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Cholesterol displacement by ceramide in sphingomyelin-containing liquid-ordered domains, and generation of gel regions in giant lipidic vesicles

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Abstract Fluorescence confocal microscopy and differential scanning calorimetry are used in combination to study the phase behaviour of bilayers composed of PC:PE:SM:Chol equimolecular mixtures, in the presence or absence of 10 mol% egg ceramide. In the absence of ceramide, separate liquid-ordered and liquid-disordered domains are observed in giant unilamellar vesicles. In the presence of ceramide, gel-like domains appear within the liquid-ordered regions. The melting properties of these gel-like domains resemble those of SM:ceramide binary mixtures, suggesting Chol displacement by ceramide from SM:Chol-rich liquid-ordered regions. Thus three kinds of domains coexist within a single vesicle in the presence of ceramide: gel, liquid-ordered, and liquid-disordered. In contrast, when 10 mol% egg diacylglycerol is added instead of ceramide, homogeneous vesicles, consisting only of liquid-disordered bilayers, are observed.

Keywords: Cholesterol; Ceramide; Sphingomyelin; Membrane domains; Fluorescence confocal microscopy; Differential scanning calorimetry

1. Introduction

The last decade has witnessed an outburst of research activity concerning the significance of lateral heterogeneity, i.e. existence of domains, in cell membranes. Once denied, then fiercely debated, it is now generally accepted that biomembranes are compositionally and functionally heterogeneous along their surface [1]. Studies in pure lipid vesicles (liposomes) have demonstrated that, even with simple two-lipid mixtures, domain formation can occur. The coexistence of gel and fluid [2,3], gel and liquid-ordered [4] and that of liquid-ordered and liquid-disordered domains [5] has been shown by a variety of physical methods (see reviews by Edidin [6] and Simons and Vaz [7]). The advent of giant unilamellar vesicles (GUV) [8,9] and the application of confocal fluorescence microscopy [10] has allowed visualization of domains and domain coexistence in a variety of lipid membranes. In particular, observation of three-domain coexistence in single vesicles has been achieved, either in POPC:Cer:Chol mixtures [11], or in DPPC:DOPC:Chol mixtures [12]. Chiantia et al. [13] observed coexistence of three kinds of domains in SM:PC:Chol:Cer supported bilayers.

Among membrane domains, those enriched in ceramide have received particular attention because Cer formation is one of the early steps in the apoptotic pathway [14,15] (see review by Taha et al. [16]). Ceramide is hardly miscible with other membrane lipids, thus it tends to segregate laterally into gel-like Cer-enriched domains [17] (see reviews in [18,19]). London and co-workers [20,21] have made the interesting observation that Cer displaces Chol from ordered lipid domains containing phosphatidylcholines, or SM, and Chol. This has been confirmed by other investigators [13,22,23]. The phenomenon is probably related to the previous observation of cholesterol displacement from plasma membranes induced by sphingomyelinase activity [24,25].

In the present study, we have applied confocal fluorescence microscopy to the study of GUV's composed of PC:PE:SM:Chol (1:1:1:1, mole ratio), a lipid mixture used by us in previous studies with the aim of mimicking the complex cell plasma membranes [26,27]. The images reveal that in these vesicles, liquid-ordered and liquid-disordered phase separation occurs. Moreover, addition of Cer gives rise to gel-like domains, that exhibit cooperative melting by differential scanning calorimetry, while addition of the structurally similar diacylglycerol blurs all interdomain boundaries.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE) and egg diacylglycerol (DAG) were purchased from Lipid Products (South Nutfield, UK). Egg SM, egg Cer and Chol were from Avanti Polar Lipids (Alabaster, AL). 1,1′-Dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiI) and Alexa Fluor 488 C_5-maleimide were from Invitrogen (Eugene, OR). NBD-Cer was a kind gift of Dr. G. Fabrias (IIQAB, CSIC, Barcelona, Spain).

2.2. GUV preparation and fluorescence microscopy

GUVs were prepared using the electroformation method developed by Angelova et al. [8]. For GUV observation at room temperature, a chamber supplied by L.A. Bagatolli (Odense, Denmark) was used, that allows direct GUV visualization under the microscope [11]. A PRET-GUV 4 Chamber supplied by Industrias Técnicas ITC (Bilbao, Spain) was used for vesicle preparation when observation of GUVs at 37 °C or 43 °C was required. Stock solutions of lipids (0.2 mg/ml total lipid containing either 0.2 mol% DiI or 0.2 mol% DiI and 0.4 mol% NBD-Cer) were prepared in a chloroform:methanol (9:1, v/v) solution. Three microlitres of the appropriate lipid stocks were added on the surface of

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Pt electrodes and solvent traces were removed by evacuating the chamber under high vacuum for at least 2 h.

2.2.1. Direct visualization of GUVs at room temperature

The Pt electrodes were covered with 400 μl of 10 mM HEPES, pH 7.4 previously heated at 60 °C. When required, 3 μM Alexa Fluor 488 was added to the HEPES Buffer. The Pt wires were connected to an electric wave generator (TG330 function generator, Thurlby Thandar Instruments, Huntington, UK) under AC field conditions (10 Hz, 1 V) for 2 h at 60 °C. In some cases, in order to obtain a more clear domain separation, after 2 h incubation at 60 °C heating was switched off, while the generator was switched off only when the temperature of the chamber reached 37 °C. When Alexa 488 was added, the excess dye was removed by washing the chamber 7 times with 10 mM HEPES, pH 7.4.

2.2.2. Observation of GUVs at 37 °C or 43 °C

The Pt electrodes were covered with 400 μl of 200 mM sucrose, previously heated at 60 °C. The Pt electrodes were connected to a generator (TG330 function generator, Thurlby Thandar Instruments) under AC field conditions (10 Hz, 1 V for 2 h, followed by 1 Hz, 1 V, 10 min) at 60 °C. Finally, the AC field was turned off and the vesicles (in 200 mM Sucrose) were collected from the PRETGUV 4 chamber with a pipette and transferred to a Micro-Incubator Platform DH-40i supplied by Warner Instruments (Hamden, USA) containing an equiosmolar buffer solution 95 mM NaCl, 10 mM HEPES, pH 7.4. Due to the different density between the two solutions the vesicles sedimented at the bottom of the chamber, which facilitated observation under the microscope.

After GUV formation, the chambers were located in an inverted confocal fluorescence microscope (Nikon D-ECLIPSE C1, Nikon Inc., Melville, NY). The excitation wavelengths were 430 nm for NBD-Cer, 488 nm for Alexa 488 and 561 nm for DiI. The images were collected through two different channels using band-pass filters of 515 ± 15 nm for the NBD-Cer and Alexa 488, and 593 ± 20 nm for the DiI. Image treatment and quantification was performed using the software EZ-C1 3.20 (Nikon Inc.). No difference in domain size, formation or distribution was observed in the GUVs along the observation period or after laser exposure.

2.3. Multilamellar vesicle preparation

For multilamellar (MLV) liposome preparation the lipids were dissolved in chloroform/methanol (2:1) and mixed as required, and the solvent was evaporated to dryness under a stream of nitrogen. Traces of the solvent were removed by evacuating the samples under high vacuum for at least 2 h. The samples were hydrated at 45 °C in 20 mM PIPES, 150 mM NaCl, 1 mM EDTA, pH 7.4, helping dispersion by stirring with a glass rod. The final lipid concentration was measured as lipid phosphorus.

2.4. Differential scanning calorimetry

For differential scanning calorimetry MLV were used. Both lipid suspension and buffer were degassed before being loaded into the sample or reference cell of a VP-DSC Microcalorimeter (MicroCal, Northampton, MA). The final concentration of PC:PE:SM:Chol (1:1:1:1, mole ratio) was 10 mM. Four heating scans, and occasionally a cooling one, at 45 °C/h were recorded for each sample. After the first one, successive heating scans on the same sample gave always superimposable thermograms. Transition temperatures, enthalpies, and widths at half-height were determined using the software ORIGIN (MicroCal) provided with the calorimeter.

3. Results and discussion

3.1. Vesicle morphology

GUV's composed of PC:SM:PE:Chol (1:1:1:1, mole ratio), prepared by electroformation, and stained with fluorescent probes were examined by confocal fluorescence microscopy (Fig. 1). At room temperature (columns A and B) the vesicles present low mobility, and different planes of the same vesicle can be micrographed, so that three-dimensional reconstruction is readily achieved. PC:SM:PE:Chol vesicles display lateral lipid heterogeneity, i.e. distinct lipid domains are seen. Vesicles in column A have been stained with DiI, which has a preference for the more fluid and disordered phases [28]. When vesicle preparation includes Alexa 488, a water-soluble stain that remains partly entrapped in the vesicles [9] (Fig. 1, column B), other domains become visible. Under our conditions, most vesicles appear to contain two domains, the more disordered one being larger in size (Fig. 1 and Supplementary Material Fig. S1). At 37 °C, the vesicles are not stuck to the coverglass as in the case of the direct visualization protocol when the

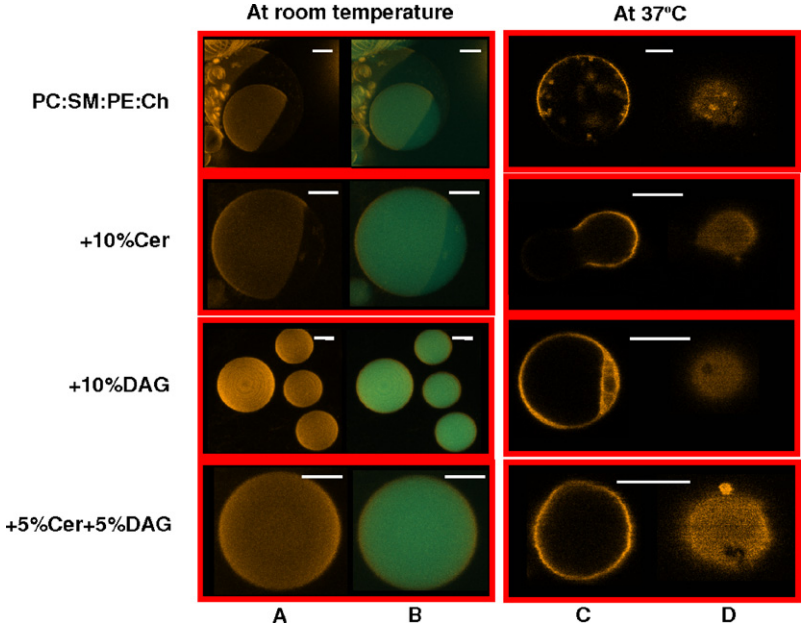

Fig. 1. Confocal microscopy of representative GUVs. Composition is given in the left-hand column. (A, B) Three-dimensional reconstructions at room temperature; (C, D) respectively, equatorial and polar planes at 37 °C. (A, C, D) DiI stain; (B) DiI + Alexa 488 stain. Bar = 10 μm.