Biochimica et Biophysica Acta 1848 (2015) 1699–1705

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Cholesterol-induced changes in hippocampal membranes utilizing a phase-sensitive fluorescence probe

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ARTICLE INFO

Article history:
Received 23 February 2015
Received in revised form 20 April 2015
Accepted 5 May 2015
Available online 8 May 2015

Keywords:
Hippocampal membrane
Membrane cholesterol
MβCD
NR12S
REES

ABSTRACT

The function of membrane receptors in the nervous system depends on physicochemical characteristics of neuronal membranes such as membrane order and phase. In this work, we have monitored the changes in hippocampal membrane order and related parameters by cholesterol and protein content utilizing a Nile Red-based phase-sensitive fluorescent membrane probe NR12S. Since alteration of membrane cholesterol is often associated with membrane phase change, the phase-sensitive nature of NR12S fluorescence becomes useful in these experiments. Our results show that fluorescence spectroscopic parameters such as emission maximum, anisotropy, and lifetime of NR12S display characteristic dependence on membrane cholesterol content. Interestingly, cholesterol-dependent red edge excitation shift is displayed by NR12S under these conditions. Hippocampal membranes exhibited reduction in liquid-ordered phase upon cholesterol depletion. These results provide insight into changes in hippocampal membrane order in the overall context of cholesterol and protein modulation.
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1. Introduction

Organization of neuronal membranes is crucial in the context of the function of receptors embedded in them, particularly in view of the unique membrane lipid composition in the nervous system [1,2]. In this context, cholesterol is a physiologically relevant lipid since brain cholesterol has been implicated in a number of neurological disorders [3–5]. It is known that the function of neuronal receptors is modulated by cholesterol [6–9], which affects neurotransmission and gives rise to mood and anxiety disorders [10]. A number of neurological disorders share a common etiology of defective cholesterol metabolism in the brain [11]. A hallmark of membrane cholesterol is its nonrandom localization in domains in biological and model membranes [12–14]. Cholesterol is unique in its ability to form liquid-ordered-like phases in higher eukaryotic plasma membranes [15]. In this overall context, exploring neuronal membrane organization in relation to membrane cholesterol modulation assumes relevance.

We recently reported the location, dynamics, and environment-sensitive properties of a novel Nile Red-based phase-sensitive membrane probe (NR12S) [16]. We further showed, utilizing model membranes of varying phases, that important fluorescence parameters such as emission maximum, red edge excitation shift (REES), anisotropy and lifetime of NR12S exhibit sensitivity to the membrane phase. In our laboratory, we have established hippocampal membranes as a primary source for studying the interaction of neuronal receptors such as the serotonin$_{1A}$ receptor with membrane lipids [17,18]. An important finding from these studies is that cholesterol-induced membrane organization is necessary for the function of neuronal receptors [7–9,18]. Since alteration of membrane cholesterol is often associated with membrane phase change, in this work, we utilized the phase-sensitive membrane probe NR12S to explore the changes in organization and dynamics of hippocampal membranes under conditions of varying cholesterol content.

2. Materials and methods

2.1. Materials

Cholesterol, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), EDTA, EGTA, $MgCl_2$, $MnCl_2$, Na_2HPO_4, iodoacetamide, PMSF, sucrose, sodium azide, Tris and methyl-β-cyclodextrin (MβCD) were purchased from Sigma Chemical Co. (St. Louis, MO). Bicinchoninic acid (BCA) assay reagent for protein estimation was from Pierce (Rockford, IL). Amplex Red cholesterol assay kit was from Molecular Probes/Invitrogen (Eugene, OR). NR12S was synthesized as described previously [19]. The concentration of a stock solution of NR12S prepared in DMSO was estimated using its molar extinction coefficient (ε) of 45,000 M^{-1} cm^{-1} at 550 nm in ethanol. All other chemicals used were of the highest purity available. Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout. Fresh bovine brains were obtained from a local slaughterhouse within

Abbreviations: BCA, bicinchoninic acid; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; MβCD, methyl-β-cyclodextrin; Nile Red, 9-diethylamino-5H-benzo[α]phenoxazine-5-one; PMSF, phenylmethylsulfonyl fluoride; REES, red edge excitation shift; Tris, *tris*-(hydroxymethyl)aminomethane

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http://dx.doi.org/10.1016/j.bbamem.2015.05.001
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10 min of death and the hippocampal region was carefully dissected out. The hippocampi were immediately flash frozen in liquid nitrogen and stored at −70 °C until further use.

2.2. Methods

2.2.1. Preparation of native hippocampal membranes

Native hippocampal membranes were prepared as described previously [18], flash frozen in liquid nitrogen and stored at −70 °C. Protein concentration was assayed using BCA reagent with bovine serum albumin as standard [20].

2.2.2. Cholesterol depletion of native hippocampal membranes

Native hippocampal membranes were depleted of cholesterol using MβCD as described previously [18,21]. Cholesterol content was estimated using the Amplex Red assay kit [22].

2.2.3. Lipid extraction from native and cholesterol-depleted membranes

Lipid extraction was carried out from native and cholesterol-depleted hippocampal membranes as described previously [23] using a modified Bligh and Dyer method [24]. The lipid extract was finally dissolved in a chloroform–methanol mixture (1:1, v/v).

2.2.4. Estimation of phospholipids

Concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid using Na_2HPO_4 as standard [25]. DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings.

2.2.5. Sample preparation

Membranes (native and cholesterol-depleted) containing 100 nmol of total phospholipid were suspended in 2 ml of 10 mM Tris buffer (pH 7.4). NR12S was added from a stock solution in DMSO such that the final probe concentration was 1 mol% with respect to the total phospholipid content. The resultant probe concentration was 0.5 μM in all cases and the DMSO content was always low (<0.1%, v/v). This ensures optimal fluorescence intensity with negligible membrane perturbation. NR12S was added to membranes while being vortexed for 1 min at room temperature (~23 °C). Samples were kept in the dark for 1 h before measurements. Background samples were prepared the same way except that NR12S was omitted.

Lipid extracts containing 100 nmol of total phospholipid in chloroform–methanol (1:1, v/v) were mixed well with 1 nmol of NR12S in DMSO. Samples were mixed well and dried under a stream of nitrogen while being warmed gently (~45 °C). After further drying under a high vacuum for at least 6 h, 2 ml of 10 mM Tris, pH 7.4 buffer was added, and lipid samples were hydrated (swelled) at ~70 °C while being intermittently vortexed for 3 min to disperse the lipid and form homogeneous multilamellar vesicles (MLVs). The MLVs were kept at ~70 °C for an additional hour to ensure proper swelling as the vesicles were formed. Such high temperatures were necessary for hydrating the samples due to the presence of lipids with high melting temperature in neuronal tissues [26]. Samples were kept in the dark at room temperature (~23 °C) overnight prior to fluorescence measurements.

2.2.6. Steady state fluorescence measurements

Steady state fluorescence measurements were performed with a Hitachi F-7000 spectrofluorometer (Tokyo, Japan) using 1 cm path length quartz cuvettes. Excitation and emission slits with a bandpass of 5 nm were used for all measurements. Background intensities of samples in which NR12S was omitted were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak and other scattering artifacts. The spectral shifts obtained with different sets of samples were identical in most cases. In other cases, the values were within ±1 nm of those reported.

Fluorescence anisotropy measurements were performed at room temperature (~23 °C) using a Hitachi Glan–Thompson polarization accessory. Anisotropy values were calculated from the equation [27]:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the fluorescence intensities (after appropriate background subtraction) measured with the excitation polarizer oriented vertically and the emission polarizer vertically and horizontally oriented, respectively. G is the grating factor and is the ratio of the efficiencies of the detection system for vertically and horizontally polarized light, and is equal to I_{HV}/I_{HH}.

2.2.7. Time-resolved fluorescence measurements

Fluorescence lifetimes were calculated from time-resolved fluorescence intensity decays using IBH 5000F NanoLED equipment (Horiba Jobin Yvon, Edison, NJ) with DataStation software in the time-correlated single photon counting (TCSPC) mode. A pulsed light-emitting diode (LED) (NanoLED-01) was used as an excitation source. This LED generates optical pulse at 490 nm with pulse duration 1.2 ns and is run at 1 MHz repetition rate. The LED profile (instrument response function) was measured at the excitation wavelength using Ludox (colloidal silica) as the scatterer. To optimize the signal-to-noise ratio, 10,000 photon counts were collected in the peak channel. All experiments were performed using emission slits with bandpass of 8 nm. The sample and the scatterer were alternated after every 5% acquisition to ensure compensation for shape and timing drifts occurring during the period of data collection. This arrangement also prevents any prolonged exposure of the sample to the excitation beam, thereby avoiding any possible photodamage of the fluorophore. Data were stored and analyzed using DAS 6.2 software (Horiba Jobin Yvon, Edison, NJ). Fluorescence intensity decay curves so obtained were deconvoluted with the instrument response function and analyzed as a sum of exponential terms

$$F(t) = \Sigma_i \alpha_i \exp(-t/\tau_i) \quad (2)$$

where F(t) is the fluorescence intensity at time t and α_i is a pre-exponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime of τ_i such that $\Sigma_i \alpha_i = 1$. The program also includes statistical and plotting subroutine packages [28]. The goodness of the fit of a given set of observed data and the chosen function was evaluated by the χ^2 ratio, the weighted residuals [29], and the autocorrelation function of the weighted residuals [30]. A fit was considered acceptable when plots of the weighted residuals and the autocorrelation function showed random deviation about zero with a minimum χ^2 value not more than 1.5. Intensity-averaged mean lifetimes $\langle\tau\rangle$ for biexponential decays of fluorescence were calculated from the decay times and pre-exponential factors using the equation [27]:

$$\langle\tau\rangle = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2}{\alpha_1\tau_1 + \alpha_2\tau_2}. \quad (3)$$

3. Results and discussion

Cholesterol content of hippocampal membranes can be conveniently modulated using MβCD by selective extraction of cholesterol from hippocampal membranes by including it in the central nonpolar cavity [18,31]. Fig. 1 shows that cholesterol content in hippocampal membranes gets progressively reduced upon treatment with increasing concentrations of MβCD. Upon treatment with 10 mM MβCD, cholesterol content was reduced to ~83% of the control. The extent of cholesterol depletion was highest when 40 mM MβCD was used, with cholesterol content being reduced to ~15% of the control. Fig. 1 shows

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