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Phase separation is induced by phenothiazine derivatives in phospholipid/sphingomyelin/cholesterol mixtures containing low levels of cholesterol and sphingomyelin

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Abstract

Lipid rafts are membrane structures enriched in cholesterol, sphingomyelin and glycolipids. In majority raft-mimicking model systems high contents of cholesterol and sphingomyelin (approximately 30 mol%) are used. Existence of raft-like structures was, however, reported also in model and natural membranes containing low levels of cholesterol and sphingomyelin. In the present work differential scanning calorimetry and fluorescence spectroscopy with the use of Laurdan probe was employed to demonstrate the existence of phase separation in model systems containing DPPC with addition of 5 mol% or 10 mol% of both cholesterol and sphingomyelin. Additionally, the influence of three phenothiazine derivatives on phase separation in mixed DPPC/cholesterol/sphingomyelin bilayers was investigated. Chlorpromazine, thioridazine and trifluoperazine were able to induce phase separation in DPPC and DPPC/cholesterol/sphingomyelin bilayers in temperatures below lipid main phase transition. However, only trifluoperazine induced phase separation in temperatures close to or above main phase transition. Trifluoperazine also induced phase separation in bilayers composed of egg yolk PC or DOPC mixed with cholesterol and sphingomyelin. We concluded that presence of lipid domains can be observed in model membranes containing low levels of cholesterol and sphingomyelin. Among three phenothiazine derivatives studied, only trifluoperazine was able to induce a permanent phase separation in phosphatidylcholine/cholesterol/sphingomyelin systems.

Keywords: Lipid rafts; Phenothiazine derivatives; Laurdan generalized polarization; Phase separation

1. Introduction

During the last decade lipid rafts attracted attention of many laboratories investigating biological membranes because they offer an excellent possibility to explain mechanisms of many membrane-related processes like transport, sorting, signal transduction and others [1,2]. Rafts are observed in natural membranes but they can be also obtained in models like giant unilamellar vesicles [3], supported lipid monolayers or bilayers [4]. In case of lipid rafts heterogeneous distribution of their main components: sphingomyelin and cholesterol is caused by the differences in physical properties of those compounds and their surrounding. For this reason in majority of model systems mimicking raft behavior besides sphingomyelin and cholesterol, highly fluid (at the temperature of experiment) lipids are also used. Such a composition of model system provides favorable circumstances for phase separation. Another feature of most commonly explored raft model systems is that cholesterol and sphingomyelin are present in high concentrations (approx. 30% of total lipid content, see [5] for most recent study in this field). It has been shown, however, that phase separation can be observed in model membranes containing much smaller cholesterol and sphingomyelin amounts. Using fluorescence correlation spectroscopy Kahya et al. [6] observed phase separation in giant unilamellar vesicles containing even less than 10% of cholesterol in DOPC/SM/Chol mixture. This finding demonstrates that presence of lipid rafts (or more general phase separation) is possible even in situations in which cholesterol

Abbreviations: Chol; cholesterol; CPZ; chlorpromazine; DOPC; dioleoyl phosphatidylcholine; DPPC; dipalmitoyl phosphatidylcholine; EYPC; egg yolk phosphatidylcholine; GP; generalized polarization; SM; sphingomyelin; TDZ; thioridazine; TFP; trifluoperazine.

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content in the membrane is small. Low cholesterol level was observed in disk membranes of the rod cells of retina [7]. Such a composition of disks does not exclude, however, the presence of lipid rafts in these membranes [8,9]. On the other hand, it was also found that low levels of membrane cholesterol appear in certain stages of development of B cells [10].

Phase separation can be observed in systems containing at least two lipid components differing in physical properties but can also be induced in mono-component systems under certain experimental conditions. As we have shown some years ago phenothiazine derivative — trifluoperazine, can induce phase separation in phosphatidylcholine bilayers in temperatures below the main phase transition [11]. The fact that phenothiazines alter the properties of lipid bilayers may correlate with their ability to modulate the activity of multidrug-related transport proteins [12] and therefore they became the object of our studies. In present work we describe the studies on the influence of three phenothiazine derivatives (trifluoperazine, chlorpromazine and thioridazine) on phase separation in mixtures containing low levels of cholesterol and sphingomyelin. The fluorescent indicator Laurdan was employed, whose generalized polarization is an ideal tool to follow the formation of domains in lipid systems [13,14]. It was found that all studied drugs induced phase separation, however the appearance of this effect depended on the phase state of the bilayer.

2. Materials and methods

2.1. Chemicals

Drugs used in the present study: chlorpromazine (CPZ), trifluoperazine (TFP) and thioridazine (TDZ) were products of ICN Biomedicals (Costa Mesa, CA, USA). Lipids: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), L-α-phosphatidylcholine from egg yolk (EYPC), sphingomyelin (SM) from egg yolk and cholesterol (Chol) were purchased from Sigma (St. Louis, MO, USA). Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) was from Molecular Probes (Eugene, OR, USA). All other chemicals used in experiments were of analytical grade.

2.2. Microcalorimetry

DPPC was mixed with chloroform solutions of cholesterol and/or sphingomyelin to obtain the desired molar ratios. Lipid mixture was then evaporated under stream of nitrogen and placed under vacuum for a minimum of 2 h to remove the traces of organic solvent. Next, each sample (1.5 mg of lipid) was hydrated with 15 μl of buffer (20 mM Tris–HCl 0.5 mM EDTA, 150 mM NaCl, pH 7.4) containing the appropriate amount of CPZ, TFP or TDZ. The samples were heated to the temperature *c.a.* 10 °C higher than the temperature of main phase transition of DPPC and vortexed. Optically homogenous lipid mixtures were transferred into aluminum pans and sealed.

Calorimetric measurements were performed using Rigaku calorimeter, which was partially rebuilt in our laboratory. Samples were scanned immediately after preparation with scanning speed of 1.25 °C/min. For each lipid mixture studied at least two samples were prepared, each sample was scanned at least four times.

2.3. Fluorescence spectroscopy

Phospholipids (DPPC, DOPC or EYPC) were mixed with appropriate amounts of cholesterol and/or sphingomyelin in chloroform. Organic solvent was then evaporated under stream of nitrogen and the samples were kept under vacuum for at least 2 h. Unilamellar liposomes were obtained by sonication of lipid suspensions in 20 mM Tris–HCl buffer, 0.5 mM EDTA, 150 mM NaCl (pH 7.4). Sonication was performed using UP 200s sonicator (Dr Hilscher, Berlin, Germany) operating at 50/50 pulse mode. Samples were sonicated on ice until the liposome suspension became opalescent. Sonicated samples were not checked for remaining multilamellar vesicles and integrity. Laurdan (1 mM) was dissolved in dimethyl sulfoxide, all drugs (10 mM) were dissolved in water. Liposomes (final phospholipid concentration 200 μM) were incubated with fluorescent probe (concentration 3 μM) in darkness for 30 min at room temperature. Next phenothiazine derivative was added (in amounts that gave final drug concentration in samples of 100 μM) and the incubation was continued for a further 15 min under the same conditions.

Laurdan emission spectra were collected using LS 50B spectrofluorimeter (Perkin-Elmer Ltd., Beaconsfield, UK). Emission and excitation slits were set to 5 nm. Temperature was controlled by a water-circulating bath and the actual temperature was measured directly in the cuvette using a platinum thermometer. The content of the cuvette was continuously mixed. In GP(λ_{ex}) experiments excitation wavelength for Laurdan was 320–400 nm and fluorescence emission spectra were recorded in the range of 410–540 nm, while in GP(T) experiments Laurdan was excited at 390 nm and emission spectra were collected in the wavelength range 410–540 nm. Data were collected and processed with FLDM Perkin-Elmer software. Laurdan generalized polarization was calculated according to the equation [15]:

$$GP = \frac{I_B - I_R}{I_B + I_R} \quad (1)$$

where I_B and I_R were the fluorescence emission intensities at the blue and red edges of the emission spectrum, respectively. The GP values were calculated using emission intensities at 440 nm (I_B) and 490 nm (I_R). Since studied phenothiazines showed only negligible fluorescence in the wavelengths range used in experiments (less than 1% of Laurdan intensity) and we have not recorded any significant influence of these compounds on Laurdan spectral properties, the recorded data were not corrected for the presence of phenothiazines.

2.4. Partition coefficient calculations

Octanol/water partition coefficients of phenothiazine derivatives studied in present work were calculated by computer