Abolition of the Thermotropic Transition of Charged Phospholipids Induced by a Cardiotoxin from *Naja mossambica mossambica* As Detected by Fluorescence Polarization, Differential Scanning Calorimetry, and Raman Spectroscopy†

Jean-François Faucon,* Jean Dufourcq, Evelyne Bernard, Luc Duchesneau, and Michel Pézolet

ABSTRACT: The effects of a *Naja mossambica mossambica* cardiotoxin on the thermotropic properties of charged phospholipids have been studied by fluorescence polarization, differential scanning calorimetry, and Raman spectroscopy. The binding of the toxin is only governed by the net charge at the interface and is not affected by the polar head group structure of the phospholipids or by the acyl chains physical state, degree of insaturation, or length. The effect of the toxin on the phospholipid structure is drastic. In all cases, the gel to liquid-crystalline phase transition monitored by fluorescence and Raman spectroscopies is progressively abolished without notable shift in temperature as the proportion of toxin is increased. The endothermic peaks detected by differential scanning calorimetry decrease in intensity as the toxin content is increased but always remain sharp. All the techniques used give complementary results, and none of them reveals the presence of secondary transitions at higher or lower temperatures. We thus believe that the lipid molecules that are perturbed by the toxin, approximately 10 ± 2 molecules, do not undergo a phase transition. Raman results demonstrate that these "boundary" lipids display a population of gauche rotamers that is as high as the one found in the liquid-crystalline phase of the pure phospholipid and this even well below the phase transition temperature. On the other hand, fluorescence results are interpreted as due to a partial immobilization of the lipids in contact with the toxin above the transition temperature. Thus, even though the interaction is governed by electrostatic forces, the toxin penetrates at least partially into the bilayers, inducing a disorganization of the aliphatic chains and changes in their mobility; this could explain their lytic activity.

Cardiotoxins from snake venom are strongly basic proteins. They have a molecular weight of about 7000 and they contain approximately 60 residues that are well reticulated by four disulfide bridges (Louw, 1974). Even though cardiotoxins display a lower toxicity than neurotoxins, they generally represent the major protein content of snake venoms. They are described as cytotoxins or direct lytic factors since they can disrupt the organization of biological membranes although this is not their sole effect on membranes. Phospholipids were first implicated in their mechanism of action since they can inhibit cardiotoxins lytic activity (Patel et al., 1969) and also because axonal membranes can bind amounts of toxins that are much greater than those expected from the number of membrane protein binding sites (Vincent et al., 1976). Furthermore, it has been shown that in model systems, cardiotoxins interact strongly with negatively charged lipids either in bilayers (Dufourcq & Faucon, 1978; Vincent et al., 1978) or in monolayers (Bougis et al., 1981; Ksenzshek et al., 1978). Finally, their interaction with phospholipids results in severe lipid morphological changes in membrane as well as in the release of species trapped within phospholipid vesicles (Faucon et al., 1979; Gulik-Krzywicki et al., 1981).

In order to improve our understanding of how cardiotoxins induce the lysis of membranes, it was necessary to investigate their steady-state perturbations on phospholipid bilayers. In this paper we present results on the effect of a cardiotoxin on the thermotropic behavior of charged phospholipids as detected by differential scanning calorimetry, fluorescence polarization, and Raman spectroscopy. These results show that for all investigated charged phospholipids, no matter their chain length and degree of insaturation, the thermotropic transition is totally abolished when the lipid to protein molar ratio is approximately 10. Furthermore, the conformation and the dynamic of the perturbed phospholipid chains are drastically changed. All the results obtained by three different techniques are in good agreement between themselves and with previously published data on the interaction of membrane proteins with model membranes.

Materials and Methods

Cardiotoxin II (V_2^{II}; Louw, 1974) used throughout this study was generously supplied by P. Bougis and H. Rochat (Marseille). It was purified from *Naja mossambica mossambica* venom (Bougis et al., 1981) and treated to eliminate phospholipase contamination (Delori & Tessier, 1980). However, because of the spectacular effects of phospholipase on the binding of the toxin to phospholipids (Dufourcq & Faucon, 1978) and on the morphological perturbation of membranes (Gulik-Krzywicki et al., 1981), all experiments were carried out in EDTA[1] in order to prevent any possibility of phospholipid degradation even during long experiments.

† From the Centre de Recherches Paul Pascal, CNRS, Domaine Universitaire, 33405 Talence, France (J.-F.F., J.D., and E.B.), and the Département de Chimie, Université Laval, Québec G1K 7P4, Canada (L.D. and M.P.). *Received October 7, 1982.* This research was supported in part by the Université de Bordeaux II (J.-F.F. and J.D.) and by the National Sciences and Engineering Research Council Canada (M.P.). Preliminary results of this paper have been presented at the Congrès de la Société de Physique, Clermont-Ferrand, France, July 81, and at the Third Biophysical Discussion, Airlie Center, VA, Oct 1981.

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; *P*, degree of polarization; DSC, differential scanning calorimetry; T_m, gel–liquid-crystalline acyl chain melting phase transition temperature; CTX, cardiotoxin II; R_i, lipid to protein molar ratio; DPPG, dipalmitoylphosphatidylglycerol; DMPG, dimyristoylphosphatidylglycerol; PS, natural phosphatidylserine; PI, natural phosphatidylinositol; DMPA, dimyristoylphosphatidic acid; DPPA, dipalmitoylphosphatidic acid.

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Synthetic phosphatidylserines were prepared and purified in the laboratory from their homologous phosphatidylcholines by head-group exchange in the presence of phospholipase D (Confurius & Zwaal, 1977). Synthetic phosphatidylglycerol and dipalmitoylphosphatidic acid were purchased from Medmark while dimyristoylphosphatidic acid was obtained from Senn Chemicals.

Intrinsic fluorescence measurements of the single Trp of cardiotoxin II at position 11 were done on a FIKA 55 MK II spectrofluorometer. The excitation wavelength was always set at 280 nm, and the excitation and emission slit widths were 7.5 nm. Complexes were formed in situ by successive addition of aliquots of a given lipid dispersion to the toxin solution.

Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH; Aldrich Chemical Co.) inserted as a probe into phospholipid bilayers was followed on an apparatus built in the laboratory according to the optical scheme of Weber & Bablouzian (1966). Since this apparatus is interfaced to a Digital LSI II PDP 11/03 minicomputer, the effect of the temperature on the fluorescence polarization P was measured automatically. Samples were prepared according to the procedure described previously (Bernard et al., 1982).

Differential scanning calorimetry (DSC) experiments were carried out on a Du Pont 990 differential calorimeter at scanning rates between 1 and 5 K/min. Thermograms were digitalized and integrated numerically in order to calculate enthalpy changes of transitions. Samples were prepared from two different procedures which gave similar results: (i) Lipids were first dissolved in benzene and labeled with [^{14}C]phosphatidylcholine (New England Nuclear) and lyophilized. They were then suspended at a concentration of 5 mg/mL in 100 mM phosphate buffer, pH 7.5, and 1 mM EDTA, and then sonicated above the transition temperature (T_m). The appropriate amount of toxin was added, and the system was incubated a few minutes above T_m and then centrifuged to yield pellets that were transferred in the DSC pans. The amount of material was checked after each experiment by opening the pans (Bernard et al., 1982). (ii) In a simpler procedure, appropriate amounts of dry phospholipids (approximately 1 mg) were weighted directly in DSC pans and hydrated by 10–15 μL of concentrated toxin solution. Sealed pans were then incubated for 1 h above T_m prior to the experiment.

The Raman spectra were excited with the 514.5-nm line of a Spectra Physics Model 165 argon ion laser and measured with a Spex Model 1400 double monochromator equipped with a cooled photomultiplier tube (RCA-C1034). The monochromator was calibrated with a neon discharge lamp, and the frequencies cited later are believed to be accurated to ± 2 cm^{-1} for sharp peaks. The spectral slit width was 5 cm^{-1}, and the laser power at the sample was between 150 and 200 mW. Capillaries (1.5-mm diameter) containing the samples were placed in a thermostated copper jacket whose temperature was monitored at ± 0.2 °C with a copper–constantan thermocouple. The incident beam was aligned perpendicularly to the capillary axis, and the scattered light was collected at right angle to the incoming radiation. Raman data were acquired with an IMSAI 8080 microcomputer which allows for multiple scanning and various forms of data treatment as described elsewhere (Savoie et al., 1979). Spectra were corrected for a slight fluorescent background by subtracting the appropriate polynomial function (Savoie et al., 1979).

Phospholipid/cardiotoxin complexes were prepared by mixing appropriate amounts of a 1% lipid dispersion with a 1% cardiotoxin solution in 100 mM phosphate buffer, pH 7.5, containing 1 mM EDTA. Mixtures were then vortexed at 50 °C for 10 min, transferred in Raman cells, and centrifuged to yield white pellets used to obtain spectra.

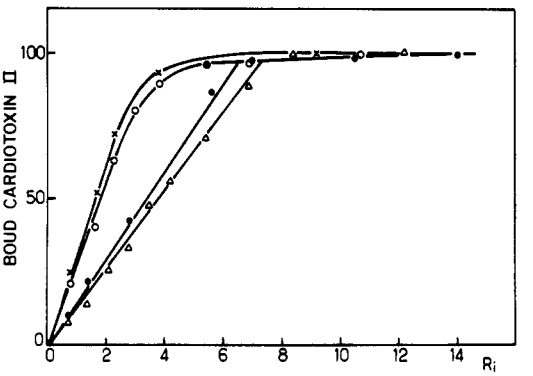

FIGURE 1: Percent of phospholipid-bound cardiotoxin calculated from the relative change of the fluorescence intensity at 330 nm of cardiotoxin II upon addition of negatively charged phospholipids, at 25 °C and pH 7.5 (20 mM phosphate buffer and 1 mM EDTA). R_i is the lipid to protein molar ratio. Excitation wavelength 280 nm. (△) Sonicated DPPG vesicles; (●) DPPG dispersions; (○) DMPA dispersions at pH 7.5; (×) DMPA dispersions at pH 9.2.

Results

Binding of CTX to Acidic Phospholipids. It has already been shown that the intrinsic fluorescence of the single tryptophan residue of CTX at position 11 is very sensitive to the formation of lipid/protein complexes (Dufourcq & Faucon, 1978; Vincent et al., 1978). Therefore, in a first step, we have used this simple technique to check whether the binding of CTX is sensitive to the physical state of phospholipids. The results obtained for two phospholipids are summarized in Figure 1 where the relative change in fluorescence intensity of CTX at 330 nm is plotted vs. the lipid to protein molar ratio R_i. When either single-shell vesicles or unsonicated dispersions of DPPG in the gel state at 25 °C are added to the CTX solution, the fluorescence intensity increases linearly and reaches a plateau around $R_i = 7$. These results are quite similar to the ones previously obtained with vesicles of natural lipids such as PS or PI at room temperature, that is, with aliphatic chains in the fluid state (Dufourcq & Faucon, 1978). Therefore, neither the physical state of the acyl chains nor the multilayer arrangement of unsonicated dispersions affects the binding of CTX to phospholipids. Similar results were obtained with DMPA at 25 °C, i.e., 25 °C lower than the transition temperature. Furthermore, for DMPA at pH 7.5 as well as at pH 9.2, the plateau is reached at approximately half the R_i value found for single-charged phospholipids. This indicates that DMPA behaves like a diacid even at pH 7.5 and that the presence of the toxin significantly lowers its normal pK value of about 8.5 (Traüble & Eibl, 1974; Eibl & Blume, 1979).

Fluorescence Polarization. The effect of temperature on the fluorescence polarization of DPH embedded in dimyristoyl- and dipalmitoylphosphatidylglycerol dispersions in the presence of increasing amounts of CTX is shown in Figure 2. The main transitions for the pure lipid bilayers are observed at 24 and 42 °C for DMPG and DPPG, respectively, while for DPPG a pretransition is also detected around 32 °C which is in good agreement with published results on these phospholipids (Findlay & Barton, 1978; Watts et al., 1978). Two main features emerge clearly from these results: (i) there is no apparent change of the transition temperatures in the presence of the toxin; (ii) there is a progressive decrease of the amplitude of the observed transitions as the lipid to protein molar ratio

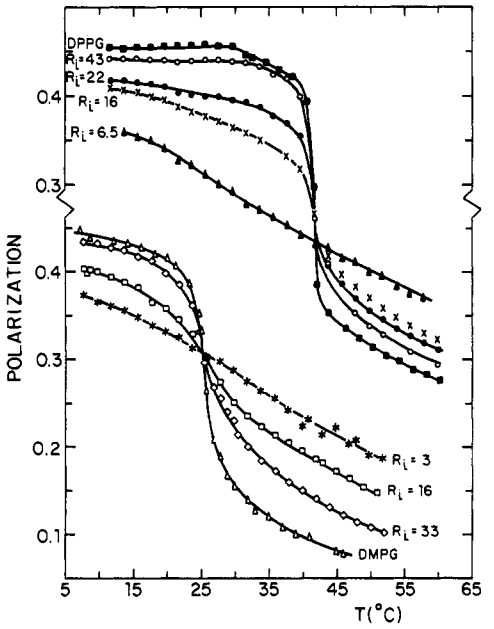

FIGURE 2: Effect of temperature on the degree of fluorescence polarization of DPH embedded in DMPG and DPPG at various lipid to cardiotoxin II molar ratios (R_i) and pH 7.5 (20 mM phosphate buffer and 1 mM EDTA).

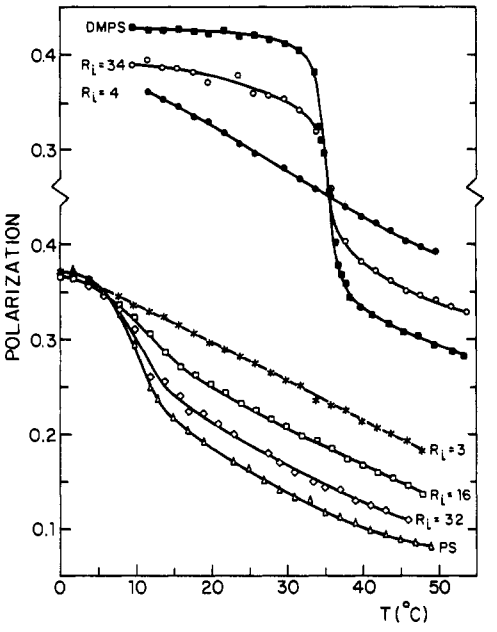

FIGURE 3: Effect of temperature on the degree of fluorescence polarization of DPH embedded in DMPS and natural PS at different lipid to cardiotoxin II molar ratios (R_i) and pH 7.5 (20 mM phosphate buffer and 1 mM EDTA).

decreases. The transition is even totally abolished when the lipids are saturated by the toxin around $R_i = 7$. The monotonic decrease of the polarization ratio of the DPH probe in the presence of excess toxin shows that when all the lipid molecules are bound to the toxin, the polarization of DPH is reduced below the transition temperature of the pure lipid bilayers while it is significantly increased above the transition.

Similar results are presented in Figure 3 for dimyristoylphosphatidylserine which gives a thermotropic transition at 35 °C and for natural PS which shows a broader transition around 10 °C. For both phospholipids, the addition of CTX induces changes that are very similar to those already shown for PG; similar results were also obtained for DPPS. It is interesting to mention here that for natural PS, the initial P

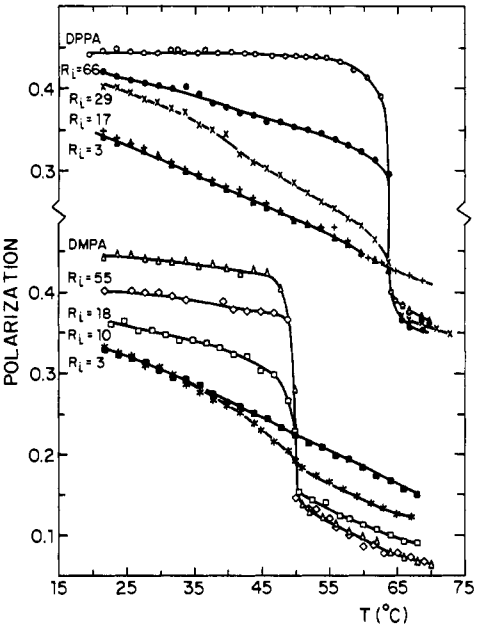

FIGURE 4: Effect of temperature on the degree of fluorescence polarization of DPH embedded in DMPA and DPPA at different lipid to cardiotoxin II molar ratios (R_i) and pH 7.5 (20 mM phosphate buffer and 1 mM EDTA).

values are smaller than those normally observed in the gel phase of synthetic phospholipids. This effect is most likely related to the polydispersity of the aliphatic chains of the natural lipid that show some disorder and/or fluidity around 0 °C. The effect of CTX is therefore much more striking on the fluid phase temperature domain where there is a progressive and significant increase of P when the amount of bound toxin is increased.

Finally, the melting curves of synthetic phosphatidic acids at pH 7.5 are reproduced in Figure 4. As shown in Figure 1, the gel to liquid-crystalline transition for the pure lipid bilayers occurs at 50 and 64 °C for DMPA and DPPA, respectively, which is in good agreement with previously published temperatures (Van Dijck et al., 1978; Jacobson & Papahadjopoulos, 1975). As for all the other investigated lipids, no change in the transition temperature is detected in the presence of the toxin, but the amplitude of the transitions progressively decreases as the toxin to phospholipid molar ratio increases.

Differential Scanning Calorimetry. The effect of CTX on the thermograms measured for DMPA and DPPG is shown in Figure 5. It is obvious from this figure that the transition amplitude decreases as more toxin is added to phospholipids so that only a very small peak remains at low lipid to protein molar ratio. On the other hand, the toxin does not cause a severe broadening of the transition that is completed within a range of a few degrees. Even in samples with high protein content, the thermograms do not show any additional peak or shoulder that could be attributed to the toxin unfolding which is in good agreement with the known thermal stability of CTX (Pézolet et al., 1982).

The careful examination of the thermograms of Figure 5 reveals that the temperature of the endothermic peak of DPPG is not affected by the presence of the toxin while for DMPA it is slightly increased by approximately 2 °C.

When the enthalpy of the transitions relative to those of the pure lipids are plotted vs. the protein to lipid molar ratio R_i^{-1} (Figure 8 under Discussion), a linear decrease is observed. The initial absolute enthalpy value (ΔH_0) is 9.7 kcal mol^{-1} for DPPG and 7.6 kcal mol^{-1} for DMPA.

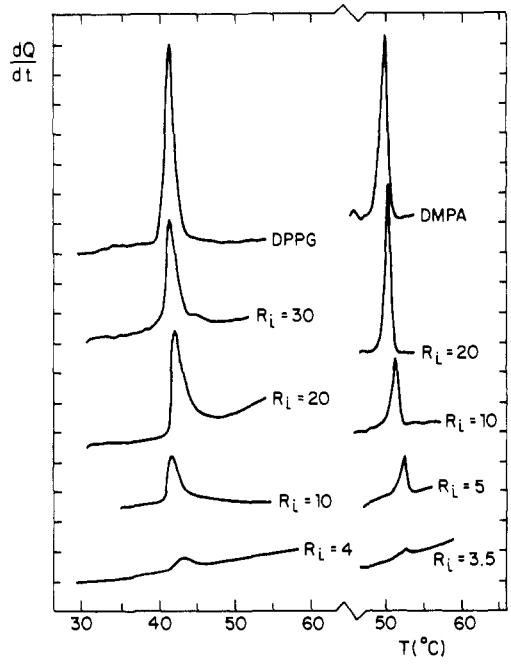

FIGURE 5: Differential scanning calorimetry thermograms of DMPA and DPPG in the presence of increasing amounts of cardiotoxin II at pH 7.5 (100 mM phosphate buffer and 10 mM EDTA). Plotted thermograms are normalized to 1 mg of lipid. Scanning rate 1 K/min for DMPA and 2 K/min for DPPG.

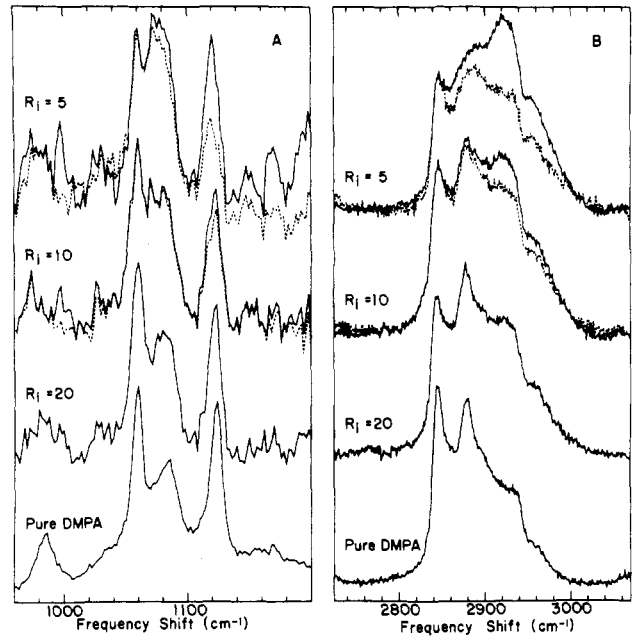

FIGURE 6: Effect of cardiotoxin II on the C–C (A) and C–H (B) stretching mode regions of the Raman spectrum of DMPA dispersions at 37 °C and pH 7.5 (100 mM phosphate buffer and 10 mM EDTA). Each spectrum is an average of six scans at 2 s/cm^{-1} for the C–C region and of three scans at 2 s/cm^{-1} for the C–H region. Spectra drawn in broken lines were corrected for the contribution of the toxin.

Raman Spectroscopy. The C–C skeletal stretching mode region (1000–1200 cm^{-1}) of the Raman spectra of phospholipids is particularly useful since it provides direct information on the intramolecular order of the aliphatic chains (Yellin & Levin, 1977; Wallach et al., 1979). Figure 6A illustrates the effects of cardiotoxin II on the C–C stretching region of DMPA at 37 °C for different lipid to protein molar ratios. The pure DMPA bilayer spectrum displays three well-defined bands at 1063, 1100, and 1130 cm^{-1} that are characteristics of the almost all-trans conformation of the highly ordered acyl chains found in the gel phase of saturated phospholipids. When CTX is added to the bilayers, the intensity of these bands decreases, and the 1130-cm^{-1} band shifts to lower frequency, whereas a new broad band at about 1080 cm^{-1} increases in intensity. All these spectral changes show unambiguously that CTX perturbs the intramolecular chain order in the gel phase of DMPA by increasing the number of gauche conformers. The intensity of the 1130-cm^{-1} band does not decrease as much as that of the 1063-cm^{-1} band upon addition of the toxin since the latter contributes to the scattering intensity in this region for lipid to protein molar ratios lower than 20. Therefore, before temperature profiles based upon peak height intensity ratios were determined, the spectra of the complexes were corrected according to the following procedure.

The Raman spectrum, between 950 and 1200 cm^{-1}, of aqueous solutions of CTX II displays bands at 1001, 1028, and 1171 cm^{-1} that come from vibrations of the phenylalanine residue of the toxin (Pézolet et al., 1982). In addition, there is a band at 1126 cm^{-1} that is assigned to a C–C stretching mode. We have already shown that the intensity and the frequency of the phenylalanine bands of CTX II are insensitive to temperature and to the binding of the toxin to DMPA (Pézolet et al., 1982). Consequently, a spectrum of an aqueous solution of CTX II at 20 °C was subtracted from the spectra of CTX II/DMPA complexes at various R_i values and temperatures to minimize the contribution of the toxin at 1001, 1028, and 1171 cm^{-1}. The result of such a subtraction is shown in Figure 6. As can be seen, the correction is significant only when the lipid to protein molar ratio is lower than 10.

Temperature profiles obtained from the peak height intensity ratio of the 1080-cm^{-1} band over the 1130-cm^{-1} band, measured on corrected and uncorrected spectra, are given in Figure 7A for both the pure DMPA dispersion and CTX/DMPA complexes with different R_i values. As shown in this figure, the addition of CTX shifts the gel to liquid-crystal phase transition of DMPA slightly toward higher temperatures which is in good agreement with the DSC results of Figure 5. The transition also decreases in amplitude when more toxin is added and even completely disappears at high cardiotoxin content.

Although the formation of toxin/phospholipid complexes increases drastically the number of gauche bonds for the gel phase, the effect is much less important in the liquid-crystalline phase. When the spectra are corrected for the protein contribution at 1130 cm^{-1}, the temperature profiles demonstrate that, within the experimental error, the number of gauche conformers above the phase transition is the same whether the toxin is present or not. It thus appears that, as opposed to the gel state, the intramolecular chain disorder of the liquid-crystalline phase of DMPA is much less affected by the presence of the toxin. We have observed a similar behavior for complexes of CTX with dimyristoylphosphatidylglycerol bilayers (M. Pézolet et al., unpublished results).

As shown in Figure 6B, the acyl chain C–H stretching region, which is sensitive to the chain–chain intermolecular interactions, is also affected by the addition of CTX to DMPA bilayers. The first spectral alteration that is observed, even at a lipid to protein molar ratio of 50, is the increase of the intensity of the 2880-cm^{-1} band relative to the one at 2850 cm^{-1} that are respectively assigned to the methylene antisymmetric and symmetric C–H stretching modes (Yellin & Levin, 1977; Gaber & Peticolas, 1977). It is well-known that this intensity ratio is sensitive to the intermolecular vibrational coupling and thus to the lateral packing of the hydrocarbon chains (Gaber & Peticolas, 1977; Snyder et al., 1980). The h_{2880}/h_{2850} ratio increases from 0.89 for pure DMPA to 1.1 when 1 molecule of cardiotoxin is added to 50 molecules of

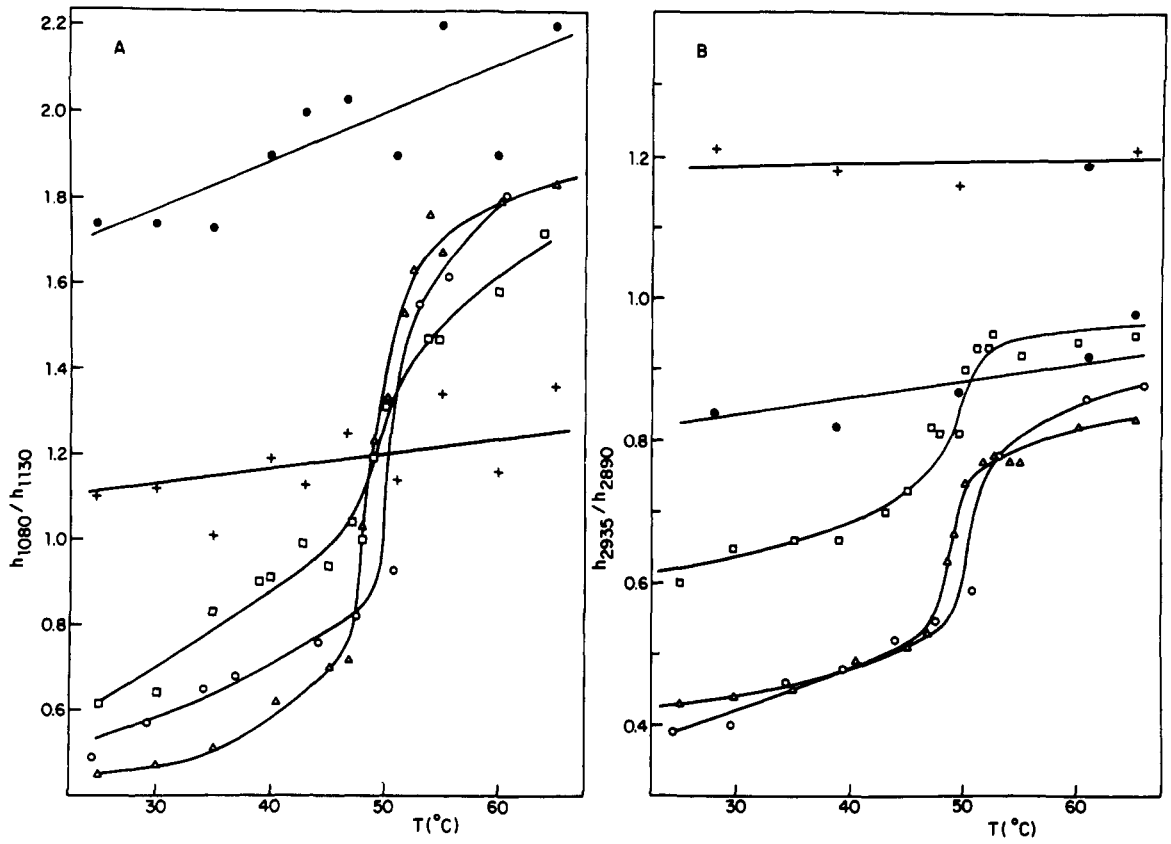

FIGURE 7: Effect of temperature on (A) the h_{1080}/h_{1130} and (B) the h_{2935}/h_{2890} intensity ratios of the Raman spectrum of cardiotoxin II/DMPA complexes at different lipid to protein molar ratios and pH 7.5 (100 mM phosphate buffer and 10 mM EDTA). (△) Pure DMPA; (○) R_i = 50; (□) R_i = 20; (+) R_i = 5; (●) R_i = 5 corrected.

DMPA, thus indicating a marked increase in intermolecular coupling. This closer chain packing of the aliphatic chains most likely results from the partial neutralization of the negative charge borne by the polar head group of DMPA at pH 7.5 by electrostatic interactions with the positively charged basic residues of the toxin. A similar change of the h_{2880}/h_{2850} ratio has been observed when Ca^{2+} was added to DMPA (R. Kouaouci et al., unpublished results) or DPPG (Susi, 1981) bilayers.

The addition of the CTX to DMPA bilayers also causes a marked increase of the 2935-cm^{-1} band (Figure 6) which results in part from underlying infrared active methylene asymmetric stretching modes that become Raman active as the intramolecular chain disorder increases (Bunow & Levin, 1977). As for the C–C region, the toxin contributes to the scattering intensity in the C–H region around 2922 cm^{-1} for lipid to protein molar ratios lower than 20. Spectra were thus corrected for the toxin contribution. However, since there is no sharp band in the C–H region of the Raman spectrum of CTX II that can be used as an internal intensity standard, and because of the weakness of the phenylalanine bands compared to the C–H bands, we have used the following criterion for the subtraction. When too much protein is subtracted from the spectrum of CTX/DMPA complex, a trough appears around 2922 cm^{-1}, a feature that is usually not observed for pure phospholipid dispersions whether they are in the gel or in the liquid-crystalline phase. The results of the subtraction are shown in Figure 7B. Temperature profiles from the h_{2935}/h_{2880} ratio that is sensitive to the intermolecular chain disorder were also constructed from the corrected and uncorrected spectra, and some of the resulting curves are shown in Figure 7B. As for the C–C region, the gel to liquid-crystal phase transition of DMPA is slightly shifted to high temperatures by the toxin. The transition also looks broad at low R_i values and completely disappears. It is interesting to note here that even though CTX induces a significant intramolecular disorder in the gel phase of DMPA at R_i = 50, it does not increase the intermolecular disorder which remains the same in both the gel and liquid-crystalline phases. On the other hand, the R_i = 20 complex exhibits a significantly greater intermolecular disorder in comparison to the pure bilayers whether it is below or above the phase transition.

Discussion

Three main conclusions can be drawn from the above results: (i) Cardiotoxin II binds to charged phospholipids independently of their polar head group structure, their chain length, or degree of insaturation and whether the chains are in the gel or in the liquid-crystalline state. The only parameter that governs the binding is the net charge at the interface. These results are in good agreement and further reinforce the conclusions first proposed from the binding of cardiotoxins to natural phospholipids (Dufourcq & Faucon, 1978; Vincent et al., 1978). (ii) For all the lipids investigated in this study, the gel to liquid-crystalline phase transition is abolished when the system is saturated in toxin. This is observed independently by the three techniques used in this study. Therefore, as opposed to mellitin that is only able to disorganize completely short-chain charged phospholipids which bear one negative charge (Bernard et al., 1982), cardiotoxin drastically perturbs the bilayer structure of all charged phospholipids. This is probably the first event which leads to cardiotoxin-induced permeability and morphological changes of lipid vesicles and membranes (Faucon et al., 1979; Gulik-Krzywicki et al., 1981). (iii) Since these effects have been observed on both synthetic lipids and on natural PS, for concentrated solutions needed

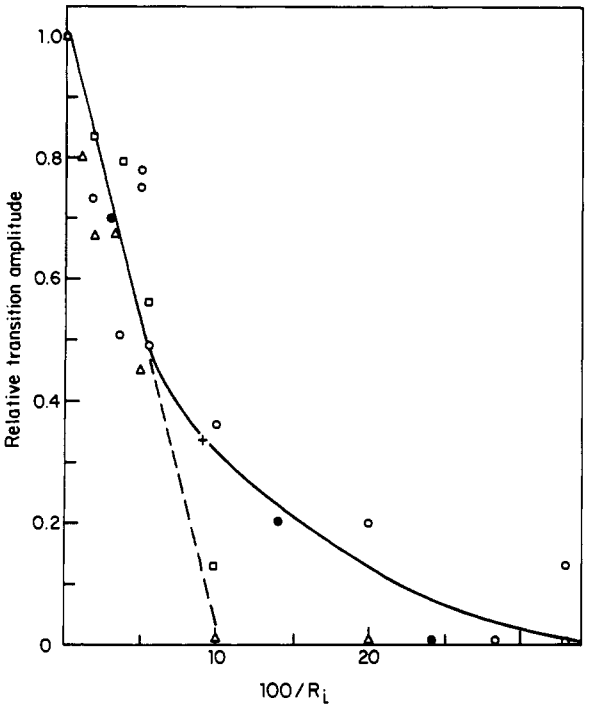

FIGURE 8: Relative transition amplitude as a function of the cardiotoxin to lipid molar ratio (R_i^{-1}). (○) DMPA by differential scanning calorimetry; (△) DMPA by Raman spectroscopy from the h_{1080}/h_{1130} ratio; (□) DMPA by fluorescence polarization; (+) DPPG by differential scanning calorimetry.

in Raman spectroscopy and in differential scanning calorimetry, as well for the 10^{-5} M range of fluorescence, we can conclude that such perturbations occur in natural membranes as soon as they contain charged phospholipids. In lipid mixtures, it has already been shown that CTX binds only to charged species (Dufourcq & Faucon, 1978; Faucon et al., 1981).

Transition amplitudes relative to those of pure lipids were calculated from the data obtained by fluorescence polarization and Raman spectroscopy. As seen in Figure 8, these results agree quite well with the enthalpy loss measured by differential scanning calorimetry. It should be mentioned here that in the case of Raman results in the C–C region, the amplitude of the observed transitions is directly related to the change of the number of gauche conformers of the acyl chains. On the contrary, in the case of fluorescence polarization, these amplitudes do not reflect any simple physical parameter. Moreover, the partition coefficient of the DPH probe between the two lipid environments is assumed constant (Lentz et al., 1976).

Figure 8 shows that the relative amplitude of the transition decreases monotonically according to the same relationship for DMPA and DPPG as the lipid to protein molar ratio decreases, even though these two lipids have different binding properties (Figure 1). From the linear section of this curve at low protein content, one can extrapolate that the enthalpy change of the transition vanishes at $R_i = 10 \pm 2$. Therefore, in the case of DMPA, the gel to liquid-crystalline transition is abolished at a lipid to protein molar ratio that is twice as big as the one determined from the binding curves (Figure 1). This means that even when all the lipids are perturbed, they are still able to bind more toxin. A similar behavior has already been observed in the case of melittin (Bernard et al., 1982).

The transition temperature for the investigated phospholipids was generally not affected by the presence of CTX; only a slight increase of T_m has been detected by DSC and Raman spectroscopy in the case of DMPA, which could be due to a decrease of the charge of the polar head group or of the local pH of the phospholipid. This interpretation is further reinforced by the C–H stretching region of the Raman spectra of CTX/DMPA complexes that show that like calcium (R. Kouaouci and M. Pézolet, unpublished results), CTX increases the intermolecular coupling between the acyl chain methylene vibrations even at such a high lipid to protein molar ratio as 50, which implies long-range perturbations.

The effects of CTX on the phase transitions of phospholipids can be interpreted by assuming that each toxin molecule strongly perturbs approximately 10 lipid molecules. The observed endothermic peaks that always remain sharp would then be due to the unperturbed phospholipids that melt with the same enthalpy change as that of the pure phospholipid. The remaining phospholipids that are in contact with the toxin would have no contribution to the observed thermograms. Such an all or nothing model, for which only the relative amount of perturbed or unperturbed lipids varies, has already been used for melittin (Bernard et al., 1982), for gramicidin (Chapman et al., 1977), and for some membrane proteins such as glycophorin (Van Zoelen et al., 1978; Mendelsohn et al., 1981) and the proteolipid from myelin (Curatolo et al., 1978). Our conclusion is further supported by Raman results in the C–C region that show that the incorporation of CTX to DMPA bilayers induces an intramolecular disordering that is equivalent to the one found in the liquid-crystalline phase of pure DMPA, and this well below the phase transition temperature. Therefore, these perturbed lipids are already disordered and cannot undergo a cooperative transition. A similar conclusion can be drawn from the fluorescence polarization results. Thus, in contrast to Ca^{2+} (Van Dijck et al., 1975) and poly(L-lysine) (Hartmann & Galla, 1978) which bind to the phosphate group at the interface and lead to a better packing of the acyl chains, CTX which has a strongly reticulated tertiary structure imposes a particular topology to its charged residues that does not favor such a packing reinforcement.

When the positively charged lysyl groups of the toxin interact with the polar head phosphate groups of the phospholipids, the net charge of the toxin and of its surrounding lipids becomes very small. Then, hydrophobic interactions can take place and the protein penetrates and disorganizes the phospholipid chains. As far as the transition temperature and enthalpy changes are concerned, the effects of cardiotoxins on phospholipid bilayers are similar to those of intrinsic proteins. However, the resulting structure of the lipid/toxin complexes may not be of the bilayer type as opposed to membrane protein/phospholipid complexes. In fact, it has been proposed (Gulik-Krzywicki et al., 1981) that cardiotoxins induce nonlamellar phases. This is also supported by the fact that at high protein content, some lipids still melt in a highly cooperative way which could be interpreted as a phase separation at least in the temperature range $T < T_m$.

Both fluorescence polarization and Raman spectroscopies can provide structural information on the lipids that are in contact with the toxin. Raman results that were obtained at the 10^{-13}-s time scale show that the phospholipid chains that are in contact with the toxin display a gauche conformer population that is almost the same as the one found in the liquid-crystalline phase of the pure phospholipid. On the other hand, fluorescence polarization data that were obtained at the time scale of 10^{-8} s reflect changes both in the order parameter and in the chain dynamics of the environment of the DPH

probe (Kawato et al., 1977; Hare et al., 1979). Despite the fact that no time-resolved anisotropy measurements have been made, the effect of temperature on *P* suggests that for a given phospholipid, the chain order of the "boundary" lipids is smaller than that of the pure lipid in the gel phase temperature domain, while it is larger in the liquid-crystalline phase. Therefore, for the gel phase, fluorescence and Raman spectroscopies lead to the same conclusion: lipids in contact with the toxin are less ordered. However, for the fluid phase, no significant ordering is detected by Raman spectroscopy, as opposed to what could be concluded from fluorescence results. Several reasons could be responsible for such an apparent discrepancy: (i) the time scale of these two methods is very different; (ii) Raman spectroscopy reflects order at the C–C or C–H level of the lipid chains themselves while the order estimated from fluorescence spectroscopy corresponds to an average value over the molecular length of the probe; (iii) Raman spectroscopy is sensitive to the state of all the C–C bonds while the exact location of DPH can be questioned. However, this probe has been shown to be equally partitioned between different lipid phases in phospholipid mixtures (Lentz et al., 1976) and is supposed to be similarly distributed in lipid/protein systems (Gomez-Fernandez et al., 1979, Jähnig, 1979).

In conclusion, it should be emphasized that the use of three different techniques has provided a coherent view on the cardiotoxin-induced perturbations of the thermotropic transitions of charged phospholipids and has improved our knowledge on the physical state of the lipids that are in contact with the toxin.

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