Biochimica et Biophysica Acta 1828 (2013) 2099–2110

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

A combined fluorescence spectroscopy, confocal and 2-photon microscopy approach to re-evaluate the properties of sphingolipid domains

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A R T I C L E   I N F O

Article history:
Received 21 March 2013
Received in revised form 10 May 2013
Accepted 13 May 2013
Available online 20 May 2013

Keywords:
Ceramide-platforms
Fluorescence spectroscopy and microscopy
Lipid rafts
Membrane lipid domains
Sphingolipids
2-photon microscopy

A B S T R A C T

The aim of this study is to provide further insight about the interplay between important signaling lipids and to characterize the properties of the lipid domains formed by those lipids in membranes containing distinct composition. To this end, we have used a combination of fluorescence spectroscopy, confocal and two-photon microscopy and a stepwise approach to re-evaluate the biophysical properties of sphingolipid domains, particularly lipid rafts and ceramide (Cer)-platforms. By using this strategy we were able to show that, in binary mixtures, sphingolipids (Cer and sphingomyelin, SM) form more tightly packed gel domains than those formed by phospholipids with similar acyl chain length. In more complex lipid mixtures, the interaction between the different lipids is intricate and is strongly dictated by the Cer-to-Chol ratio. The results show that in quaternary phospholipid/SM/Chol/Cer mixtures, Cer forms gel domains that become less packed as Chol is increased. Moreover, the extent of gel phase formation is strongly reduced in these mixtures, even though Cer molar fraction is increased. These results suggest that in biological membranes, lipid domains such as rafts and ceramide platforms, might display distinctive biophysical properties depending on the local lipid composition at the site of the membrane where they are formed, further highlighting the potential role of membrane biophysical properties as an underlying mechanism for mediating specific biological processes.

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1. Introduction

Over the past couple of decades, renewed attention has been given to membrane lipids. It is now widely recognized that lipids are more than mere structural components, participating actively in cellular processes as lipid second messengers [1,2], by interacting directly and specifically with certain proteins [3] and/or by providing specialized membrane regions (lipid domains) [4] that have the capacity to segregate or colocalize membrane proteins. The composition and physical properties of these membrane regions differ from the bulk membrane and are the key factor responsible for their specialized roles in biological processes, as in trafficking and signal transduction [5]. Several types of membrane domains have been identified including lipid rafts [6] and ceramide-platforms [7], which were shown to be important players in a number of cellular processes.

From a biophysical perspective, lipid rafts are characterized by their fluid ordered (liquid-ordered, l_o) nature [8] while Cer-platforms consist of more rigid domains with gel-like properties [9–12]. While there are some controversies regarding the existence of these domains in cellular membranes [13–15], it is nevertheless commonly accepted that regions with l_o-like properties might be present in biomembranes in a temporal- and spatial-dependent manner [16]. In contrast, the existence of gel domains under physiological conditions was a matter of dispute since their biophysical properties would limit diffusion of lipids and proteins in the biomembrane, which would compromise cell functioning [17]. However, it was recently shown that gel domains enriched in sphingolipids are present in the cellular membrane of yeast and that these might be involved in fundamental cell processes [18].

It has been hypothesized that alterations in membrane properties might be one of the trigger mechanisms for cellular responses and that minor changes in the properties of lipid domains might activate

Abbreviations: Cer, Ceramide; Chol, Cholesterol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; GUV, Giant unilamellar vesicles; Laurdan, 6-dodecanoyl-2-(dimethylamino)naphthalene; MLV, Multilamellar vesicles; l_d, Liquid disorder; l_o, Liquid ordered; NBD-DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(nitro-2-1,3-benzoxadiazol-4yl); POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PCer, N-palmitoyl-D-*erythro*-sphingosine; PSM, *N*-palmitoyl-*D*-erythro-sphingosylphosphorylcholine; Rho-DOPE, N-rhodamine-dipalmitoylphosphatidylethanolamine; SLs, Sphingolipids; SM, Sphingomyelin; *t*-PnA, *trans*-parinaric acid

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http://dx.doi.org/10.1016/j.bbamem.2013.05.011

specific cellular processes [19]. In this way, it is important to understand the physical properties underlying lipid domain formation and the characteristics of the phases that are formed. It is widely known that different lipids are able to form gel- and l_o-like phases. However, limited information is available regarding the differences in the properties of the lipid domains formed in membranes containing different lipid composition. In the present study, fluorescence spectroscopy, confocal and two-photon microscopy were used to further evaluate the characteristics of phases formed in different lipid mixtures that are known to present gel/fluid and/or l_o/l_d (liquid-disordered) phase separation. The use of model membranes that can be accurately manipulated, by changing the lipid components and their molar ratio, allowed the biophysical characterization of membranes composed of phospholipids, sphingolipids and/or cholesterol. Using a stepwise approach and a combination of fluorescent probes with different phase-related properties it was possible to identify i) the distinctive characteristics of the domains formed in those lipid mixtures and ii) the interplay between lipid rafts and Cer-platforms, giving further insights into the paradigm of Chol–Cer interactions.

2. Materials and methods

2.1. Materials

POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), PSM (*N*-palmitoyl-*D*-erythro-sphingosylphosphorylcholine), PCer (N-palmitoyl-D-*erythro*-sphingosine) and Rho-DOPE (N-rhodamine-dipalmitoyl-phosphatidylethanolamine) were from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Chol) was from Sigma (Leiden, The Netherlands). DPH (1,6-diphenyl-1,3,5-hexatriene), *t*-PnA (*trans*-parinaric acid), Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) and NBD-DPPE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxa-diazol-4yl)) were from Molecular Probes (Leiden, The Netherlands). All organic solvents were UVASOL grade from Merck (Darmstadt, Germany). The concentration of the lipid and of the probes stock solutions was determined as described [20].

2.2. Fluorescence spectroscopy

Multilamellar vesicles (MLV) (total lipid concentration of 0.1 and 0.6 mM) were prepared as described [20]. The suspension medium was 10 mM sodium phosphate, 150 mM NaCl, and 0.1 mM EDTA (pH 7.4). Steady state fluorescence measurements of *t*-PnA, DPH and Laurdan (at a probe/lipid ratio of 1/500, 1/250 and 1/400, respectively) were performed in a SLM Aminco 8100 series 2 spectrofluorometer with double excitation and emission monochromators, MC400 (Rochester, NY). All measurements were performed in 0.5 cm × 0.5 cm quartz cuvettes. The excitation (λ_{exc})/emission (λ_{em}) wavelengths were 305/405 nm for *t*-PnA, 360/430 for DPH and 350/435 nm for Laurdan. A constant temperature was maintained using a Julabo F25 circulating water bath controlled with 0.1 °C precision directly inside the cuvette with a type-K thermocouple (Electrical Electronic Corp., Taipei, Taiwan). For measurements performed at different temperatures, the heating rate was always below 0.2 °C/min.

Laurdan GP (generalized polarization) was determined using [21]:

$$GP = \frac{I_{435} - I_{500}}{I_{435} + I_{500}} \quad (1)$$

where I_{435} and I_{500} are the emission intensities at those wavelengths, corresponding to the maximum emission in the gel and in the liquid crystalline phase, respectively [21,22]. Theoretically this parameter varies from +1 to −1, however, experimentally it ranges from 0.7 to −0.3 both for pure lipids or mixtures e.g. [21–24].

Time-resolved fluorescence measurements with *t*-PnA were performed using $\lambda_{exc} = 305$ nm (using a secondary laser of Rhodamine 6G) and $\lambda_{em} = 405$ nm. The decays were analyzed using TRFA software (Scientific Software Technologies Center, Minsk, Belarus). The fluorescence decay is described by a sum of exponentials, where α_i is the normalized pre-exponential (or amplitude) and τ_i is the lifetime of the decay component *i*. The mean fluorescence lifetime is given by:

$$\langle \tau \rangle = \sum_i \alpha_i \tau_i^2 / \sum_i \alpha_i \tau_i. \quad (2)$$

2.3. Determination of the partition coefficient of the probes between two phases

The partition coefficient of the probes (DPH, Laurdan, *t*-PnA) between gel and fluid ($K_p^{g/f}$) or liquid ordered and liquid disordered phases ($K_p^{lo/ld}$) in binary and ternary mixtures was determined from the variation of the photophysical parameters of these probes with the mole fraction of the fluid (*f*), X_f, the gel phase (*g*), X_g, the liquid disordered (l_d), Xl_d, and liquid ordered (l_o), Xl_o. The composition of the mixtures and the mole fraction of each phase (X_i) were taken from the tie-line at 23 °C of the respective phase diagram. The partition coefficient is an equilibrium constant that quantifies the partition of the probe between the two distinct phases present in these mixtures (*f* and *g*, l_o and l_d), and consequently is independent of the particular composition of the mixtures.

The partition coefficient was calculated according to the following expression [25]:

$$\langle r \rangle = \frac{\varepsilon_g \langle r \rangle_g K_p X_g + \varepsilon_f \overline{\tau}_f / \overline{\tau}_g \langle r \rangle_f X_f}{\varepsilon_g K_p X_g + \varepsilon_f \overline{\tau}_f / \overline{\tau}_g X_f} \quad (3)$$

where X_i is the phase mole fraction, ε_i is the molar absorption coefficient, $\overline{\tau}_i$ and $\langle r \rangle_i$ are the amplitude-weighted fluorescence lifetime and steady-state fluorescence anisotropy of the probe in phase *i*, respectively. K_p is obtained by fitting the equation to the data as a function of X_i.

2.4. Confocal fluorescence microscopy

Giant unilamellar vesicles (GUVs) containing the appropriate lipids and probes, were prepared by electroformation, as previously described [20]. A probe/lipid ratio of 1:500 for Rho-DOPE and 1:200 for NBD-DPPE and Laurdan was used. The GUVs were then transferred to 8 well Ibidi® μ-slides and confocal fluorescence microscopy was performed using a Leica TCS SP5 (Leica Microsystems CMS GmbH, Mannheim, Germany) inverted microscope (DMI6000) with a 63× water (1.2 numerical aperture) apochromatic objective. NBD-DPPE and Rho-DOPE excitation was performed using the 458 nm and 514 nm lines from an Ar^+ laser, respectively. The emission was collected at 480–530 nm and 530–650 nm, for NBD-DPPE and Rho-DOPE, respectively. Confocal sections of thickness <0.5 μm were obtained using a galvanometric motor stage. Two-dimensional (2D) projections were obtained using the Leica Application Suite-Advanced Fluorescence software.

Two photon excitation data were obtained by using the same Leica TCS SP5 inverted microscope but with a titanium-sapphire laser as the excitation light source. The excitation wavelength was set to 780 nm and the fluorescence emission was collected at 400–460 nm and 470–530 nm to calculate the GP images. Laurdan GP images were obtained through a homemade software based on a MATLAB environment. Briefly, both channel intensities are corrected for background intensities and Laurdan GP values are determined from:

$$GP = \frac{I_{400-460} - GI_{470-520}}{I_{400-460} + GI_{470-520}} \quad (4)$$

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