Biochimica et Biophysica Acta 1828 (2013) 1822–1828

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Organization of fluorescent cholesterol analogs in lipid bilayers — Lessons from cyclodextrin extraction

Sigrid Milles [a,1,2], Thomas Meyer [b,2], Holger A. Scheidt [b], Roland Schwarzer [a], Lars Thomas [b], Magdalena Marek [c], Lajos Szente [d], Robert Bittman [e], Andreas Herrmann [a], Thomas Günther Pomorski [c], Daniel Huster [b,*], Peter Müller [a,**]

[a] Humboldt-University Berlin, Department of Biology, Invalidenstr. 42, D-10115 Berlin, Germany
[b] University of Leipzig, Department of Medical Physics and Biophysics, Härtelstr. 16-18, D-04107 Leipzig, Germany
[c] Center for Membrane Pumps in Cells and Disease — PUMPKIN, Danish National Research Foundation, Department of Plant and Environmental Sciences, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark
[d] CYCLOLAB LTD., P.O. Box 435, H-1525 Budapest, Hungary
[e] Department of Chemistry and Biochemistry, Queens College of CUNY, Flushing, NY 11367-1597, USA

ARTICLE INFO

Article history:
Received 14 December 2012
Received in revised form 15 March 2013
Accepted 3 April 2013
Available online 11 April 2013

Keywords:
Cholesterol
Fluorescent cholesterol
Cyclodextrin
Membrane structure

ABSTRACT

To characterize the structure and dynamics of cholesterol in membranes, fluorescent analogs of the native molecule have widely been employed. The cholesterol content in membranes is in general manipulated by using water-soluble cyclodextrins. Since the interactions between cyclodextrins and fluorescent-labeled cholesterol have not been investigated in detail so far, we have compared the cyclodextrin-mediated membrane extraction of three different fluorescent cholesterol analogs (one bearing a NBD and two bearing BODIPY moieties). Extraction of these analogs was followed by measuring the Förster resonance energy transfer between a rhodamine moiety linked to phosphatidylethanolamine and the labeled cholesterol. The extraction kinetics revealed that the analogs are differently extracted from membranes. We examined the orientation of the analogs within the membrane and their influence on lipid condensation using NMR and EPR spectroscopies. Our data indicate that the extraction of fluorescent sterols from membranes is determined by several parameters, including their impact on lipid order, their hydrophobicity, their intermolecular interactions with surrounding lipids, their orientation within the bilayer, and their affinity with the exogenous acceptor.

1. Introduction

Cholesterol is one of the most intensively investigated biological molecules [1]. This high interest is justified by the essential physiological roles cholesterol plays in eukaryotic cells. Examples are the role of cholesterol for the formation of lateral membrane domains [2,3], maintenance of membrane permeability, and membrane trafficking (see [4]). To improve our understanding about the physiological roles of cholesterol on the molecular level, fluorescent analogs have been studied in fluorescence microscopy and spectroscopy (see [5,6] and references cited therein). Since water-soluble cyclodextrins (CDs), especially methyl-β-cyclodextrin (MβCD), are able to form complexes with endogenous cholesterol [7], it is possible to efficiently manipulate the level of cholesterol and its analogs in model and biological membranes. However, the ability of the fluorescent analogs to reflect the properties of endogenous cholesterol [8] and the influence of the fluorophore on CD-cholesterol interaction must be critically considered. For the latter, various studies have shown that CDs are also able to complex fluorescence groups [9–12]. Therefore, we have characterized the interaction of fluorescent (NBD- and BODIPY-) labeled sterols (structures see Fig. 1) with CDs by investigating the ability of CDs to extract the analogs from

Abbreviations: BCh-1, 23-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diazas-indacen-8-yl)-24-norchol-5-en-3β-ol; BCh-2, 22-[4-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacen-8-yl)-butyroxy]-23,24-bisnorchol-5-en-3β-ol; CDs, cyclodextrin(s); CHO cells, Chinese hamster ovary cells; CLSM, confocal laser scanning microscopy; DHE, dehydroergosterol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DMEM, Dulbecco's modified eagle medium; FAIM, fluorescence anisotropy imaging microscopy; FLIM, fluorescence lifetime imaging microscopy; FRET, Förster resonance energy transfer; 5-/10-/16-doxyl-PC, 1-palmitoyl-2-stearoyl-(5/10/16-doxyl)-*sn*-glycero-3-phosphocholine; HBS, HEPES buffered solution; HPβCD, 2-hydroxypropyl-β-CD; LUVs, large unilamellar vesicles; MβCD, 2,6-di-*O*-methyl-β-CD; MLVs, multilamellar vesicles; NBDCh, 25-{N-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl]amino}-27-norcholesterol; PE, phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPC-d_{31}, 1-palmitoyl-d_{31}-2-oleoyl-*sn*-glycero-3-phosphocholine; Rh, rhodamine; Rh-PE, N-(lissamine rhodamine B sulfonyl)-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; Tempo-PC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(Tempo)choline

* Correspondence to: D. Huster, Institute of Medical Physics and Biophysics, University of Leipzig, Härtelstr. 16-18, 04107 Leipzig, Germany. Tel.: +49 345 5524942; fax: +49 345 5527013.
** Corresponding author. Tel.: +49 30 20938691; fax: +49 30 20938585.
E-mail addresses: daniel.huster@medizin.uni-leipzig.de (D. Huster), peter.mueller.3@rz.hu-berlin.de (P. Müller).
[1] Present address: EMBL Heidelberg, Meyerhofstr. 1, D-69117 Heidelberg, Germany.
[2] These authors equally contributed to this work.

0005-2736/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2013.04.002

large unilamellar vesicles (LUVs). Previous studies showed that one BODIPY-labeled analog (BODIPY-cholesterol, referred to here as BCh-1) mimics the properties of native cholesterol rather well, in that it, similarly to cholesterol, partitions preferentially into liquid-ordered lipid domains [13]. The other BODIPY-labeled analog (BCh-2), which has an artificial ester linkage within the side chain and was synthesized as a negative control, was shown (i) in monolayer studies not to mix ideally with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and not to result in condensation of the mixed monolayer area and (ii) in lipid vesicles not to partition into liquid-ordered lipid domains [13,14]. The NBD-labeled cholesterol (NBDCh) has been shown not to specifically mimic the molecular behavior of native cholesterol [8]. We established an assay to measure the kinetics of CD-mediated cholesterol extraction from membranes based on Förster resonance energy transfer (FRET) between the fluorescent cholesterol analogs as the donor and rhodamine-labeled phosphatidylethanolamine (Rh-PE) as the acceptor. We found that the cholesterol analogs are extracted differently from lipid membranes. In order to search for reasons of these differences, we measured the orientation of cholesterol analogs within the lipid membrane and their lipid condensation effect in comparison with that of endogenous cholesterol.

2. Materials and methods

2.1. Materials

POPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1-palmitoyl-d_{31}-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC-d_{31}), 1-palmitoyl-2-stearoyl-(5/10/16-doxyl)-*sn*-glycero-3-phosphocholine (5-/10-/16-doxyl-PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho (Tempo)choline (Tempo-PC), 25-{N-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl]amino}-27-norcholesterol (NBDCh), N-(lissamine rhodamine B sulfonyl)-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (Rh-PE), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). The BODIPY-labeled analogs of cholesterol, 23-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diazas-indacen-8-yl)-24-norchol-5-en-3β-ol (BCh-1) and 22-[4-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacen-8-yl)-butyroxy]24-bisnorchol-5-en-3β-ol (BCh-2) were synthesized using the method described in [14]. BCh-1 is also referred to as BODIPY-cholesterol and TopFluorCholesterol. αCD, βCD, (2-hydroxy)propyl-βCD (HPβCD), 2,6-di-*O*-methyl-β-CD (MβCD), and γCD were provided from Cyclolab (Budapest, Hungary). All other chemicals were from Sigma-Aldrich (Taufkirchen, Germany). The HEPES buffered solution (HBS) contained 145 mM NaCl and 10 mM HEPES (pH 7.4).

2.2. Preparation of LUVs and multilamellar vesicles (MLVs)

LUVs were prepared by the extrusion method [15]. The desired amount of lipids (DOPC and fluorescent lipids at 0.5 mol% if not stated otherwise) was dissolved in chloroform and dried in a rotating round-bottom flask under vacuum until a lipid film was formed. To evaporate the remaining solvent completely, the film was dried under high vacuum for at least 15 min. Lipids were hydrated with a small volume of ethanol and HBS, giving a final concentration of 1 mM (final ethanol concentration, <1% (v/v)). To prepare LUVs, the MLV suspension was subjected to five freeze–thaw cycles followed by extrusion of the lipid suspension at room temperature 10 times at 40 °C through two 0.1 μm polycarbonate filters (mini-extruder from Avanti Polar Lipids; filters from Costar, Nucleopore, Tübingen, Germany).

MLVs were used in the NMR experiments. After the lipids were mixed in chloroform, the solvent was evaporated and the samples were dissolved in cyclohexane and lyophilized to provide a fluffy powder that was hydrated with 40 wt.% deuterium-depleted water for ^{2}H NMR or with D_2O for ^{1}H NMR measurements. For equilibration, the samples were subjected to ten freeze–thaw cycles and centrifuged ten times. The specimens were transferred into 5-mm glass vials for ^{2}H NMR experiments and into 4-mm high-resolution MAS rotors for ^{1}H MAS NMR.

2.3. Measurement of extraction of cholesterol analogs

The assay for measuring the extraction of cholesterol analogs from membranes was based on the approach described by John et al. [16] in which FRET between dehydroergosterol (DHE) and dansyl-labeled phosphatidylethanolamine (PE) was used. Here, we used FRET between the NBD or BODIPY moiety linked to cholesterol and the rhodamine (Rh) moiety linked to PE. LUVs were prepared containing DOPC, Rh-PE, and the respective cholesterol analog (BCh-1, BCh-2, or NBDCh); excitation of the BODIPY or NBD moiety results in Rh fluorescence when the fluorophores are proximal to each other. A specific extraction of labeled cholesterols to an extravesicular acceptor, e.g. CD, results in a decrease of FRET. Therefore, the FRET signal of LUVs upon the addition of CDs is used to monitor the release of cholesterol from the bilayers.

50 μl of LUV solution (containing 1 mM of lipids, 0.5 mol% Rh-PE, and the respective cholesterol analog) was mixed with 1.45 ml of HBS in a fluorescence cuvette. The time-dependent fluorescence intensity was recorded (excitation 470 nm, emission 590 nm, slit widths 4 nm) at 37 °C. After 30 s, CDs were added at the concentrations shown in the respective figures. After 300 s, Triton X-100 (final concentration, 0.5% (w/v)) was added to diminish the FRET completely. Curves were normalized by setting the fluorescence intensities before addition of CD to one and the intensities after addition of Triton X-100 to zero. Fluorescence kinetics were recorded using an Aminco Bowman Series 2 spectrofluorometer (Urbana, IL).

Quantitative data of the kinetics were obtained by fitting the curves to a model applied for the extraction of short-chain phospholipids from membranes [17]. The model considers the flip-flop of cholesterol analogs between the inner and outer leaflets of the vesicle membranes and the extraction of cholesterol analogs from the outer leaflet by an acceptor as well as its back insertion. In the case of very rapid extraction kinetics, the model was over-parameterized. Therefore, in these cases, the curves were fitted to a single-exponential equation, giving the rate constant and the plateau of extraction (see Results).

2.4. Fluorescence quenching experiments

The membrane orientation and distribution of the fluorescent cholesterol analogs were determined by measuring the extent of fluorescence quenching in the presence of different spin-labeled PCs. LUVs were prepared containing 5 mol% of Tempo-PC, 5-Doxyl-PC, 10-Doxyl-PC, or 16-Doxyl-PC, respectively, 90 mol% POPC, and 5 mol% of the cholesterol

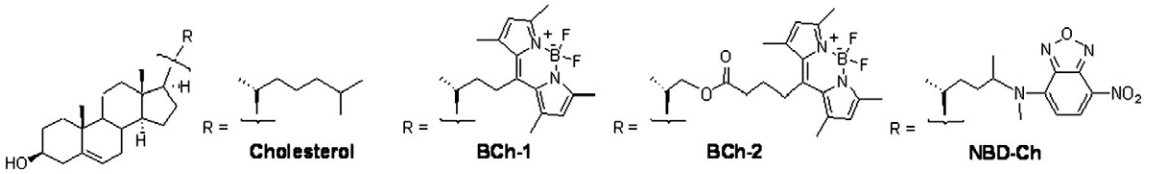

Fig. 1. Structures of the fluorescent cholesterol analogs used in this study.