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A RESTATEMENT OF MELITTIN-INDUCED EFFECTS ON THE THERMOTROPISM OF ZWITTERIONIC PHOSPHOLIPIDS

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Perturbations induced by melittin on the thermotropism of dimyristoyl-, dipalmitoyl-, distearoylphosphatidylcholine and natural sphingomyelin are investigated and rationalized from data obtained by fluorescence polarization, differential scanning calorimetry and Raman spectroscopy. Depending on the technique and/or experimental conditions used, the observed effects differ at the same lipid to protein molar ratio, due to partial binding of melittin. The binding is more efficient for tetrameric than for monomeric melittin, but in both cases its affinity is weaker for phosphatidylcholine dispersions in the gel phase than for sonicated vesicles. For temperatures $T \geqslant T_m$ efficient binding occurs whatever the initial state of the lipids is. One can summarize the effects induced by melittin on the transition temperature as follows: (i) No upward shift is observed on synthetic phosphatidylcholines when lipid degradation is avoided. This is achieved by using highly purified melittin, phospholipase inhibitors, and/or non-hydrolysable lipids. (ii) Melittin monomer does not change T_m. (iii) When melittin tetramer is stabilized, it decreases T_m by 10–15 deg. C. The transition broadens, and is finally abolished for $R_i \leqslant 2$. Very similar results are found for natural sphingomyelin. Fluorescence polarization indicates similar changes in order and dynamics of the acyl chains for all lipid studied. For $T \leqslant T_m$, fluorescence and Raman show that melittin decreases the amount of CH_2 groups in *'trans'* conformation and the intermolecular order of the chains. According to fluorescence data, there is an increase of the rigid-body orientational order at $T \geqslant T_m$, while from Raman the positional intermolecular order decreases without significant change in the CH_2 groups *'trans'/'gauche'* ratio.

Introduction

Melittin is a well known amphiphilic peptide which accounts for half of the dry weight of bee venom [1] and whose structure is now solved down to 2Å resolution [2]. Since it interacts readily with lipids of both natural and synthetic membranes, it is becoming a popular model system to look at lipid-protein interactions.

Reviewing the recent literature concerning melittin effects on the thermotropic behaviour of lipids, puzzling and even contradictory results are found.

(i) No shift of the transition temperature (T_m) of dipalmitoylphosphatidylcholine (DPPC) was first observed by Mollay [3] and confirmed afterwards [4–6].

(ii) A dramatic increase in T_m of dimyristoylphosphatidylcholine (DMPC) was de-

Abbreviations: DMPC, DPPC, DSPC, respectively dimyristoyl-, dipalmitoyl- and distearoylphosphatidylcholine; lysoPC, lysophosphatidylcholine; T_m, transition temperature; R_i, incubation lipid to protein molar ratio; P, fluorescence polarization ratio.

tected by Raman spectroscopy [7,8] and further emphasized by other techniques [9,10]. This result, interpreted as the melting of boundary lipids [11], is almost unique among the abondant literature on lipid-protein interactions.

(iii) Finally for charged lipids, we observed that, depending on the chain length and the net charge at the lipid interface [12], melittin either induces a large decrease in T_m or the total abolition of the transition.

Since the value of a model system depends first on non-equivoqual experimental facts, and since melittin is proposed as a 'simple' system illustrative for lipid-protein interactions, this controversial situation urges to be clarified.

In this paper, we emphasized the consequences of the the main peculiarities of melittin systems. First, melittin acts synergistically with bee venom phospholipase A_2 [13,14] so that a great care has to be taken to avoid any phospholipase contamination. Although this has yet been claimed for a long time, it was often neglected. Second, the binding of melittin to zwitterionic phospholipids depends on their physical state, that is whether they form vesicles or dispersions, in the gel or liquid-crystalline state [10,15,16]. Third, the monomer/tetramer equilibrium in solution we proposed [17,18] is now well documented [19,20,21]. Therefore, depending on the experimental conditions, which are usually imposed by the technique used, one may be dealing with two different systems which have been shown to interact differently with lipids [19].

Materials and Methods

We have used either melittin available commercially from Bachem, Serva and Sigma, or melittin purified in the laboratory. As mentioned by manufacturers, in all cases residual phospholipase activity was detected. Melittin was purified both from the commercial samples and directly from the venom using an HPLC procedure related to that of De Grado et al. [22], and to those published during the course of this study [10,23]. Crude bee venom from Sigma (300 mg) is first dissolved in 3 ml buffer 1 (CH_3CN/H_2O, 56 : 44, v/v with 1% $KClO_4$), shaken and centrifuged, the upper phase being recovered. To the lower oily phase, 1 ml of buffer 1 and 0.5 ml of 20 mM Tris acetate (pH 7.5) are added. After centrifugation, the supernatant is recovered and pooled. In a second step, solutions are loaded on a semi-preparative Waters C18 reversed phase column, and eluted with buffer 1 at 1 ml/min. All early and late peaks are discarded while the major peak due to melittin is recycled three times. Pure methanol is flushed on the column after each run. Fractions corresponding to melittin peaks are pooled, lyophilized and desalted on Sephadex G-10 in 1 M acetic acid/acetonitrile (80 : 20, w/w). In a third step, melittin resuspended in water is treated by sulfite as proposed by Mollay [13], and is reinjected on the C18 column and processed as described in step 2. This whole procedure is done twice. Pure melittin is obtained as clear colorless solutions at 40 mM, it elutes in HPLC as a single sharp symmetrical peak. Its residual phospholipase activity was < 1 mU as measured on egg yolk phosphatidylcholine. The amino-acid composition after acid hydrolysis gave the following results: Thr 1.89 (2); Ser 0.97 (1); Glx 2.06 (2); Pro 0.97 (1); Gly 3.10 (3); Ala 2.09 (2); Val 1.95 (2); Ile 2.89 (3); Leu 4.17 (4); Lys 3.15 (3); Arg 1.94 (2). Cys, His, Met, Tyr, Phe were not significantly detected. This gives a 4% standard deviation for the purest melittin; without sulfite treatment, this deviation was approx. 9%.

DMPC, DPPC and DSPC were purchased from Sigma; the ether analog 1,2-ditetradecyl, *rac*-3-phosphorylcholine, i.e. DMPC diether was purchased from Medmark (Darmstadt, F.R.G.). Bovine brain sphingomyelin was from Lipid Products (Nutfield, U.K.). Diphenylhexatriene was obtained from Aldrich Chem. Co.

Calorimetric experiments were performed on a Dupont 990 differential scanning calorimeter as previously described [12], at a scanning rate of 5 deg. C/min. The samples were prepared by direct hydration in the DSC pans of about 1 mg of lipids by a concentrated melittin solution (30 mM) in a 100 mM phosphate buffer, pH = 7.5 in the presence or not of 10 mM EDTA. In order to ensure the homogeneity of the samples, they were incubated for 30 min at 37 °C and centrifuged several times at 3000 rpm, except for kinetic studies. Then, repetitive DSC scans were run until the obtained thermograms did not show any evolution. This

was generally achieved within a few hours with purified melittin in the presence of EDTA.

Fluorescence spectra were routinely obtained on either a Fika 55 MK II or an SLM 8000 spectrofluorometer. Fluorescence polarization experiments were done on a spectrometer built in the laboratory as previously described [12], at a scanning rate of 20 deg. C/h. Dried phospholipids ($\cong$ 10 mg) suspended in 1 ml of a 20 mM phosphate buffer (pH = 7.5)/1 mM EDTA, were incubated for a few minutes above T_m, and shaked on a vortex. A few microliters of a 6 mM solution of diphenylhexatriene in THF was added up to a probe/lipid molar ratio of 0.5% [24]. For measurements, aliquots of these dispersions were diluted in 3 ml buffer and after addition of appropriate amounts of concentrated melittin solution ($\cong$ 1 mM), samples were incubated for 20 min above T_m.

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 computerized spectrometer [25] with a spectral resolution of 5 cm^{-1}. The laser power at the sample was approx. 150 mW. The lipid-peptide samples were prepared as usually [26] by mixing equal volumes of a lipid dispersion at 20% (w/w) and a melittin solution of appropriate concentration to obtain the desired R_i value, both in 50 mM Tris-HCl buffer (pH 7.5).

Results

Effect of phospholipase activity on the thermotropism of DMPC

The effects of commercial and purified melittin on the phase behaviour of DMPC at a lipid to melittin molar ratio $R_i = 15$ with or without EDTA were first investigated by DSC. Repetitive scans were performed in this case immediately after mixing melittin and DMPC. It can be seen in Fig. 1b that with commercial melittin the sharp transition of pure DMPC gradually broadens and a new high temperature transition appears at about 35 °C. The amplitude of this transition increases until at the end the 24 °C one is abolished. In the presence of EDTA, which is a phospholipase A_2 inhibitor, no shift of the transition is detected, even after several hours (Fig. 1a). However, at time scales of 10 h, a significant modification of the high temperature region is always observed. The process observed without EDTA is thus severely slowed down

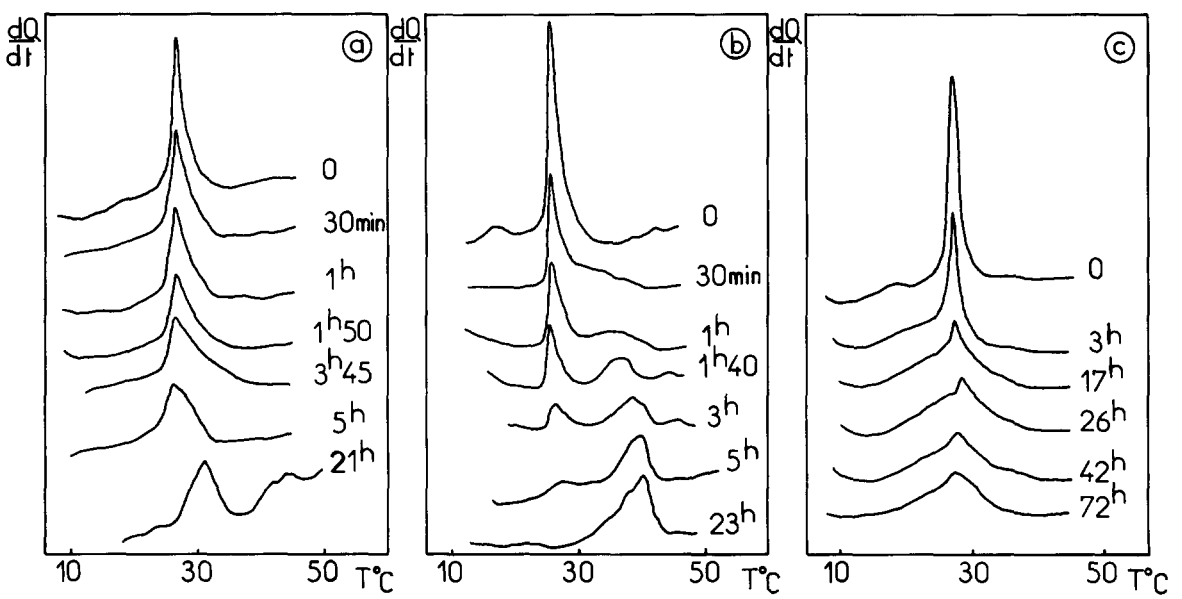
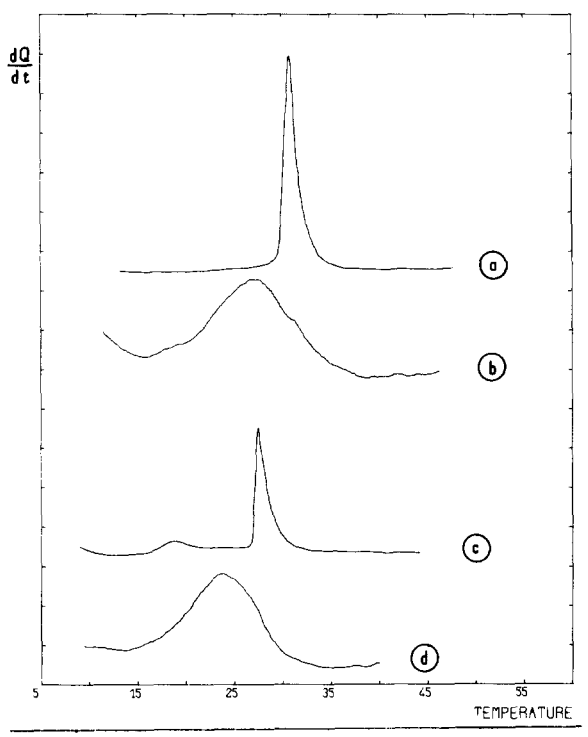


Fig. 1. Differential scanning calorimetry of DMPC in the presence of melittin at a lipid to protein molar ratio $R_i = 15$. Thermograms were recorded at various times, as indicated in the Figure, after mixing of melittin and DMPC in the DSC pan. Scanning rate = 5 deg. C/min. (a) Commercial melittin in the presence of 1 mM EDTA. (b) Commercial melittin without EDTA. (c) Purified melittin without EDTA.



but not totally inhibited.

Highly purified melittin under similar conditions but in the absence of EDTA does not lead to any shift of the transition towards high temperatures but a significant broadening of the transition occurs (Fig. 1c and 2). This broad endothermic transition starts at about 12–15 °C, i.e. 10 deg. C lower than for pure DMPC.

Using the analog DMPC diether, which is not a substrate for phospholipase, similar results were obtained without EDTA for the purest melittin as well as for those which were contaminated. At $R_i = 5$ the transition profile (Fig. 2) is constituted by a broad component which starts at about 15 °C. No kinetic leading to change in the high tempera-

Fig. 2. Differential scanning calorimetry of DMPC and DMPC diether in the presence or not of melittin at a lipid to protein molar ratio $R_i = 5$. Scanning rate = 5 deg. C/min. Thermograms are normalized to 1 mg of lipid. (a) Pure DMPC diether. (b) DMPC diether + melittin ($R_i = 5$). (c) Pure DMPC. (d) DMPC + melittin ($R_i = 5$). Thermograms (b) and (d) are plotted with a 5-fold higher sensitivity than curves (a) and (c).

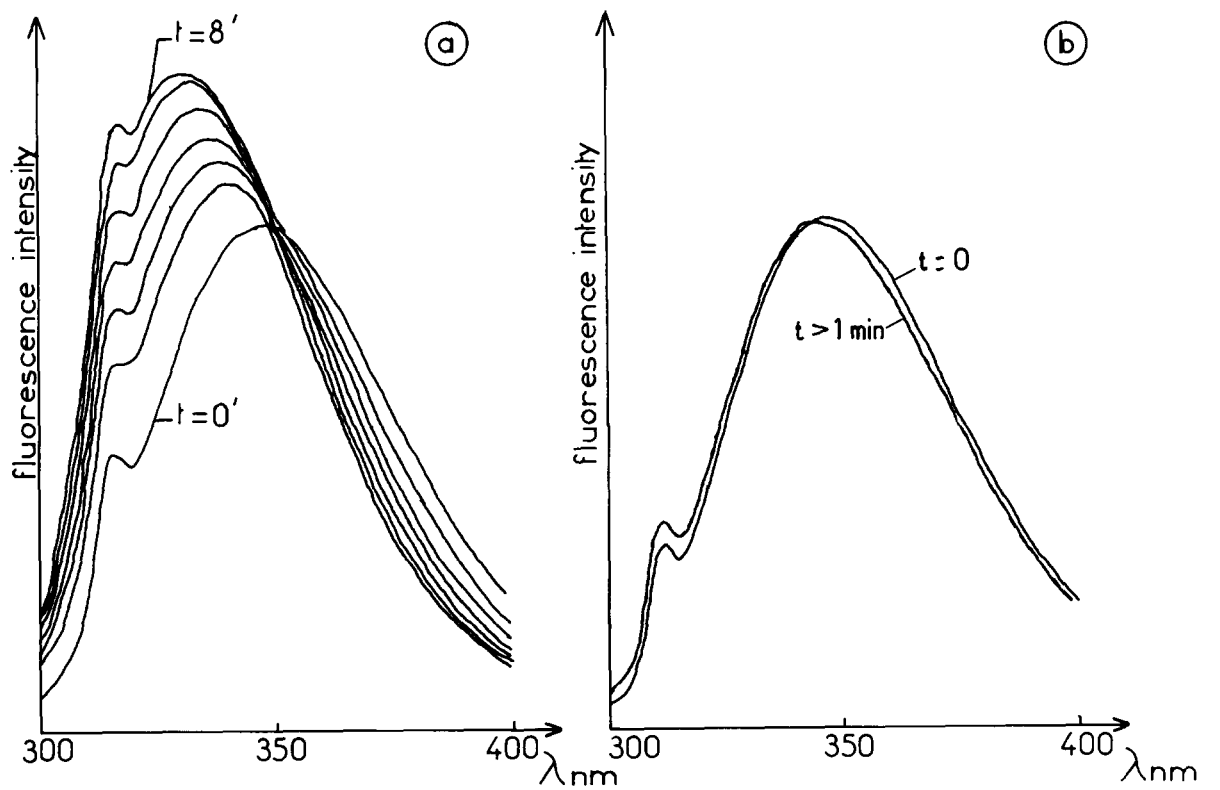


Fig. 3. Evolution of the fluorescence spectra of commercial melittin in the presence of (a) DMPC and (b) DMPC diether at a lipid to melittin molar ratio $R_i = 4$. Tris acetate buffer (pH = 7.5), $[Ca^{2+}] = 3$ mM, [melittin] = 7 μM. Temperature = 25°C.

ture range was detected.

Intrinsic fluorescence of melittin has already been used to monitor its binding to phospholipids [15,16]. Interaction of monomeric melittin with DMPC diether at low R_i values gives a small blue shift corresponding to a partial binding of melittin, since the amount of lipids is limitant [15]. This binding occurs readily and no further change of the fluorescence spectrum is observed even after prolonged incubation (Fig. 3b). The same results were obtained for highly purified melittin and DMPC in the presence and absence of EDTA.

On the contrary, for commerical melittin in the absence of EDTA, a slow kinetic occurs on a time scale ranging from minutes to hours, depending on the melittin origin, i.e. on the amount of phospholipase (Fig. 3a). The marked blue shift then observed in the tryptophan fluorescence reflects an increased binding of melittin to the occurring degradation products for which it has a higher affinity [15]. We yet proposed very similar conclusions for cardiotoxins interacting with phosphatidylcholine bilayers [27].

After completion of the experiments, the purity of the lipids of most samples was checked by thin-layer chromatography on silicic acid plates. When commercial melittin was used or for any time consuming experiments, lysoPC was detected as a contaminant which, in the extreme cases, can account for up to 50% of the lipids. Ternary mixtures of DMPC/fatty acid/lyso PC with increasing amounts of C_{14} lysoPC/fatty acid (1 : 1) were also studied by DSC. The transition temperature of these lipid mixtures increased from 24 to 35 °C as soon as the molar ratio of LysoPC reaches 0.3, and complex endothermic peaks were detected. In addition, the temperature profiles derived from the C-C and C-H stretching regions of the Raman spectrum of an equimolar mixture of lysoDMPC and myristic acid displayed a major transition at approx. 30 °C with a weaker one around 24 °C. All these results are in total agreement with those of Jain et al. [28,29] on similar mixtures.

Perturbations induced by monomeric and tetrameric melittin on the thermotropism of DMPC, followed by fluorescence polarization

All the fluorescence polarization experiments were performed with purified melittin in the presence of EDTA, at two fixed lipid concentrations ($1.2 \cdot 10^{-5}$ M and $1.2 \cdot 10^{-4}$ M). Such concentra-

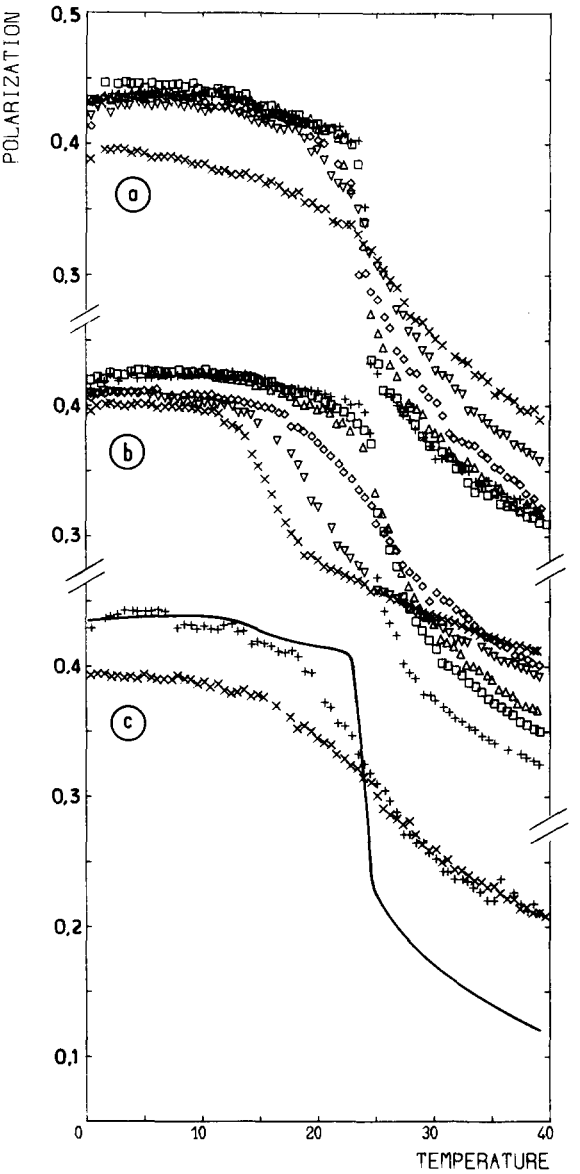


Fig. 4. Effect of temperature on the degree of fluorescence polarization P of diphenylhexatriene embedded in unsonicated dispersions of DMPC at different lipid to melittin molar ratios (R_i). 20 mM phosphate buffer (pH = 7.5)/1 mM EDTA.
(a) [NaCl] = 0 M, [DMPC] = $1.2 \cdot 10^{-5}$ M: (+) DMPC alone, (□) $R_i = 26$, (△) $R_i = 10$, (◇) $R_i = 5$, (▽) $R_i = 2$, (×) $R_i = 0.5$.
(b) [NaCl] = 2 M, [DMPC] = $1.2 \cdot 10^{-4}$ M: (+) DMPC alone, (□) $R_i = 30$, (△) $R_i = 16$, (◇) $R_i = 5$, (▽) $R_i = 2$, (×) $R_i = 0.5$.
(c) [NaCl] = 0 M, [DMPC] = $1.2 \cdot 10^{-5}$ M, $R_i = 0.5$: (+) first heating scan, (×) cooling scan. Solid line, pure DMPC.

tions allowed to keep melittin predominently in the form of monomer or tetramer over the whole range of R_i and temperature studied. This was checked by looking at the polarization and wavelength of tryptophan emission, which are very sensitive to the auto-association process [17–19].

The fluorescence polarization ratio P of diphenylhexatriene embedded in unsonicated dispersions of DMPC is plotted in Fig. 4 versus temperature at various R_i values. At low lipid concentration and ionic strength, melittin is mainly monomeric, and one can then observe that the transition temperature T_m of DMPC remains practically unchanged for R_i values ranging from 25 to 0.5 (Fig. 4a), while the pretransition disappears. Moreover P increases markedly in the liquid-crystalline phase whereas it monotonously decreases in the gel one. As seen in Fig. 5 this decrease of P in the gel phase remains very small down to $R_i = 5$, and becomes really significant only in the presence of a very large excess of melittin, i.e. at $R_i = 0.5$. This can explain the lack of effect recently reported by Jähnig et al. [6], since they have restricted their study to $R_i > 10$. Finally, no saturation effect is observed on the phase transition of DMPC in the presence of increasing amounts of melittin (Fig. 4a), thus, its total abolition at R_i values far below 0.5 cannot be ruled out.

At high lipid concentration and ionic strength, melittin is mainly in its tetrameric form. The perturbations then observed on unsonicated DMPC dispersions (Fig. 5) are similar to those obtained with melittin monomer both for $T \gtrless T_m$, although the changes in P are larger at high R_i values. Moreover, an important new feature occurs: the transition temperature progressively shifts down from 25 to about 12–15 °C for $R_i < 5$ (Fig. 4b). Finally, it is interesting to notice that the lipid suspension, initially very turbid, becomes quite clear in the presence of melittin, especially below T_m, which shows that the multilamellar onion-like lipid structures are probably changed into very small size particles.

All the above results were obtained using the melittin/DMPC mixtures incubated above T_m before performing the measurements. Identical results were then always obtained for successive heating and cooling scans done within 24 h after sample preparation.

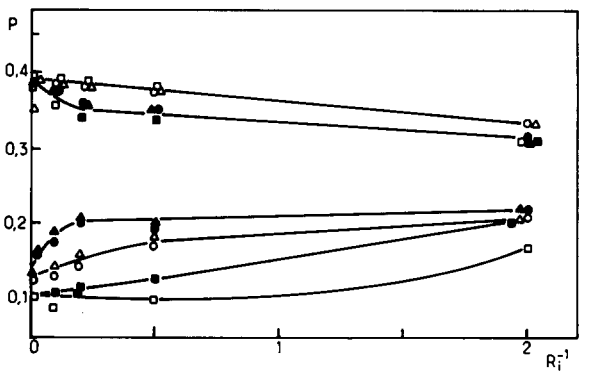


Fig. 5. Variation of the degree of fluorescence polarization P of diphenylhexatriene versus the melittin to lipid molar ratio (R_i^{-1}). Results obtained by pointing the P values either 10 deg. C above (lower curves) or below (upper curves) the transition temperature of the lipid. 20 mM phosphate buffer (pH = 7.5)/1 mM EDTA. (○) Unsonicated DMPC, (△) sonicated DMPC, (□) unsonicated DSPC. Open symbols: NaCl = 0 M. Filled symbols: NaCl = 2 M.

On the contrary, when the sample is not pre-incubated above T_m, the first heating scan in the presence of melittin at $R_i = 0.5$, is similar to that of the pure lipid up to about 15 °C, a temperature close to that of the pretransition of pure DMPC (Fig. 4c). In the liquid-crystalline phase, a large increase of P is observed. Upon cooling, the temperature profile differs from that of the first heating scan below T_m, an important decrease of P being observed in the gel phase. Further heating and cooling scans always lead to curves superimposable and identical to that in Fig. 4a.

Finally, the results obtained by fluorescence polarization at high ionic strength look similar to those obtained by DSC on DMPC at $R_i = 15$ and 5 (Figs. 1c and 2). This indeed has to be expected since, due to the experimental procedure to prepare DSC samples, the high melittin concentrations (20 to 30 mM in 100 mM phosphate buffer) lead to the stabilization of melittin tetramer [19]. However, it must be mentioned that no transition can be detected at $R_i = 2$ by DSC (data not shown). In addition, at R_i values higher than 10, a complex endothermic behaviour is generally observed: a sharp peak, centered at the transition temperature of pure DMPC, is superimposed to a low temperature broad transition. Such a behaviour is never clearly resolved by fluorescence polarization but

the existence of two partially overlapping transitions could account for the severe broadening observed at intermediate R_i values.

Unsonicated DMPC dispersions have been used in all the preceding experiments, so one can question the accessibility of the lipid molecules to melittin. Then, the same set of measurements has been carried out on sonicated DMPC vesicles which exhibit, in the absence of melittin, a broad phase transition due to their small curvature radius [30,31]. In the presence of melittin, even at high R_i values ($R_i = 30$), the observed changes of P (Fig. 5) are similar to those previously shown for unsonicated dispersions.

Perturbations induced by melittin on the thermotropism of DPPC, DSPC and sphingomyelin

In order to ensure some generalization of the effects observed on DMPC, fluorescence polarization experiments were performed on three other zwitterionic phospholipids, namely DPPC, DSPC and natural sphingomyelin. In the case of DPPC (Fig. 6) and DSPC (data not shown), the maximum effects induced by melittin on P for both the gel and the liquid-crystalline phases are in very good agreement with those obtained with DMPC. However, as shown in Fig. 5, the change of P versus R_i^{-1} above T_m is considerably less steep for DSPC than for DMPC, while similar changes are observed below T_m. Moreover, the temperature profile of P at high ionic strength (Fig. 6), for R_i values ranging from 5 to 2, seems to indicate the presence of two successive overlapping phase transitions.

In order to check a possible direct effect of ionic strength on the thermotropic behaviour of melittin tetramer-phospholipid complexes, some experiments were run at low ionic strength, i.e. 20 mM phosphate buffer, melittin tetramer being then obtained by using toxin concentrations in the millimolar range [17]. A decrease in T_m is again detected (Fig. 6c), but it is weaker than that observed at high ionic strength (Fig. 6b): at $R_i = 0.5$, the shift is only 5 deg. C instead of about 10 deg. C. Furthermore, the transition profile is more broadened than in 2 M NaCl, and large amounts of melittin ($R_i = 0.026$) almost abolished the transition, as also observed by DSC at $R_i = 2$.

Natural sphingomyelin, which is not a phos-

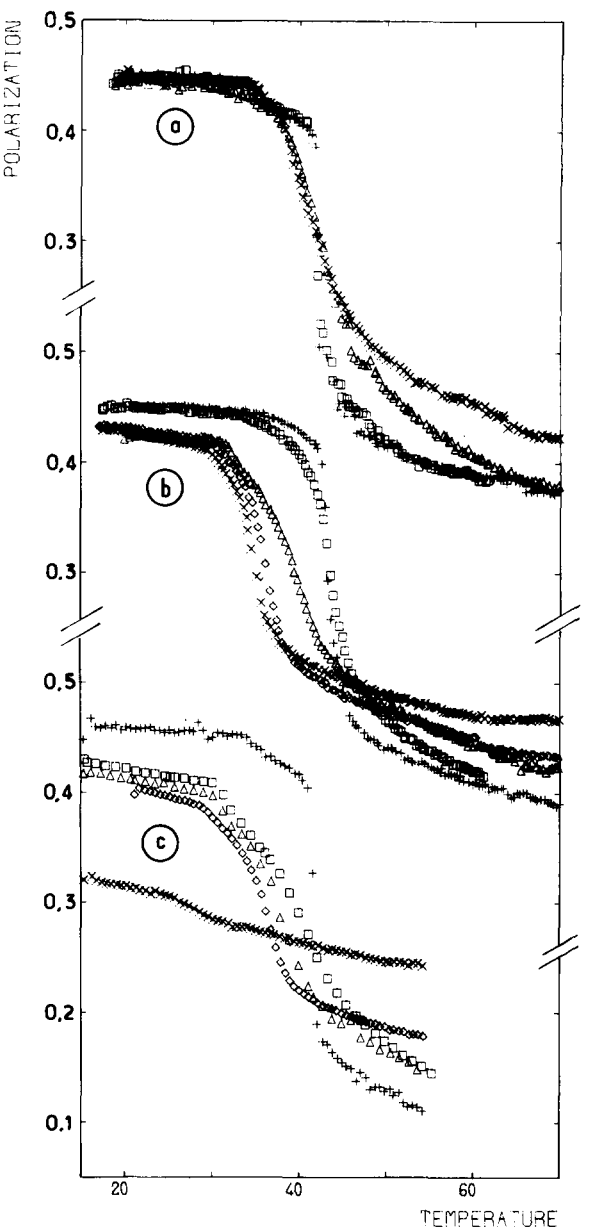


Fig. 6. Effect of temperature on the degree of fluorescence polarization P of diphenylhexatriene embedded in unsonicated dispersions of DPPC at different lipid to melittin molar ratios (R_i). 20 mM phosphate buffer (pH = 7.5)/1 mM EDTA.
(a) [NaCl] = 0 M, [melittin] = $1.2 \cdot 10^{-5}$ M, (+) DPPC, (□) $R_i = 15$, (△) $R_i = 4$, (×) $R_i = 1.6$.
(b) [NaCl] = 2 M, [melittin] = $1.3 \cdot 10^{-4}$ M, (+) DPPC, (□) $R_i = 27$, (△) $R_i = 3.7$, (◇) $R_i = 1.1$, (×) $R_i = 0.65$.(c) [NaCl] = 0 M, [melittin] = 1.45 mM, (+) DPPC, (□) $R_i = 5$, (△) $R_i = 2$, (◇) $R_i = 0.5$, (×) $R_i = 0.026$.

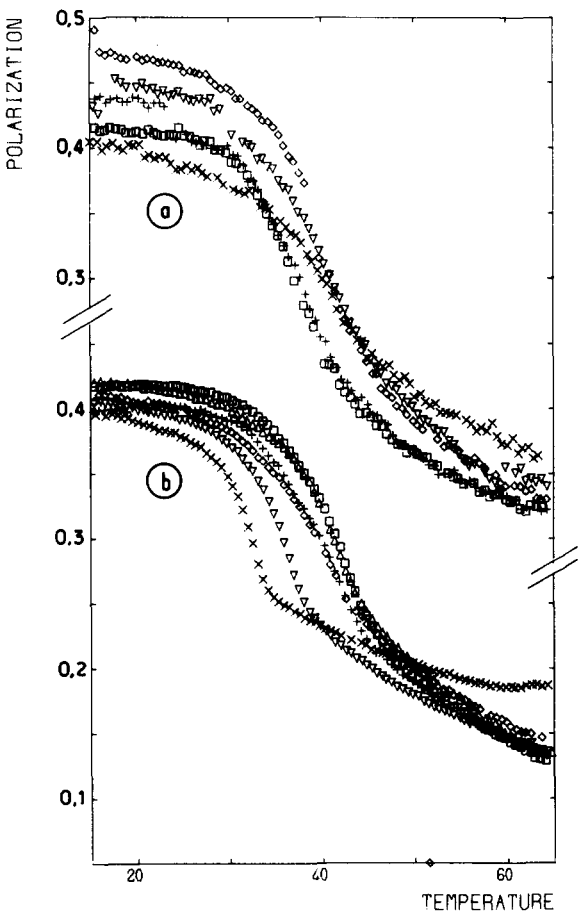


Fig. 7. Effect of temperature on the degree of fluorescence polarization P of diphenylhexatriene embedded in unsonicated dispersions of natural sphingomyelin at different lipid to melittin molar ratios (R_i). 20 mM phosphate buffer (pH = 7.5)/1 mM EDTA. (a) [NaCl] = 0 M, (b) [NaCl] = 2 M. (+) Sphingomyelin, (□) $R_i = 30$, (△) $R_i = 10$, (◇) $R_i = 5$, (▽) $R_i = 2$, (×) $R_i = 0.5$.

pholipase A_2 substrate, has a broad transition spreading from 30 to 40 °C [24,32,33]. It behaves quite similarly to phosphatidylcholines (Fig. 7), but the effects observed on P are weaker than for phosphatidylcholines, which could be due to the chain unsaturation. The broad transition of the pure lipid centered around 35–40 °C is shifted by about 3 deg. C to higher temperatures in the presence of melittin monomer at $R_i = 5$. A 1 deg. C upward shift seems also to occur with melittin tetramer, but only at R_i values greater than 10. Further melittin addition always leads to a decrease in T_m by about 9 deg. C.

Perturbations induced by melittin on the thermotropism of DMPC followed by Raman spectroscopy

Due to the high concentration required to obtain good Raman spectra (10% by weight), melittin is always tetrameric. This was clearly evidenced from the Raman spectrum of pure melittin in solution that shows that the toxin is mainly in the α helical conformation. In the presence of melittin, the Raman spectrum of DMPC undergoes several changes. Fig. 8 shows the temperature dependence of the C-C and C-H stretching regions for pure DMPC dispersions compared to that of melittin/DMPC complexes at $R_i = 10$.

The C-C skeletal optical mode region (960 to 1200 cm^{-1}) provides direct information about the intramolecular order of the aliphatic chains of DMPC [34–36]. The spectrum of pure DMPC at 5 °C (Fig. 8a) displays three well defined bands at 1062, 1040 and 1128 cm^{-1} that are characteristics of segments of the all-*trans* conformation in the highly ordered acyl chains. As the temperature is raised, the intensity of the 1062 and 1128 cm^{-1} bands decreases and simultaneously the 1090 cm^{-1} band shifts to 1079 cm^{-1} and increases in intensity, thus reflecting the growing population of *gauche* conformations.

The effect of melittin on the C-C region of the spectrum of DMPC differs whether the lipid is in the gel or liquid-crystalline state (Fig. 8a). At low temperature, melittin increases the number of *gauche* bonds while at temperatures above T_m, practically no change is observed. This is also clearly seen on the temperature profiles obtained from the h_{1079}/h_{1120} and h_{1079}/h_{1062} ratios taken as spectral indexes of intrachain disorder (Fig. 9). Moreover on these curves only a single transition for the melittin/DMPC complex is always observed without any transition around 30 °C as opposed to what reported on previous Raman studies [7,8]. The remaining transition is broad in the presence of the toxin and starts around 10 to 12 °C which is in fairly good agreement with the above DSC and fluorescence data.

Difference spectra also allows to estimate the effect of melittin on the conformation of the acyl chains. In the C-C region the difference spectrum at 3 °C between the pure DMPC dispersion and the melittin/DMPC complex at $R_i = 10$ is very similar to that obtained by performing the dif-

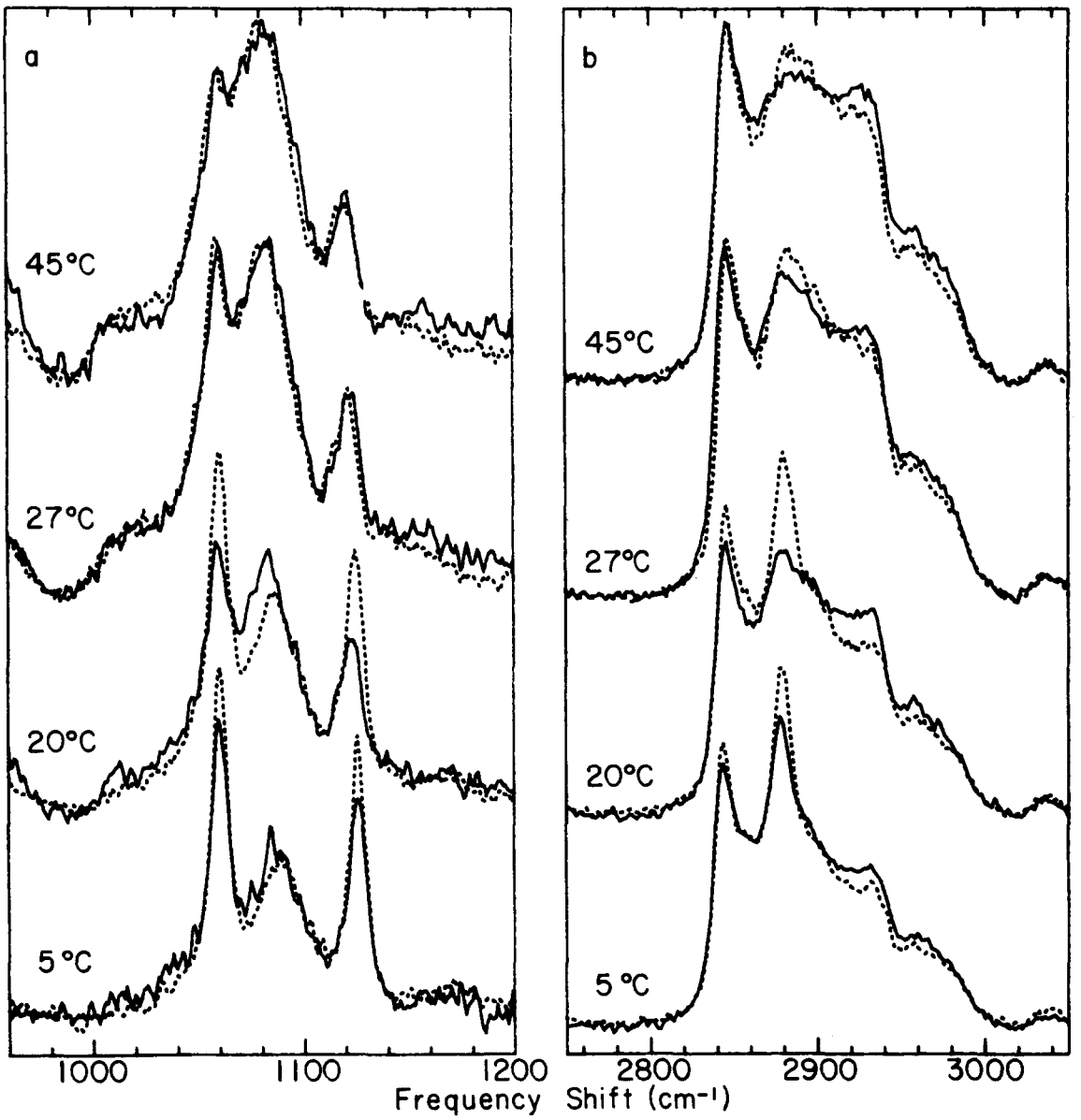


Fig. 8. Effect of melittin on (a) the C-C and (b) the C-H stretching mode regions of the Raman spectra of a pure DMPC dispersion (dashed line) and of a DMPC/melittin complex at $R_i = 10$ (solid line) at different temperatures. Each spectrum is an average of 2 scans at 2 s/cm^{-1} for the C-C region and 1 scan at 1 s/cm^{-1} for the C-H region. In addition, spectra of the complex in the C-C region are five point smoothed. Spectra at a given temperature were plotted in such a way to have the same surface.

ference spectrum for pure DMPC between the gel state at 3°C and the $P_{\beta'}$ phase at 20°C. Therefore, the intramolecular disorder induced by melittin tetramer at $R_i = 10$ in the gel phase of DMPC at 3°C is comparable to the disorder occurring at the pretransition. However, such difference spectra differ significantly in the C-H stretching mode region (2750 to 3050 cm^{-1}) and in the CH bending mode region (1400 to 1500 cm^{-1}). Since the latter region is sensitive to the lateral packing of the acyl chains [37,38], one has to conclude that the effect of melittin on the chain packing of

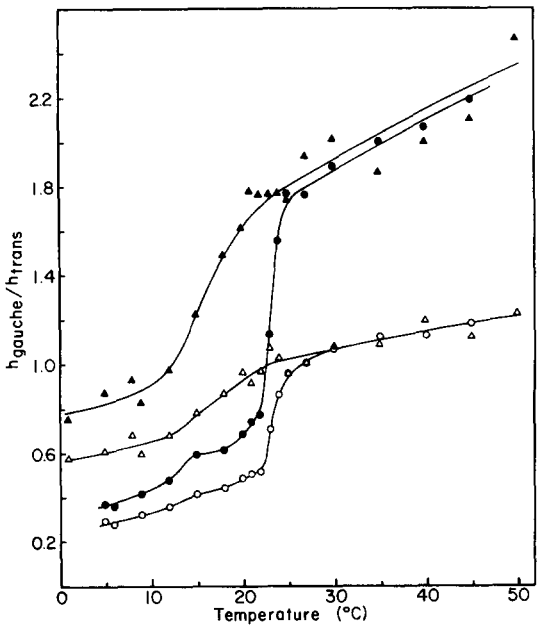


Fig. 9. Temperature profile of pure DMPC (○) and DMPC/melittin at $R_i = 10$ (△) derived from the Raman spectral h_{1079}/h_{1120} (filled symbols) and h_{1079}/h_{1062} (open symbols) peak height intensity ratios.

DMPC at 3°C is much more pronounced than the change observed at the pretransition for pure DMPC.

The C-H stretching vibrations of the hydrocarbon chains of DMPC are also sensitive to the presence of melittin (Fig. 8b). But as opposed to what is observed for the C-C region (Fig. 8a), the perturbation occurs in the whole temperature range. Among the spectral changes detected, the approx. 2880 cm^{-1} band, assigned to the antisymmetric C-H stretching vibration, broadens and its peak intensity decreases in the presence of melittin. This broadening depends on several factors, such as interchain vibrational interactions [39], conformational disorder [37] and chain rotational mobility [40]. At the opposite, the 2930 cm^{-1} band intensity increases in the presence of melittin. This change is attributed to an underlying infrared active CH_2 asymmetric stretching mode that becomes Raman allowed as the chain symmetry is lowered [41]. Therefore, the h_{2930}/h_{2880} intensity ratio, is an index of the overall disorder of the lipid acyl chain matrix.

Fig. 10 displays the temperature profiles obtained from the h_{2930}/h_{2880} ratio for pure DMPC and for DMPC/melittin complexes at $R_i = 10$ and 4. As for the C-C region (Fig. 9), only a single transition is observed in the presence of melittin, but in this case it is so severely broadened that it is very difficult to define a transition temperature, especially at $R_i = 4$. These results qualitatively agree with the previously reported ones [8–11] except that we do not observe any transition at high temperature, above T_m, in the presence of melittin. The h_{2930}/h_{2880} ratio increases markedly when melittin is added to DMPC, thus showing an increase of the overall disorder of the system in the gel phase as well as in the liquid crystalline one. At $R_i = 4$, the measured ratios have to be corrected for the peptide contribution. This correction does not change the temperature profile but only the absolute value of the intensity ratio, and it is negligible for the $R_i = 10$ complex.

The whole Raman spectra of DMPC/melittin complexes was also examined in order to detect DMPC-bound melittin bands. At $R_i = 10$ they were

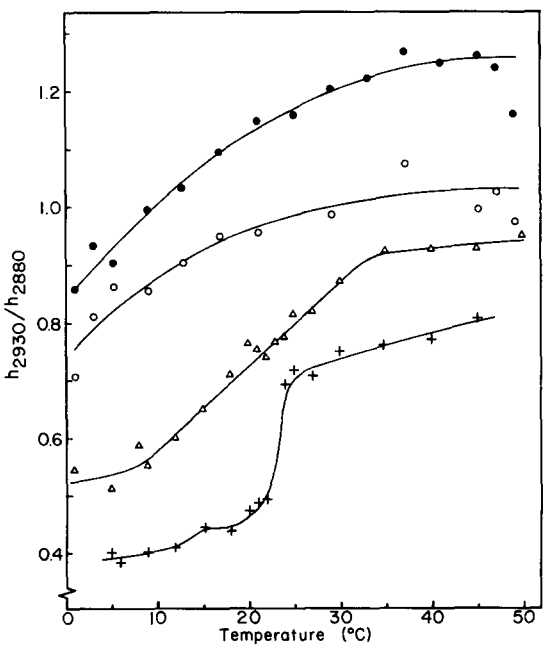


Fig. 10. Temperature profiles of pure DMPC (+) and of DMPC/melittin complexes at $R_i = 10$ (△), $R_i = 4$ (●) and $R_i = 4$ after correction for the protein contribution (○) derived from the Raman spectral h_{2930}/h_{2880} peak height intensity ratio. The corrected spectrum was computed using the 700 to 800 cm^{-1} region as an intensity standard.

very weak and only the amide I band was resolved. However, at $R_i = 4$, bands at 760, 1010, 1330 and 1550 cm^{-1} arising from tryptophan, and the strong amide I band at 1650 cm^{-1}, were observed and did not vary with temperature. From the amide I frequency, one can conclude that bound melittin is mainly in the α-helical conformation, in agreement with previous ORD and CD data [1,19]. In addition, this conformation is not affected by the phase transition of DMPC.

Discussion

The results presented here illustrate the pitfalls of simple model systems and allow to reinterpret and rationalize most of the work done on the perturbations of the thermotropic properties of phosphatidylcholines induced by melittin.

First, the shift in T_m to higher temperature previously reported [7–10] and attributed to the melting of 'boundary lipids' surrounding the peptide, is not due to any direct effect of melittin, but is the consequence of lipid degradation due to phospholipase A_2 contamination. So, one must again emphasize that the greatest care must be taken in order to prevent this artefact. This can be achieved by using: (i) highly purified melittins, (ii) phospholipase inhibitors such as EDTA (iii) lipids which are not substrates, i.e. DMPC diether and sphingomyelin.

Dealing now with the effects of melittin itself on the thermotropic behaviour of phospholipids, it appears clearly that they are governed by two basic parameters, namely the auto-association state of melittin and the amount of melittin effectively bound to the lipid structure.

If one assumes that lipid perturbations are related to the amount of bound melittin, the results presented here above, together with those of previous studies [15,18], allow to propose the following picture for the binding of melittin to phosphatidylcholines. First, it appears that the lipid-peptides structures formed on isotherm bindings are greatly depending of the supramolecular assembly of the lipid, i.e. single-shell vesicles or unsonicated dispersions. Indeed, vesicles readily bind melittin, even in the gel state [15], whereas dispersions only interact around and above T_m as it can be inferred from Fig. 6, and has equally been checked by intrinsic fluorescence (data not shown). On the contrary, identical results are obtained when the samples are first incubated above T_m, then cooled (Fig. 5).

So, it seems that metastable PC/melittin complexes are formed on isotherm binding and that the thermodynamic equilibrium is reached only on going through the lipid transition. This could induce a disruption of the vesicular structure or an increased binding to dispersions, which would finally lead to identical PC/melittin structures, whatever the initial state of the lipid is. The reproducibility of the results on successive heating and cooling scans supports this conclusion. One can recall that such conditions correspond to a maximal density of lipid structural defects [42] which have been proved to ensure a higher binding efficiency for membrane proteins [43] and apolipoproteins [44].

Another important feature concerning the binding of melittin to PC is that it is reversible and can be described by dissociation constants K_d. K_d values are of the same order of magnitude as the concentrations used in fluorescence, but vary noticeably according to the experimental conditions: K_d is larger in the gel phase than in the fluid one [15,16], and for melittin monomer compared to tetramer (Fig. 5), although this could be also due, at least in part, to an ionic strength effect as it has been observed on phosphatidylcholine black membranes [45].

Such an equilibrium provides a simple explanation for the apparent discrepancies observed between the three different techniques used. Indeed, fluorescence requires diluted solutions in the micromolar range, whereas DSC and Raman spectroscopy need about 10^3–10^4 higher concentrations. So, at a same R_i value, most of the melittin does interact with lipids when studied by DSC and Raman, whereas in fluorescence only a weak fraction of melittin is really bound, explaining why no saturation effect is detected even at $R_i = 0.5$.

The binding of melittin to phosphatidylcholine is therefore strikingly different from that reported with negatively charged phospholipids [12]. In that case, electrostatic effects are the major driving force to form complexes where charge complementarity of melittin and lipids is fullfilled. This leads to such a high affinity between the two

partners that stoichiometric binding happens in all cases: high and low concentrations, gel and liquid-crystalline phases, vesicles and dispersions, monomeric and tetrameric states [12]. This allows direct comparison of experiments done with different techniques. The screening of the net charge of melittin and dipalmitoylphosphatidylglycerol by 2 M NaCl further reinforces such a picture. Instead of the phase separation yet observed [12], a behaviour similar to the one reported here above for DPPC occurs (data not shown).

Let us now consider the perturbations induced by melittin on the order and dynamics of the aliphatic chains. In all cases, a decrease of the order is detected in the gel phase from diphenylhexatriene fluorescence polarization, and Raman spectroscopy indicates an important increase in the number of *'gauche'* conformers, in agreement with results yet reported by Jähnig et al. [6]. This also agrees with the decrease of the intermolecular coupling of C-H vibrations, reflecting a positional disorder in the arrangement of the chains from the regular hexagonal packing with more spacing between the chains. At the opposite, in the liquid-crystalline phase no effect is detected by Raman spectroscopy on the C-C vibrations while from fluorescence it is concluded that the order increases. Such a discrepancy between both techniques has yet often been described in the case of lipid-protein systems, and it could arise, as previously discussed [26], from several reasons: the time scales of these two techniques, the localization of the diphenylhexatriene probe, and the intrinsic difference, at the molecular level, between the measured 'orders'. Raman spectroscopy would be related to the conformational order the lipid chains, whereas fluorescence polarization is sensitive to their rigid-body orientational order [6]. Then, in the fluid phase, melittin does not change the *trans-gauche* ratio but it decreases the amplitude of the wobbling motion of the whole chain.

Another aspect of the perturbations induced by melittin concerns its effect on the lipid thermotropism. At R_i values greater than 30, the main observed effects are always the abolition of the pretransition and the breakdown of single-shell lipid vesicles. Only weak effects are detected on T_m and on the transition width, which agrees with the foremost results of Mollay et al. on DPPC [3] and with those of Jähnig et al. [6]. This also agrees with the recent study of Posh et al. [46], who observed significant changes in the molar volume of the lipid and so emphasized the perturbation induced by very low amounts of melittin.

With monomeric melittin, increasing the amount of bound peptide severely broadens the transition without noticeable change change in T_m. Again this agrees with the results of Jähnig et al. [6] obtained by fluorescence polarization and Raman spectroscopy, although the peculiar procedure used for the preparation of their Raman samples does not allow to specify the state of auto-association of melittin.

In the experimental conditions where melittin tetramer is stabilized, a broadening of the transition without change of T_m is first observed by fluorescence polarization, then a progressive lowering of T_m by 10–15 deg. C in 2 M NaCl and smaller at low ionic strength occurs. For DMPC, DSC and Raman results also lead to similar conclusions. The resulting transition has an enthalpy lower than that of pure lipid, $\Delta H \cong 2$–3 kcal, and its cooperativity is severely decreased. Moreover, the clearing of the solutions indicates the breakdown of large particles (unpublished results). Similar features were yet described for other amphipatic peptides [48,49]. This has also to be paralleled to the rouleaux and disc-shaped structures yet observed by Prendergast et al. [10], while in this case, phospholipase activity could perhaps account for their occurence at very high R_i values.

A further increase in the amount of bound tetrameric melittin finally enables to induce the total abolition of the transition as detected by DSC and fluorescence polarization. This could be related to the formation of mixed micelles of melittin tetramer swollen by a few lipid molecules as yet proposed by Talbot et al. [19]. The Raman results at $R_i = 4$ also reinforce this hypothesis since the corrected h_{2930}/h_{2880} ratios are very close to the values measured for micelles of lysoPC at 20 °C (unpublished results).

For natural sphingomyelin the weak upward shift first observed at high R_i values, probably reflects a preferential perturbation of the shorter and/or more unsaturated species which have the lower T_m. On increasing the amount of melittin bound, all lipids become perturbed and T_m de-

creases similarly to what described for synthetic phosphatidylcholine.

The effects observed on the thermotropism of zwitterionic phospholipids could be accounted for by assuming a detergent-like behaviour of melittin. As increasing amounts of peptide are added to the lipid suspension, melittin could be first incorporated into the lipid bilayer then a lamellar-micellar phase transition could occur and, at last, further increases of melittin amount could lead to peptide-enriched micelles of decreasing size, until mixed micelles of melittin tetramer swollen by a few lipid molecules have been reached. Similarities between physico-chemical properties of melittin and detergents have been stressed for a long time [52], however, it must be recalled that important differences have also been emphasized [53], one of them being the concentration range used.

Finally, one can ask for the reasons of the difference in behaviour of the two states of melittin. This could be due to the intrinsic structural differences between melittin monomer and tetramer. However, the major reason certainly originates from the experimental conditions required to stabilize the tetramer, which are per se sufficient to explain its better binding and, consequently, a larger perturbation of the bilayer, at a given R_i: indeed, high melittin concentration can allow to overcome the repulsive forces due to the net positive charge developped at the interface when melittin binds to zwitterionic lipids, while high ionic strength totally screens such electrostatic repulsion.

Another important question to be answered is the relevance of melittin as a model peptide in the general problem of interactions between lipids and membrane proteins. Intrinsic membrane proteins generally induce a broadening of the transition: the mid-point transition temperature remains generally unchanged, although the melting of protein-enriched domains starts at a lower temperature [54]. It has also recently been reported by Lentz et al. [55] that, in the case of Ca^{2+}-ATPase, a new broad transition appears well below that of pure lipid at low lipid to protein molar ratios. So, the perturbation induced by melittin on the lipid thermotropism looks similar to that observed with membrane proteins. Furthermore, Jähnig et al. [6] have recently drawn up an "unifying description" of the effect of membrane proteins on lipid order based on spectroscopic data obtained with melittin in the fluid lipid phase. It is suggested that melittin induces a tilt of about 20–30° on the lipid boundary chains. Our experimental results also support this proposal, since they agree well with those of Jähnig et al. [6]. Furthermore as previously shown in the case of phosphatidylglycerols [56], such a change in the tilt angle should lead to a decrease in T_m, which actually occurs with tetrameric melittin. It could also induce a decrease in the bilayer thickness, resulting in an enhancement of the permeability [57]. However, it is assumed in this model that all the incorporated melittin molecules span the lipid bilayer, which seems to be unlikely taking into account the reversibility of the interaction we observed when melittin is added to preformed lipid suspensions. Furthermore, a wedge-effect as previously proposed in the case of both detergents [50] and melittin [58] could well account for the formation, with zwitterionic phospholipids, of small-size particles when the amount of bound melittin is sufficient.

In conclusion, melittin displays a wide variety of interactions with lipid structures, depending on the experimental conditions. Then, they have to be rigorously controlled and varied in a very broad range in order to acquire an overall picture, and it seems unlikely that a single molecular model allows to account for all the observed phenomena. Melittin could thus illustrate many kinds of situations encountered in biological systems and in anyway, it provides from a physico-chemical point of view, an attractive model to elucidate the mechanism of lipid-protein interactions at the molecular level.

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