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Cholesterol-dependent thermotropic behavior and organization of neuronal membranes

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ABSTRACT

The composition of neuronal membranes is unique with diverse lipid composition due to evolutionary requirement. The organization and dynamics of neuronal membranes are crucial for efficient functioning of neuronal receptors. We have previously established hippocampal membranes as a convenient natural source for exploring lipid-protein interactions, and organization of neuronal receptors. Keeping in mind the pathophysiological role of neuronal cholesterol, in this work, we used differential scanning calorimetry (DSC) and small angle X-ray scattering (SAXS) to explore thermotropic phase behavior and organization (thickness) of hippocampal membranes under conditions of varying cholesterol content. Our results show that the apparent phase transition temperature of hippocampal membranes displays characteristic linear dependence on membrane cholesterol content. These results are in contrast to earlier results with binary lipid mixtures containing cholesterol where phase transition temperature was found to be not significantly dependent on cholesterol concentration. Interestingly, SAXS data showed that hippocampal membrane thickness remained more or less invariant, irrespective of cholesterol content. We believe that these results constitute one of the early reports on the thermotropic phase behavior and organizational characterization of hippocampal membranes under varying cholesterol content. These results could have implications in the functioning of neuronal receptors in healthy and diseased states.

1. Introduction

The nervous system is enriched with a diverse variety of lipids. The lipid diversity has been proposed to arise in response to evolutionary requirement of higher cognition in primates [1,2]. Cholesterol is an important lipid in the nervous system since it is known to affect neurotransmission by regulating the function of neuronal receptors [3–7], thereby giving rise to mood and anxiety disorders [8]. Brain cholesterol is implicated in a number of neurological disorders [9–11]. An interesting aspect of brain cholesterol is that it is exclusively synthesized *in situ* and there is no evidence of cholesterol transport from the blood plasma to the brain [12], at least in humans [13]. Defective cholesterol metabolism in the brain therefore results in a number of neurological disorders [14]. In this overall scenario, exploring neuronal membrane organization in the context of membrane cholesterol modulation assumes relevance.

In our laboratory, we have previously established bovine hippocampal membranes as a primary source for studying the interaction of membrane lipids with neuronal G protein-coupled receptors (GPCRs) such as the serotonin$_{1A}$ receptor [15,16]. A crucial finding from these studies is that cholesterol-induced membrane organization is necessary for the function of neuronal receptors [3,5–7,16]. With the overall objective to correlate cholesterol-dependent functional changes of neuronal receptors with alterations in membrane organization and dynamics, we previously utilized approaches based on fluorescence spectroscopy [17–20], and electron spin resonance spectroscopy [21]. Although spectroscopic approaches provide a wealth of information on the probe environment, the information obtained is local (short range) in nature.

Membrane phase is an important determinant of membrane organization and function [22,23]. Alteration of membrane cholesterol is often associated with changes in membrane phase [24–26] and thickness [27,28]. Differential scanning calorimetry (DSC) [29–31] and small angle X-ray scattering (SAXS) [32] are commonly used techniques to characterize thermotropic phase behavior, membrane organization and thickness in model and biological membranes. In this work, we used DSC and SAXS to probe the changes in thermotropic behavior and organization of hippocampal membranes under conditions of varying cholesterol content. Our results constitute one of the first reports on changes in thermotropic behavior and organization of

Abbreviations: BCA, bicinchoninic acid; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; GPCR, G protein-coupled receptor; MβCD, methyl-β-cyclodextrin; PMSF, phenylmethylsulfonyl fluoride; SAXS, small angle X-ray scattering; Tris, *tris*-(hydroxymethyl)aminomethane.

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hippocampal membranes with respect to cholesterol content which could provide novel insight into functional changes under these conditions.

2. Materials and methods

2.1. Materials

Cholesterol, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), methyl-β-cyclodextrin (MβCD), EDTA, EGTA, iodoacetamide, phenylmethylsulfonyl fluoride (PMSF), sucrose, sodium azide, Na_2HPO_4, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). Bicinchoninic acid (BCA) reagent for protein estimation was from Pierce (Rockford, IL). Amplex Red cholesterol assay kit was from Molecular Probes/Invitrogen (Eugene, OR). 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 10 min of death, and the hippocampal region was carefully dissected out. The hippocampi were immediately flash frozen in liquid nitrogen and stored at −80 °C till further use.

2.2. Methods

2.2.1. Preparation of native hippocampal membranes

Native hippocampal membranes were prepared as described previously [16], flash frozen in liquid nitrogen and stored at −80 °C. Protein concentration was assayed using BCA reagent with bovine serum albumin as standard [33].

2.2.2. Cholesterol depletion from native hippocampal membranes

Native hippocampal membranes were depleted of cholesterol using MβCD as described previously [16,34]. Hippocampal membranes resuspended at a protein concentration of 2 mg/ml were treated with different concentrations of MβCD in 50 mM Tris buffer (pH 7.4) at room temperature (25 °C) with constant shaking for 1 h. Membranes were then spun down at 50,000 ×*g* for 5 min, washed once with 50 mM Tris buffer (pH 7.4) and resuspended in the same buffer. Cholesterol content was estimated using the Amplex Red cholesterol assay kit [35].

2.2.3. Estimation of phospholipids

Lipid phosphate was assayed subsequent to total digestion by perchloric acid using Na_2HPO_4 as standard [36]. DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings.

2.2.4. Sample preparation

Native and cholesterol-depleted hippocampal membranes containing ~5–15 mg of total protein were suspended in 1 ml of 50 mM Tris buffer (pH 7.4) and used for DSC and SAXS experiments. Each sample was vortexed for 3 min before carrying out experiments.

2.2.5. Differential scanning calorimetry

Thermotropic behavior of native and cholesterol-depleted hippocampal membranes was investigated using a MicroCal VP-DSC microcalorimeter (Northampton, MA). Before running the DSC scan, each sample was degassed for ~10 min at 20 °C to avoid bubbles. All samples were subjected to two heating and two cooling scans between 1 and 110 °C at a scan rate of 1 °C/min. In each experiment, the first heating scan was considered for the determination of apparent phase transition temperature in the thermogram. Thermograms were overlaid to display the phase transition peaks and the overlaid plots were generated using Origin version 7.0 (OriginLab, Northampton, MA).

2.2.6. Small angle X-ray scattering

Scattering profiles of native and cholesterol-depleted hippocampal membranes were recorded for 1 h at 20 °C using S3 Micro (Hecus X-ray Systems GmbH, Graz, Austria). Scattering profiles were obtained using methods described previously [37]. The repeat distance or *d*-spacing of native and cholesterol-depleted hippocampal membranes were calculated using the following equation [38,39]:

$$d = 2\pi/q_{peak} \quad (1)$$

where *d* is the repeat distance (unit cell periodicity) and q_{peak} denotes the maximum of the scattering peak. Here, *d* represent the sum of the membrane bilayer (including membrane proteins) and water (hydration) layer thickness [39]. We calculated the average *d*-spacing of hippocampal membranes from the *d*-spacing values obtained from the first and second order scattering peaks.

3. Results and discussion

3.1. Change in cholesterol content in hippocampal membranes upon MβCD treatment

We have previously shown that MβCD, a water-soluble oligomer, acts as an efficient agent to selectively extract cholesterol from hippocampal membranes by including cholesterol in its central nonpolar cavity [16,40]. Fig. 1 shows that cholesterol content in hippocampal membranes progressively decreases upon treatment with increasing concentration of MβCD. Treatment with 10 mM MβCD was able to reduce cholesterol content to ~86% of the control membrane. The extent of cholesterol depletion increases with increase in MβCD concentration and was highest when 40 mM MβCD was used, where cholesterol content was reduced to ~26% of the control. Fig. 1 shows that the membrane phospholipid level remained mostly invariant upon MβCD treatment. The inset in Fig. 1 shows the corresponding values of cholesterol to phospholipid ratio (mol/mol) in hippocampal

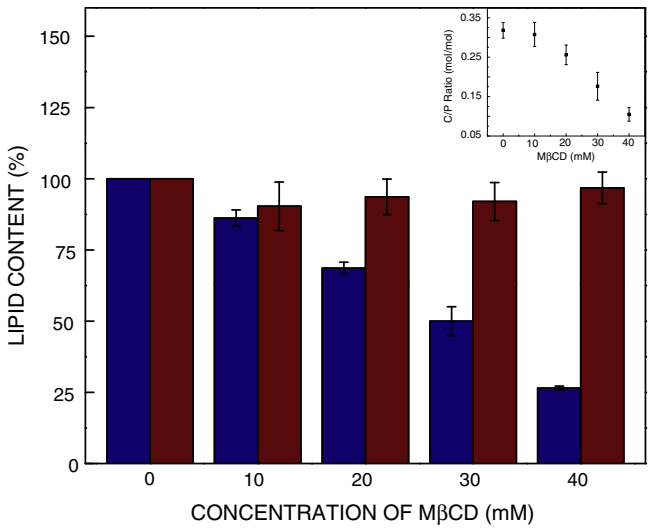

Fig. 1. Cholesterol (blue bars) and phospholipid (maroon bars) contents upon depletion of cholesterol in native hippocampal membranes with increasing concentration of MβCD. Values are expressed as percentages of the respective lipid content in native hippocampal membranes in the absence of MβCD. The inset shows the change in cholesterol to phospholipid ratio (C/P) (mol/mol) with increasing concentration of MβCD in hippocampal membranes. Data shown represent means ± S.E. of three independent experiments. See Materials and methods section for other details. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)