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

Yuhong Pang, Hua Zhu, Ping Wu, Jianwen Chen*

National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Datun Road 15, Chaoyang District, Beijing 100101, China

Received 8 February 2005; revised 13 March 2005; accepted 14 March 2005

Available online 29 March 2005

Edited by Sandro Sonnino

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.

*Corresponding author. Fax: +86 10 64840672.
E-mail address: chenmaci@sun5.ibp.ac.cn (J. Chen).

0014-5793/$30.00
doi:10.1016/j.febslet.2005.03.038

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.

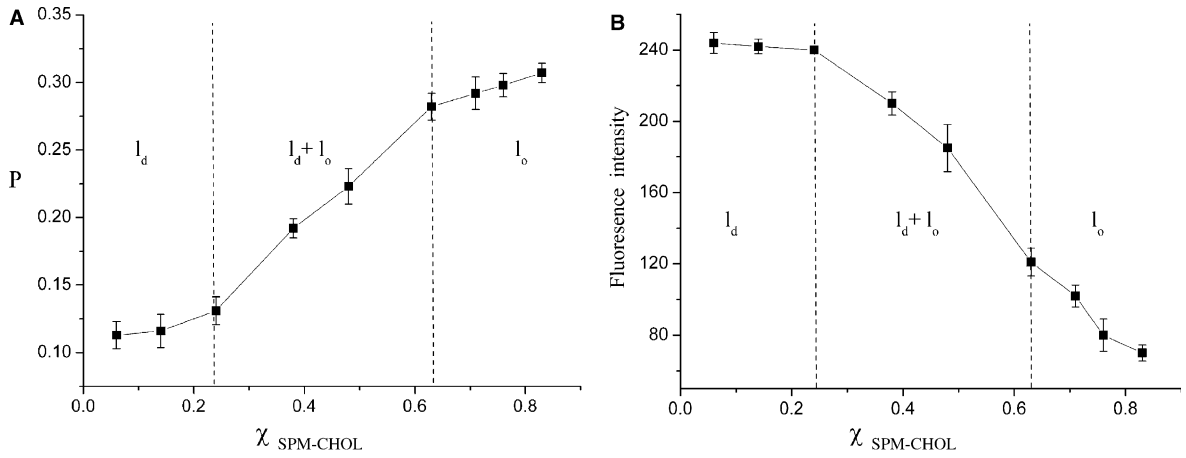

Fig. 1. SPM–CHOL/PC phase diagram. The fluorescence polarization (*P*) of DPH (A) and the fluorescence intensity of MC-540 (B) in various molar ratios of SPM–CHOL/PC liposomes containing 5 mol% of PS and PMCA. The ratio of SPM/CHOL was kept to 1:2 (mol). The experiments were carried out at 37 °C. The data were averages of three identical independent experiments.

3.2. PMCA activity

The structure of the biological membrane is very important for functions of membrane proteins. Any structure changes in lipid membrane, therefore, may have a profound influence on membrane proteins. After reconstitution of PMCA into the various designed liposomes, the lipid phase-dependence of PMCA activity was assayed. As shown in Fig. 2A, the activity of the enzyme was relatively high in the liposome mixtures containing low concentration of SPM–CHOL. However, the activity sharply decreased with the increase of SPM–CHOL content, in particular, the activity significantly decreased when the concentration of SPM–CHOL was over 0.72. This implied that the significant reduction in PMCA activity is due to lipid-phase transition. It should be noted that CaM, an important activator for PMCA, had a much weaker activation for PMCA in high SPM–CHOL content liposome than in low content.

Ca^{2+} transmembrane transport through PMCA plays a very important role in maintaining intracellular Ca^{2+} homeostasis. And it is imagined that the effect of lipid structure changes on PMCA activity should be direct. Fig. 2B shows the PMCA Ca^{2+} uptake activity in the presence of various concentrations of SPM–CHOL. The Ca^{2+} uptake activity decreased progressively with gradual increase of SPM–CHOL content (Fig. 2B, a–c). But the activity dropped abruptly when the SPM–CHOL concentration was increased to 72% (Fig. 2B, d). Further increase of SPM–CHOL concentration had only a slight effect on the activity. This indicates that l_o phase is an unfavorable environment for both ATPase activity and Ca^{2+} uptake activity of PMCA.

3.3. Intrinsic fluorescence measurement

Fig. 3 shows the intrinsic fluorescence emission spectra of PMCA reconstituted into liposomes containing various concentrations of SPM–CHOL. It can be seen that the emission maximum of PMCA was not significantly changed, but the emission intensity gradually increased with increasing SPM–CHOL concentration. These results suggest that the tryptophan residues of PMCA in l_o phase are already in a more hydrophobic environment than in l_d phase.

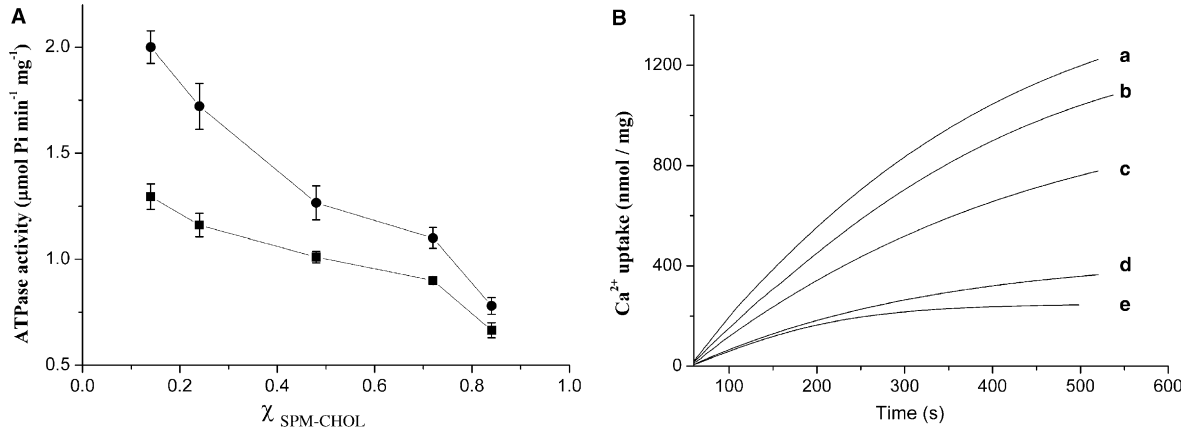

Fig. 2. Activity of PMCA. PMCA was reconstituted into various molar ratios of SPM–CHOL/PC liposomes containing 5 mol% PS. (A) ATPase activity in the absence (■) or presence (●) of 2 μg/ml CaM at 37 °C, respectively. The data were averages of three independent experiments. (B) Ca^{2+} uptake activity of PMCA at 37 °C. The experiments were done at least three times with similar results.

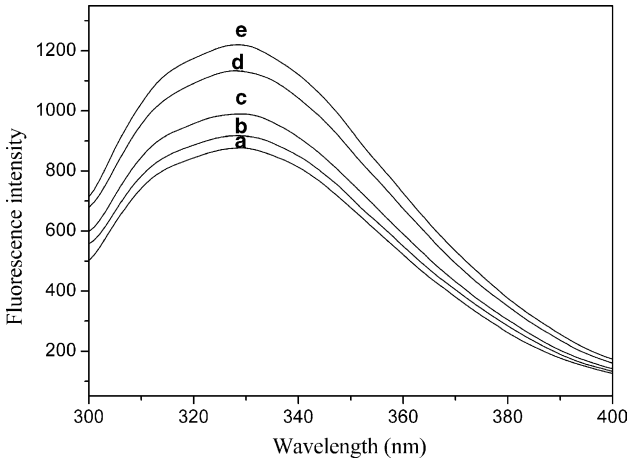

Fig. 3. Tryptophan intrinsic fluorescence. Fluorescence spectra of PMCA reconstituted into various SPM–CHOL/PC liposomes at 37 °C. The content of SPM–CHOL was 14, 24, 48, 72 and 84 mol% from a to e, respectively. The data were representative of three identical independent experiments.

3.4. Fluorescence quenching studies

Acrylamide is a polar, uncharged compound that has been shown to quench the fluorescence of tryptophan indole ring predominately. This probe has the ability to quench any excited tryptophanyl residues, regardless of whether the residue are located on the surface or the apolar area [24,30]. PMCA is a multi-tryptophan protein. In this protein, the fluorescence of certain tryptophans must be quenched much more readily than others because of their localization in the protein. The sensitivity of acrylamide quenching to exposure tryptophans suggested that it should be very useful for monitoring protein conformational changes [24]. Fig. 4A shows the Stern–Volmer plots of the tryptophan fluorescence quenching of PMCA reconstituted into liposomes. The slope of the Stern–Volmer plots reflects quenching degrees. As shown in Fig. 4A, a marked increase in quenching degree was observed in lower SPM–CHOL concentration, indicating that more tryptophanyl residues in the protein are becoming greatly exposed to acrylamide.

Table 1
Acrylamide and HB quenching constant, K_{sv}, for tryptophan in PMCA reconstituted into liposomes containing various SPM–CHOL concentrations

[SPM–CHOL]	K_{sv}	
	Acrylamide (M^{-1})	HB (mM^{-1})
14 mol%	4.54 ± 0.21	59.5 ± 0.78
24 mol%	4.25 ± 0.15	58.0 ± 1.71
48 mol%	3.89 ± 0.22	54.8 ± 1.10
72 mol%	3.50 ± 0.19	50.0 ± 1.83
84 mol%	2.74 ± 0.17	44.9 ± 1.95

The values of K_{sv} shown were averages from at least three separate independent experiments.

It has been reported that hydrophobic HB is mainly located in the lipid bilayer of membranes, so it is lipid-soluble and is often used to quench fluorescence emitted from tryptophan located in the hydrophobic domain of the proteins [31,32]. The Stern–Volmer plots of HB quenching of reconstituted PMCA with liposomes is shown in Fig. 4B. A gradual increase of quenching degree can be observed with increase of HB content and lower quenching appeared at higher SPM–CHOL concentrations.

Listed in Table 1 is an effective quenching constant, K_{sv}, for tryptophan in PMCA reconstituted into liposomes containing various SPM–CHOL concentrations. It can be seen clearly that the K_{sv} of either acrylamide or HB decreased with the increase of SPM–CHOL content.

3.5. MIANS fluorescence assay

MIANS binds covalently to cysteinyl residues in proteins and only becomes fluorescent after the maleimide undergoes a covalent reaction with sulfhydryl groups [25,33]. In this study, MIANS was used to detect conformational changes in PMCA embedded in various liposomes. The addition of MIANS to lipoproteins led to a rapid increase in fluorescence intensity (Fig. 5). The rate of increase in fluorescence intensity declined steadily over a period of about 3 min. But, as shown in Fig. 5, SPM–CHOL in the liposomes resulted in a reduction in final fluorescence intensity and the rate of reaction of PMCA with MIANS, which was dependent on the added SPM–CHOL concentration (i.e., Fig. 5a–e), indicating that

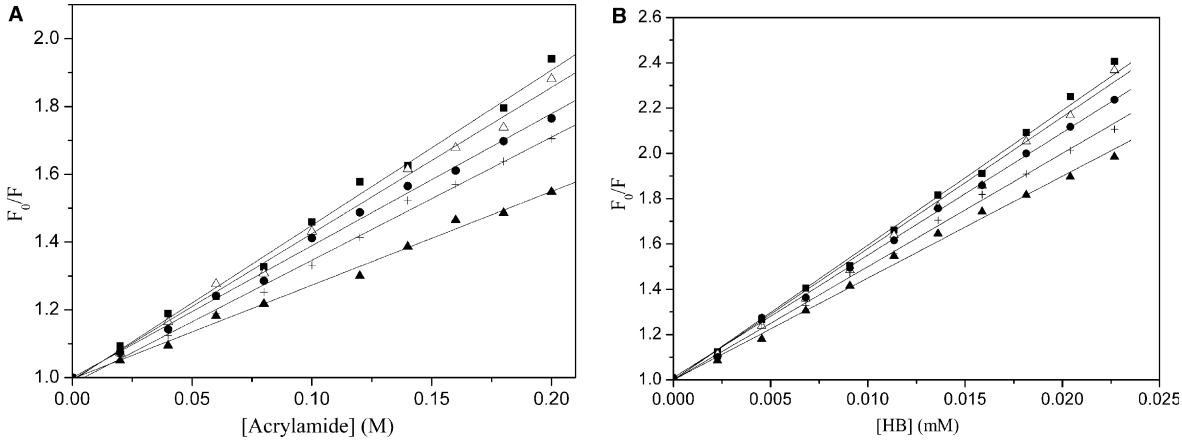

Fig. 4. Fluorescence quenching. PMCA intrinsic fluorescence quenching by acrylamide (Fig. 3a) and HB (Fig. 3b) at 37 °C, respectively. The SPM–CHOL content in liposomes was 14 mol% (■), 24 mol% (△), 48 mol% (●), 72 mol% (+) and 84 mol% (▲), respectively. The data were representative of three identical independent experiments.

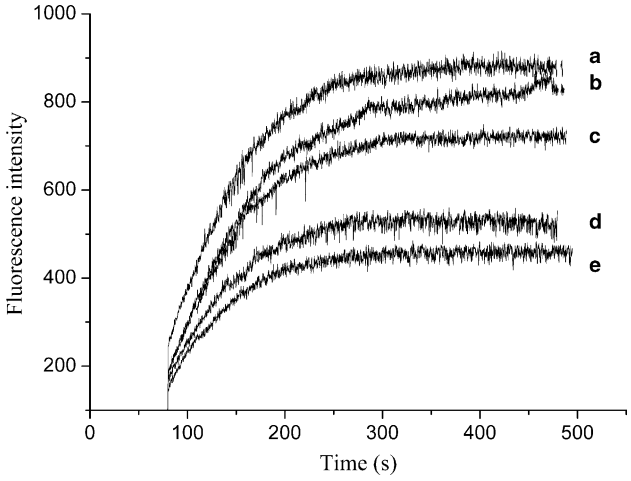

Fig. 5. MIANS Fluorescence. MIANS fluorescence spectra of PMCA reconstituted into various liposomes at 37 °C. The content of SPM–CHOL was 14, 24, 48, 72 and 84 mol% from a to e, respectively. The spectra represent three identical independent experiments.

more of the cysteiny residues of PMCA in l_o phase might be binded to MIANS.

4. Discussion

Domains rich in SPM and CHOL have been a subject of great interest recently in cell biology because some important integral membrane proteins may be preferentially located within them [34]. Such domains in some cells that do not express caveolin are called lipid rafts. According to the Simons's hypothesis, the lateral assembly of sphingolipids and CHOL creates rafts floating in a glycerophospholipid-rich environment [35]. Many of the same proteins found in the caveolae of caveolin-expressing cells are enriched in lipid rafts, too [36]. In the present study, a model membrane system of SPM–CHOL/PC was used to detect the structure and function of PMCA embedded in the lipid rafts. We used natural PC as a representative of lipid in cell plasma membrane. In fact, the model membrane we used was a ternary system containing three binary systems: CHOL/PC, SPM/CHOL and SPM/PC. Since the presence of CHOL could induce the formation of a l_o phase from l_d or S_o phase both below and above Tm of the phospholipids [13,14,37–39] and SPM–CHOL are the main lipid components of rafts/caveolae, in this paper, we gradually increased SPM–CHOL (1:2, mol/mol) content in the PC liposome in order to easily construct lipid rafts. Here, both DPH and MC-540 fluorescence were used only to investigate the progress of the transition from l_d to l_o phase. Experiments indicated that the lipid raft domains (l_o phase) in liposomes enhanced with the increase of SPM–CHOL content. The results were consistent with those of other laboratories described above. We did not study, in the present investigation, the precise mechanism of interaction among PC, SPM and CHOL, or characterization of their dynamics.

There is abundant evidence that many important proteins are dynamically associated with caveolae or raft domains in plasma membranes, including PMCA; glycosylphosphatidylinositol (GPI)-anchored proteins; protein kinase C; G protein-coupled membrane receptor; doubly acylated kinases of the Src family and inositol 1,4,5,-trisphosphate receptor [9,10,40–44]. If these proteins preferentially reside in rafts in the natural state, it would mean that rafts promote protein-protein interactions, and this in turn could be important for cellular processes such as signal transduction and intracellular trafficking [34]. It was very surprising that SPM/CHOL lipid membranes (l_o phase) were available for protein kinase C (PKC) binding. But the specific domains were not a favorable location for its activity [45]. The major object of our study was to probe the behaviors of PMCA in l_o phase. We employed the following three complementary approaches to characterize the physical appearance of PMCA. (1) More SPM–CHOL concentration results in an increase of tryptophan intrinsic fluorescence, indicating PMCA conformation tends to be more folding (compact) in l_o phase. (2) Studies of both acrylamide and HB fluorescence quenching could give more information on the conformational changes of the protein occurring in both hydrophilic and hydrophobic domains. The quenching experiments demonstrate that the conformation of PMCA embedded in lipid l_o phase is more compact than in lipid l_d phase, in either hydrophilic or hydrophobic domains. (3) The MIANS fluorescence experiment supports the results of intrinsic fluorescence and fluorescence quenching by acrylamide and HB described above: more compact conformation of PMCA in l_o phase will appear. In view of the relationship between the activity and the molecular conformation of PMCA, knowledge of the conformational basis for the effect of lipid phase on the activity of PMCA is essential to elucidate the mechanism for the interaction of phospholipids with PMCA. Our results showed that PMCA activity decreased accompanied by an increase of SPM–CHOL concentration, in other words, by an increase of l_o phase, where a more compact conformation of PMCA would appear.

Since the enzyme does not work well in rich SPM–CHOL domains, why is PMCA concentrated in rafts/caveolae? Findings previously reported indicated that a variety of acidic phospholipids including PS, cardiolipin (CL), phosphatidylinositol (PI), phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidic acid (PA) stimulated the V_{max} and decreased the K_m of PMCA isolated from erythrocyte and pig stomach smooth muscle [21,46]. The stimulated-modes can also be observed in other calmodulin-stimulated enzymes [47–49], so they may be general to all such enzymes. Physiologically, it may be of interest to discuss how its function is performed in rafts/caveolae. Firstly, although caveolae and the so called rafts are believed to be enriched in the bulk membrane lipids such as CHOL and SPM, PA, PS, PI and PIP_2 are also believed to be preferentially located in rafts/caveolae [50–53]. Therefore, the active center of PMCA may be surrounded in vivo by such acidic phospholipids so that the enzyme activity regulated by small changes in the acidic phospholipid content in caveolae may be possible. In particular, calmodulin, a very important activator for PMCA, is enriched in caveolae [54]. It is very convenient to cross-talk between PMCA and calmodulin in caveolae. Secondly, it has been demonstrated that epidermal growth factor receptor (EGFR) is initially concentrated in caveolae but rapidly moves out of this membrane domain in response to EGF [55]. Therefore, PMCA may be similar to EGFR, in that it is initially located in preexisting caveolae which acts as storage. When the enzyme is stimulated by

external signals, it will migrate from the caveolae to certain active positions of plasma membranes to assist in response to cell behavior.

Evidence from the in vitro studies presented in this paper indicates that the promotion of l_o phase formation is likely to be an important function of sphingolipid and CHOL because these lipids are enriched in the plasma membrane to form raft microdomains. But the microdomain environment containing rich SPM and CHOL is possibly not a favorable place for PMCA activity. In addition, there is a growing body of evidence that a number of proteins in caveolae, such as Ras, Src, protein kinase C, eNOS, EGFR, can directly interact with caveolin-1, an essential structural component of caveolae, which consequently results in a strong inhibition of their biological activities [56,57]. Therefore, we have to address the questions of how these signaling molecules and enzymes perform their function in caveolae, and how these molecules are regulated in caveolae. The consequences of inhibition of PMCA activity in raft domains described above are likely but much additional work will also be required to fully elucidate it.

Acknowledgments: This research was supported by the Natural Science Foundation of China (30030186 and 30230120). We thank Zhongnian Zhou and Meirong Zhang for their expert technical assistance.

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