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Formation of micro-domains as functional regions in biomembranes: specific interactions inferred by differential scanning calorimetry and microscopic imaging of membrane fluidity

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Abstract

Using differential scanning calorimetry, preferential interaction between melittin and dimyristoylphosphatidylcholine was observed in various binary mixtures of phospholipids. It is shown that matching of the hydrophobic regions between melittin and fatty acyl chains of phospholipids is the most important factor. Using a microscopic imaging instrument for membrane fluidity, specific interaction between cholesterol and sphingomyelin in rafts was confirmed in living CHO cells. An environment sensitive fluorescence dye, laurdan, was used in this home-built instrument. The membrane fluidity of the cells was scarcely affected with the treatment of sphingomyelinase up to 0.1 U ml^{-1}. On the other hand, increase of the membrane fluidity was observed in CHO cells treated with methyl-beta-cyclodextrin, which removes cholesterol molecules from biomembranes of the cells in a concentration dependent manner up to 10 mM. But a low concentration of methyl-beta-cyclodextrin (1 mM) did not raise the membrane fluidity. However, increase of membrane fluidity was observed in CHO cells treated with sphingomyelinase and then with 1 mM methyl-beta-cyclodextrin. These results suggest specific interaction between sphingomyelin and cholesterol in the rafts.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

The cell, an essential unit of life, is constructed with various membrane structures. And the biomembranes are assemblies of lipids, proteins and various constituents. The membrane proteins require lipids for their function and the function is under the influence of structure and

physical property of the lipid membrane. The interactions of membrane proteins with lipid bilayers are principally hydrophobic and electrostatic in nature. Integral proteins crossing the bilayer once or several times interact hydrophobically with the hydrocarbon chains of membrane lipids. The hydrophobic force is the major factor in constructing the biomembrane structures, and so the hydrophobic part of the integral protein traverses or penetrates the hydrophobic core of the lipid bilayer. Owicki *et al* have theoretically manifested how an integral membrane protein perturbs the order and composition of lipid bilayers [1, 2] and Mouritsen and Bloom have proposed a thermodynamic model of protein–lipid interactions from a viewpoint of mismatch of the hydrophobic regions of the lipids and proteins in membranes [3]. The cell is a system in which a large number of various functions are integrated into the micrometre scale, and the formation of the micro-domains plays an important role for this integration by making heterogeneous regions in a small area. Recently, micro-domains have been found in the biomembranes as 'rafts' where sphingolipids, cholesterol and GPI-anchored proteins are concentrated to form sites for signal transduction [4].

The raft is functional micro-domain found in cell membranes formed by thermo dynamical process, and specific proteins such as GPI-anchored protein and haemagglutinin are concentrated into this micro-domain. For the specific interaction of membrane proteins and lipids, matching of the hydrophobic region is proposed from a theoretical viewpoint [3]. On the other hand, it is shown in a lipid model system that specific interaction between sphingomyelin and cholesterol is important for the formation of the raft [5]. In the present study, the basic mechanism of sorting (concentrating specific proteins into rafts) was investigated using melittin as a model of membrane protein, and the matching of hydrophobic regions was experimentally shown by use of differential scanning calorimetry. Furthermore, the specific interaction between sphingomyelin and cholesterol for the formation of the raft was examined in living CHO cells by use of the microscopic imaging instrument of membrane fluidity.

2. Materials and methods

Synthetic phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Melittin (purity 91% by HPLC) and cholesterol were purchased from Sigma Chemical Co. (St Louis, MO). Laurdan (6-dodecanoyl-2-dimethylaminonaphtalene) was purchased from Molecular Probes (Eugene, OR). The desired binary mixture of phospholipids (2.0 μmol) was taken from the chloroform solution into a small test tube and the solvent evaporated first by a nitrogen stream then under reduced pressure overnight. The lipids were dispersed in a bath-type sonifier, Iuchi VS10U (Tokyo, Japan), at 80 °C for 2 min into 1 ml of 50 mM HEPES buffer (pH 7.3) containing various concentrations of melittin. All preparations contained 5 mM EDTA in order to inhibit phospholipase activity included in melittin [6]. Calorimetric scans were performed with a differential adiabatic micro-calorimeter, Privalov calorimeter DASM-4, at a heating scan rate of 0.5 K min^{-1} with 0.5 ml volume cells under a pressure of 2.0 atm to prevent bubble formation [7], and the data were processed by an NEC PC-9801RX personal computer (Tokyo, Japan). The heat absorption was calibrated by Jule's heat from the internal equipped circuit. Densities of lipid bilayer membranes were measured with an Anton Paar DMA60/602 density meter at various temperatures [8]. After the measurement, the concentration of the lipid dispersion was determined by phosphorus assay.

The membrane fluidity of living CHO cells was imaged with home-built microscopic imaging instrument using laurdan. This instrument images the generalized polarization defined by Parassasi *et al* [9] at video rate.

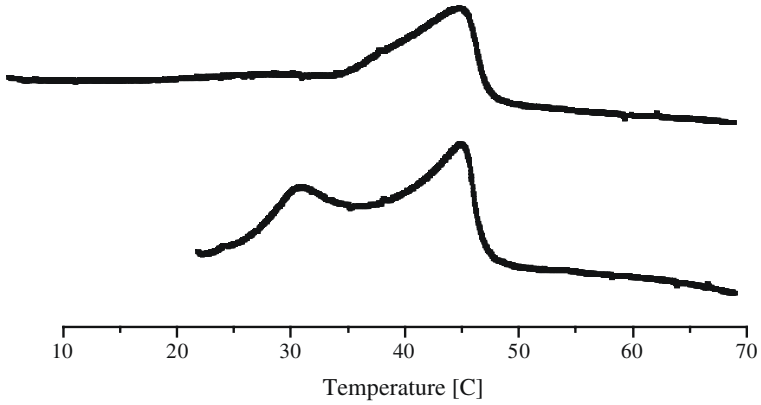

Figure 1. Thermograms of binary mixture of dimyristoylphosphatidylcholine (DMPC) and distearoylphosphatidylcholine (DSPC) (bottom) in the absence of melittin and (top) in the presence of 5 mol% of melittin in 50 mM HEPES buffer (pH 7.3, containing 5 mM EDTA).

3. Results

3.1. Preferential interaction of melittin with dimyristoylphosphatidylcholine

Melittin is a major component of bee venom, consists of 26 amino acid residues [10] and has an amphipathic nature [11, 12]. The amino acid sequence is shown as follows. Gly–Ile–Gly–Ala–Val–Leu–Lys–Val–Leu–Thr–Thr–Gly–Leu–Pro–Ala–Leu–Ile–Ser–Trp–Ile–Lys–Arg–Lys–Arg–Gln–GlnNH$_2$.

When the polypeptide interacts with phospholipids, it contains about 70% of alpha-helix structure [13], and so melittin has been extensively investigated as a model peptide of lipid–protein interactions in biomembranes [14]. Interaction between melittin and phospholipid membranes is supposed to go through two steps sequentially, binding of melittin with phospholipid membranes and then penetration of melittin into the membranes [15]. In the present study, we have investigated the effect of melittin on binary mixtures of phospholipids. Each phospholipid species in membranes has its own feature of phase transition characterized by temperature and enthalpy, so their miscibility, phase transition temperatures and phase transition enthalpies determine the thermogram of the phospholipid mixture [7]. In order to elucidate the preferential interaction between melittin and a phospholipid species, the thermograms of various binary mixtures of phospholipids were analysed in the absence and the presence of melittin. First, we examined the effect of melittin on binary mixture of dimyristoylphosphatidylcholine (DMPC), having saturated acyl chains of 14 carbons, and distearoylphosphatidylcholine (DSPC), having saturated acyl chains of 18 carbons. The combination of DMPC and DSPC is a low-miscibility one. In the absence of melittin, the mixture gave a broad transition with two peaks at 30.1 and 44.5 °C (figure 1). The low- and high-temperature peaks correspond to DMPC- and DSPC-rich regions, respectively. Addition of melittin to the mixture reduced the low-temperature peak exclusively. This result indicates that the melittin molecules interact preferentially with DMPC molecules in the binary mixture of DMPC and DSPC. For the second combination of binary mixture, DSPC was substituted by dipalmitoylphosphatidylcholine (DPPC), having saturated acyl chains of 16 carbons. The molecular structure of DPPC is different from that of DMPC only by a 2-methylene moiety in the acyl chains. This combination is a miscible one, and one transition peak was observed in the control thermogram without melittin. Addition of melittin induced separation of the peak

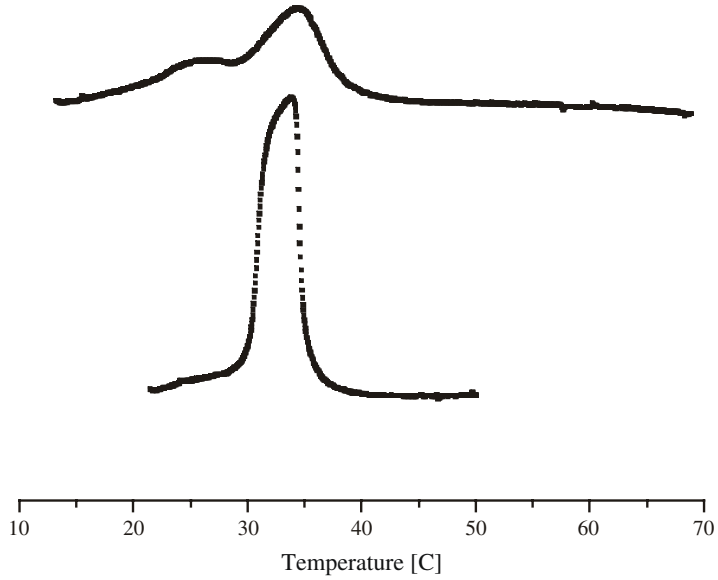

Figure 2. Thermograms of binary mixture of dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) (bottom) in the absence of melittin and (top) in the presence of 5 mol% of melittin in 50 mM HEPES buffer (pH 7.3, containing 5 mM EDTA).

into two peaks. This result implies that melittin molecules interact with DMPC preferentially and pull DMPC molecules out from the mixture. Moreover, the transition peak separates into two peaks (figure 2).

3.2. Specific interaction between sphingomyelin and cholesterol in living CHO cells

In order to investigate the specific interaction between sphingolipid and cholesterol in rafts, we used CHO cells and investigated specific interaction between sphingomyelin and cholesterol in the living cells. To examine the specific interaction, CHO cells were treated with methyl-β-cyclodextrin to remove cholesterol or treated with sphingomyelinase to convert sphingomyelin to ceramide. It is expected that removal of cholesterol changes membrane fluidity, so we measured the membrane fluidity of the CHO cells with a home-built microscopic imaging instrument. For the microscopic imaging of membrane fluidity, we used an environment sensitive fluorescence dye, laurdan. The property of laurdan is well explained in DPPC liposomes. In gel phase the emission peak is observed at 440 nm, and in fluid phase the peak shifts to 490 nm. In the gel phase water molecules cannot penetrate the lipid bilayer. However, in the fluid phase, water molecules can penetrate the membrane, the laurdan begins to feel a polar environment, and solvent relaxation induces a red shift of the emission spectrum. This physical property related to membrane fluidity is expressed by the generalized polarization (GP) as defined by the following equation.

$$\mathrm{GP} = \frac{I_{440\ \mathrm{nm}} - I_{490\ \mathrm{nm}}}{I_{440\ \mathrm{nm}} + I_{490\ \mathrm{nm}}}.$$

The fluorescence image was separated into an image at 440 nm and an image at 490 nm by the combination of dichroic mirrors, and each image was focused on the focus plane of a CCD camera side by side. The two images are recoded on digital video recorder and they were

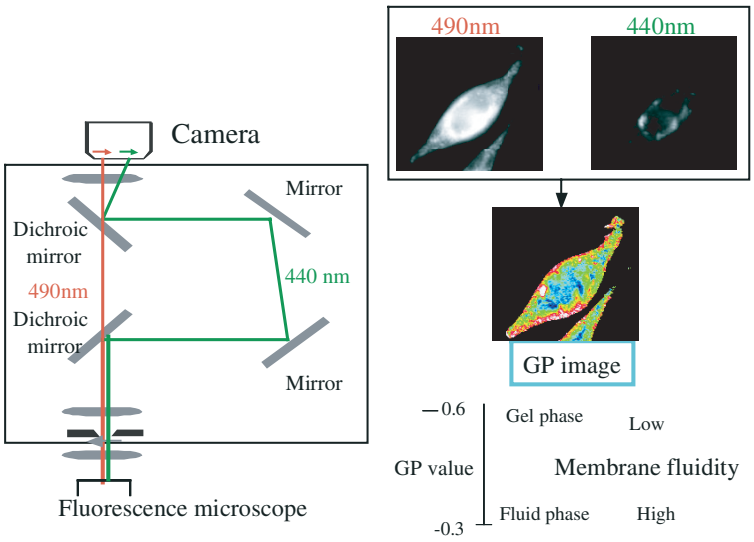

Figure 3. Scheme of optical system in microscopic imaging instrument of membrane fluidity and calculation of generalized polarization (GP) image from the fluorescence images at 490 and 440 nm.

processed to a generalized polarization image by personal computer (figure 3). First, change of membrane fluidity by treatment with sphingomyelinase was examined. Sphingomyelinase treatment showed almost no effect on the membrane fluidity. Next, we examined change of membrane fluidity by treatment with methyl-β-cyclodextrin. Removal of cholesterol by methyl-β-cyclodextrin raised the membrane fluidity in a dose dependent manner up to 15 mM. However, 1 mM of methyl-β-cyclodextrin hardly affected the membrane fluidity. Figure 4(a) shows microscopic images of membrane fluidity in living CHO cells treated with sphingomyelinase and methyl-β-cyclodextrin sequentially. CHO cells were treated with these series of sphingomyelinase, and then treated with 1 mM of methyl-β-cyclodextrin. In figure 4(b), averaged GP values of cell fractions are plotted for the concentration of sphingomyelinase. The result indicates that increase of membrane fluidity (removal of cholesterol by methyl-β-cyclodextrin) depended on the concentration of sphingomyelinase pre-treatment.

4. Discussion

The present experiments indicate that melittin interacts preferentially with DMPC molecules in mixed phospholipids bilayers. When peptides or proteins bind to the surface of acidic phospholipid membranes electrostatically, the phase transition temperature is raised and the transition enthalpy is not reduced by the binding [15, 16]. On the other hand, the phase transition enthalpy is much reduced when hydrophobic molecules penetrate the phospholipid membranes [17, 18]. The hydrophobic region of the DMPC bilayer membrane is 3.32 nm in all-*trans* configuration [18]. The effective acyl chain length from carbonyl carbon to terminal methyl carbon calculated from the segmental order parameter is 1.07 nm for DMPC [18], and the head-to-tail distance of 20 amino acids of melittin except six hydrophilic amino acids is approximately 3.0 nm in the alpha-helix structure with a kink of 120°–160° [19]. Pott and Dufourc have reported that hydrophobic thickness of DPPC-cholesterol membrane surrounded

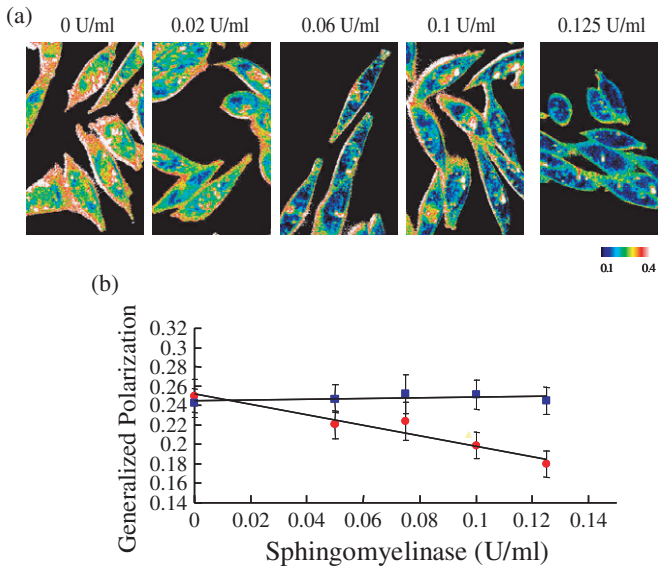

Figure 4. Change of membrane fluidity by sequential treatment of CHO cells. (a) Microscopic GP images of CHO cells by sequential treatment with sphingomyelinase then methyl-beta-cyclodextrin. CHO cells were treated with indicated concentrations of sphingomyelinase at 37 °C for 10 min and then treated with 1 mM of methyl-beta-cyclodextrin. The rainbow colour bar indicates the GP value. (b) Averaged GP values of cell regions are plotted for the concentration of sphingomyelinase. Square, the cells were treated with sphingomyelinase. Circle, the cells were treated with sphingomyelinase and then with methyl-beta-cyclodextrin.

by melittin is nearly 2.9 nm [20]. Lavialle *et al* investigated the interaction of melittin with DMPC membranes by Raman spectroscopy [21]. Although their experiments were performed under a condition that phospholipase A_2 activity was not inhibited, they provided evidence for the fatty acyl chains were immobilized by melittin molecules. As for penetration of melittin into lipid bilayers, electron microscopy has manifested that DMPC liposomes containing 5 mol% melittin yield intramembranous particles on the freeze-fractured faces [22]. Considering these reports and the present results, I could propose a possible process of the melittin–DMPC interaction as follows. Melittin added to liposomal dispersions binds to the surface of lipid bilayer membranes electrostatically, and then it penetrates into the hydrophobic core in the form of tetramer if the region-matched hydrophobic interaction is satisfied. In the process, it is most important that the thickness of the hydrophobic core of the lipid bilayer fits the length of the hydrophobic region of the melittin molecules.

For the second purpose, we used CHO cells and investigated the specific interaction between sphingomyelin and cholesterol. In order to examine the specific interaction, CHO cells were treated with methyl-β-cyclodextrin to remove cholesterol or treated with sphingomyelinase to convert sphingomyelin to ceramide. It is expected that removal of cholesterol changes membrane fluidity, so we measured the membrane fluidity of the CHO cells. A raft was found as detergent—resistant membrane fragments isolated from cells. The role of the raft in the signal transfer is noticed in various cells [23, 24]. Formation of the raft is based on dynamic clustering of sphingolipids and cholesterol in cell membranes, and cholesterol is the most important factor for formation of micro-domains [25]. Detergent resistant rafts are formed with a ternary mixture of phosphatidylcholine, sphingomyelin and cholesterol [26], so we investigated the specific interaction between sphingomyelin and

cholesterol in living cells, and we succeeded in manifesting the specific interaction by use of a microscopic imaging instrument of membrane fluidity.

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