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Effect of glucosylceramide on the biophysical properties of fluid membranes

Ana R.P. Varela [a,b,c], Amélia M.P.S. Gonçalves da Silva [d], Alexander Fedorov [b], Anthony H. Futerman [c], Manuel Prieto [b], Liana C. Silva [a,*]

[a] iMed.UL - Research Institute for Medicines and Pharmaceutical Sciences, Faculdade de Farmácia, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal
[b] Centro de Química-Física Molecular and Institute of Nanoscience and Nanotechnology, Instituto Superior Técnico, Universidade Técnica de Lisboa, Complexo I, Av. Rovisco Pais, 1049-001 Lisboa, Portugal
[c] Department of Biological Chemistry, Weizmann Institute of Sciences, Rehovot 76100, Israel
[d] Centro de Química Estrutural, Instituto Superior Técnico, Universidade Técnica de Lisboa, Complexo I, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

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ABSTRACT

Glucosylceramide (GlcCer), a relevant intermediate in the pathways of glycosphingolipid metabolism, plays key roles in the regulation of cell physiology. The molecular mechanisms by which GlcCer regulates cellular processes are unknown, but might involve changes in membrane biophysical properties and formation of lipid domains. In the present study, fluorescence spectroscopy, confocal microscopy and surface pressure–area (π–A) measurements were used to characterize the effect of GlcCer on the biophysical properties of model membranes. We show that C16:0-GlcCer has a high tendency to segregate into highly ordered gel domains and to increase the order of the fluid phase. Monolayer studies support the aggregation propensity of C16:0-GlcCer. π–A isotherms of single C16:0-GlcCer indicate that bilayer domains, or crystal-like structures, coexist within monolayer domains at the air–water interface. Mixtures with POPC exhibit partial miscibility with expansion of the mean molecular areas relative to the additive behavior of the components. Moreover, C16:0-GlcCer promotes morphological alterations in lipid vesicles leading to formation of flexible tubule-like structures that protrude from the fluid region of the bilayer. These results support the hypothesis that alterations in membrane biophysical properties induced by GlcCer might be involved in its mechanism of action.

1. Introduction

Glycosphingolipids (GSLs) are ubiquitous components of eukaryotic cell membranes. GSLs are synthesized by sequential addition of saccharides to the hydroxyl group at the C-1 position of the ceramide (Cer) backbone [1–4]. GSLs are mainly located in the extracellular leaflet of the plasma membrane, where they are involved in several functions such as cell-to-cell interaction and recognition [1,5,6]. Glucosylceramide (GlcCer), one of the simplest GSLs, is widely distributed in mammalian tissues. GlcCer is formed at the cytosolic leaflet of Golgi apparatus by glycosylation of Cer by GlcCer synthase (GCS) [7]. After its synthesis, GlcCer is transported to the luminal side of the Golgi apparatus where is converted into more complex GSLs, or transported by FAPP2 (4-phosphate adaptor protein-2) [8] to the cytoplasmic leaflet of the plasma membrane (PM) or of the endoplasmic reticulum (ER) [9–11]. In the PM, GlcCer may remain on the cytoplasmic leaflet or translocate to the cell surface [12].

GlcCer is an important intracellular messenger that plays key roles in cell maintenance and regulation [13–17]. However, the molecular mechanisms by which GlcCer regulates cellular processes are unknown. GSLs are thought to be involved in the formation of lipid rafts which, because of their specific biophysical properties, can act as signaling platforms [5,6]. In the last decade, much effort has been made to understand the biophysical properties of lipid rafts [18–21]; however, little attention has been given to GlcCer. GlcCer has a high main transition temperature (T_m) and a complex thermotropic phase behavior, with multiple transitions between different stable and metastable phases [22]. Due to their high T_m and extensive hydrogen-bond network, GSLs with small uncharged headgroups are expected to segregate from low T_m phospholipids and form tightly packed domains [23–27], with a higher packing density than sphingomyelin with a similar backbone [28–30]. These observations suggest that GlcCer might promote similar changes to those induced by Cer in the biophysical properties of fluid membranes [31–33]. Previous studies from our group have showed that Cer has a complex phase behavior promoting

Abbreviations: C16:0-Cer, N-palmitoyl-D-*erythro*-sphingosine; C24:0-Cer, *N*-lignoceroyl-D-*erythro*-sphingosine; C24:1-Cer, *N*-nervonoyl-D-*erythro*-sphingosine; C16:0-GlcCer, D-glucosyl-ß-1,1′ N-palmitoyl-D-*erythro*-sphingosine; Cer, Ceramide; Chol, Cholesterol; DPH, 1,6-diphenyl-1,3,5-hexatriene; FAPP2, 4-phosphate adaptor protein-2; GalCer, Galactosylceramide; GCS, GlucosylceramideSynthase; GlcCer, Glucosylceramide; GSL, Glycosphingolipid; GUV, Giant unilamellar vesicles; MMA, Mean Molecular Area; MLV, Multilamellar Vesicles; NBD-DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl); POPC, 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine; Rho-DOPE, 2-Dioleoyl-*sn*-Glycero-3-Phosphoethanolamine-N-(LissamineRhodamine B Sulfonyl); SL, Sphingolipids; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; T_m, Main Transition Temperature; *t*-PnA, *trans*-parinaric acid (octadeca-9,11,13,15-tetraenoic acid)

* Corresponding author. Tel.: +351 217 946 400x14245; fax: +351 217 937 703.
E-mail address: lianacsilva@ff.ul.pt (L.C. Silva).

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extensive alterations in the biophysical properties of membranes that are dependent both on Cer content and membrane lipid composition, particularly on cholesterol (Chol) content [34–36].

In the present study, fluorescence spectroscopy, confocal microscopy and surface pressure - area (π–A) measurements were used to characterize lipid lateral distribution, ability to segregate into tightly-packed domains and membrane morphological alterations induced by C16:0-GlcCer in fluid model membranes. Our results show that C16:0-GlcCer has a high tendency to segregate into highly ordered gel domains and to increase the order of the fluid phase, although to a lower extent than the corresponding Cer (C16:0-Cer). Thermodynamic analysis of POPC/C16:0-GlcCer mixed monolayers indicates (partial) miscibility with positive deviations from the ideal behavior. Moreover, C16:0-GlcCer promotes morphological alterations in lipid vesicles leading to the formation of flexible tubule-like structures that protrude from the fluid membrane.

2. Materials and methods

2.1. Materials

POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), GlcCer (D-glucosyl-ß-1,1' N-palmitoyl-D-*erythro*-sphingosine), Rho-DOPE (N-rhodamine-dipalmitoylphosphatidylethanolamine) and DOPE-biotin (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(biotinyl)) were from Avanti Polar Lipids (Alabaster, AL). 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 were determined as previously described [33].

2.2. Fluorescence spectroscopy

To evaluate the effect of GlcCer on membrane biophysical properties, multilamellar vesicles (MLV) (total lipid concentration of 0.1 mM) were prepared as previously described [33]. The suspension medium was 10 mM sodium phosphate, 150 mM NaCl, 0.1 mM EDTA (pH 7.4). Fluorescence anisotropy of *t*-PnA, DPH and Rho-DOPE, and Laurdan emission spectra (at a probe/lipid ratio of 1/500, 1/200, 1/500 and 1/400, respectively) were measured in a SLM Aminco 8100 series 2 spectrofluorimeter 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 320/405 nm for *t*-PnA; 358/430 nm for DPH; 350/435 nm for Laurdan; 570/593 for Rho-DOPE. 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. The fluorescence anisotropy <*r*> was calculated from [37]:

$$<r>=\frac{I_{vv}-GI_{vh}}{I_{vv}+2GI_{vh}} \quad (1)$$

where the different intensities (I_{ii}) are the steady state vertical and horizontal components of the fluorescence emission with the excitation vertical (I_{vv} and I_{vh}) and horizontal (I_{hv} and I_{hh}) for the emission axis. The latter pair of components is used to calculate the *G* factor ($G = I_{hv}/I_{hv}$). An appropriate blank was subtracted from each intensity reading before calculation of the anisotropy value.

Laurdan GP (generalized polarization) was determined using [38]:

$$GP=\frac{I_{440}-I_{490}}{I_{440}+I_{490}} \quad (2)$$

where $I_{440\ and}$ I_{490} are the emission intensities at 440 and 490 nm respectively, reporting the maximum emission in the gel and in the liquid crystalline phase, respectively [38,39]. Theoretically this parameter varies from +1 to −1, however, experimentally ranges from 0.7 to −0.3 both for pure lipids or mixtures [38].

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 experimental decays were analyzed using TRFA software (Scientific Software Technologies Center, Minsk, Belarus).

2.3. Confocal fluorescence microscopy

Giant unilamellar vesicles (GUVs) containing the appropriate lipids, DOPE-biotin (at a biotinylated/non-biotinylated lipid ratio of $1{:}10^6$), (Rho-DOPE and NBD-DPPE) (at a probe/lipid ratio of 1:500 and 1:200, respectively). These were prepared by electroformation, as previously described [32,40,41]. The GUVs were then transferred to 8 well Ibidi® μ-slides that had been previously coated with avidin (at 0.1 mg/ml) to improve GUV adhesion to the plate [42]. Confocal fluorescence microscopy was performed using a Leica TCS SP5 (Leica Mycrosystems 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 below 0.5 μm were obtained using a galvanometric motor stage. Three-dimensional (3D) projections were obtained using Leica Application Suite-Advanced Fluorescence software.

2.4. Lipid monolayers and surface pressure–area measurements

Surface pressure–area (π–A) isotherm measurements were carried out on a KSV 5000 Langmuir–Blodgett system (KSV Instruments, Helsinki) installed in a laminar flow hood. Procedures for π–A measurements and cleaning care were described elsewhere [43]. Monolayers were spread drop-wise as chloroform solutions using a microsyringe, on the subphase of a buffer solution. Unless specified, the concentration of spreading solution was 0.5 mM. The temperature of the subphase was controlled by water circulation from a thermostat within an error of ±0.1 °C. The barrier speed of symmetric compression was 10 mm min^{-1} (3.3 $Å^2$ $molecule^{-1}$ min^{-1}). π–A isotherms were measured at least three times from fresh spreading solutions to confirm reproducibility.

The isothermal two-dimensional compressibility modulus, or elastic modulus, is calculated from the π–A isotherms as $C_s^{-1} = -A(\partial A/\partial \pi)_T$.

The Gibbs energy of mixing, ΔG_{mix}, is taken from:

$$\Delta G_{mix}(\pi) = \Delta G_{ideal} + G^E(\pi), \quad (3)$$

Where ΔG_{ideal} is the Gibbs energy of ideal mixing at low surface pressure ($\pi \rightarrow 0$)

$$\Delta G_{ideal} = RT(X_1 lnX_1 + X_2 lnX_2), \quad (4)$$

and $G^E(\pi)$ is the excess Gibbs energy of mixing:

$$G^E(\pi) = \int A^E(\pi) d\pi, \quad (5)$$