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Interaction of Myotoxin *a* with Artificial Membranes: Raman Spectroscopic Investigation†

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ABSTRACT: Myotoxin *a* from the venom of *Crotalus viridis viridis* (prairie rattlesnake) is a small protein which is responsible for myonecrosis. It is a basic protein with 42 amino acid residues of known sequence. Three disulfide bonds give it a highly compact structure. Microscopic examination of the toxin's effects reveals that the most pronounced and earliest visible damage occurs intracellularly, in the sarcoplasmic reticulum membrane system of skeletal muscle. A better understanding of its mechanism of action is therefore of particular interest. The interaction of myotoxin *a* with artificial membranes (multibilamellar phospholipid dispersions) was investigated by using dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylserine (DMPS). Two regions of the Raman spectrum were examined for information: the C–H stretching region between 2800 and 3000 cm^{-1} and the C–C stretching region between 1000 and 1300 cm^{-1}. The effects of myotoxin *a* on the thermotropic phase behavior of the artificial membranes were determined. This was done by monitoring three structurally sensitive Raman intensity ratios, $I_{2932/2880}$, $I_{2880/2850}$, and $I_{1088/1126}$. It was found that myotoxin *a* destabilized the ordered structure of the gel phase of phospholipid bilayers. This effect was seen with both DMPC and DMPS. The pretransition of DMPC was perturbed by myotoxin *a*, while the main gel to liquid-crystal phase transition temperature was decreased. The effect of myotoxin *a* on the phase behavior of DMPS was found to be pH dependent with the least effect observed at low pH values. These results suggest the involvement of negatively charged phosphate groups of phospholipids in the interaction of myotoxin *a* with artificial membranes.

Myotoxin *a*, a low molecular weight basic protein from the venom of *Crotalus viridis viridis*, is a well-characterized myonecrotic factor found widely among rattlesnake venoms (Ownby et al., 1976; Pool et al., 1981; Tu, 1982). It is representative of the small myotoxins, whose biological activity appears to be quite distinct from the large myotoxins (Gutierrez et al., 1984). Vacuolization of the sarcoplasmic reticulum is the first observable effect small myotoxins have on muscle tissue. Recent electron microscopic evidence shows that horseradish peroxidase conjugated myotoxin *a* is located at the sarcoplasmic reticulum membrane (Tu & Morita, 1983). The mechanism of the toxin's action is of considerable interest since the first site of tissue damage is at an intracellular location. The effect of myotoxin *a* on the Ca^{2+}-ATPase of sarcoplasmic reticulum is of particular interest.

Phospholipids in aqueous systems have characteristic phase behaviors which depend on the nature of the polar head groups, as well as chain length and degree of unsaturation of the acyl chains. They exist in a densely packed array in the gel phase below the main transition temperature (T_m).[1] This is a highly ordered state with maximal lateral packing between the acyl chains from neighboring phospholipids. Above the T_m, lipids are in a less ordered state known as the liquid-crystal phase. Under these conditions, lateral interactions between acyl chains are reduced and hydrocarbon chain packing decreases.

A number of Raman spectral parameters, including frequency and intensity, have been shown to be sensitive to the structural dynamics of phospholipids in aqueous dispersion. The hydrocarbon skeletal C–C stretching region provides information about the conformation of the acyl chains (Lippert & Peticolas, 1972), while the C–H region reflects the packing of the hydrocarbon chains (Lavialle & Levin, 1980). Numerous studies have been reported on the effect of various perturbants [including cholesterol (Bush et al., 1980), proteins (Curatolo et al., 1978), peptides (Verma & Wallach, 1976), and ions and ionophores (Hank & Ho, 1980; Mendelson et al., 1982)] on the phase transition characteristics of phospholipid dispersions.

We report here studies on the effects of myotoxin *a* on the thermotropic phase behavior of multibilamellar dispersions of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylserine (DMPS). These effects were observed by

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DMPS, dimyristoylphosphatidylserine; *I*, Raman peak intensity; T_m or T_{m_2}, primary gel to liquid-crystal phase transition; T_{m_1}, pretransition or head-group transition seen with DMPC.

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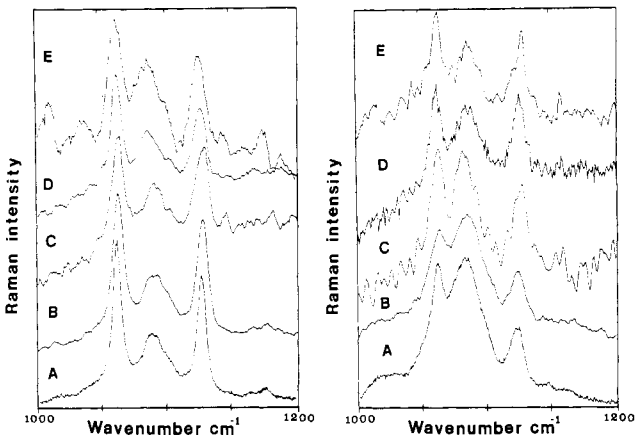

FIGURE 1: Effect of myotoxin *a* on the C–C stretching region of DMPC at 5 (left panel) and 33 °C (right panel). (A) Control spectra of DMPC multibilamellar dispersion; (B) lipid to myotoxin *a* molar ratio of 80/1, (C) 40/1, (D) 20/1, and (E) 5/1.

monitoring certain temperature-sensitive C–H and C–C stretching vibrations by laser Raman spectroscopy.

Experimental Procedures

DMPC and DMPS were purchased from Avanti Polar Lipids Co., Birmingham, Al. Further purification was not required as determined by thin-layer chromatography. Myotoxin *a* was isolated by the method of Cameron & Tu (1977). Purity of the protein was monitored by disc gel electrophoresis and analytical isotachoelectrophoresis (LKB). The phospholipid–water dispersion was prepared by the procedure of Gaber et al. (1978). Appropriate amounts of myotoxin *a* were subsequently added, and the mixture was vortexed 4 times during a 1-h incubation at 10 °C above the main gel to liquid-crystal phase transition temperature. The dispersion was concentrated by centrifugation at 15000*g* in a Kimax melting point capillary tube using Teflon adaptors designed for the HB-4 rotor of a Sorvall RC2-B centrifuge.

Samples sealed in capillaries were allowed to equilibrate at 4 °C for 4 h under low-intensity laser illumination prior to data collection. Temperature in a thermostated cell was controlled with a cryostatic circulator (Haake Model A-80). The temperature adjacent to the sample was monitored with a copper–constantan thermocouple. Experimental points were monitored in approximately 3 °C increments. Samples were held for 30 min at each experimental temperature before spectra were recorded to allow for thorough equilibration.

Laser Raman spectra of the dispersions were obtained by excitation of the sample with the 514.5-nm line of an argon ion laser (Spectraphysics Model SP-164) at 50–100-mW power. Data were collected in 0.5 wavenumber increments for 0.5 s at each point. Signal averaging was accomplished with a Spex Ramalog 5 spectrophotometer and a Spex SCAMP computer. Typically, 3 scans were averaged for the C–H region, and 5–10 scans were required for the C–C region. The band intensity ratios were obtained from the peak high values.

Results

DMPC is known to exhibit a characteristic pretransition (T_{m_1}) near 10 °C. This low-temperature transition reflects the head-group packing of DMPC into the ripple phase at temperatures below 10 °C. It is also known to undergo its primary phase transition (T_{m_2}) from the gel to liquid-crystal phase between 22 and 25 °C (Spiker & Levin, 1976). The phase behavior of DMPC was determined by measuring the

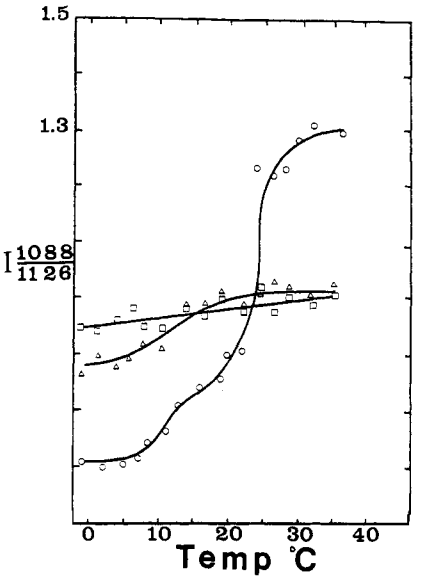

FIGURE 2: Effect of myotoxin *a* on the thermotropic phase behavior of DMPC. (○) Control phase transition of DMPC; (△) lipid to toxin molar ratio of 40/1 and (□) 20/1.

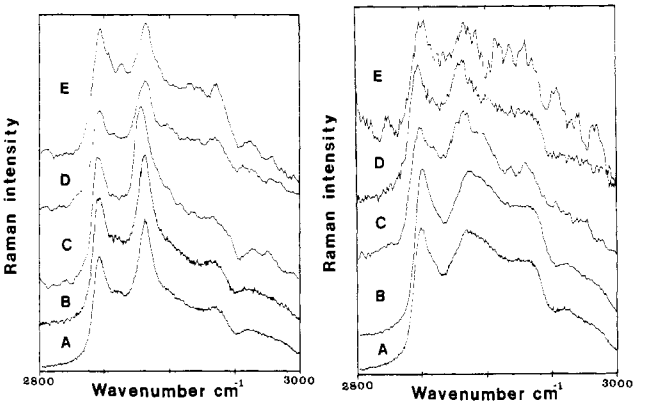

FIGURE 3: Effect of myotoxin *a* on the C–H stretching region of DMPC at 5 (left panel) and 33 °C (right panel). (A) Control spectra of DMPC multibilamellar dispersion; (B) lipid to myotoxin *a* molar ratio of 80/1, (C) 40/1, (D) 20/1, and (E) 5/1.

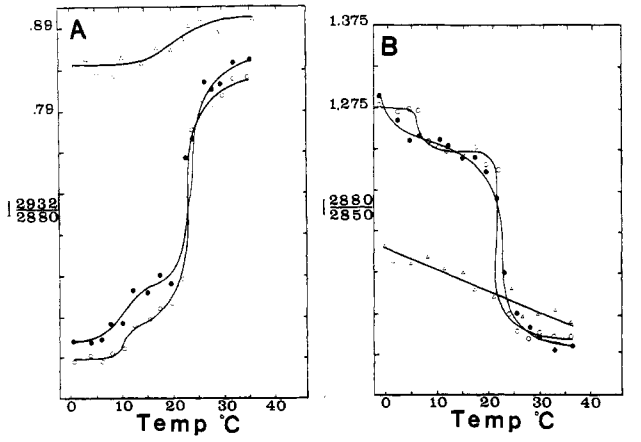

FIGURE 4: Effect of myotoxin *a* on the thermotropic phase behavior of DMPC as monitored by the temperature-sensitive C–H ratio (A) $I_{2932/2880}$ and (B) $I_{2880/2850}$: (○) control phase transition of DMPC; (●) lipid to myotoxin *a* molar ratio of 80/1 and (△) 40/1.

Raman spectra of two different regions, the C–C and C–H stretching regions. The C–C stretching regions of DMPC at 5 and 33 °C are shown in Figure 1A. The peak at 1088 cm^{-1} reflects C–C bonds of the acyl chains in the gauche confor-

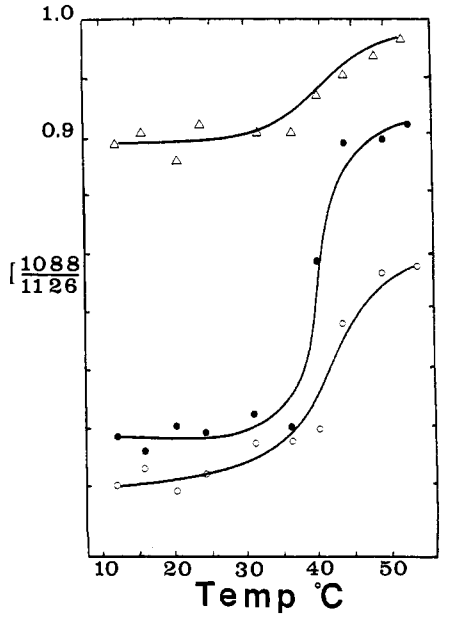

FIGURE 5: Effect of pH on the thermotropic phase behavior of DMPS at pH (●) 7.0, (△) 4.0, and (○) 11.0.

mation, while the peak at 1126 cm^{-1} indicates C–C bonds in the trans conformation. The number of gauche conformations along the C–C backbones of the aliphatic chains increases with temperature. This is reflected in a plot of the intensity ratio $I_{1088/1126}$ over the entire range of temperatures examined which shows T_{m_1} at 11–12 °C and T_{m_2} at 23–24 °C (Figure 2) for DMPC in the absence of myotoxin *a*.

The C–H stretching vibration region between 2800 and 3000 cm^{-1} was also monitored. The C–H regions of DMPC alone at 5 and 33 °C are shown in Figure 3A. Certain C–H vibrational bands are sensitive to environmental temperature effects. The intensity ratio of $I_{2932/2880}$ represents the C–H symmetric stretching vibration of the terminal methyl groups at the hydrophobic center of the bilayer, while the ratio $I_{2880/2850}$ is sensitive to the methylene group asymmetric C–H stretch (Verma & Wallach, 1976). The transition as determined by $I_{2932/2880}$ is usually slightly higher than the value determined from the ratio $I_{2880/2850}$. The ratio $I_{2932/2880}$ gave 10 and 23 °C for T_{m_1} and T_{m_2}, respectively (Figure 4A), compared to values of 7 and 22 °C as determined by $I_{2880/2850}$ (Figure 4B).

Myotoxin *a* exhibits observable effects on the normal phase behavior of DMPC at molar ratios as low as 80/1 lipid to toxin. At this low level, the pretransition is obviously perturbed (Figure 4A) or no longer observable (Figure 4B). At higher levels of myotoxin *a*, the normal pretransition behavior is entirely eliminated (Figures 2 and 4). Levels of myotoxin *a* of only 1 part in 40 show the nearly total elimination of any phase behavior. A small degree of cooperativity, as shown by the typically sigmoidal-shaped curve characteristic of a phase transition, is still seen with the ratios $I_{1088/1126}$ (Figure 2) and $I_{2932/2880}$ (Figure 4A). The cooperativity apparent from the ratio of $I_{1088/1126}$ is eliminated at a lipid to toxin ratio of 20/1 (Figure 2). Thus, myotoxin *a* increases the more disordered state of DMPC. The degree of this disorder and the extent of the disruption of the lipid's normal phase behavior are dependent upon the toxin concentration.

The phase behavior of DMPS is also well established (Browning & Seelig, 1980). It is an interesting model compound for negatively charged phospholipids because of the nature of its head group, and also because of its natural occurrence in the sarcoplasmic reticulum. While DMPS does not show the pretransition seen with DMPC, the magnitude of its gel to liquid-crystal phase transition is greatest at pH 7.0 using any of the three Raman intensity ratios (Figures 5 and 6). No consistent shift in the transition temperature was seen as a result of the pH changes.

The observation that the phase transition of pure DMPS at pH 4.0, 7.0, or 11.0 continues to show some sigmoidal characteristics implies that altering only the pH within this range does not eliminate the phenomena of intermolecular cooperativity (Figures 5 and 6). The ratio $I_{1088/1126}$ indicates that pH 4.0 (Figure 7A) favors the formation of gauche conformers along the myristoyl chains even below the normal phase transition (38 °C at pH 7.0). The same thing occurs at pH 11.0 (Figure 7C) where the gauche conformation is increased on both sides of the transition in the presence of myotoxin *a*.

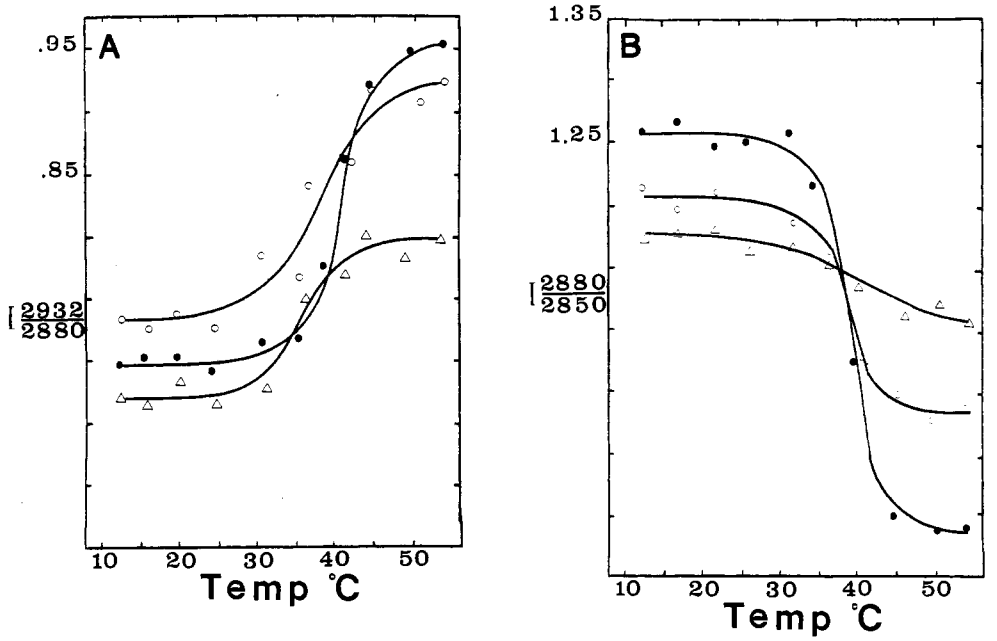

FIGURE 6: Effect of pH on the thermotropic phase behavior of DMPS as determined by the temperature-sensitive C–H intensity ratios (A) $I_{2932/2880}$ and (B) $I_{2880/2850}$ at pH values of (●) 7.0, (△) 4.0, and (○) 11.0.

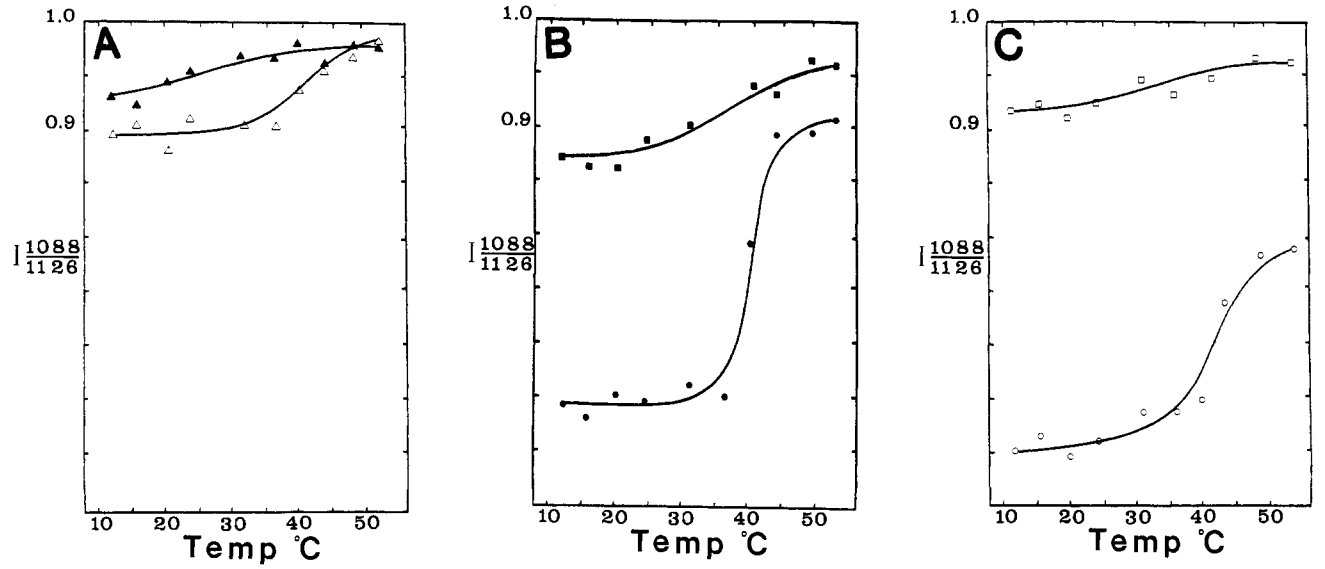

FIGURE 7: Effect of myotoxin *a* on the thermotropic phase behavior of DMPS as determined by the intensity ratio $I_{1088/1126}$: in the absence of myotoxin *a* at pH values of (●) 7.0, (△) 4.0, and (○) 11.0; in the presence of myotoxin *a* at a lipid to toxin molar ratio of 40/1 at pH values of (■) 7.0, (▲) 4.0, and (□) 11.0.

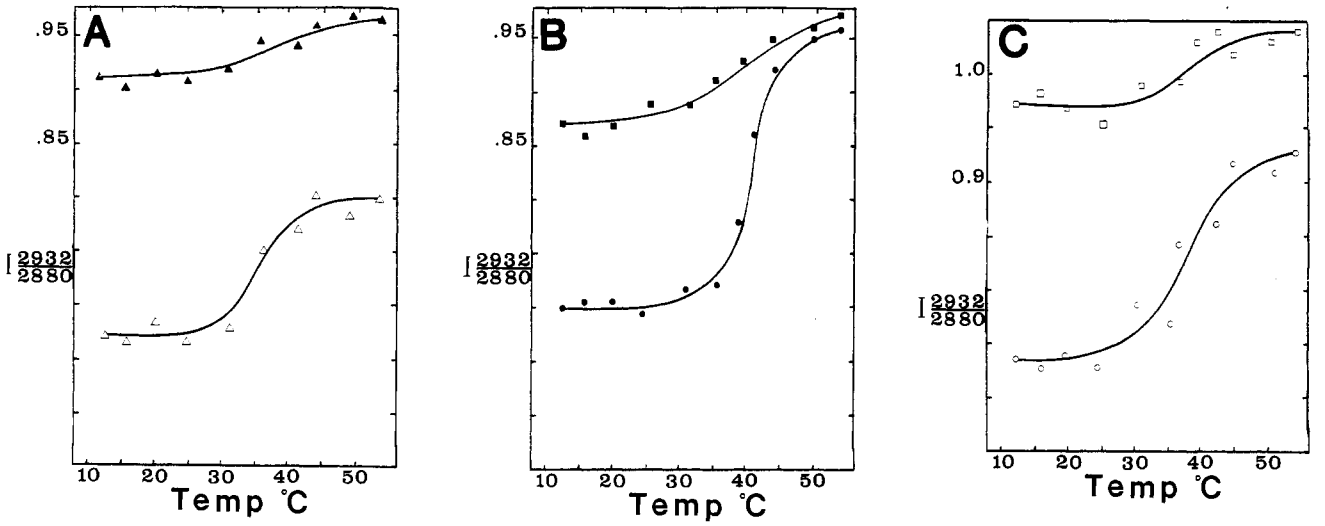

FIGURE 8: Effect of myotoxin *a* on the thermotropic phase behavior of DMPS as determined by the intensity ratio $I_{2932/2880}$: in the absence of myotoxin *a* at pH values of (●) 7.0, (△) 4.0, and (○) 11.0; in the presence of myotoxin *a* at a lipid to toxin molar ratio of 40/1 at pH values of (■) 7.0, (▲) 4.0, and (□) 11.0.

As in the case of DMPC, the transition temperature appears slightly higher by the ratio $I_{2932/2880}$ (40 °C) (Figure 6A) than by $I_{2880/2850}$ (Figure 6B), which gives a value of 38 °C. Both of these indicate that there is a slight increase in the ordered state above the T_m at both pH 4.0 and pH 11.0.

Myotoxin *a* at the molar ratio of 40/1 lipid to toxin showed pH-dependent effects on the phase behavior of DMPS by all the Raman ratios examined (Figures 7, 8, and 9). At pH 7.0, myotoxin *a* had the effect of decreasing the order parameters by the greatest amount at temperatures below the T_m (Figures 7B, 8B, and 9B). The ratio $I_{1088/1126}$ also shows an increase in gauche conformations, especially below the T_m (Figure 7). All three Raman ratios also indicate that myotoxin *a* has the effect of reducing cooperativity at all the pH values examined. Myotoxin *a* had the least effect upon the transition at pH 4.0 (Figures 7A, 8A, and 9A).

DISCUSSION

Membrane-active proteins are commonly found in the venoms of many poisonous snakes, as well as in a variety of other organisms. One such toxin, cardiotoxin from *Naja* (cobra) venom, has a sequence and structure similar to that of postsynaptic neurotoxins (Pezolet et al., 1982) but quite distinct from that of myotoxin *a* (Fox et al., 1979). Although the two toxins are clearly not related, both are low molecular weight basic proteins. Cardiotoxin from *Naja atra atra* has a molecular weight of 7000 and an isoelectric point of 10.6 (Chen et al., 1982); myotoxin *a* has a molecular weight of 4200 with a p*I* of 9.6. While cardiotoxins act on the cardiac muscle, causing cardiac arrest, myotoxin *a* has entirely different effects on skeletal muscle. Cardiotoxins are known to bind the heart cell membranes (Tonsing et al., 1983), while myotoxin *a* is localized in the sarcoplasmic reticulum of skeletal muscle. Cardiotoxin has been shown to disrupt the thermotropic phase transition of phospholipid model membranes (Faucon et al., 1983). It induces disorganization of the aliphatic chains and alters their mobility.

Other molecules known to affect the phase behavior of artificial membranes include cholesterol, melittin, and myelin proteolipid apoprotein. These examples are interesting for the sake of comparison. Cholesterol has been shown to broaden

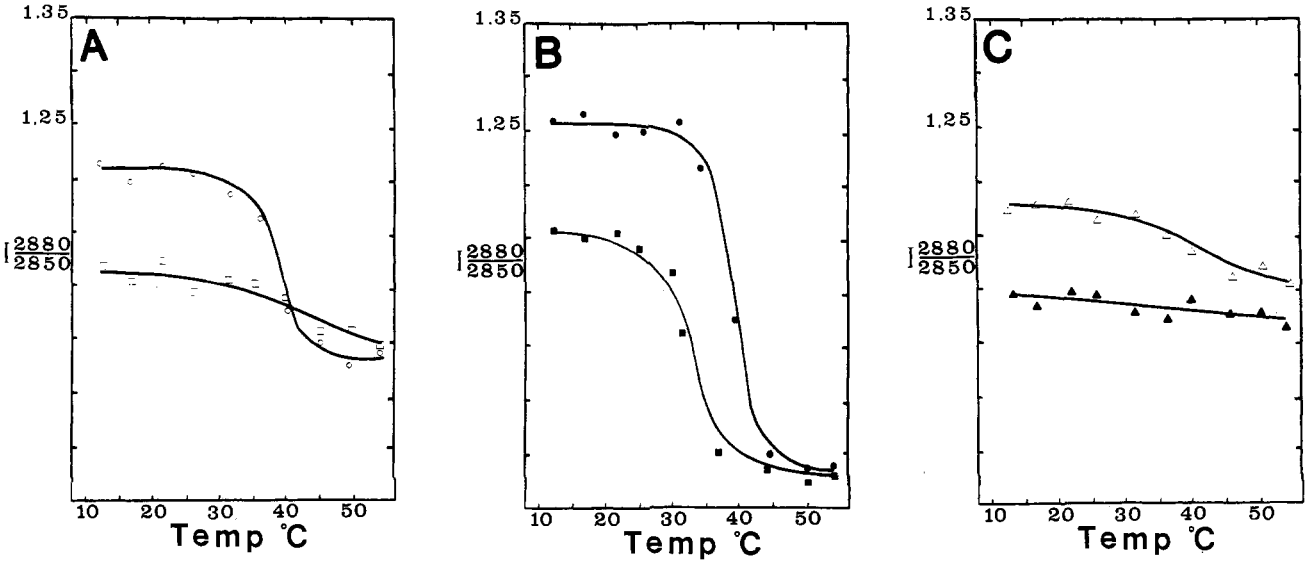

FIGURE 9: Effect of myotoxin *a* on the thermotropic phase behavior of DMPS as determined by $I_{2880/2850}$: in the absence of myotoxin *a* at pH values of (●) 7.0, (▲) 4.0, and (○) 11.0; in the presence of myotoxin *a* at a lipid to toxin molar ratio of 40/1 at pH values of (■) 7.0, (▲) 4.0, and (□) 11.0.

the phase transition by decreasing interactions between hydrophobic chains, thus reducing cooperativity (Lippert & Peticolas, 1971). Melittin, a small polypeptide isolated from *Apis mellifera* (honeybee) venom, strongly alters the phase characteristics of artificial membranes (Verma & Wallach, 1976). Melittin induces an annular lipid region surrounding the toxin (lavialle & Levin, 1984), essentially creating two lipid domains each with its own recognizable phase behavior. Myelin proteolipid apoprotein, the major protein component of myelin, also has complex effects on membrane phase behavior (Curatolo et al., 1978). It causes both a decrease in the T_m and considerable broadening of the normal transition of artificial membranes composed of saturated phospholipids (Dasseux et al., 1984).

Early work in our lab provided evidence that the weak membrane-lytic action of myotoxin *a* upon erythrocytes was inhibited by the addition of calcium ion (Cameron & Tu, 1977). This suggests the involvement of myotoxin *a* with negatively charged lipids. Our current findings support the idea that myotoxin *a* interacts with certain phospholipids present in the sarcoplasmic reticulum in such a way as to alter their phase behavior. Since the sarcoplasmic reticulum membrane of skeletal muscle controls calcium ion flux within the muscle via Ca^{2+}-ATPase, it is possible that this toxin–lipid interaction affects the activity of the Ca^{2+}-ATPase by disturbing certain lipids necessary to maintain its active conformation.

The sarcoplasmic Ca^{2+}-ATPase is known to require a particular lipid environment to maintain its activity MacLennan, 1970). This is believed to exclude cholesterol from the region of the ATPase complex (Warren et al., 1975). It is interesting to note that the greatest magnitude of the phase transition of DMPS occurred at pH 7.0. If indeed the membrane phase or that of particular lipids regulates the activity of Ca^{2+}-ATPase, it is not surprising that the greatest changes occur at near-physiological pH values. The major lipid components of the sarcoplasmic reticulum from various muscle types are phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (Borchman et al., 1982), while Ca^{2+}-ATPase is the most abundant protein. Therefore, it is possible that myotoxin *a* might be useful to elucidate the molecular mechanisms of Ca^{2+}-ATPase regulation in sarcoplasmic reticulum.

Registry No. DMPC, 18194-24-6; DMPS, 64023-32-1.

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Influence of Trinitrophenylation on the Structure and Dynamics of Phosphatidylethanolamine-Containing Model Membranes

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ABSTRACT: The influence of trinitrophenylation on hydration, acyl chain melting characteristics, and polymorphism of phosphatidylethanolamine- (PE-) containing model membranes has been investigated. ^2H nuclear magnetic resonance spectroscopy (NMR) was used to study the hydration properties of (trinitrophenyl)phosphatidylethanolamine (TNPPE). ^{31}P NMR techniques, including saturation transfer and the use of phosphatidylcholine thio analogues, were employed to investigate the TNPPE head-group conformation. The thermotropic behavior of PE/TNPPE mixtures was studied by means of differential scanning calorimetry. The macroscopic organization of the phospholipids was monitored by ^{31}P NMR, small-angle X-ray diffraction, and freeze–fracture electron microscopy. The results indicate that TNPPE is anhydrous in character and does not form ordered structures by itself. Furthermore, even in excess of water, the phosphate region of the fluid TNPPE molecules cannot undergo long-axis rotation, presumably due to intermolecular ring interactions. On the other hand, in aqueous dispersions TNPPE molecules are to a limited extent ($\simeq$20 mol %) soluble in PE. From the decrease in enthalpy of the gel to liquid-crystalline phase transition the TNPPE molecules appear to interact with PE molecules in a 1:4 stoichiometry. In hydrated mixtures with phosphatidylcholine (PC) or PE, the phosphate moiety of the TNPPE molecule undergoes long-axis rotation. However, by comparison with PC and PE in these samples, TNPPE has a different head-group conformation. This is possibly caused by a trinitrophenyl ring orientation either at the bilayer/water interface or perpendicular to the plane of the bilayer. TNPPE hardly influences the temperature of the gel to liquid-crystalline phase transition in PE bilayers. In contrast, TNPPE strongly destabilizes the bilayer organization in both PE and PC model membranes, most likely due to dehydration effects and interbilayer trinitrophenyl ring interactions.

During the last decade the polymorphism of various phospholipid classes has been studied intensively [for a recent review, see De Kruijff et al. (1984)]. PE[1] is probably the best studied phospholipid that can undergo a reversible transition from bilayer to hexagonal (H_{II}) phase (Luzzati et al., 1966; Cullis & De Kruijff, 1978; Rand et al., 1971; Seddon et al., 1983, 1984). The suggestion that nonbilayer phospholipid structures are involved in physiologically important events has been made since the discovery of lipid polymorphism (Luzzati, 1968; Lucy, 1964; Shipley, 1973). There are several indirect arguments to suggest that transiently occurring nonlamellar lipid structures or hexagonal (H_{II}) phase prefering phospholipids, by the nature of their low head-group hydration, could play an important role in membrane function. For instance, the ^{31}P NMR characteristics of rabbit and rat liver microsomes (Stier et al., 1978; De Kruijff et al., 1980c) and the phase preference of the isolated endogenous PE (De Kruijff et al., 1980c) suggest that nonbilayer phospholipid phases might occur in this membrane and that these are possibly related to functional properties of the microsomal membrane like, e.g., membrane fusion and the transmembrane movement of phospholipids.

One approach in investigating the possible biological importance of PE polymorphism is to study the structural and functional aspects of PE head-group modifications in membranes. The best known and most convenient probe to modify and detect aminophospholipids is TNBS (Litman, 1974, 1975; Higgins & Pigott, 1982; Sleight & Pagano, 1983; Hoekstra & Martin, 1982), which reacts with PE under the formation of a trinitrophenyl derivative (structure **1**). A prerequisite for such an approach is to know whether TNBS labeling affects the physical properties of PE. As a first step in this direction, we investigated the effects of TNBS labeling on acyl-chain melting characteristics, hydration, and polymorphism of model membranes composed of PE, mixtures of PC and PE, and microsomal lipids, respectively, by using ^{31}P NMR, DSC, SAXS, and freeze–fracture electron microscopy. It will be shown that PE and TNPPE show a drastically

[1] Abbreviations: PE, phosphatidylethanolamine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; NMR, nuclear magnetic resonance; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; TNBS, trinitrobenzenesulfonic acid; DSC, differential scanning calorimetry; TNPPE, (trinitrophenyl)phosphatidylethanolamine; DEPE, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine; DEPC, 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine; HPTLC, high-performance thin-layer chromatography; Pipes, 1,4-piperazinediethanesulfonic acid; SAXS, small-angle X-ray scattering; DETNPPE, 1,2-dielaidoyl-*sn*-glycero-3-[(trinitrophenyl)phosphoethanolamine]; DOTNPPE, 1,2-dioleoyl-*sn*-glycero-3-[(trinitrophenyl)phosphoethanolamine]; $\Delta\sigma$, residual chemical shift anisotropy.

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