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PHASE SEPARATIONS INDUCED BY MELITTIN IN NEGATIVELY-CHARGED PHOSPHOLIPID BILAYERS AS DETECTED BY FLUORESCENCE POLARIZATION AND DIFFERENTIAL SCANNING CALORIMETRY

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Interactions between melittin and a variety of negatively-charged lipid bilayers have been investigated by intrinsic fluorescence, fluorescence polarization of 1,6-diphenylhexatriene and differential scanning calorimetry. (1) Intrinsic fluorescence of the single tryptophan residue of melittin shows that binding of this peptide to negatively-charged phospholipids is directly related to the surface charge density, but is unaffected by the physical state of lipids, fluid or gel, single-shell vesicles or unsonicated dispersions. (2) Changes in the thermotropic properties of negatively-charged lipids upon melittin binding allow to differentiate two groups of lipids: (i) A progressive disappearance of the transition, without any shift in temperature, is observed with monoacid C_{14} lipids such as dimyristoylphosphatidylglycerol and -serine (group 1). (ii) With a second group of lipids (group 2), a transition occurs even at melittin saturation, and two transitions are detected at intermediate melittin content, one corresponding to remaining unperturbed lipids, the other shifted downward by 10–20°C. This second group of lipids is constituted by monoacid C_{16} lipids, dipalmitoylphosphatidylglycerol and -serine. Phosphatidic acids also enter this classification, but it is the net charge of the phosphate group which allows to discriminate: singly charged phosphatidic acids belong to group 2, whereas totally ionized ones behave like group 1 lipids, whatever the chain length. (3) It is concluded that melittin induces phase separations between unperturbed lipid regions which give a transition at the same temperature as pure lipid, and peptide rich domains in which the stoichiometry is 1 toxin per 8 phospholipids. The properties of such domains depend on the bilayer stability: in the case of C_{16} aliphatic chains and singly charged polar heads, the lipid-peptide domains have a transition at a lower temperature than the pure lipid. With shorter C_{14} chains or with two net charges by polar group, the bilayer structure is probably totally disrupted, and the new resulting phase can no longer lead to a cooperative transition.

Introduction

Melittin is actually one of the most studied peptides as a model for lipid-protein interactions. It is an amphipathic and strongly basic peptide of 26 residues, extracted from bee venom [1], which gives rise in solution to a monomer-tetramer equilibrium [2]. The main physiological effects of melittin are its lytic action towards living cells and artificial lipid bilayers [3], and its capability to stimulate phospholipase A_2 [4,5].

Interactions of melittin with phospholipids and natural membranes have already been studied by a wide variety of techniques, as recently reviewed by Habermann [6]. These works were dealing with the characteristics of the binding (specificity towards various phospholipids, effect of fluidity...), the structure of the lipid-peptide complex, and also with the perturbations of the fluidity and thermotropic properties of the bilayer. This last type of

data is certainly essential for a better understanding of the mechanism by which melittin is able to induce lysis upon binding to the lipid bilayer. However, most of the available information is actually restricted to the effects of melittin on zwitterionic phospholipids, mainly synthetic phosphatidylcholines, and results obtained by different authors seem to be contradictory: either the lipid transition temperature is unaffected [7–9], shifted upwards [10], or two distinct transitions are detected in the presence of melittin [11], the perturbed lipids having then a higher melting temperature. Furthermore, perturbations of negatively-charged lipids by melittin have never been studied, whereas it has been shown that they can bind this peptide much more efficiently than the neutral ones: for example, 25 molecules of dipalmitoylphosphatidylcholine are needed to bind one melittin molecule, whereas only four molecules of phosphatidylserine are required [12]. Acidic lipids could then play a particularly important role in the lysis phenomenon: this is supported by the fact that cardiotoxins, another class of direct lytic factors, exhibit a strict specificity towards negatively-charged lipids [13].

Here, we report on the effects of melittin on the fluidity and the thermotropic properties of a variety of negatively-charged phospholipids. Experiments have been carried out using both differential scanning calorimetry and fluorescence polarization of the 1,6-diphenylhexatriene probe embedded in the bilayer. It is shown that melittin induces drastic changes in the characteristics of the order-disorder transitions, depending on the chain length and the net electric charge of the lipid.

Materials and Methods

Dimyristoylphosphatidylserine and dipalmitoyl-phosphatidylserine were synthesized in the laboratory, according to the method of Confurius and Zwaal [14], and purified by CM-cellulose column chromatography. Dipalmitoylphosphatidylglycerol, dimyristoylphosphatidylglycerol, and dipalmitoylphosphatidic acid were purchased from Medmark, and dimyristoylphosphatidic acid from Senn Chemicals. Melittin was from Serva, and was used without further purification: it could be contaminated by some phospholipase A_2, so 1 mM EDTA was always added to the samples in order to inactivate any trace of hydrolytic activity [15].

Differential scanning calorimetry. Phospholipids were dissolved in benzene, and an aliquot of phosphatidyl[^{14}C]choline (New England Nuclear) was added to give a specific activity of $4.8 \cdot 10^4$ dpm per mg; this solution was then lyophilized. Dried phospholipids were suspended in 100 mM phosphate buffer, pH = 7.5, 1 mM EDTA, or 20 mM acetate buffer, pH = 3.5, 1 mM EDTA, and sonicated for 5 min above the lipid transition temperature with an Annemasse F 50 sonicator. Melittin was added to labelled lipid at the selected molar ratio, and this suspension was incubated for some minutes above the lipid transition temperature, then centrifuged at 40000 rpm for 1 h at 20°C in a SW 50.1 Beckmann rotor. About half the wet pellet was transferred to the calorimeter pans. The rest of the pellet resuspended in buffer was used for fluorescence polarization measurements. Calorimetric experiments were carried out on a Dupont 990 differential calorimeter at scanning rates of 5 or 2 K/min and sensitivities ranging from 0.02 to 0.1 $\text{mcal} \cdot \text{s}^{-1} \cdot \text{inch}^{-1}$. The obtained thermograms were first converted in a digital form, then numerically integrated using a Digital computer, in order to calculate enthalpy values.

Fluorescence measurements. The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene obtained from Aldrich Chem. Co., was introduced in the lipid bilayer at a molar ratio of 1% or less by adding, according to Shinitzky and Barenholz [16], some microliters of a solution in tetrahydrofuran to the lipids suspended in 20 mM phosphate buffer, pH = 7.5, or 20 mM acetate buffer, pH = 3.5, containing 1 mM EDTA. The lipid concentration was always in the range of 10 to 40 μM. A concentrated melittin solution was added in order to reach the selected lipid/peptide molar ratio (R_i), then the sample was incubated above the transition temperature of the lipid for 10 min. The degree of fluorescence polarization P was measured with an apparatus built in the laboratory, following the optical scheme proposed by Weber and Bablouzian [17], and connected to a minicomputer Digital LSI 11 PDP 11/03 which allowed the complete automatization of measurements.

Intrinsic fluorescence measurements of the

single tryptophan of melittin were carried out with a FIKA 55 MK II spectrofluorometer: excitation wavelength was always at 280 nm, excitation and emission slits being of 7.5 nm.

Results

Binding of melittin to negatively-charged phospholipid vesicles and dispersions

Before studying the effects of melittin on lipid bilayers, it was first necessary to investigate how interaction occurs in the experimental conditions used. For this purpose, changes in intrinsic fluorescence of melittin upon addition of lipids have been followed, this method being very sensitive to the formation of lipid-peptide complexes, as previously shown [12,18].

Addition of sonicated dipalmitoylphosphatidylglycerol vesicles to a melittin solution at 25°C, i.e. below the transition temperature of the lipid, leads to a blue shift of the emission maximum from 348 to 332 nm, and to an increase in the fluorescence intensity up to 80% at 330 nm. The percentage of the relative change in intensity at 330 nm is plotted in Fig. 1 versus the lipid to protein molar ratio R_i. After a quasi-linear increase, a plateau is reached for $R_i \simeq 4$, all the melittin present in solution being then bound to lipids. These results are quite similar to those previously reported in the case of natural phosphatidylserine at 25°C, i.e. in the fluid state [12]. Furthermore, as equally shown in Fig. 1, unsonicated dispersions of dipalmitoylphosphatidylglycerol lead to a binding curve identical to that obtained with vesicles.

Similar results have been obtained with another negatively-charged lipid, dimyristoylphosphatidic acid (Fig. 1b). It is well-known that phosphatidic acid can give rise to two dissociation equilibria, corresponding to the loss of one or two protons by the phosphate group, with pK_a values of about 3 and 8.5, respectively, [19,20]. Thus, at pH = 3.5, this lipid bears only one negative charge, and it can be seen in Fig. 1b that the binding curve of melittin is very close to that obtained with dipalmitoylphosphatidylglycerol. On the contrary, at pH = 9, the polar head bears two negative charges, and the full binding of melittin then requires only 2 or 2.5 lipid molecules per peptide molecule.

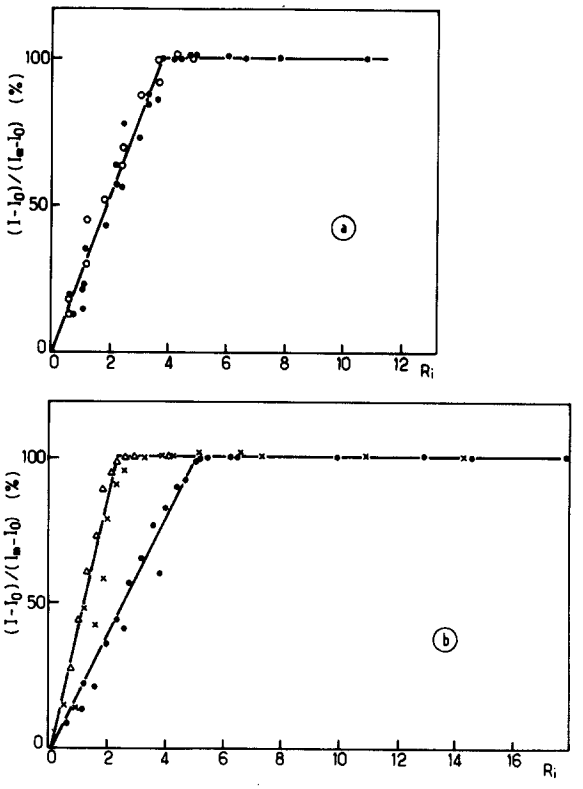

Fig. 1. Relative change in the fluorescence intensity at 330 nm of melittin (5 μM) upon addition of negatively-charged phospholipids at 25°C and pH=7.5, versus the lipid to peptide molar ratio R_i. I_0 is the fluorescence intensity of pure melittin, I_m the intensity at lipid saturation, and I the intensity at the molar ratio R_i. (a) Dipalmitoylphosphatidylglycerol: ○, Single-shell vesicles; ●, unsonicated dispersions. (b) Dimyristoylphosphatidic acid dispersion at various pH values: ●, 3.5; ×, 7.5; △, 9.2.

Moreover, the binding curve obtained at pH = 7.5 is quite similar to that at pH = 9 (Fig. 1b). So, even at pH = 7.5, dimyristoylphosphatidic acid behaves like a diacid in the presence of melittin.

These experiments thus show that melittin interactions with negatively-charged lipids are regulated by the surface charge density, but are independent of the physical state of the lipids, i.e. gel or liquid-crystalline vesicles or unsonicated dispersions. Since transitions occurring in the latter systems are sharper and better defined than those of vesicles [21,22], most of the data in the following sections deal with experiments done with unsonicated dispersions.

Effect of melittin on the thermotropic properties of phosphatidic acids

Taking in account the above results, experiments have been performed at two different pH values, 3.5 and 7.5. The changes versus temperature in the degree of polarization, P, of diphenylhexatriene embedded in dipalmitoylphosphatidic acid dispersions are plotted in Fig. 2. At pH 7.5, the addition of melittin leads to a drastic effect (Fig. 2a): the amplitude of the transition decreases progressively when the content of melittin increases, and the transition totally disappears at saturation in melittin. This occurs without any change in the transition temperature which remains at 65°C, i.e. that of the pure lipid [23].

At pH 3.5 changes in the thermotropic properties of dipalmitoylphosphatidic acid induced by melittin are more complex as seen on Fig. 2b. At low melittin contents ($R_i > 10$) one can notice the presence of two well-defined transitions the amplitudes of which vary in an opposite way: on one hand, the amplitude of the transition which occurs at the temperature of the pure lipid (about 66°C) decreases monotonously, whereas, on the other hand a new transition at about 11°C below appears and its amplitude concomitantly increases. At a lipid to protein molar ratio, R_i, of about 10, only the low-temperature transition remains. Then further increase of the melittin content ($R_i < 10$) induces a progressive broadening of this single transition and a shift from 54–55°C to about 42°C. Therefore in this case, even at melittin saturation the lipids still give rise to a phase transition.

The behaviour of dimyristoylphosphatidic acid, a shorter chain length phosphatidic acid, has also been investigated both by fluorescence polarization and differential scanning calorimetry using the same samples, as described in Materials and Methods. The obtained results at pH 3.5 and 7.5 are summarized in Fig. 3 and Fig. 4. The transition temperatures of the pure lipid, 51°C and 55°C at pH 7.5 and 3.5, respectively, are in good agreement with those of the literature [24,25] and the effects of melittin addition are quite similar to those obtained with dipalmitoylphosphatidic acid. At pH 7.5, the transition totally disappears without any important shift in its temperature (Fig. 3a). At pH 3.5 on the contrary melittin induces first the

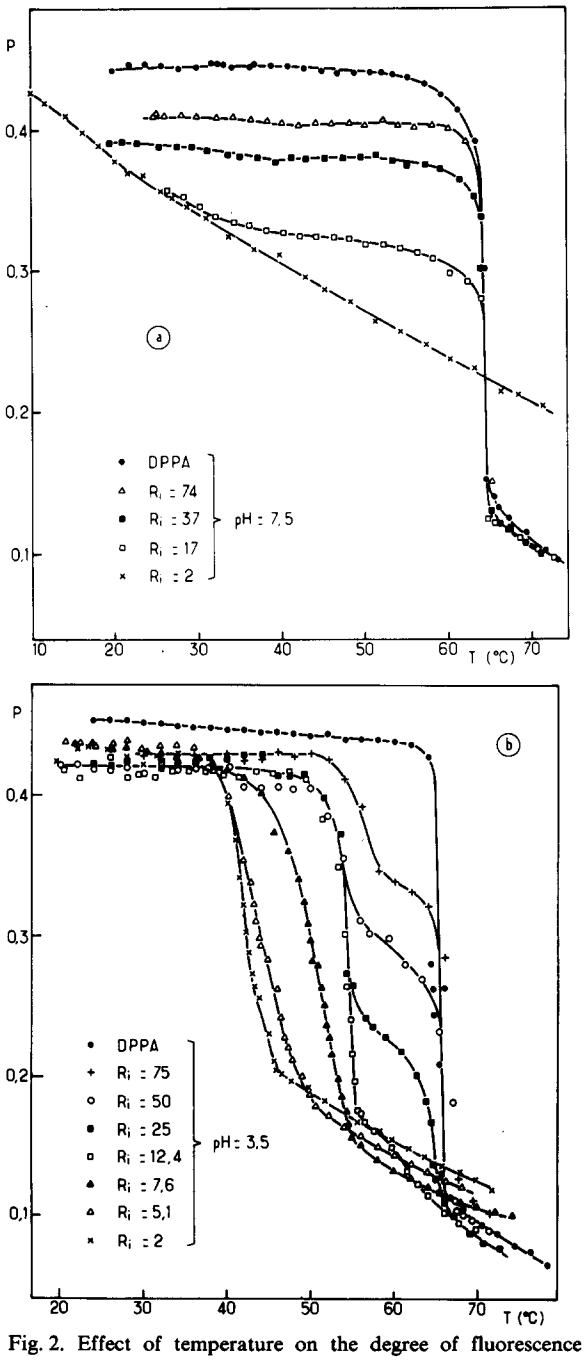

Fig. 2. Effect of temperature on the degree of fluorescence polarization P of diphenylhexatriene embedded in dipalmitoylphosphatidic acid dispersions, at various lipid to melittin molar ratios R_i. a, pH=7.5; b, pH=3.5.

coexistence of two well-defined transitions: one at the temperature of the pure lipid and the other about 12°C lower (Fig. 4a). Afterwards for R_i

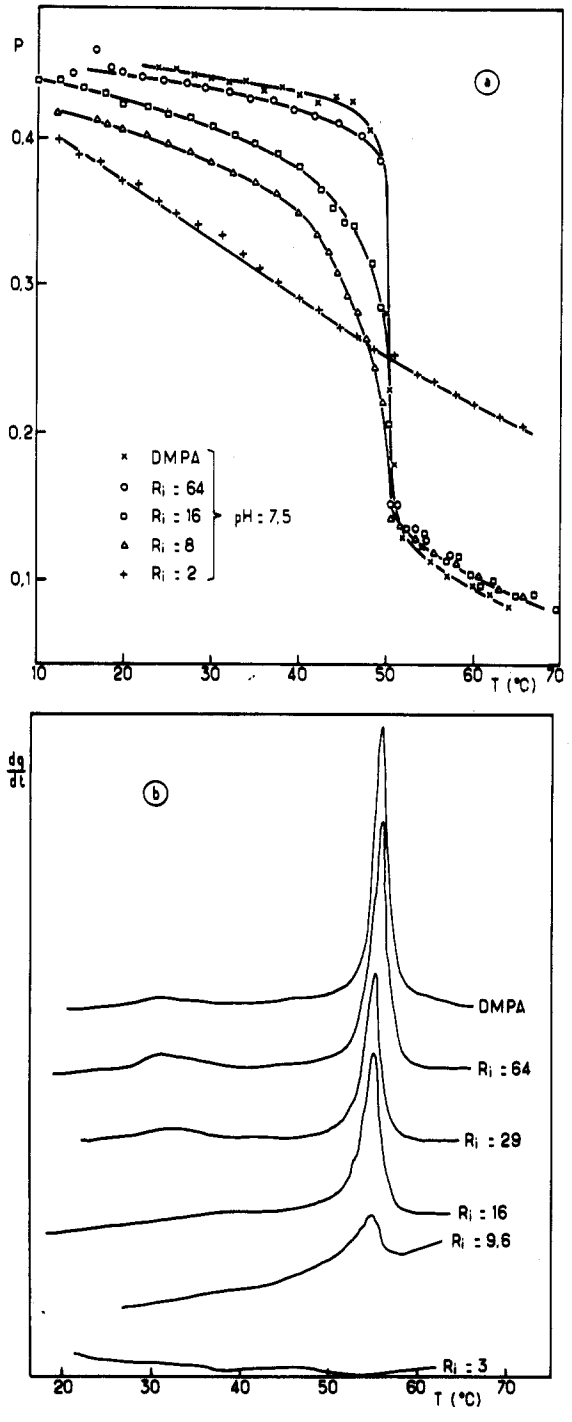

Fig. 3. Effect of melittin at various molar ratios R_i on the phase transition of dimyristoylphosphatidic acid at pH=7.5, as detected (a) by fluorescence polarization and (b) by differential scanning calorimetry. Plotted thermograms are reduced to 1 mg lipid; scanning rate: 2 K/min.

values lower than 10 only the low-temperature transition remains and shifts progressively to 25–26°C when R_i decreases.

As it can be seen in Fig. 3b and Fig. 4b the results obtained by differential scanning calorimetry agree very well with the fluorescence polarization data. One has to emphasize that at pH 3.5 two transitions are clearly resolved at low contents of melittin ($R_i > 10$). The transition enthalpies are plotted in Fig. 5 versus R_i^{-1}, the peptide to lipid molar ratio. The decreases in the enthalpy of the transition occurring at the temperature of the pure lipid are similar whatever the pH is (Fig. 5a). Concomitantly the enthalpy of the low-temperature transition detected at pH 3.5 increases almost linearly up to a value of about 9–10 kcal · mol^{-1} at $R_i = 8$ (Fig. 5b).

Effect of melittin on the thermotropic properties of synthetic phosphatidylglycerol and phosphatidylserine

Experiments have been carried out in this case at pH = 7.5, where both phosphatidylglycerol and phosphatidylserine bear a single negative charge. With the dimyristoyl (C_{14}) derivatives, a behaviour close to that of phosphatidic acids at pH = 7.5 is observed: the transition is progressively broadened, then disappears, without important changes in its temperature (Figs. 6a and 7a). On the contrary, dipalmitoyl (C_{16}) derivatives lead to results similar to those obtained with phosphatidic acids at pH = 3.5 (Figs. 6b and 7b): even at melittin saturation, a transition is observed, at a temperature lower than that of the pure lipid. Furthermore, at intermediate contents in melittin, two transitions are detected, one at the transition temperature of the pure lipid, the other occurring at a lower temperature.

It must be mentioned that these two transitions are also observed when sonicated vesicles of dipalmitoylphosphatidylglycerol are used instead of unsonicated dispersions. This rules out artefacts such as heterogeneities in the lipid-peptide complex due to the multilayer arrangement of lipids in dispersions.

The behaviour of dimyristoyl- and dipalmitoylphosphatidylglycerol and of dipalmitoylphosphatidylserine has also been investigated by differential scanning calorimetry, and the observed results are

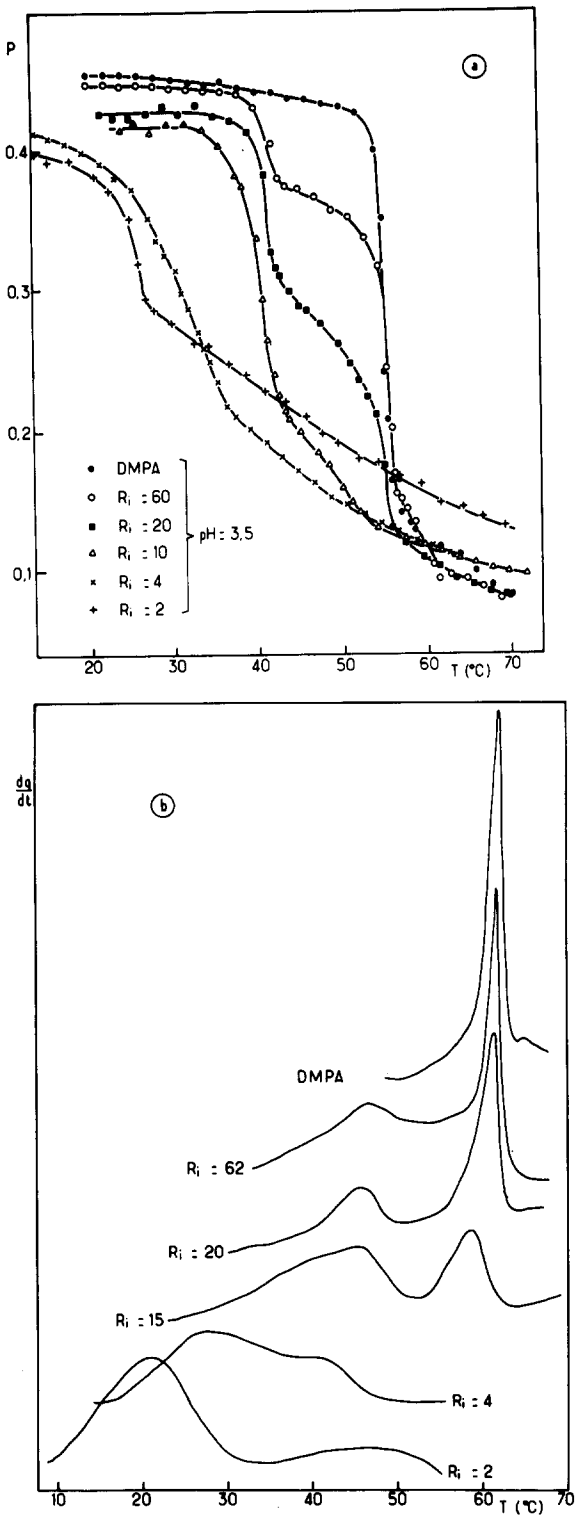

Fig. 4. Effect of melittin at various molar ratios R_i on the phase transition of dimyristoylphosphatidic acid at pH=3.5, as detected (a) by fluorescence polarization and (b) by differential scanning calorimetry. (Thermograms corresponding to pure DMPA and $R_i=62$ are reduced to 1 mg lipid, the others to 2 mg; scanning rate: 2 K/min.)

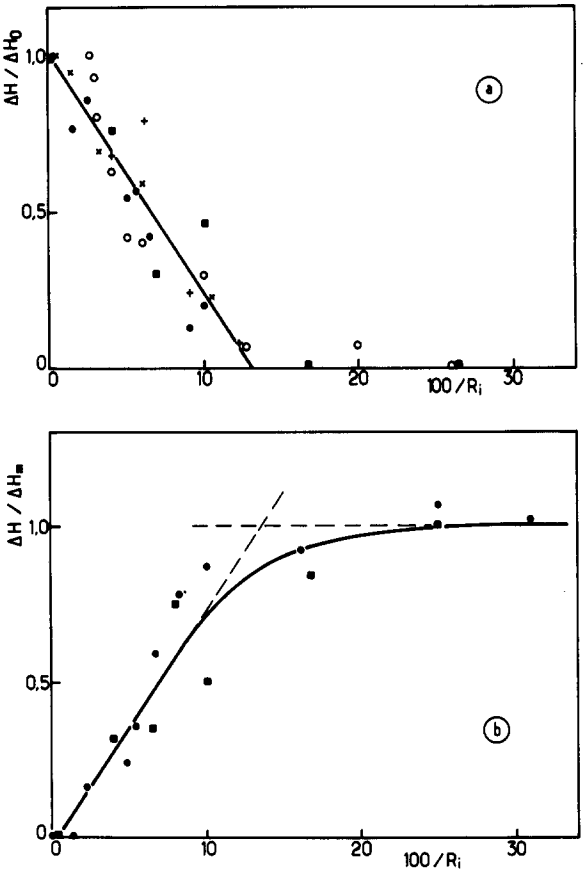

Fig. 5. (a) Relative change in the transition enthalpy ΔH of the 'unperturbed lipids' as a function of the melittin to lipid molar ratio (R_i^{-1}). ΔH_0 represents the transition enthalpy in the absence of melittin. ●, dimyristoylphosphatidic acid at pH=3.5; ×, dimyristoylphosphatidic acid at pH=7.5; ■, dipalmitoylphosphatidylserine at pH=7.5; +, dimyristoylphosphatidylglycerol at pH=7.5; ○, natural phosphatidylserine at pH=7.5 (data from Ref. 9). (b) Relative change in the transition enthalpy ΔH of 'perturbed lipids' versus the melittin to lipid molar ratio (R_i^{-1}), in the case of dimyristoylphosphatidic acid at pH=3.5 (●), and dipalmitoylphosphatidylserine at pH=7.5 (■). ΔH_m represents the enthalpy value reached at melittin saturation ($\Delta H_m=8.1$ kcal·mol^{-1} for dipalmitoylphosphatidylserine and 10 kcal·mol^{-1} for dimyristoylphosphatidic acid). Several scans were run for each pan, the most often upon increasing temperature. Two or more independent samples have generally been studied for each R_i, and the averaged values of ΔH are reported here.

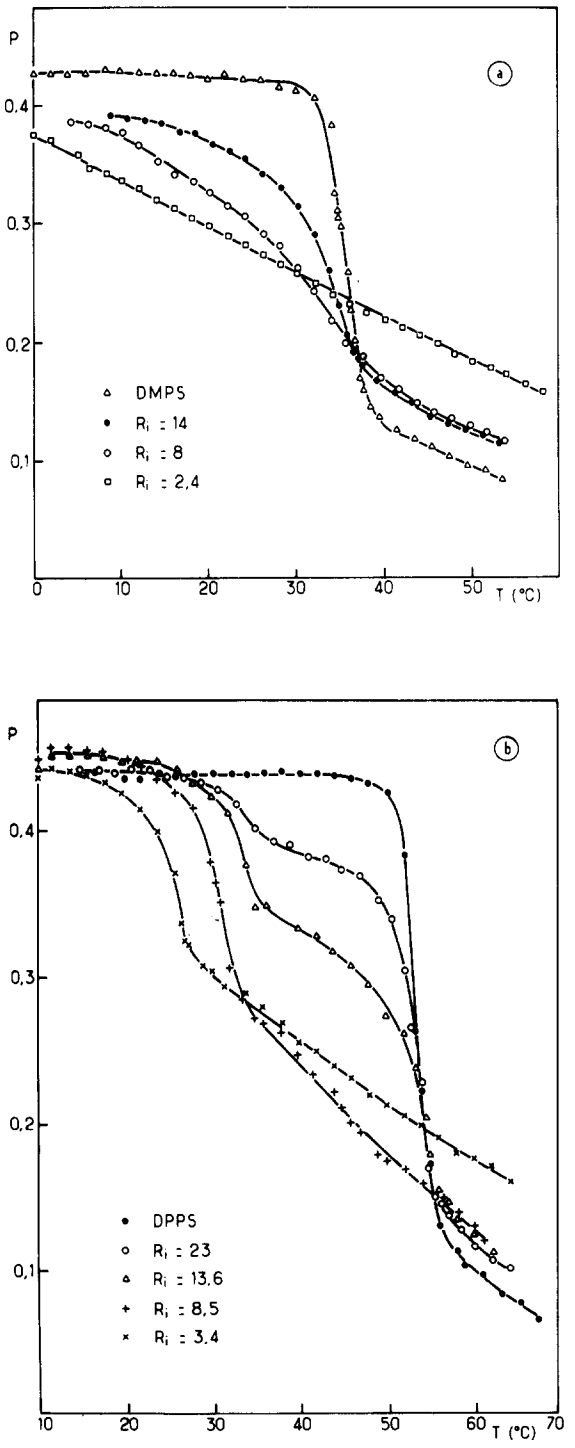

Fig. 6. Effect of melittin at various molar ratios R_i on the phase transition of synthetic phosphatidylserines at pH=7.5, as detected by fluorescence polarization: (a) dimyristoylphosphatidylserine; (b) dipalmitoylphosphatidylserine.

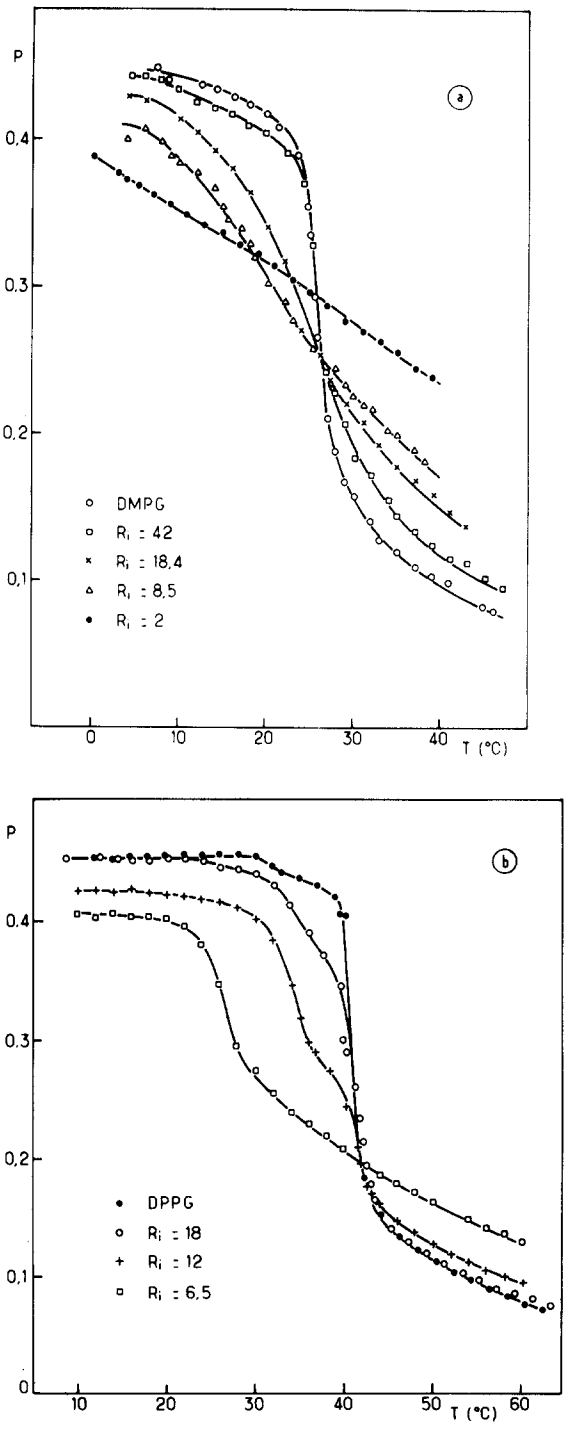

Fig. 7. Effect of mellitin at various molar ratios R_i on the phase transition of synthetic phosphatidylglycerol at pH=7.5, as detected by fluorescence polarization: (a) dimyristoylphosphatidylglycerol; (b) dipalmitoylphosphatidylglycerol.

in agreement with the fluorescence polarization ones. Furthermore, as can be seen in Fig. 5, the variations of the transition enthalpies versus R_i^{-1} are very similar to those obtained above with dimyristoylphosphatidic acid. In the case of dipalmitoylphosphatidylserine, two transitions are resolved, the enthalpy of the low-temperature one increasing up to 8–9 kcal · mol^{-1} at melittin saturation. Two transitions are also detected in the case of dipalmitoylphosphatidylglycerol. However, due to the overlap of the two peaks, transition enthalpies could not be determined and therefore are not reported in Fig. 5.

Results previously obtained with natural phosphatidylserine [9] have also been plotted in Fig. 5a, and it is quite noticeable that all these data can be fitted, within experimental error by a single straight line which intercepts the abscissa axis at $R_i = 8 \pm 2$.

Discussion

Results obtained by intrinsic fluorescence agree well with the previously reported ones [12], and allow to define in more detail interactions of melittin with negatively-charged phospholipids. Firstly, the physical state, gel or liquid crystal, of the lipid has no effect on melittin binding, contrary to what happens in the case of zwitterionic lipids [12,18,26]. This shows that electrostatic forces play a predominant role in the formation of the complex, and seems to rule out an important penetration of the hydrophobic part of the peptide within the bilayer. This conclusion is supported by the fact that the stoichiometry of complexes is directly related to the surface charge density of the bilayer, as shown by pH effects on dimyristoylphosphatidic acid. Moreover, the binding of melittin at the interface leads to a downward shift of the apparent pK_a of this lipid, as already observed in the case of cardiotoxins [13]. It has also to be mentioned that the presence of a positive charge on the N-terminal of melittin does not seem to be a crucial parameter for the binding. This group has a p$K = 7.6$ [27], and, although this value could be changed at the interface, the Gly − 1 position is probably uncharged at pH = 9.2, where binding readily occurs. This agrees with the well-known fact that formylated melittin exists naturally and is also active [1]. Finally, intrinsic fluorescence experiments show that single-shell vesicles and unsonicated dispersions behave in the same way towards melittin, which means that all the lipids, even located in the inner layers of 'onion-like' structures, are quite accessible to the peptide, in agreement with its lytic properties.

Differential scanning calorimetry and fluorescence polarization experiments show that melittin induces in all cases a drastic perturbation of bilayers constituted of negatively-charged phospholipids. Concerning its effects on the bilayer fluidity, melittin acts in opposite ways as a function of the physical state of the lipid: an increase of the degree of polarization P of diphenylhexatriene is indeed observed when the lipid is in the liquid crystalline state, above the transition temperature T_c, whereas P decreases when aliphatic chains are in the gel state, below T_c. However, it has previously been shown by time-resolved anisotropy measurements [28,29] that P of diphenylhexatriene embedded in lipid bilayer is depending both on the microviscosity and on the degree of order of its environment. It is thus not possible to interpret quantitatively the results obtained here from steady state measurements [30].

From the effects observed on the thermotropic properties of bilayers in the presence of melittin, negative lipids can be divided into two different groups: (i) for the first group, the order-disorder transition progressively disappears, without any significant shift in temperature, whereas (ii) lipids of the second group always undergo a transition shifted to lower temperature, even at melittin saturation. Moreover, in this last case, two transitions are detected at high lipid to protein molar ratios ($R_i > 10$). Group 1 is constituted of monoacid C_{14} lipids (dimyristoylphosphatidylglycerol and -phosphatidylserine), of diacid C_{14} and C_{16} lipids (dimyristoyl- and dipalmitoylphosphatidic acid at pH = 7.5), and, as previously shown [9] of natural negatively-charged lipids such as phosphatidylserine. On the contrary, monoacid C_{16} lipids (dipalmitoylphosphatidylglycerol, -phosphatidylserine, and dipalmitoylphosphatidic acid at pH = 3.5) belong to group 2. Dimyristoylphosphatidic acid at pH = 3.5 is the only exception since it behaves like a lipid of this last group, although being a monoacid C_{14} lipid.

From this classification, it is clear that the main difference between the two groups of lipids lies in the stability of the formed bilayer: interaction energy between lipid molecules is indeed directly related to the length of aliphatic chains and to the electric charge of the polar head, and thus it is larger for lipids of group 1 than for those of group 2. The apparent anomalous behaviour of dimyristoylphosphatidic acid at pH = 3.5 can in fact be explained by the weak steric hindrance of its polar head, which could lead to stronger chain-chain interactions, compared to phosphatidylserine or phosphatidylglycerol. Thus, the action of melittin towards lipids of groups 1 and 2 seems to differ according to the stability of the bilayer: if its cohesion is sufficient, the lipid assembly preserves a well-defined structure, even in the presence of an excess of melittin, and is still able to give rise to a transition. Otherwise, the bilayer is probably totally disrupted, as has been shown in the case of natural phosphatidylserine by electron microscopy [9] and the new resulting phase cannot lead to a cooperative phenomenon.

Two models have been widely used to account for lipid-protein interactions at the molecular level: (i) The protein perturbs a limited number of lipids at its contact, constituting an 'annulus', the remaining lipids being unaffected [31]. (ii) The protein and lipids at contact aggregate together forming a domain which constitutes a phase different from that of the pure lipid [32,33].

According to the first model, the annulus would be constituted of about eight lipid molecules in the case of melittin, and it seems unlikely that such a few lipids could give rise to a cooperative transition process. Therefore, this model could account only for the progressive disappearance of a single transition, as observed for most of the systems studied in literature, like gramicidin [34], apolipoproteins [35] and membrane proteins [32].

However, our results with lipids of group 2 clearly demonstrate that perturbed lipids give rise to a cooperative transition. Such an occurrence of two well-defined transitions implies that at least in the gel state two distinct phases exist. Therefore, in this case, melittin induces a phase separation between unperturbed regions giving rise to a sharp transition with characteristics (T_c, ΔH) identical to those of pure lipids, and peptide rich regions which lead to a new well-defined transition (T_c', $\Delta H'$) at lower temperature. Such a behaviour has yet been observed in a few cases such as the basic myelin protein [36,37], polymyxin [38] or polylysine [37,39].

Due to similarities of melittin effects on the disappearance of pure lipid transitions whatever the lipid group is (Fig. 5a), it can be proposed that this last model (phase separation) also holds for group 1 lipids: in this case, the separation would occur between a pure lipid phase and a peptide rich region in which lipids are not able to melt cooperatively. Thus, from group 1 to group 2, only the structure of the lipid-toxin domains would change, according to the lipid-lipid molecular interactions.

As seen in Fig. 5a, the enthalpy of the pure-lipid transition decreases down to zero for $R_i \simeq 8$ (i.e. below the melittin saturation level) whatever the lipid used. So, as soon as melittin binds, it squeezes eight lipid molecules out of the pure lipid phase, whatever the chain length and the charge of the lipids are. Further binding of melittin ($R_i < 8$) simply leads to an increased perturbation of the lipid system, as shown by the downward shift of T_c' in the case of lipids of group 2. The calorimetric data by Mollay on dipalmitoylphosphatidylcholine [7] were also indicative of a similar process, since they lead to roughly 10 lipid molecules excluded from the main transition. A similar conclusion was inferred by Lavialle et al. [11] from Raman spectroscopy experiments on melittin-dimyristoylphosphatidylcholine. Finally, one could mention that data obtained with glycophorin, the well-known membrane protein from erythrocytes, are very similar since it always perturbs 80 lipid molecules [40], despite the fact that in these systems, the protein incorporation never allowed to abolish the transition.

Results in Fig. 5 equally show that the populations of perturbed and unperturbed lipids detected by calorimetry are quite complementary within the experimental errors. Moreover, the transition enthalpy of perturbed lipids, which is related to the entropy change associated with this transition, is equal to or even higher than that of pure lipids.

As already mentioned results obtained with group 2 lipids agree fairly well with those previously reported in the case of basic myelin protein

[36,37] and polymyxin [38], which are known to interact both electrostatically and hydrophobically with lipids. In these cases indeed, two transitions were detected, and perturbed lipids melted at a lower temperature compared to pure lipids. On the contrary, polylysine, which interacts mainly through charged groups, induced phase separation with perturbed lipids having a higher melting temperature [37,39]. This again reinforces our opinion that electrostatic forces play an important role in modulating the affinity of melittin towards lipids [12], but that an hydrophobic contact, probably restricted to a few methylene groups near the polar head [41], is the main cause of the perturbations of the bilayer properties. A simple charge neutralisation should give opposite effects to those really observed.

At that point, it has to be recalled that our data agree well with the fluorescence data of H. Vogel on dimyristoylmethylphosphatidic acid [8]. However, they differ notably from those of Lavialle et al. [11] by Raman spectroscopy, who detected two transitions with melittin-dimyristoylphosphatidylcholine complexes, but attributed the high temperature component to perturbed lipids. This seems rather surprising and further studies are still needed to clarify such an effect of melittin on synthetic phosphatidylcholines.

In summary, our results strongly support the occurrence of phase separations induced by melittin on negatively-charged lipid bilayers. The next step should be to give information on the structure of such domains in relation to the fact that the most striking effect of melittin is to induce lysis. It could be speculated that in natural membranes, where the aliphatic chains of lipids are mostly unsaturated, the melittin action would be similar to that observed with group 1 lipids: the formation of peptide rich domains could then lead to a local breakdown of the membrane, as it has been recently suggested in the case of cardiotoxins from electron microscopy experiments [42]. Studies on lipid mixtures, which are now in progress, should be an essential step for a better understanding of the mechanism of lysis.

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