The characterization of plasma membrane Ca^{2+}-ATPase in rich sphingomyelin–cholesterol domains

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Abstract **According to the raft hypothesis, sphingolipid–cholesterol (CHOL) microdomains are involved in numerous cellular functions. Here, we have prepared liposomes to simulate the lipid composition of rafts/caveolae using phosphatidylchone, sphingomyelin (SPM)–CHOL in vitro. Experiments of both 1,6-diphenyl-1,3,5-hexatriene and merocyanine-540 fluorescence showed that a phase transition from l_d to l_o can be observed clearly. In particular, we investigated the behavior of a membrane protein, plasma membrane Ca^{2+}-ATPase (PMCA), in lipid rafts (l_o phase). Three complementary approaches to characterize the physical appearance of PMCA were employed in the present study. Tryptophan intrinsic fluorescence increase, fluorescence quenching by both acrylamid and hypocrellin B decrease, and MIANS fluorescence decrease, indicate that the conformation of PMCA embedded in lipid l_o phase is more compact than in lipid l_d phase. Also, our results showed that PMCA activity decreased with the increase of SPM–CHOL content, in other words, with the increase of l_o phase. This suggests that the specific domains containing high SPM–CHOL concentration are not a favorable place for PMCA activity. Finally, a possible explanation about PMCA molecules concentrated in caveolae/rafts was discussed.**

Keywords: Plasma membrane Ca^{2+}-ATPase; Conformation; Lipid rafts; Fluorescence.

1. Introduction

The significance of lipids and of structural diversity of the self-assembled lipid bilayer membrane is still poorly comprehended, in contrast to the immense progress in the understanding of the functions of proteins and nucleic acid in living cells. It has been known that lipids can serve as second messengers, regulators of membrane proteins and can perform other functions in biological membranes [1,2]. Sphingolipid, typified by sphingomyelin (SPM), is one of the major classes of membrane phospholipids in eukaryotic organisms [3]. SPMs and phosphatidylcholines (PCs) constitute more than 50% of membrane phospholipids and are strongly enriched in the external plasma membrane leaflet of cells [3]. Cholesterol also is a major constituent of plasma membrane. It is an alicyclic lipid molecule, consisting of four fused rings, a 3β-hydroxyl and a hydrophobic tail, all of which are significant in interacting with phospholipids [4]. The most exciting discovery about lipids in recent years is that they were found to play a key role in the formation of functional membrane rafts or caveolae, which are sphingolipid- and CHOL-rich domains. Rafts/Caveolae are postulated to function as platforms involved in the lateral sorting of certain proteins during their trafficking within cells, as well as during signal transduction events [5–7].

Plasma membrane Ca^{2+}-ATPase (PMCA) plays a key role in the fine control of the cytoplasmic free Ca^{2+} concentration and maintaining intracellular Ca^{2+} homeostasis [8]. It has been reported that the Ca^{2+} pump of the plasma membrane is localized in caveolae, and the Ca^{2+}-ATPase was found to be concentrated 18- to 25-fold in the caveolae membrane compared with the non-caveolae portion of the plasma membrane [9,10]. These observations led us to investigate the structure and function of the Ca^{2+}-ATPase in the specific lipid environment of lipid rafts/caveolae.

In the present paper, we have prepared liposomes to simulate the lipid composition of rafts/caveolae using PC, SPM and cholesterol (CHOL) in vitro. Here, the ratio of SPM and CHOL was kept constant: 1:2 (mol:mol) [11]. PC containing major lipid component 1-palmitoyl, 2-oleoyl-sn-glycerophosphocholin (POPC) and dipalmitoyl-phosphatidylcholine (DPPC) [12] was chosen to represent the low- and high-melting component because POPC and DPPC have the ability to form a liquid-ordered (l_o) phase in the presence of CHOL both below and above Tm [13,14]. We used a graduated ratio of SPM–CHOL/PC to observe the transitions from liquid-disordered (l_d) phase to l_o phase, and in particular, to study the plasma membrane Ca^{2+}-ATPase behaviors in various lipid phases which could be thought to represent non-raft and raft membrane domains, respectively.

2. Materials and methods

2.1. Materials

SPM (from chick egg), CHOL, PC, phosphatidylserine (PS), and acrylamide, pyruvate kinase (PK), L-lactic dehydrogenase (LDH), phospho-enol pyruvate (PEP), and EGTA were obtained from Sigma. Triton X-100 and ATP were obtained from Fluka. SM_2 Bio-Beads was obtained from Bio-Rad. Calmodulin–Sepharose 4B was purchased from Pharmacia. 1,6-diphenyl-1,3,5-hexatriene (DPH), merocyanine-540 (MC-540) and 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid, sodium salt (MIANS) were purchased from Molecular Probes. Hypocrellin B (HB) was prepared by Prof. Jiachang Yue of the Institute of Biophysics, Academia Sinica according to the method described in [15]. β-Nicotinamide adenine dinucleotide (NADH), reduced disodium salt, was obtained from Boehringer. All other reagents were commercially available in China and were of analytical grade.

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2.2. Purification of PMCA from porcine brain

The PMCA was purified from porcine brain by calmodulin affinity column chromatography according to the method of Salvador and Mata [16] and stored at −80 °C in a elution buffer containing 20 mM HEPES/KOH, 130 mM KCl, 2 mM EDTA, 0.06% Triton X-100 and 2 mM 2-mercaptoethanol (pH 7.4). The purity of the PMCA was checked by SDS–PAGE. The protein concentration was measured by the modified Lowry method [16], using bovine serum albumin as standard.

2.3. Reconstitution of purified PMCA into proteoliposomes by the Bio-Beads

PMCA was reconstituted into various molar ratios of SPM–CHOL/PC liposomes containing 5 mol% PS (the molar ratio of SPM to CHOL was kept at 1:2) according to the protocol described by Levy et al. [17]. Lipids were suspended in buffer (40 mM HEPES–KOH, 120 mM KCl, and 5 mM $MgCl_2$, pH 7.4) containing 2% TX-100 and sonicated on ice for about 2 min. Purified PMCA was mixed with the lipid sample to give a 1:5000 ratio of protein:lipid (mol/mol). Detergent was removed by direct addition of three aliquots of 80 mg/ml wet SM_2 Bio-Beads every hour, and the mixture was agitated slowly at room temperature to give the final preparation of sealed vesicles.

2.4. DPH and MC-540 labeling and fluorescence measurement

The fluorescence probe of DPH was dispersed into proteoliposome suspension at a probe-to-lipid molar ratio of l:500, then incubated at 37 °C for 1 h in the dark. Fluorescence polarization measurements were determined on a Hitachi F-4500 spectro-fluorimeter fitted with a polarization attachment as described by Shinitzky and Barenholz [18]. The samples were excited at 360 nm, and the emissions at 430 nm were recorded. Both excitation and emission slits were set at 5 nm. The degree of fluorescence polarization (P), which reflects the motion and viscosity of lipid molecules, was calculated according to the following formula: $P = (I_{VV} - GI_{VH})/(I_{VV} + GI_{VH})$ [19], where I_{VV} and I_{VH} are the fluorescence intensities measured with parallel and perpendicular oriented polarizers, respectively, and G is the calibration factor. Here, $G = I_{HV}/I_{HH}$, $V = 90$, $H = 0$.

The final concentration of the MC-540 fluorescence probe of 4 μM was thoroughly mixed with proteoliposome suspension at a probe-to-lipid molar ratio of l:200, then incubated at 37 °C for 1 h in the dark. Fluorescence measurements were determined on a Hitachi F-4500 spectro-fluorimeter with an excitation wavelength of 540 nm and the emissions at 590 nm were recorded [20]. Both excitation and emission slits were set at 5 nm.

2.5. Determination of ATPase avtivity and Ca^{2+} uptake activity of reconstituted PMCA

For ATP hydrolysis assays of PMCA, the absorbance of NADH at 340 nm was monitored with a calcium-regenerating, coupled enzyme system [21]. Briefly, 5 μg protein of proteoliposome was added to the reaction mixture that contained 40 mM HEPES–KOH, 120 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.15 mM NADH, 0.42 mM PEP, 1 mM ATP, 10 IU PK, and 28 IU LDH in a final volume of 1 ml. After 2 min incubation at 37 °C, the reaction was started by the addition of $CaCl_2$ (15 μM free Ca^{2+}). Defined concentrations of free Ca^{2+} were established by a $CaCl_2$/EGTA solution and calculated by using the binding affinities previously described [22] with the aid of a computer program: Winmax (see website http://www.stanford.edu/~cpatton/).

The calcium uptake was measured with a Hitachi 557 dual wavelength spectrophotometer at 37 °C which monitored the absorbance of arsenazo III at 675–685 nm by using ATP-regeneration in a solution of 40 mM HEPES, 100 mM KCl, 5 mM $MgSO_4$, 70 μM arsenazoIII, 50 μM Ca^{2+}, 25 mM ATP (pH 7.2) [23], and the protein concentration of proteoliposome was 20 μg/ml.

2.6. Intrinsic fluorescence measurement

The intrinsic fluorescence of reconstituted PMCA was measured on a Hitachi F 4500 spectro-fluorimeter at 37 °C with an excitation wavelength of 285 nm. The fluorescence emission spectra in the 300–400 nm range were recorded. The protein concentration was 20 μg/ml in the same medium of 40 mM HEPES–KOH, 120 mM KCl, and 5 mM $MgCl_2$, pH 7.4. Both excitation and emission slits were set at 5 nm.

2.7. Measurements of intrinsic fluorescence quenching by acrylamide and HB

The intrinsic fluorescence intensity of reconstituted PMCA in the same medium was measured as above with the excitation wavelength at 285 nm and emission wavelength at 335 nm in the absence and presence of quenchers (acrylamide, HB) which were sequentially added to the samples with a concentrated solution.

The quenching constants (K_{sv}) were calculated using the Stern–Volmer equation: $F_0/F = 1 + K_{sv}[Q]$ [24], where K_{sv} is the Stern–Volmer quenching constant, F_0 is the fluorescence in the absence of the quencher, F is the fluorescence in the presence of the quencher, and [Q] is the molar concentration of the quencher. The data presented were averages of at least three different experiments.

2.8. MIANS labeling and fluorescence measurement

The proteoliposomes were centrifuged at 100 000×g for 30 min and resuspended with the same buffer several times to remove 2-mercaptoethanol. The measurement of MIANS-binding fluorescence intensity was carried out on a Hitachi 4500 spectro-fluorimeter by addition of 8 μM MIANS to the sample with a protein concentration of 20 μg/ml at 37 °C [25]. The excitation wavelength was 322 nm and the emission wavelength was 420 nm with both excitation and emission slits of 5 nm.

3. Results

3.1. Phase transition of SPM–CHOL/PC system

DPH, a hydrophobic probe can be used to shows a double hard-cone wobbling movement to incorporate hydrocarbon inside the membrane bilayer. And orientation of the probe in the membrane is related to the physical state of the lipid bilayer. The polarization of DPH (P value) in model membranes is used to study the phase transition from l_d to l_o phase [13]. Fig. 1A shows the changes of polarization of DPH in SPM–CHOL/PC mixtures with increasing SPM–CHOL concentration at 37 °C. The phase diagram indicated that the P value was slightly increased at 0.14–0.24 of $\chi_{SPM-CHOL}$. After that, the P value abruptly increased with the increase of $\chi_{SPM-CHOL}$. Over 0.63, the increase tended to level off. The results from our and other research groups suggested that the lipid mixtures underwent a phase transition from l_d to l_o phase [13,26]. There was l_d/l_o coexistence in the lipid mixtures between 0.24 and 0.63 of $\chi_{SPM-CHOL}$ (Fig. 1A). The significance of this result is that l_d/l_o phase separation is very likely to occur in cell membranes with 30–40% CHOL. The SPM–CHOL necessary to attain a single l_o phase is quite high [27,28]. In the present study, the concentration may be over 0.63.

The experiments of the lipophilic probe merocyanine-540 (MC-540) provided further evidence about lipid-phase transition in addition to that described above. Binding of MC-540 to artificial bilayer membranes was assessed by measuring the enhancement of fluorescence when dye enters the hydrophobic environment of the membranes [20]. MC-540 is able to sense the degree of lipid packing of bilayers and inserts preferentially into bilayers whose lipids are more widely spaced. The stronger the fluorescence intensity of MC-540, the looser the pack density of lipid molecules. In Fig. 1B, the fluorescence intensity fell with increasing $\chi_{SPM-CHOL}$, in particular, 0.24–0.63 of $\chi_{SPM-CHOL}$ induced an abrupt decrease of fluorescence intensity, indicating the lipid-phase transition from l_d to l_o in the liposome because MC-540 appeared to bind to fluid-phase vesicles much more strongly than to gel-phase vesicles [29]. The results provided more quantitative data of the behavior of this lipid structure.