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Short communication

Ceramide modulates the lipid membrane organization at molecular and supramolecular levels

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Abstract

The role of lipids in membranes has changed rapidly from static to dynamic and emphasized their involvement in information transduction, linking temporal and topological structuring through compositionally driven effects. Ceramide has been described as an important modulator of different membrane functions. In mixtures with ganglioside GM1, the condensation induced by ceramide increases intermolecular interactions, leading to an increase of the phase transition temperature and size of the self-assembled structure. In mixtures with phosphatidylcholines, ceramide segregates laterally in the gel state, forming domains whose thickness depend on global concentration and chain asymmetry of the sphingolipid.

Keywords: Ceramide; AFM; Interdigitation; Topology; Phase diagram; Lipid domains

1. Introduction

Through the formation of ceramide, the sphingomyelin cycle (Kronke, 1999) relates the metabolism of sphingomyelin to that of glycosphingolipids that are important modulators of membrane function. In transient conditions in living cells, ceramide can reach 3–20 mol% of total phosphatidylcholine (Obeid et al., 1993).

Depending on constraints determined by molecular geometry (Carrer and Maggio, 2001) ceramide favors hexagonal II (HII) phase formation by segregating in domains with negative curvature that affect membrane fission, fusion and bilayer permeability (Holopainen et al., 2000; Kolesnick et al., 2000). Also, depending on the relative proportions of HII phase-forming lipids (such as ceramide, phosphatidylethanolamine, and diacylglycerol) and gangliosides (HI phase-forming lipids), topological compensation of membrane tensions modulate non-bilayer to bilayer transitions (Maggio, 1994; Veiga et al., 1999). In natural membranes liquid-ordered state regions, enriched in ceramide, sphingomyelin, cholesterol and glycosphingolipids resistant to detergent dissolution and known under the name of 'rafts'(Kolesnick et al., 2000), are assumed to exist as laterally segregated domains. To understand the physico-chemical bases of the effects of ceramide, it is necessary to

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characterize its participation in generating segregated phases in mixed lipid systems. In the present work, we have studied the thermodynamic and structural effects of different proportions of ceramide in mixtures with ganglioside GM1 or with phosphatidylcholines.

2. Materials and methods

Ganglioside GM1 was purified from bovine brain (Fidelio et al., 1991). Bovine brain ceramide (Type III) was from Sigma–Aldrich (St. Louis, MO). dpPC, dmPC and C8Ceramide (*N*-octanoyl sphingosine) (C8Cer) were from Avanti Polar Lipids. HPTLC showed over 99% purity. Lipids were premixed from chloroform–methanol (2:1) solutions (with 5% water for GM1), dried under N_2, hydrated (after high vacuum for 4 h) with 145 mM NaCl, submitted to four freeze-thaw cycles of 5 min, and mechanically dispersed at 70 °C. For calorimetry, the dispersions were scanned at 45 or at 30 °C/h (for GM1–Cer mixtures) in a Microcal MC-2D Differential Scanning Calorimeter. Dispersion size distributions were measured at 23 °C by dynamic light scattering (DLS) in a submicron photon correlation spectrometer (Nicomp-380). Negatively stained (2% uranyl acetate) dispersions were inspected in a JEM1200 EXII-JEOL electronic transmission microscope. For X-ray diffraction, lipid dispersions were introduced into glass capillaries, sealed, mixed at 70 °C by repeated inversion/centrifugation, exposed at room temperature at a detector distance of 12 cm for 2 h, to a generator operating at 50 kV and 160 mA with a Cu–K radiation source, a graphite crystal monocromator, and 0.5 mm diameter collimator for exposures. Still photographs were recorded, and the intensity of the diffraction lines was measured by an averaged radial densitometry. For Atomic Force Microscopy (AFM), the multilamellar vesicles were extruded through 0.1 μm pore polycarbonate membranes at 65 °C to obtain unilamellar vesicles 80 ± 30 nm diameter (LUVs); 100–150 μl of LUVs (0.05–0.1 mg/ml) were incubated for 5–30 min over freshly cleaved mica in a custom-made sample cell at the desired temperature. After extensive rinsing with pure water, the sample was observed with a Pico Scan AFM-STM equipment under water. Silicon cantilevers with Al coating, nominal force constant 0.35 N/m were used.

3. Results and discussion

3.1. Structural features of ceramide-containing gel-state bilayers

The temperature–composition phase diagram of mixtures of dpPC with ceramide was reported previously (Carrer and Maggio, 1999). We show here that the thermotropic behavior, X-ray diffraction pattern and lateral structuring shown by AFM of self-assembled dispersions coincide in indicating the coexistence of gel-state phases with different structural features.

Fig. 1A shows the variation of the onset temperature for gel-phase melting (Tg) in mixtures of dpPC with ceramide up to a proportion of 40 mol%. Tg remains constant up to 8 mol% ceramide showing that dpPC in the gel-phase state is excluded from another gel phase enriched in ceramide. At a mole fraction of 0.12, ceramide becomes partially miscible with dpPC in the gel phase. The isothermal Tg of the mixture between 12 and 30 mol% ceramide indicates melting of a gel-state solution, coexisting with another gel phase enriched in the sphingolipid. Between a ceramide mole fraction of 0.3 and 0.4, only one gel phase is present. The mixture of dpPC with 8–12 mol% ceramide represents a gel phase (S_1) that is immiscible with the gel phase of pure dpPC and with the higher melting gel phase (S_2) formed above 30 mol% ceramide (Fig. 1A).

The X-ray diffraction studies at 25 °C were performed on pure dpPC and mixtures with ceramide at 4, 20 and 38 mol%. All samples showed between 2 and 4 low-angle reflections whose indexing correspond to lamellar structures. Fig. 2 shows the intensity vs. the dispersion vector scans corresponding to the different samples. For pure dpPC, the small angle region shows a lamellar spacing of 66 Å, and the wide angle region a lattice spacing of 4.08 Å, in agreement with literature for the $L_{\beta'}$ phase. At 4 mol%, the

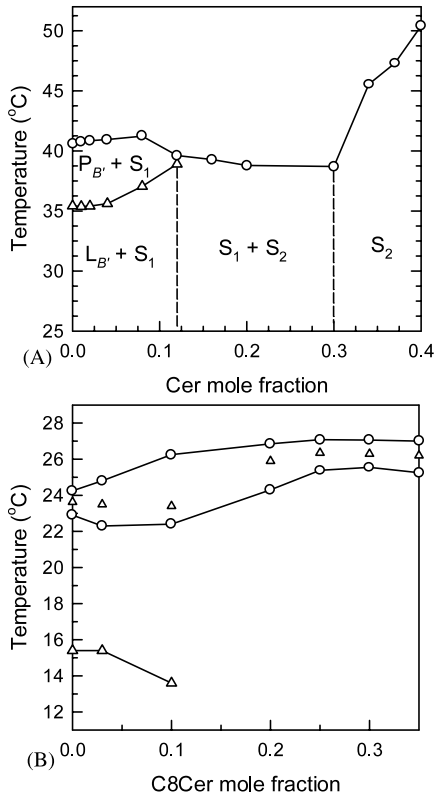

Fig. 1. Partial calorimetric phase diagrams of phospholipid–ceramide mixtures. (A) Variation of the onset temperature for gel-phase melting in mixtures of dpPC with bovine brain ceramide. (B) Partial calorimetric phase diagram of dmPC–C8Cer mixtures. Circles are onset and completion temperatures, triangles are Tm (temperature of maximal Cp) values.

intensity peaks in the small angle region are wider than for pure dpPC, probably indicating some phase coexistence, but the lamellar spacing (66 Å) and the lattice spacing (4.06 Å) indicate a similar bilayer structure than that of dpPC. This is in agreement with the isothermal melting (for both the pre-transition and the main transition of dpPC) observed in the gel-phase diagram at this composition (see Fig. 1A). At 20 mol% ceramide, the diffraction pattern reveals fewer and more diffuse reflections (suggesting less ordered structures). The small angle region shows only two reflections corresponding to increased spacings of 71 Å, and the wide angle spacing shows a broad reflection at 4.13 Å (Fig. 2). At 38 mol% ceramide, the intensity peaks at the low angle show three

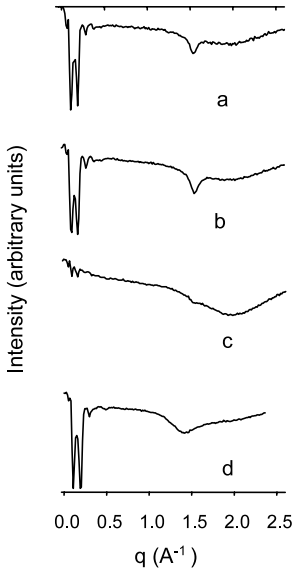

Fig. 2. X-ray diffraction data of gel-phase dpPC–bovine brain ceramide samples. The intensity scans corresponding to pure dpPC (a), 4 (b), 20 (c) and 38 mol% ceramide (d) are shown as a function of the dispersion vector.

broad reflections corresponding to lamellar spacings of 66 Å (that of pure dpPC) and one broad, weak, isolated reflection at 12.7 Å which, being single, does not allow assignment to a defined long-range structure; in the wide angle region there is a very broad reflection centered at about 4.55 Å, typical of disordered chain lattice. The broadest and diffuse, single reflection centered at about 3.3 Å, occurring in all samples, corresponds to the aqueous solution and is also found in the absence of lipid.

3.2. Ceramide-induced thermodynamic–topological transduction

Ganglioside GM1 forms a liquid-expanded monolayer. Mixed monolayers with ceramide show condensation, favoring lateral interactions and raising the phase transition temperature. The critical packing parameter, calculated from monolayer data and reflecting the mean molecular geometry, predicts that the increase in the proportion of ceramide should induce a topological change from micelles to bilayer vesicles of increasing size (Carrer and Maggio, 2001).

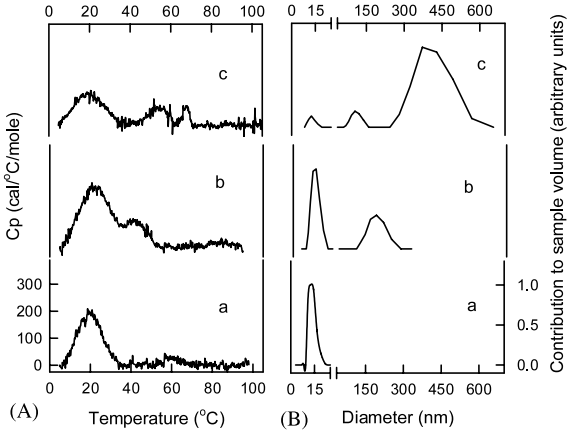

Fig. 3. Calorimetry and DLS data form aqueous dispersions of mixtures GM1–bovine brain ceramide. Excess heat capacity vs. temperature (A) and contribution to sample volume vs. diameter (B) for pure GM1 (a), 5 Cer (b) and 27 mol% Cer (c).

Fig. 3 shows that the broad and low enthalpy (0.8 kcal/mol) thermotropic transition of pure GM1 is centered at about 20 °C. DLS shows a size distribution of 13 ± 2 nm. By increasing the proportion of ceramide to 3 mol%, the micelle size distribution remains similar but the midpoint phase transition shifts up by about 8 °C, reflecting the more condensed intermolecular packing induced by ceramide. At 5 mol% ceramide in the mixture and above, a second and third transition peaks at progressively higher temperatures are found in the calorimetric scans. At 5 mol% DLS indicates the existence of micelles of 13.8 ± 2.2 nm contributing to 70% of the total particle volume, coexisting with multilamellar bilayer vesicle structures of 190 ± 30 nm as confirmed by negative staining electron microscopy (Carrer and Maggio, 2001). At higher proportion of ceramide, the size, polidispersity and percentage of total particle volume contributed by the larger structures increase (Fig. 3).

The combined results obtained by calorimetry and DLS show that the condensation induced by ceramide on ganglioside GM1, which increases favorable intermolecular interactions, is reflected in an increase of the phase transition temperature and size of the self-assembled structure. In addition, the results point out the amplified transduction taking place from the level of the lipid molecular geometries to thermodynamic–geometric compensations in the supramolecular structure.

3.3. Gel-phase immiscibility and bilayer thickness in short-chain ceramide-enriched domains

In bovine brain ceramide, the average length of the longest amide-linked fatty acyl moiety to the sphingosine base introduces hydrocarbon chain asymmetry in a considerable proportion of the molecules of the natural sample. On the other hand, it is known that acyl chain asymmetry can lead to chain interdigitation which, in turn, affects the thermotropic transitions and possibilities for lateral phase separation of domains having different thickness (Huang and Mason, 1986). In order to investigate the influence of ceramide hydrocarbon chain asymmetry on the thermotropic transitions and the structure of phase separated domains we studied the behavior of mixtures of dmPC with C8-ceramide by calorimetry and AFM.

The partial (up to 35 mol% C8Cer) temperature–composition phase diagram of dmPC–C8Cer (Fig. 1B) reveals that these lipids are miscible in the fluid phase and show gel-phase immiscibility at proportions of C8Cer below 10 mol%. Preliminary observations by AFM were performed on the mixtures with 10 and 30 mol% C8Cer, in both cases at 17–20 °C, that is in the gel phase. For the mixture with 10 mol%, the surface as shown by AFM showed patches of bilayer over the mica, with a fairly uniform thickness of 45–50 Å (Fig. 4A). No domains of different thickness were observed that could be reliably distinguished from noise and baseline drift. The maximum thickness of a bilayer of pure gel dmPC should be of 52 Å as calculated by measuring the distance between the extended choline and the terminal methyl group for a molecule constructed with a molecular editor. These observations indicate that, in the mixture with 10 mol% C8Cer, the bilayer thickness is determined by the non-interdigitated, dmPC-enriched gel phase, and the C8Cer-enriched gel phase is probably not interdigitated. For the mixture with 30 mol% C8Cer, the AFM images show bilayer patches that appear to display lateral phase separation (Fig. 4B). The two main thick-

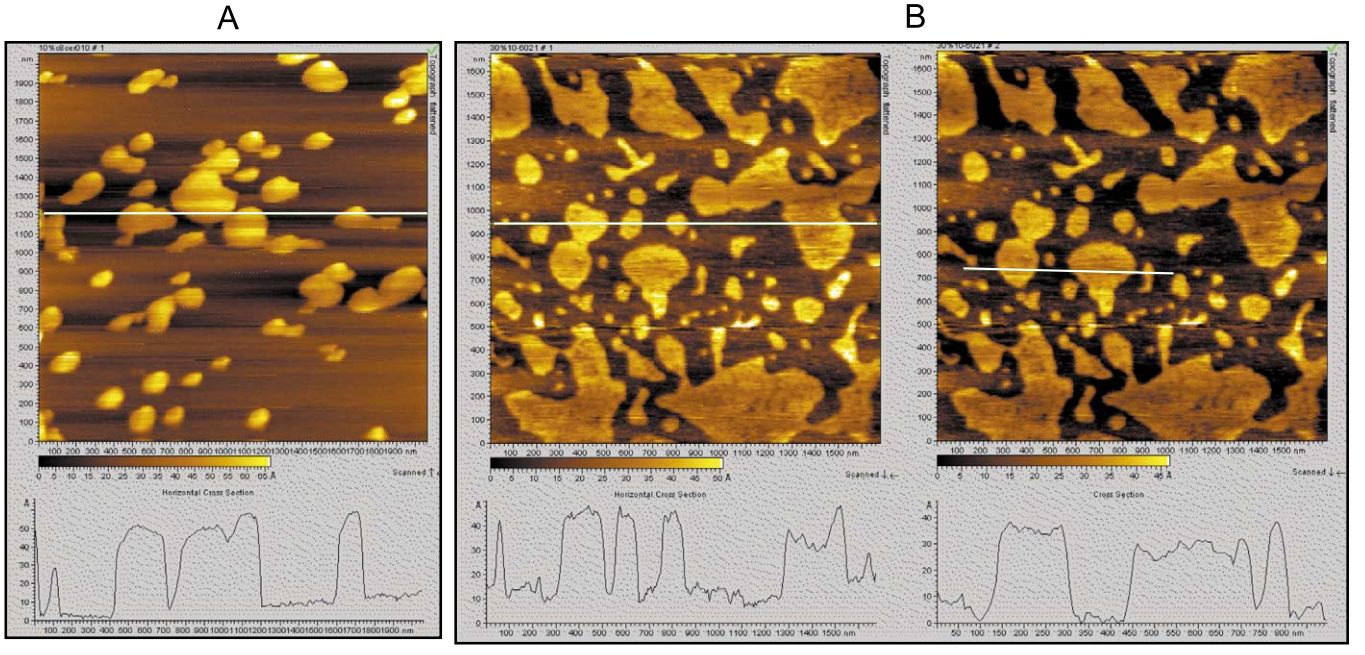

Fig. 4. AFM images of mixtures dmPC–C8Cer. Representative images of 10 mol C8Cer (A) and 30 mol% C8Cer (B) LUVs deposited on mica. The height profiles beneath the images correspond to the horizontal lines drawn in the images.

nesses measured are 24 ± 4 and 38 ± 4 Å. The thickness of a bilayer formed by mixed-interdigitated, all-trans C8Cer should be of 23–25 Å. The observations suggest the existence of mixed-interdigitated, C8Cer-enriched domains in coexistence with dmPC-enriched domains. The latter are somewhat thinner than the bilayer formed at lower C8Cer content, probably indicating the effect of the hydrophobic mismatch generated by the presence of the thinner domains, that would expose the hydrocarbon chains to the water phase.

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