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Palmitoyl ceramide promotes milk sphingomyelin gel phase domains formation and affects the mechanical properties of the fluid phase in milk-SM/DOPC supported membranes

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ABSTRACT

Ceramides are minor structural components of membranes involved in biological functions. In the milk fat globule membrane (MFGM), ceramides are susceptible to affect the lateral packing of polar lipids, especially the milk sphingomyelin (MSM). To investigate this, palmitoylceramide (PCer) was added to MSM/DOPC (dioleoylphosphatidylcholine) in order to form hydrated lipid bilayers. Differential scanning calorimetry evidenced interactions of PCer with the MSM in the solid-ordered phase to form MSM/PCer structures with a higher thermostability than MSM. Atomic force microscopy revealed that PCer modified lipid packing in both the liquid-disordered DOPC phase where it increased thickness and mechanical stability, and the solid-ordered MSM phase where it recruited MSM molecules yet initially in the liquid phase at 26 °C and then increased the area of the MSM/PCer domains. The effect of PCer on the mechanical properties of the MSM-rich domains remains to be elucidated. These results bring new insights on the role of ceramides in the control of biophysical and biological properties of the MFGM. They also open perspectives for the design of emulsions and liposomes, using milk polar lipids as food-grade ingredients.

1. Introduction

Biological membranes are a complex mixture of numerous proteins and lipid molecules (e.g. glycerophospholipids, sphingolipids, cholesterol) in which lipid-driven membrane domains are formed. Sphingosine-based lipids, e.g. sphingomyelin and ceramides, are potent lateral phase segregation inducers. Over the last decades, a high level of interest has concerned ceramides that are minor components of biological membranes and known as intermediates in the metabolism of more complex sphingolipids (e.g. sphingomyelin, cerebrosides, gangliosides) involved in cellular processes and in diseases [1,2,3]. The biological functions of ceramides are thought to depend on their unique biophysical properties which promote alteration of membrane properties.

Ceramides are sphingolipids with a long chain base linked via its amino group to a fatty acid chain and are among the more hydrophobic lipids in membranes because of their small polar head group and hydroxyls found on the long-chain base [4]. The amide group on carbon 2, together with hydroxyls, provides ceramides and sphingolipids in general with efficient hydrogen bonding capability [5,6]. The fact that ceramide acyl chains are mostly saturated and long (16 carbon atoms or longer) and lack a proper large head group gives them some unique membrane properties. Ceramides exhibit a high gel/solid-ordered to liquid-disordered (gel/s_o-to-l_d) phase transition temperature (Tm), generally of over 60 °C [7,8]. The unusual structure and biophysical properties of ceramides contribute to their strong impact on membrane properties, even at very low concentrations [1]. In cellular plasma membranes, endogenous ceramides reach levels of 0.1–1 mol% of total phospholipids but can increase to concentrations up to 10 mol% in apoptotic membranes [9,10]. Ceramide molecules increase the molecular order of polar lipid mixtures and promote lateral phase separation with formation of gel/s_o phase ceramide-rich domains [1,3,11,12]. Ceramides have caught attention in recent years for their significant roles in metabolic signals involved in many cellular events, e.g. as promoters of cell growth arrest or apoptosis, or as coordinators of transmembrane signaling through the formation of ceramide-rich platforms under cellular stress [4,13,14].

Of all biological membranes, the one enveloping the milk fat globule (called the milk fat globule membrane, MFGM [15]) is of particular interest since it corresponds to about 160 m^2 per liter of milk and it is involved in many functions, e.g. immunity in the gastrointestinal tract of the neonate and mechanisms of milk lipid digestion [16,17,18]. The

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main polar lipids of the MFGM are PE, PC, PI, PS and the sphingolipids, mainly milk sphingomyelin (MSM; about 30 wt% of milk polar lipids, Tm ~ 33–34 °C [19,20]). Ceramides are present in the MFGM at significant levels compared to plasma membrane, ranging 1.5–5 mol% of the total phospholipids [21,22], but whether they form local ceramide-rich platforms as in plasma membranes [4] is currently unknown. Ceramides are also metabolites generated by sphingomyelinase enzymatic degradation of MSM (e.g. in the intestine upon digestion of milk lipids [23,24]) that have well-known bioactive properties [25]. In particular, the synthesis and activity of alkaline sphingomyelinase increase in the neonatal period of suckling mammal offsprings to yield ceramides and downstream metabolites implicated in maturation of the intestinal tract and in the development of digestive functions [26,27]. In the MFGM surrounding fat globules *in situ* in milk, authors showed the lateral segregation of MSM-rich domains in the gel phase or in the liquid-ordered phase in presence of cholesterol surrounded by a fluid phase composed of unsaturated polar lipids [28,29]. These MSM-rich domains are correlated with thickness mismatch at the phase boundary and with mechanical heterogeneity [30,31,32] that may both affect the stability of the milk fat globule toward enzymatic or physical stresses. The biophysical properties and functional role of ceramides in the MFGM and in particular toward MSM-rich domain formation remain to be elucidated.

In the last decades, the preparation of hydrated supported lipid bilayers and their exploration using atomic force microscopy (AFM) has proven a powerful approach to characterize the topography (i.e. phase separation, formation of domains, area and shape of the domains, bilayer thickness) of membranes with nanoscale resolution [33,34,35,36]. Moreover, AFM spectroscopy allows probing local nanomechanical properties of supported lipid bilayers [37]. In our earlier reports, we have demonstrated using AFM that the thermotropic properties, topography and mechanical properties of MSM/DOPC (50/50 mol%) and MFGM model membranes can vary with composition, particularly the presence of cholesterol [20,31,38]. Thus, lipid molecules naturally present in small amount in biological membranes, as the cholesterol, can modulate the topography and mechanical properties of the MFGM. The role played by other lipid molecules naturally present in the MFGM, e.g. ceramides, on the biophysical properties of the MSM domains is unknown and requires further investigations.

In the present study, we investigated the role of PCer on the thermotropic phase behavior and topography of MSM/DOPC bilayers. The thermotropic phase behavior of MSM/DOPC/PCer multilamellar vesicles was examined by differential scanning calorimetry. Also, hydrated bilayers of MSM and DOPC were prepared in presence of increasing concentrations of palmitoyl ceramide (PCer) and the topography of MSM/DOPC/PCer supported bilayers was investigated at room temperature (i.e. below the Tm of MSM) using AFM.

2. Experimental methods

2.1. Materials

Sphingomyelin from bovine milk (MSM; > 99%), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; > 99%) and the chemically well-defined synthetic *N*-palmitoyl-D-*erythro*-sphingosine (C16:0-ceramide; PCer, > 99%) were purchased from Avanti Polar Lipids (Alabaster, AL) and used as received (Fig. 1).

MSM has a phosphatidylcholine head group and a long chain base as the hydrophobic backbone (mainly d18:1 [39]), together with an amide-linked acyl chain. The N-acyl chains of MSM have various lengths and unsaturation: 19% C16:0, 3% C18:0, 1% C20:0, 19% C22:0, 33% C23:0, 20% C24:0, 3% C24:1*n*-9 [19,38]. PCer has a hydroxyl head group and the same sphingosine backbone as the main one found in MSM with C16:0 as the acyl chain. DOPC has a phosphatidylcholine head group and the monounsaturated acyl chains C18:1*n*-9 as hydrophobic backbone. PIPES 10 mM (1,4-piperazinediethane sulfonic acid; purity ≥ 99%; Sigma Aldrich, Milwaukee, WI, USA) buffer was prepared with NaCl 50 mM and 5 mM $CaCl_2$ (Sigma) in Milli-Q water and adjusted to pH 6.7 using NaOH 5 M. Lipid samples were prepared by dissolving appropriate amounts of stock solutions of MSM, DOPC and PCer in chloroform/methanol (4/1 v/v) and by mixing them in the desired molar proportions. The organic solvent was then evaporated under a stream of dry nitrogen at 50 °C, and the dried films stored at − 20 °C until used.

2.2. Differential scanning calorimetry (DSC)

The thermotropic properties of multilamellar vesicles composed of the individual polar lipids PCer, MSM, DOPC, the binary systems MSM/PCer (9.5 mol% PCer and 16.5 mol% PCer; PCer/MSM molar ratio of 10.5 and 19.8 mol%, respectively) and DOPC/PCer (16.5 mol% PCer), and the ternary systems MSM/DOPC/PCer (47.5/47.5/5 and 45.5/45.5/9 mol%; PCer/MSM molar ratio of 10.5 and 19.8 mol%, respectively) were determined by DSC using a DSC Q1000 apparatus (TA Instruments, Newcastle, DE). The calibration of the calorimeter was performed with indium standard (melting point = 156.66 °C, ΔH melting = 28.41 $J \cdot g^{-1}$). The lipids were hydrated at 70 °C (95–100 °C for PCer) with PIPES buffer with NaCl 50 mM and 5 mM $CaCl_2$ pH 6.7 to reach a final concentration of 16–18 wt% lipids for MSM, PCer, MSM/PCer mixtures and 30–40 wt% lipids for MSM/DOPC/PCer samples. The dispersions were heated at a temperature above the transition temperature of the individual lipids and mixtures and thoroughly mixed in a vortex stirrer to form multilamellar vesicles. The samples were introduced in 20 μL aluminum pans hermetically sealed after sample introduction. An empty pan was used as a reference. The samples were cooled at 1 °C·min^{-1} and heated at 5 °C·min^{-1} either in the temperature range from − 10 °C to 110 °C to avoid ice formation in the samples or from − 40 °C to 110 °C to characterize DOPC phase transition. Additional DSC experiments were performed on heating at 1 °C·min^{-1} and did not reveal any difference in the endothermic events, but the signal was very low (results not shown). All the thermal measurements were performed in triplicate. Standard parameters were calculated by the software (Universal Analysis 2000, v 4.1 D).

2.3. Atomic force microscopy (AFM)

The ternary system MSM/DOPC/PCer was investigated using AFM. The achieved concentrations of PCer were 0, 5 and 9 mol% of the total polar lipids while the molar ratio MSM/DOPC was kept constant at 50/50, which corresponds to PCer/MSM molar ratios of 0, 10.5 and 19.8 mol% (as for DSC experiments). The MSM/DOPC/PCer dried samples were hydrated with PIPES-NaCl-$CaCl_2$ buffer at 65 °C (i.e. above the Tm of lipids) to reach a final concentration of 0.1 wt% lipids then thoroughly vortexed. Small unilamellar vesicles (SUV) were produced at ~65 °C by sonication [40], diluted 10-fold (v/v) with hot PIPES-NaCl-$CaCl_2$ buffer, deposited onto freshly cleaved mica in an Asylum Research AFM liquid cell, then incubated at ~65 °C for 60 min. Slow cooling of the samples was performed using a programmed incubator at rates sequentially decreasing from ~1 °C·min^{-1} to ~0.1 °C·min^{-1}. Once equilibrated at room temperature (24.6 ± 2 °C), the bilayers were extensively rinsed with PIPES buffer. AFM imaging of the bilayers was performed in contact mode using an MFP-3D Bio AFM (Asylum Research, Santa Barbara, CA, USA), silicon MSNL probes (nominal spring constant k ~0.03 $N \cdot m^{-1}$ - Bruker Nano Surfaces, Santa Barbara, CA, USA) and loading forces typically below ~1 nN. Temperature inside the AFM liquid cell was ~26 °C. The typical scan rate was 0.5 Hz for 256 × 256 pixels images. Up to 10 images of 10 × 10 μm^2 scan size were recorded at various locations on each sample and were found similar for a given lipid composition. The images were typically planefitted at order 0, flattened at order 1 then planefitted again after masking of the protruding domains. Sections were drawn across images to measure the height difference H between