard deviation indicated by horizontal error bars.

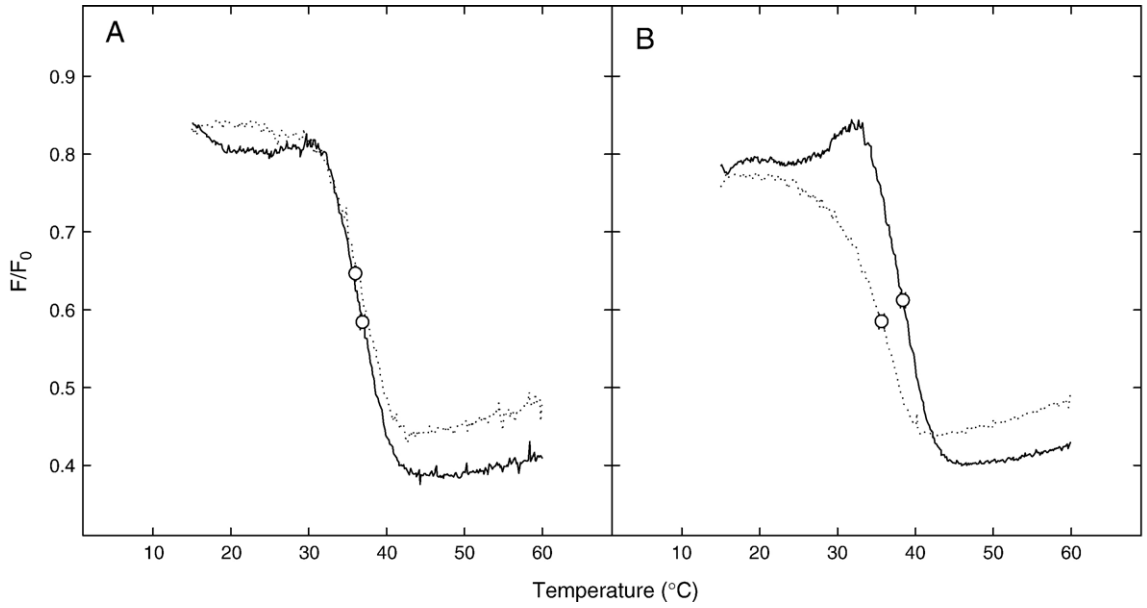

Fig. 6. Formation of ordered domains in complex lipid bilayers containing sphingomyelin, galactosylceramide and sulfatide. The quenching of tPA fluorescence was used as a measure of the amount of ordered domains formed in bilayers without (A) or with (B) sulfatide. The fraction of unquenched fluorescence (F/F_0) is plotted as a function of increasing temperature. The bilayers were prepared in TRIS–NaCl buffer to contain A. POPC (±7SLPC): PSM: PGalCer: cholesterol (60:15:10:10, molar ratio) or B. POPC (±7SLPC): PSM: PGalCer: sulfatide: cholesterol (60:15:10:5:10, molar ratio) with 1 mol% tPA used as the fluorescent probe in both panels. The quenching susceptibility of the probe in vesicles in a calcium free environment (dotted line) is compared to that in the presence of 10 mM $CaCl_2$ (solid line). The total lipid concentration was 50 μM and the temperature gradient 5 °C/min. The mean temperature of domain dissociation as reported by the release of the probe was calculated as described in the Materials and methods section from at least three different experiments and is indicated by the open circles with the standard deviation indicated by horizontal error bars.

When PSM was added to the system as a third sphingolipid component and the amounts of the glycosphingolipids were reduced to 10 mol% GalCer and 5 mol% sulfatide the properties of the lateral domains changed. As seen in Fig. 6 (panel A) membranes containing POPC (±7SLPC): PSM: PGalCer: cholesterol (60:15:10:10, molar ratio) with 1 mol% tPA exhibited ordered domains which were very similar in temperature stability as those reported earlier for closely related mixtures [15]. These domains were not significantly stabilized by the addition of calcium. Addition of 5 mol% sulfatide to the system did not stabilize the sphingolipid domains against temperature per se (Fig. 6B, dotted line), but when calcium was added to the system (Fig. 6B, solid line) a significant stabilization was detected, indicated by a 3 °C higher temperature of domain dissociation. Compared to the results reported in Fig. 5, the domains formed with PSM and glycosphingolipids were more stable against temperature induced changes than those formed by the glycosphingolipids alone.

4. Discussion

This study was conducted to characterize the lateral phase behavior of *N*-palmitoyl-sulfatide in mixed bilayer membranes. Going from more simple lipid mixtures with sulfatide as the only sphingolipid in a fluid matrix of POPC towards more complex membranes including other sphingolipids as well we have looked at 1) ordered domain formation with sulfatide, 2) sterol enrichment in such domains and 3) stabilization of the domains against temperature by the addition of calcium.

Using two distinct phase selective fluorescent probes, *trans*-parinaric acid and cholestatrienol, together with a quencher in the fluid phase, we are able to distinguish between ordered domains in general and ordered domains enriched in sterol [15,39]. According to the quenching experiments with *N*-palmitoyl-sulfatide as the sole sphingolipid it seemed to segregate from the POPC-rich phase to some extent at temperatures well below the physiological, i.e. with domain dissociation at about 25 °C (Fig. 1). The lateral domains formed did however not include sterol according to quenching of the CTL probe. These results are in good agreement with our previous findings that acyl chain-defined monoglycosylceramides form gel phase domains which exclude cholesterol in corresponding bilayer membranes [15]. According to a previous study on GalCer/cholesterol bilayers, cholesterol will not insert into the GalCer matrix until the interactions between adjacent GalCer molecules are disrupted by e.g., higher temperatures [44]. The addition of calcium which has been suggested to interfere with hydrogen bonding interactions between sulfatide molecules did however not increase the partitioning of sterol into the sulfatide-rich domains in our system.

Since sulfatide is always accompanied by other sphingolipids in biological membranes we looked at the propensity of the molecule to participate in domain formation with PSM. The partition coefficient reported here for the fluorescent tPar-sulfatide shows that it has a clear preference for the PSM-rich gel phase compared to the POPC-rich fluid phase (Fig. 2). This is in good agreement with the partitioning reported by Silvius and Wang for an indolyl-sulfatide in a brain-SM:DOPC: cholesterol (1:1:1, molar ratio) mixture [45]. We could safely

assume that the tPar-sulfatide would behave similarly to *N*-palmitoyl-sulfatide in the bilayer membranes, since we found tPar-SM and PSM to have very similar partition coefficients when studied by the same methods. Also, tPA had a clear preference for the PSM-rich phase in our system. The quenching of tPA showed that PSM-rich domains were formed in the presence of 5 mol% sulfatide (Fig. 3). Our results also showed that the PSM-rich domains with sulfatide were significantly stabilized by calcium in a concentration dependent manner. Since no such stabilization was detected for sphingomyelin-rich domains without sulfatide we could conclude that the calcium dependence was related to the presence of sulfatide in the domains. The mean temperature for domain dissociation was affected by calcium to much the same degree and at corresponding calcium concentrations as has previously been reported for the T_m of mixed DSPC:sulfatide membranes [3]. The thermotropic behavior of pure sulfatide has also previously been shown to be of a complex, calcium dependent nature [25]. The intermolecular hydrogen bonding is affected by the presence of calcium in an acyl chain dependent manner, so that the largest effect of calcium is seen with sulfatides containing hydroxylated acyl chains [30]. In our system the *N*-palmitoyl-sulfatide-containing lateral domains were stabilized by the presence of calcium. However, we also found that the calcium effects were dependent on sulfatide concentration in the bilayer.

From the quenching experiments it was also clear that sterol was included in the domains formed by PSM and sulfatide (Fig. 3). Our results thereby support the recent AFM study which indicated that sulfatide is enriched in the sterol-rich domains in a phosphatidylcholine bilayer where liquid-disordered and liquid-ordered phases coexist [23]. We have previously shown that small differences in the head group structure of neutral glycosphingolipids can influence their lateral domain formation quite drastically [15]. In this previous study we also examined if a mixture of two palmitoylated glycosphingolipids with different head groups (15 mol% of each) would be able to accommodate sterols when present in the same membrane. The quenching of CTL was studied in bilayers composed of POPC (±7SLPC): glycosphingolipids: sterol (60:15+15:10, mol%). The results showed that sterol accommodation in the domains was largely dependent on which two glycosphingolipids were present in the mixture. Here we wanted to compare our previous results with palmitoylated neutral glycosphingolipids (PGalCer, PGlcCer and PLacCer) to the results obtained with *N*-palmitoyl-sulfatide. Studying complex lipid mixtures in which sulfatide and PGalCer constituted the sphingolipid components showed that these two glycosphingolipids formed ordered domains that could be detected by quenching of tPA (Fig. 5). The temperature stability of these domains was lower than for pure PGalCer domains, but significantly higher than for pure sulfatide domains (Fig. 1 and [15]). On the other hand, it seems that the PGalCer/sulfatide mixture in this study was capable of accommodating at least somewhat more sterol in comparison to mixtures of PGalCer with other neutral palmitoylated glycosphingolipids [15]. The domains formed by sulfatide and PGalCer were not stabilized by calcium to any significant degree. Earlier model membrane studies have shown that GalCer and sulfatide in different populations of unilamellar phospholipid vesicles will cause aggregation and intermembrane interaction [34,46,47]. This interaction was highly dependent on the presence of divalent cations in the surrounding medium [9,46], indicating that quite different forces govern that interaction compared to the lateral interactions within the membrane plane.

The most complex bilayers included in this study were those composed of POPC (±7SLPC): PSM: PGalCer: sulfatide: cholesterol (60:15:10:5:10, molar ratio) with 1 mol% tPA. These bilayers were studied in comparison to similar bilayers without sulfatide (Fig. 6). The domains formed by PGalCer and PSM were largely unaffected by the addition of 5 mol% sulfatide. Without sulfatide the sphingolipid domains were also not stabilized by calcium to any significant degree. However, when sulfatide was included in the lipid mixture the addition of calcium to the surrounding buffer stabilized the sphingolipid domains significantly against temperature.

In conclusion, we have shown that *N*-palmitoyl-sulfatides form lateral domains to some extent by themselves but participate even more readily in domain formation with other sphingolipids. Therefore, we conclude that *N*-palmitoyl-sulfatide at physiological concentrations is most likely to participate in lateral domain formation with other sphingolipids also in biological membranes. We can also conclude that although individual glycosphingolipids might form gel phase domains that do not include sterol, mixtures of sphingolipids including sphingomyelin will form ordered domains in a fluid lipid matrix and these domains will be enriched in sterol as well (this study and [15]). It also seems from our results that sulfatide at physiologically relevant concentrations forms lateral domains with other sphingolipids present in the bilayer and that the stability and properties of the lateral domains will be influenced by the presence of divalent counter ions, such as calcium.

Acknowledgements

This study was supported by generous grants from the Academy of Finland, the Sigrid Juselius foundation, the Magnus Ehrnrooth foundation, the Oskar Öflund foundation, the K. Albin Johansson foundation and Medicinska Understödsföreningen Liv och Hälsa. The work of Y.J.E. Björkqvist was supported by the National Graduate School in Informational and Structural Biology at Åbo Akademi University.

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