SUPPORTING MATERIAL

Importance of the sphingoid base length for the membrane properties of ceramides

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MATERIALS AND METHODS

Materials – 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL) and cholesterol (CHL) and methyl-β-cyclodextrin (mβCD) from Sigma/Aldrich (St. Louis, MO). All lipid stock solutions were prepared in hexane:isopropanol (3:2 by vol), stored at –20°C and warmed to ambient temperature before use. *Trans*-parinaric acid (tPA) was prepared from α-linolenic acid according to (1). Cholesta-5,7,9(11)-trien-3-beta-ol (CTL) was synthesized and purified as described previously (2). 1-Palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine (7SLPC) was synthesized from 7-doxyl-stearic acid (TCI Europe N.V., Belgium) and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids) in an esterification reaction as described previously (3). tPA, CTL and 7SLPC were stored dry under argon at –87°C until dissolved in argon-purged methanol (for tPA and 7SLPC) or ethanol (for CTL), and used within a week. The water used for sample preparation was purified by reverse osmosis followed by passage through a Millipore UF Plus water-purification system (Millipore, Billerica, MA) to yield a product with final resistivity of 18.2 MΩcm. All other organic and inorganic chemicals were of highest purity available, and solvents of spectroscopic grade.

tPA fluorescence lifetime measurements – Fluorescence lifetimes of tPA were measured in multilamellar vesicles (100 μM final lipid concentration containing 0.5 mol% of tPA) of varying lipid composition (POPC/Cer, 85:15, POPC/PSM/CHL, 60/30/10 and 75/15/10, and POPC/PSM/Cer/CHL, 60:15:15:10 mol%). The vesicles were prepared by mixing the lipids and tPA and evaporating the solvent under a stream of nitrogen. Residual solvent was removed under vacuum for 1 h, and the resulting lipid films hydrated with argon-purged MQ-water at 65 °C for 30 min. The samples were then vortexed briefly, saturated with argon and sonicated for 10 min at 65°C (Bransonic 2510 bath sonicator, Branson Ultrasonics, Danbury, CT). Before fluorescence

measurements the samples were kept in the dark at RT over night. The fluorescence decays of tPA were recorded at 23°C with a FluoTime 200-spectrometer with a PicoHarp 300E time-correlated single photon counting module (PicoQuant GmbH, Berlin, Germany). tPA was excited with a 298 nm led laser source and the emission collected at 430 nm. The samples were kept under constant stirring during the measurements. Data were acquired and analyzed with the FluoFit Pro-software obtained from PicoQuant. The decay fits were obtained by a non-linear least squares iterative reconvolution method based on the Marquardt-Levenberg algorithm. The quality of the fits and the justification for the number of exponentials was assessed from the reduced χ^2 and a random distribution of the weighted residuals.

DSC – Multilamellar vesicles for DSC were prepared by mixing PSM and the ceramide analogs (1:1 by mol) and evaporating the solvent under a stream of nitrogen. Residual solvent was removed under vacuum for 1 h. The resulting lipid films were then hydrated with MQ-water (1 mM final lipid concentration) at 95°C for 30 min, followed by bath sonication at 95°C with several rounds of intermittent vigorous vortex mixing until opalescent and homogeneous preparations were obtained. The temperature for preparation of the DSC-samples was higher than in the other experiments due to the remarkably higher amount of high-melting temperature lipids. The samples were cooled down to room temperature and degassed for 5 min with a ThermoVac instrument (Microcal, Northampton, MA) before transfer to the DSC. Four consecutive heating and cooling scans were recorded between 20°C and 95°C at a temperature gradient of 1°C/min using a high-sensitivity Microcal VPDSC-instrument (Northampton, MA). Data analysis was performed using Origin-software (OriginLab, Northampton, MA). We observed the repeated heating and cooling thermograms to be essentially identical in shape. Thermograms shown in the results are the second heating scans.

Fluorescence quenching experiments – Lipid vesicles of varying composition (POPC/PSM 82/18, POPC/PSM/CHL, 70/15/15, 60/30/10, or 75/15/10, POPC/PSM/Cer 70/15/15, and POPC/PSM/Cer/CHL, 60:15:15:10 mol%) for fluorescence quenching experiments were prepared to a final lipid concentration of 50 μM. The required amounts of lipids, fluorescent probes (CTL or tPA) and the quencher (7SLPC) were mixed and dried under a stream of nitrogen. Two sets of vesicles were prepared for each desired composition, the quenched F-samples that contained the probe (1 mol% of CTL replacing an equal amount of cholesterol or 1 mol% of tPA added to the lipid solution) and the quencher (30 mol% of 7SLPC replacing an equal amount of POPC), and the F_0-samples that contained the probe but no 7SLPC. Residual solvent was removed in vacuum for at least 30 min. The dry lipid films were stored under argon at –20°C until hydrated one at a time for 30 min with pre-heated, argon-purged MQ-water at 65°C. Multilamellar vesicles were then formed at 65°C by sonicating the CTL-containing samples with a probe sonifier (W-450, Branson Ultrasonics, Danbury, CT) for 2 min (25% duty cycle and 10W power output), and the tPA-containing samples with a Branson bath sonicator for 5 min. Quenching data were collected with a QuantaMaster-1-spectrofluorometer (Photon Technology International, Lawrenceville, NJ) by measuring the fluorescence signal of tPA (Ex 305 nm, Em 405 nm) or CTL (Ex 324 nm, Em 390 nm) while heating the samples from 10°C to 65°C at a rate of 5°C/min. The excitation and emission slits were set to 5 nm for measuring CTL and, due to higher quantum yield, to 4 nm for tPA. The temperature was controlled by a Peltier element with a temperature probe immersed in the sample solution. The measurements were

made in quartz cuvettes with a light path length of 1 cm and the sample solutions were kept at a constant stirring (350 rpm/min) throughout the measurement. Fluorescence emission intensity was measured both in the F-sample (quenched) and in the F_0-sample (unquenched). Fluorescence quenching was then calculated using the PTI FeliX32-software and reported as the F/F_0 ratio against temperature.

Sterol partition assay – Sterol partitioning measurements between large unilamellar vesicles and mβCD were performed as described previously (4, 5). Briefly, lipids were mixed (POPC, POPC/PSM/CHL 60/30/10 or 75/15/10, and POPC/PSM/Cer/CHL, 60:15:15:10 mol%) with 2 mol% of CTL, and dried under a stream of nitrogen. The lipid films were then hydrated for 30 min at 65°C and vortexed briefly to form multilamellar vesicles at 1 mM lipid concentration. The multilamellar vesicles were then extruded through a polycarbonate membrane with 200 nm pores to yield unilamellar vesicles, which were used for the partitioning assay. The molar fraction partition coefficient (K_x) of CTL was determined by measuring the anisotropy of CTL (Ex 324 nm, Em 390 nm) with a QuantaMaster-1-spectrofluorometer in a series of samples with 40 μM final lipid concentration and increasing concentration of mβCD. Anisotropy was measured at equilibrium conditions at 23°C after incubation of the samples over night, or at 37°C after 2 h of incubation. During the fluorescence measurements, the temperature in the samples was controlled by a Peltier element under constant stirring. The CTL-anisotropies were then converted to molar partition coefficients as previously described in (4).

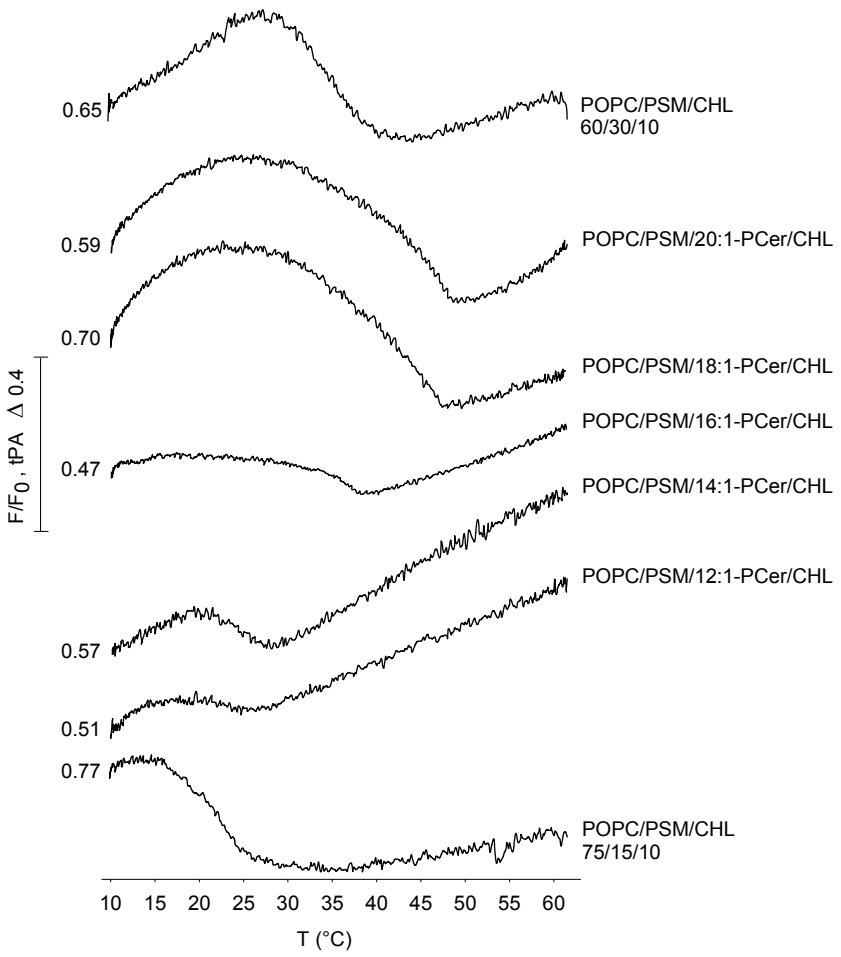

FIGURE S1 Effect of the ceramide analogs on melting of ordered domains in complex bilayers. 7SLPC-induced fluorescence quenching of tPA as a function of temperature was measured in multilamellar vesicles with 50 μM final lipid concentration. The vesicles were composed of POPC/PSM/CHL (60/30/10 or 75/15/10 mol%) or POPC/PSM/Cer/CHL (60/15/15/10 mol%). F/F_0 was defined as the relative fluorescence intensity of tPA in the F-samples that contained both 7SLPC (replacing 50% of POPC) and tPA (1 mol%) over the intensity in the F_0-samples that contained tPA (1 mol%) but no 7SLPC. Only 18:1- and 20:1-PCer were able to increase the end-melting temperature of the PSM-rich ordered domains when compared to the bilayers that contained 30 mol% of PSM. POPC/PSM/CHL 75/15/10-molar composition is shown as a reference for bilayers with reduced amount of PSM but no ceramide. As a reference to the relative changes in the F/F_0 between the different mixtures, the initial F/F_0-value is given for each measurement. Representative curves of at least four independently repeated experiments are shown.

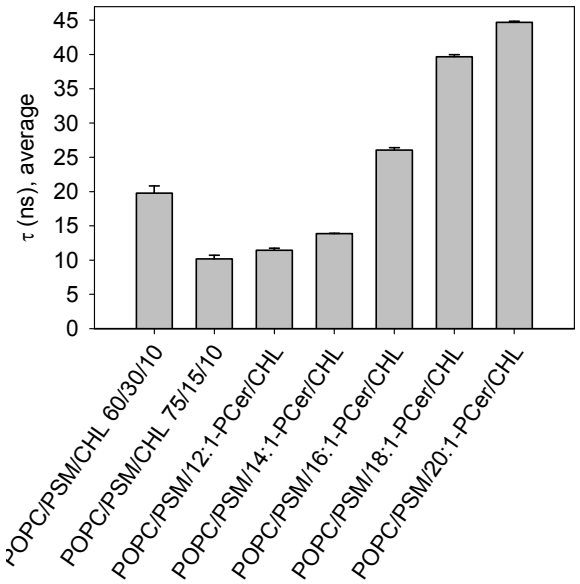

FIGURE S2 Effect of the ceramide analogs on the order of complex bilayers. Time-resolved fluorescence decays of tPA (0.5 mol%) were measured in multilamellar vesicles (100 μM final lipid concentration) composed of POPC/PSM/CHL (60/30/10 or 75/15/10 mol%) and POPC/PSM/Cer/CHL (60/15/15/10 mol%) at 23°C. When half of the PSM in the bilayers that contained 30 mol% of PSM was replaced by the ceramides, 16:1-, 18:1-, and 20:1-PCer were observed to increase the average lifetime of tPA. POPC/PSM/CHL 75/15/10-molar composition is shown as a reference for bilayers with reduced amount of PSM but no ceramide. Average intensity weighted lifetimes ±SD for triplicates are shown.

Table S1 Time-resolved tPA fluorescence decays in ceramide containing bilayers at 23°C.

	τ_1	f_1	α_1	τ_2	f_2	α_2	τ_3	f_3	α_3
POPC	5.9±0.1	86.9±0.6	67.8±0.2	1.9±0.1	13.1±0.6	32.2±0.2			
POPC/12:1-PCer	6.6±0.1	84.1±4.2	68.2±3.1	2.6±0.5	15.9±4.2	31.9±3.1			
POPC/14:1-PCer	6.8±0.1	86.3±2.2	71.1±1.9	2.7±0.3	13.8±2.2	28.9±1.9			
POPC/16:1-PCer	36.8±0.1	26.4±0.6	5.6±0.2	7.7±0.1	62.4±2.1	63.1±2.6	2.8±0.2	11.2±1.5	31.3±2.4
POPC/18:1-PCer	49.9±0.1	65.1±1.0	16.9±0.9	8.7±2.0	25.3±5.6	40.6±14.0	2.7±1.0	9.6±6.1	42.5±13.3
POPC/20:1-PCer	57.9±1.2	73.0±2.7	20.5±1.0	10.6±3.6	16.2±3.0	29.4±13.1	3.4±0.8	10.9±5.4	50.2±13.5
POPC/PSM/CHL 60/30/10	37.8±1.0	28.2±3.9	9.6±1.6	15.0±0.7	55.6±0.5	47.4±2.8	4.8±0.6	16.2±3.8	43.0±4.4
POPC/PSM/CHL 75/15/10	14.3±1.1	45.8±3.2	25.7±2.4	7.1±0.5	51.1±2.8	57.7±2.2	1.6±0.2	3.2±0.4	16.5±0.4
POPC/PSM/12:1-PCer/CHL	15.5±0.4	49.0±1.4	28.2±0.8	7.8±0.2	48.8±1.2	56.2±3.3	1.4±0.5	2.2±0.3	15.6±4.1
POPC/PSM/14:1-PCer/CHL	25.8±4.1	20.6±11.3	9.6±6.2	12.1±1.4	69.8±6.4	63.8±1.3	3.8±1.5	9.6±5.0	26.5±5.9
POPC/PSM/16:1-PCer/CHL	47.3±0.9	36.5±2.6	12.2±1.0	15.9±1.0	51.5±1.3	51.6±4.0	5.2±1.0	12.1±3.9	36.2±4.9
POPC/PSM/18:1-PCer/CHL	56.7±0.1	60.5±1.0	22.6±0.6	16.3±0.6	29.2±0.6	38.0±2.5	5.5±0.3	10.3±1.6	39.5±3.1
POPC/PSM/20:1-PCer/CHL	59.4±0.3	68.0±0.9	27.3±0.5	15.9±0.4	24.8±0.1	37.3±0.8	4.8±0.3	7.2±0.7	35.4±1.3

All binary mixtures were 85/15 (mol%), and the quaternary mixtures containing ceramides 60/15/15/10 (mol%). τ; lifetimes (ns), *f*; fractional intensities (%), α; fractional amplitudes (%). The values are averaged over three independently repeated experiments ±SD.

Supporting References

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