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Phase state and surface topography of palmitoyl-ceramide monolayers

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ABSTRACT

In cell biology (and in many biophysical) studies there is a natural tendency to consider ceramide as a highly condensed, solid-type lipid conferring rigidity and close packing to biomembranes. In the present work we advanced the understanding of the phase behavior of palmitoyl-ceramide restricted to a planar interface using Langmuir monolayers under strictly controlled and known surface packing conditions. Surface pressure–molecular area isotherms were complemented with molecular area–temperature isobars and with observations of the surface topography by Brewster Angle Microscopy. The results described herein indicate that palmitoyl-ceramide can exhibit expanded, as well as condensed phase states. Formation of three phases was found, depending on the surface pressure and temperature: a solid (1.80 nm thick), a liquid-condensed (1.73 nm thick, likely tilted) and a liquid-expanded (1.54 nm thick) phase over the temperature range 5–62 °C. A large hysteretic behavior is observed for the S phase monolayer that may indicate high resistance to domain boundary deformation. A second (or higher) order S → LC phase transition is observed at about room temperature while a first order LC → LE transition occurs in a range of temperature encompassing the physiological one (observed above 30 °C at low surface pressure). This phase behavior broadens the view of ceramide as a type of lipid not-always-rigid but able to exhibit polymorphic properties.

1. Introduction

Since when it was proposed as a key player in various cell signaling processes (Kolesnick et al., 2000), ceramide (Cer) has received considerable attention and it became increasingly clear that most of its effects are strongly related to its capacity to markedly influence the physical properties of cell membranes (van Blitterswijk et al., 2003; Goni and Alonso, 2009). The generation of Cer upon sphingomyelin enzymatic breakdown induces highly ordered segregated lateral structures in artificial systems and other membrane changes (Lopez-Montero et al., 2009; Fanani et al., 2010) that may be involved in the structural dynamics related to cell membrane signaling (Grassme et al., 2007).

Sphingomyelin is the major sphingolipid present in the outer leaflet of cell plasma membranes (Venien and Le, 1988), its fatty acid composition consisting mostly in saturated fatty acids. C16:0 is one of the fatty acids that comprise the bulk of the fatty acid pool linked to sphingomyelin isolated from natural sources (Estep et al., 1979). Therefore, sphingomyelinase-generated Cer in the plasma membrane contains the same fatty acid composition and will predominantly consist of palmitoylCer (pCer) (Goni and Alonso, 2006). The physical properties of Cer and its interaction with phospholipids, cholesterol and/or sphingomyelin have been studied in binary and ternary model system (Maggio et al., 1978; Goni and Alonso, 2006; Lopez-Montero et al., 2009). Regarding pCer, an increasing amount of information is published on the effects of this lipid in binary mixtures (Holopainen et al., 2000, 2001; Goni and Alonso, 2006; Goni and Alonso, 2009; Busto et al., 2009; Lopez-Montero et al., 2009; Karttunen et al., 2009) but comparatively little work has dealt with the physical properties of pure pCer itself (Shah et al., 1995; Chen et al., 2000), likely because technical difficulties have been reported for reaching workable homogeneous samples at proportions of pCer above 30 mol% (Holopainen et al., 2000; Busto et al., 2009), a situation also found for bovine brain Cer (Carrer and Maggio, 1999). An in-depth calorimetric and structural (X-ray diffraction) study was published by Shah et al. (1995). A complex thermotropic behavior was observed for the fully hydrated pCer. A metastable bilayer gel phase exists at low temperatures, with increasing temperature an exothermic transition occurs at 64 °C to form a stable bilayer probably chain tilted with respect to the bilayer normal. Further increase in temperature leads to a disordered (likely HII-type) chain-melted phase (T_m 90 °C). These results were further supported by Moore's work

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Abbreviations: pCer, N-palmitoylsphingosine; LE, liquid-expanded; LC, liquid-condensed; S, solid; BAM, Brewster Angle Microscopy; HII, hexagonal II phase; C_s^{-1}, compressibility modulus.

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using infrared studies (Chen et al., 2000). In a recent study of pCer/palmitoylsphingomyelin interactions in monolayers it was mentioned that pure pCer monolayers exhibit an isothermal surface pressure-induced condensed–condensed phase transition at room temperature (Busto et al., 2009). That transition occurs about 40 °C below the gel–gel transition observed for bulk aqueous dispersions of pCer (Shah et al., 1995; Chen et al., 2000). In cell biology (and in many biophysical) studies there is a traditional tendency to consider Cer as a highly condensed, solid-type, lipid conferring rigidity and close packing to biomembranes. In the present work we advanced the understanding of the phase behavior of pCer restricted to a planar interface using Langmuir monolayers under strictly controlled and known surface packing conditions. This avoids relaxation of lateral tensions causing escape to third dimension through formation of non-lamellar phases that is possible in bulk systems (Veiga et al., 1999). Surface pressure–molecular area isotherms were complemented with molecular area–temperature isobars and with observations of the surface topography by Brewster Angle Microscopy (BAM). The results indicate that, similar to other lipids [including other sphingolipids (Maggio et al., 2004)], pCer can exhibit expanded, as well as condensed phase states. Formation of three phases was found, depending on the surface pressure and temperature: a solid, a liquid-condensed and a liquid-expanded phase over the temperature range 5–62 °C. Noteworthy, a liquid-expanded–condensed transition occurs in a range of temperature encompassing the physiological one (34–39 °C).

2. Experimental procedures

2.1. Chemicals

Palmitoyl-ceramide (pCer) was purchased from Avanti Polar Lipids (Alabaster, AL), it was over 99% pure by thin-layer chromatography and was used without further purification. Solvents and chemicals were of the highest commercial purity available. The water was purified by a Milli-Q (Millipore, Billerica, MA) system, to yield a product with a resistivity of ~18.5 MU/cm. Absence of surface-active impurities was routinely checked as described elsewhere (Bianco and Maggio, 1989). The lipid stock solution was kept at −70 °C until use and at −20 °C during the working day.

2.2. Monolayer isotherms and isobars

Compression–expansion isotherms and isobars were obtained for synthetic pCer at different temperatures and surface pressures. Typically, lipid monolayers were spread from 25 μl of chloroform solution onto a 266 cm^2 Teflon trough filled with 200 mL of 145 mM NaCl, pH ~5.6. The film was relaxed for 5 min at 0 mN/m and subsequently compressed to the target pressure. Surface pressure and film area were continuously measured and recorded with a KSV Minitrough equipment (KSV, Helsinki, Finland) enclosed in an acrylic box. Subphase temperature was controlled by a Haake bath with external circulation (Thermo Electron, Karlsruhe, Germany) and automatically recorded (Lab-Trax, World Precision Instruments, Sarasota, FL) as a function of time. When second compression of the film was studied, the lipid monolayer was compressed up to 35 mN/m, relaxed by decompression to 0.7 mN/m (in about 5 min), allowed to stand at that pressure for 5 min and subsequently compressed to collapse. The collapse and other phase transition points were estimated by the third derivate method (Brockman et al., 1980). Compressibility modulus (C_s^{-1}) was calculated from the isotherm data as: $C_s^{-1} = -A(d\pi/dA)_T$ (Ali et al., 1991; Mohwald, 1995). After reaching the target pressure, isobars were recorded 5 min at 5 °C and then heated to 62 °C (final temperature) at a rate of 1 °C/min. Isobaric thermal expansivity was calculated from isobar data as follows: $\lambda = (1/A)(dA/dT)_\pi$ (Mohwald, 1995). All measurements were performed at a compression rate of 1 ± 0.5 Å^2/mol/min and under N_2 atmosphere.

2.3. BAM measurements

Monolayers were prepared as described above but using a conveniently small Model 102 M equipment (NIMA Technology Ltd, Coventry, England). The Langmuir equipment was mounted on the stage of a Nanofilm EP3 Imaging Elipsometer (Accurion, Goettingen, Germany) used in the Brewster Angle Microscopy (BAM) mode. Zero reflection was set with a polarized 532λ laser incident on the bare aqueous surfaces at the Brewster angle (53.1°). After monolayer formation, and/or compression, the reflected light was collected with a 10× objective and a reflectivity index calculated [typically $R = (\text{grey level} - 13.3) \times 2.8e - 8$]. Assuming a refraction index of 1.33 and 1.5 for the aqueous surface and the lipid film respectively, R relates to the thickness (d) of the monolayer as follows: $R = 0.07784\,(-3.416e - 3 \times d)^2$ (Lheveder et al., 2000).

3. Results and discussion

Surface pressure–mean molecular area isotherms of pure pCer were performed at different temperatures. At room temperature, the isotherms apparently show a transition-free condensed behavior as previously reported (Holopainen et al., 2001; Busto et al., 2009). However a diffuse, probably second or higher order, phase transition between two condensed phases that usually goes unnoticed can be detected as an inflection in the isotherm curve and compressibility modulus (C_s^{-1}) analysis (Fig. 1, see black arrows). The existence of this transition was mentioned in a recent publication when the abscissa axis was considerably expanded (Busto et al., 2009). At temperatures 22–27 °C, the phase present at low surface pressures was characterized by a C_s^{-1} value of about 300–500 mN/m and the phase present at high surface pressures shows C_s^{-1} values of about 600–800 mN/m. According with the C_s^{-1} values observed, these phases can be characterized as liquid-condensed (LC) and solid (S), respectively (Davies and Rideal, 1963). These C_s^{-1} values agree with published data (Holopainen et al., 2001) where a biphasic behavior of C_s^{-1} was reported but without discussion. It is worth noting that the C_s^{-1} values for the S phase of pCer found here are similar to those of the solid phase state of fatty acids and cholesterol (Davies and Rideal, 1963; Smaby et al., 1997) and are higher than the C_s^{-1} values reported for other phospholipids or sphingolipids with the only exception of galactosylceramide with saturated fatty acyl chains (Smaby et al., 1996). By contrast, the C_s^{-1} values found for the LC phase of pCer is rather similar to that reported for natural Cer (Maggio, 2004) and Cer-1 phosphate (Kooijman et al., 2009). Further features of this diffuse phase transition are provided by isobars analysis (see below).

At 45 °C and above, a first order liquid-expanded (LE)–LC phase transition is observed (see gray arrows in Fig. 1) involving a large change in molecular area (77% increase at 10 mN/m) (see also Fig. 5). This phase shows C_s^{-1} values of ≈60 mN/m, corresponding to a LE character (Davies and Rideal, 1963; Smaby et al., 1996). The presence of a LE phase for Cer was previously reported only for N-stearoyl Cer at 5.7 mN/m at 52 °C (Fidelio et al., 1986). This is not very different from what we found for pCer (at 11 mN/m at 49 °C, see Fig. 1). The difference observed indicates, as would be expected, that the addition of two methylene groups to the N-fatty acid substituent of Cer favors stabilization of the LC phase.

The isotherms for pCer at relatively low temperatures show a less condensed behavior during the first compression and become