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The Behaviors of Ca^{2+}-ATPase Embedded in Interdigitated Bilayer[1]

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We investigated the behavior of a membrane protein, Ca^{2+}-ATPase, in interdigitated phospholipid bilayers. The results showed that Ca^{2+}-ATPase does not cause significant alterations in the interdigitation of 16:0 LPC/DPPC (27.0 mol% LPC) vesicles when it is reconstituted with lipids. Intrinsic fluorescence, acrylodan fluorescent adducts, and CD spectra indicated that Ca^{2+}-ATPase, when embedded in interdigitated bilayer structures, is more exposed to the hydrophilic environment and has a looser structure than when embedded in non-interdigitated bilayers. The interdigitation of acyl chains induces a rapid loss of enzyme activity. It is suggested that interdigitated bilayer structures may play an important role as negative regulatory factors in physiological functions.

Key words: Ca^{2+}-ATPase, interdigitated bilayer, lysophosphatidylcholine.

Lysophospholipids, especially lysophosphatidylcholines (LPC), are known to be present as minor constituents in various cell membranes. They bind to cell membranes inducing morphological changes in the cell, membrane fusion, cell aggregation, hemolysis, and affect cell permeability properties (*1-3*). LPC are now recognized to be precursors of bioactive molecules that are generated during signal transduction as second messengers (*4*). Protein kinase C (PKC) is a key component in signal transduction pathways, where, its activity is regulated by certain lysophospholipids, LPC in particular (*5, 6*). LPC molecules may be regarded structurally as an extreme of the asymmetric phosphatidylcholine system in which the entire Sn-2 acyl chain is replaced by a hydrogen atom. The wedge shaped molecules can form micelles above the chain-melting temperature and interdigitated lamellar structures in the gel state (*2, 7*). Techniques such as differential scanning calorimetry (DSC) and ^{13}C-NMR (*1*), ^{31}P-NMR and freeze-fracture electron microscopy (*8*), light scattering and X-ray (*9*) have been used to compare the behavior and properties of lysophosphatidylcholine/phosphatidylcholine binary mixtures under various conditions. Recently, we used DSC, X-ray diffraction, and fluorescence polarization, a technique developed by our laboratory (*10*), to investigate the structural changes of 16:0 LPC/DPPC binary mixtures. The results indicated that 16:0 LPC induces DPPC, which is normally a non-interdigitated lipid, to form an interdigitated gel phase at low concentration (*11*). The interdigitated structures are destabilized slowly, finally becoming micellar (*11*) with increasing 16:0 LPC concentration. The formation of the interdigitated bilayer structure has not yet been observed in biological membranes *in vivo*. However, some amphiphilic molecules, such as glycerol (*12*), chlorpromazine (*13*), methanol, ethanol (*14*), polymyxin B (*15*), myelin basic protein (*16*), Tris (*17*), and anisodamine (*10*), can cause interdigitated structure formation in liposomal membranes composed of phosphatidylcholines or phosphatidylglycerols. It is believed that the existence of an interdigitated phase in a biological membrane may be much more common than previously considered. If some interdigitated domains occur in biomembranes, they may play important functional roles, as described by Slater and Huang (*18, 19*). However, they have not been investigated so far, because of the lack of methods to detect the interdigitated structure in reconstituted vesicles containing membrane proteins (*20*). In this paper, we used nAS and 16AP fluorescence polarization to detect the interdigitation of 16:0 LPC/DPPC/Ca^{2+}-ATPase reconstituted vesicles and also probed the behavior of Ca^{2+}-ATPase in interdigitated bilayer structures.

MATERIALS AND METHODS

Materials—Dipalmitoylphosphatidylcholine (DPPC) and 16:0 lysophosphatidylcholine (16:0 LPC) were obtained from Sigma. 3-(9-anthroyloxyl)-stearic acid (3AS), 9-(9-anthroyloxyl)-stearic acid (9AS), 16-(9-anthroyloxyl)-palmitic acid (16AP), and 6-acryloyl-2-dimethylaminonaphthalene (Acrylodan) were obtained from Molecular Probes. All other reagents used were of analytical grade.

Preparation of Ca^{2+}-ATPase—The purification and measurement of the functional activity of Ca^{2+}-ATPase from rabbit sarcoplasmic reticulum were carried out as described in Michelangeli and Munkonge (*21*). The protein concentration was determined by the method of Lowry *et al.* (*22*).

Reconstitution of Ca^{2+}-ATPase—Reconstitution of Ca^{2+}-ATPase in vesicles was performed as described by Wang *et al.* (*23*). One milligram of DPPC or 16:0 LPC/DPPC (27.0 mol% LPC) was dissolved in chloroform/methanol (1:1 v/v), dried under a stream of nitrogen gas to form a thin film on the bottom of a tube, then evacuated in a lyophilizer overnight. Ca^{2+}-ATPase (100 μg) and 1 ml of 10 mM

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Tris-HCl buffer (pH 7.2) were then added, and the mixture was dispersed by vigorous vortexing at 45°C for 10 min, freeze-thawed, and then vortexed further at 45°C for 10 min. The reconstitued samples were adjusted to 2 ml and stored at 4°C until used.

Fluorescence Polarization Measurement—Fluorescence probes of 3AS, 9AS, and 16AP were dissolved in methanol as stock solutions. The probes were added to reconstituted samples at a lipid/probe weight ratio of 500:1, and then incubated at 45°C for 1 h. The samples were held overnight at 4°C, and the fluorescence polarization was determined on a Hitachi F-4010 spectrofluorometer fitted with a polarization attachment. The degree of fluorescent polarization (P), which reflects the motion and viscosity of lipid molecules, was calculated according to the following formula: $P = (I_{VV} - G \cdot I_{VH})/(I_{VV} + G \cdot I_{VH})$, $V = 90°$, $H = 0°$, 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, $G = I_{HV}/I_{HH}$.

Intrinsic Fluorescence Measurements—Intrinsic fluorescence was determined on a Hitachi F-4010 spectrofluorometer. The samples were excited at 284 nm, and emission scanned from 300 to 400 nm with a bandpass of 10 nm.

Fluorescence Measurement of Thiol Adducts of Acrylodan—A stock solution of acrylodan was prepared in dimethyl formamide. Ca^{2+}-ATPase dissolved in 10 mM Tris-HCl buffer (pH 7.2) was labeled with acrylodan at a protein/acrylodan molar ratio of 1:50. The reaction was allowed to proceed at 4°C for 4–12 h, and the excess acrylodan was removed by dialysis. The labeled protein was reconstituted into lipid vesicles as described above. Fluorescence experiments were performed on a Hitachi F-4010 spectrofluorometer. The excitation wavelength was 370 nm, and the emission spectra were scanned from 400 to 600 nm with a bandpass of 10 nm.

Circular Dichroism Measurements—CD spectra of Ca^{2+}-ATPase reconstituted vesicles were collected on a Jasco J-720 Spectropolarimeter at room temperature at a protein concentration 5 μM. The scan speed was 100 nm/min.

RESULTS

1) Fluorescence Polarization Measurement of Lipid Vesicles and Proteoliposomes—In an interdigitated structure, the acyl chains of one layer are inset into the opposite lipid layer with the ends of the acyl chains resting near the polar head groups of the opposing lipid molecules. In this case, the physical properties of the methyl ends of the acyl chains are similar to those of the carbons near the polar head groups. As an application of this argument, Wang, H.Y. *et al.* used a spin label, 16-doxyl-stearic acid, to determine the interdigitated phase of DPPG induced by polymyxin B (*24*). We used the fluorescence probes n-AS and 16AP to determine the interdigitated phase of DPPG caused by anisodamine and polymyxin B (*10, 25, 26*). This method has been proved effective for studying phase transition by DPH fluorescence measurement and small angle X-ray diffraction (*11, 27*). Figure 1 shows that the polarization of pure DPPC vesicles formed in Tris buffer has a polarization gradient at all temperatures, *i.e.* 3AS > 9AS > 16AP (Fig. 1A), and that the P values decrease as the temperature rises. In the 27.0 mol% 16:0 LPC/DPPC binary mixtures, the 16AP polarization increased greatly below 40°C (Fig. 1B). This can be explained as due to the insertion of the acyl chains of one lipid layer into the other, giving the methyl ends similar physical properties to carbons 3–9. Above 40°C, the polarization of 16AP decreases dramatically and the polarization gradient was resumed (Fig. 1B). The results indicate that 27.0 mol% of 16:0 LPC induces LPC/DPPC vesicles to form an interdigitated structure in the gel phase.

Similar results can be observed in proteoliposome systems. Table I shows the fluorescence polarization of Ca^{2+}-ATPase reconstituted in DPPC and 27.0 mol% 16:0 LPC/DPPC vesicles. In a non-interdigitated structure model (DPPC/Ca^{2+}-ATPase vesicles), polarization gradients can be obtained at either 30 or 45°C. The polarization of 16AP increases and the polarization gradient is abolished at 30°C

TABLE I. **Fluorescence polarization of Ca^{2+}-ATPase reconstituted in DPPC and 27.0 mol% 16:0 LPC/DPPC vesicles.**

		P values		
		3AS	9AS	16AP
DPPC/Ca^{2+}-ATPase	30°C	0.223	0.214	0.159
	45°C	0.192	0.163	0.081
16:0 LPC/DPPC/Ca^{2+}-ATPase	30°C	0.214	0.172	0.181
	45°C	0.149	0.113	0.064

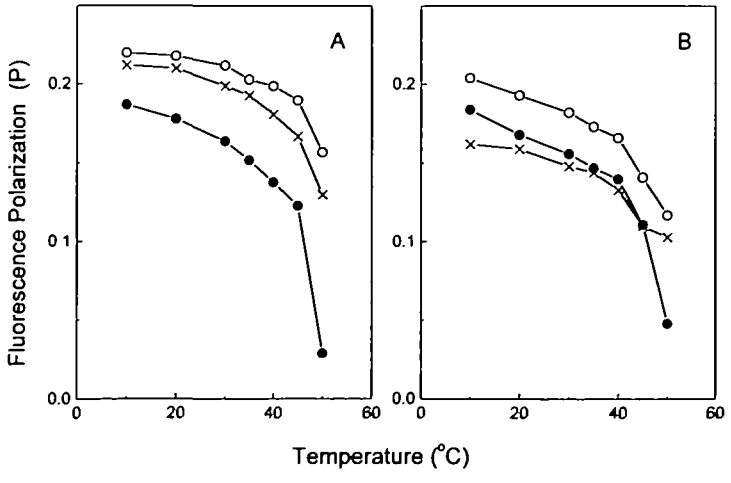

Fig. 1. Fluorescence polarization of DPPC (A) and 27.0 mol% 16:0 LPC/DPPC (B) vesicles at different temperatures. ○, 3AS; ×, 9AS; ●, 16AP.

($< T_m$) in lipoprotein vesicles comprising 16:0 LPC/DPPC/Ca^{2+}-ATPase, which is an interdigitated structure model, while lipoprotein vesicles still show a polarization gradient at 45°C ($> T_m$) (*i.e.* 3AS>9AS>16AP). It is clear from Table I that Ca^{2+}-ATPase does not significantly alter 16:0 LPC/DPPC interdigitation at gel phase when it is reconstituted with the lipids. We will use these proteoliposome models to compare the properties of Ca^{2+}-ATPase in interdigitated bilayer structures and in non-interdigitated bilayer structures.

2) Intrinsic Fluorescence Spectra of Ca^{2+}-ATPase—Figure 2 shows that the intrinsic fluorescence intensity of 16:0 LPC/DPPC/Ca^{2+}-ATPase vesicles is lower than that of DPPC/Ca^{2+}-ATPase vesicles, and that the emission peak has undergone an obvious red shift from 334 to 336 nm. Ca^{2+}-ATPase has 13 Trp residues, 10 of which are located in the hydrophobic transmembrane domain (*28*). The results of a slight red shift of the emission peak and a decrease in the emission intensity suggest different microenvironments for Trp residues of Ca^{2+}-ATPase in interdigitated and non-interdigitated bilayer structures: more tryptophan residues are exposed to a hydrophilic environment in the former than in the latter.

3) Fluorescence Measurement of Ca^{2+}-ATPase Adducts of Acrylodan—In order to investigate the conformation changes of Ca^{2+}-ATPase in vesicles of DPPC and 16:0 LPC/DPPC, Ca^{2+}-ATPase was labeled with acrylodan, which reacts efficiently and selectively with thiol groups (-SH) in proteins to form covalently-linked fluorescent adducts. The quantum yield of the fluorescence derivatives is enhanced when the probe reacts with sulfhydryl groups in protein (*29*). The emission spectrum of a protein-acrylodan adduct depends on the hydrophilicity or hydrophobicity of the environment of the fluorescent probes that bind the thiol groups. It has been reported that a red shift in the emission maximum of a protein-acrylodan adduct reflects more exposure of the fluorophore to a hydrophilic environment (*29*). Figure 3 shows the fluorescence spectra of proteoliposomes of Ca^{2+}-ATPase reconstituted in DPPC and 16:0 LPC/DPPC, respectively. It is clearly seen that the emission peak for the proteoliposomes in 16:0 LPC/DPPC is significantly red-shifted by about 6 nm compared with that for the proteoliposomes in DPPC. This indicates that the acrylodan-thiol groups of Ca^{2+}-ATPase in 16:0 LPC/DPPC are exposed to a more hydrophilic environment than they are in DPPC. Those results correspond with those of intrinsic fluorescence. It is known that there are 22 sulfhydryl groups in Ca^{2+}-ATPase (*28*). The relative fluorescence intensity of 16:0 LPC/DPPC proteoliposomes is markedly higher than that of the DPPC proteoliposomes because there is more labeling of the thiol moieties by the fluorescence probe in a hydrophilic environment.

4) Short-Wavelength UV CD Spectra—A CD spectrum usually reflects the conformational alteration of the backbone of a protein (*30*). The two negative peaks at 209 and 222 nm contribute to the α-helix structure. We used CD to compare the conformations of Ca^{2+}-ATPase in DPPC and 27.0 mol% 16:0 LPC/DPPC vesicles (Fig. 4). The ellipticity around 209-225 nm for proteoliposomes in 16:0 LPC/DPPC is always negatively lower than that in DPPC,

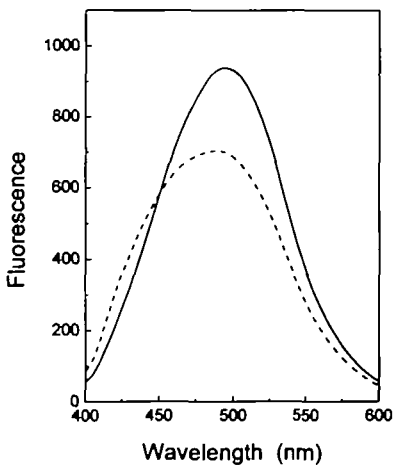

Fig. 3. **Fluorescence spectra of Acrylodan-Ca^{2+}-ATPase reconstituted in DPPC (---) and 27.0 mol% 16:0 LPC/DPPC (—) vesicles at 30°C.**

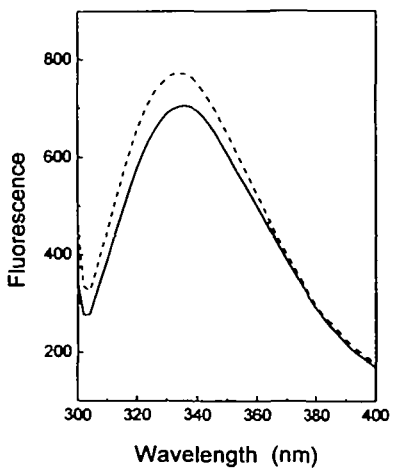

Fig. 2. **Intrinsic fluorescence spectra of Ca^{2+}-ATPase reconstituted in DPPC (---) and 27.0 mol% 16:0 LPC/DPPC (—) vesicles at 30°C.**

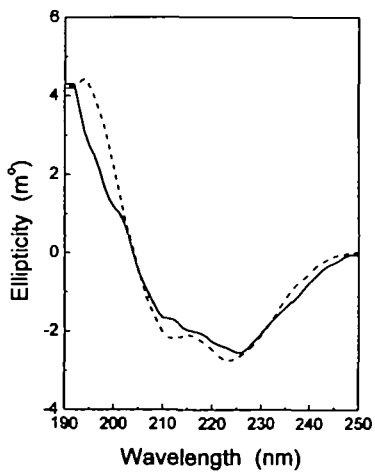

Fig. 4. **Circular dichroism spectra of Ca^{2+}-ATPase reconstituted in DPPC (---) and 27.0 mol% 16:0 LPC/DPPC (—) vesicles at room temperature.**

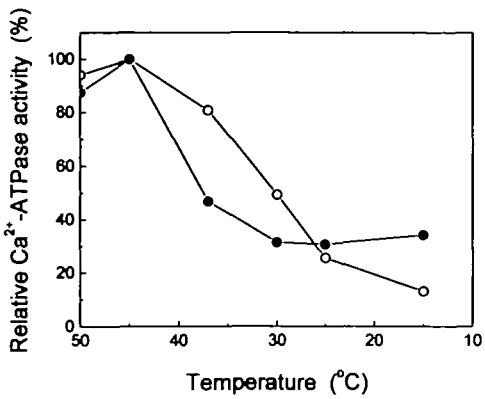

Fig. 5. Relative activity of Ca^{2+}-ATPase reconstituted in DPPC (–○–) and 27.0 mol% 16:0 LPC/DPPC (–●–) vesicles at different temperatures.

indicating a decrease in ordered structure and an increase in random coil. It is possible that Ca^{2+}-ATPase as proteoliposomes in 16:0 LPC/DPPC, *i.e.* in an interdigitated structure, is looser than in DPPC, because of the exposure of a major portion of the enzyme to a hydrophilic environment. Because lipids perturb the spectra, quantitative analysis of the spectra is difficult.

5) Assay of Ca^{2+}-ATPase Activity—The structure of the phospholipid membrane is important for many biological functions. Any structural changes in the membrane, therefore, may have a profound influence on membrane proteins. The temperature-dependence of the enzymatic activities of DPPC/Ca^{2+}-ATPase and 16:0 LPC/DPPC/Ca^{2+}-ATPase vesicles are shown in Fig. 5. The activity of the enzyme in DPPC vesicles shows a relatively slow decrease as the temperature is changed from 45 to 30°C, while a sharp reduction in enzymatic activity occurrs for 16:0 LPC/DPPC/Ca^{2+}-ATPase proteoliposomes. This is implies that a significant decrease in Ca^{2+}-ATPase activity is due to lipid interdigitation, because of the formation of an interdigitated structure in 16:0 LPC/DPPC/Ca^{2+}-ATPase vesicles at temperatures below T_m.

DISCUSSION

In a previous paper, differential scanning calorimetry, fluorescence polarization, and X-ray diffraction were used to show that at low concentration, 16:0 LPC induces DPPC to form an interdigitated bilayer from a non-interdigitated one (*11*). Here, in order to study the effects of interdigitation on Ca^{2+}-ATPase, it was first necessary to confirm that the insertion of Ca^{2+}-ATPase does not severely change the interdigitated structure in the 16:0 LPC/DPPC system. The data in Table I clearly show that Ca^{2+}-ATPase does not significantly alter 16:0 LPC/DPPC interdigitation at gel phase when reconstituted with the lipids. This is an ideal model in which to study the effects of interdigitation on the structure and function of membrane proteins.

It is necessary that the bilayer thickness be optimal for the structure and function of these integral membrane proteins exist (*31*). For example, the conformation of gramicidin in a model membrane is likely to form an equilibrium between double helix and $\beta^{6.3}$-helix, which varies as a function of membrane thickness. A channel conformation can be readily obtained when the acyl chain length is between 10 and 18 (*32, 33*). Small X-ray diffraction showed that when 27.0 mol% 16:0 LPC was incorporated into DPPC, the thickness of the bilayer decreased from 7.36 to 5.52 nm (*11*). Modulation of a bilayer by the formation of interdigitated bilayer domains may serve to influence the behavior of integral membrane proteins. A thin bilayer results in more amino acid residues that were originally embedded in the hydrophobic region of non-interdigitated membranes to be exposed to the hydrophilic environment (Figs. 2 and 3). This may be bring about perturbations in the native structure of Ca^{2+}-ATPase. Probably, this would lead to the inactivation of Ca^{2+}-ATPase. It should also be noted that lateral and vertical displacements or the rotational mobility of the embedded protein may be affected by interdigitation. Because of interdigitation, the midplane of a bilayer is lost (*18*). All of these factors may contribute to inactivity.

The wedge shaped 16:0 LPC molecules pack the interdigitated bilayer structure below 3°C. It is pointed out in this paper that although a low temperature is advantageous for the formation of interdigitation by 27.0 mol% 16:0 LPC/DPPC binary mixtures (Fig. 1 and Table I), interdigitation can be observed below 40°C (DPPC transition temperature) that is very close to physiological conditions. This suggests that the interdigitation of acyl chains plays an important roles as a negative regulatory factor in physiological functions.

Recently, it was suggested that interdigitation may be involved in the organization of sphingolipid-cholesterol rafts (*34*). Sphingolipids that have long and saturated or monounsaturated acyl chains can interdigitate with the cytoplasmic leaflet of the bilayer. Cholesterol is present in both leaflets where it functions as spacers in the cytoplasmic leaflet, filling the voids created by interdigitating fatty acid chains (*35*). Sphingolipid-cholesterol rafts that are insoluble in Triton X-100 at 4°C are involved in numerous cellular functions from membrane traffic and cell morphogenesis to cell signaling (*36*). It is not known whether the interdigitation of sphingolipid participates in these cellular functions. The physiological significance of interdigitation deserves more attention.

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