Ultrasonic Study of Melittin Effects on Phospholipid Model Membranes

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ABSTRACT Low dose effects of melittin on dilute suspensions of dipalmitoylphosphatidylcholine multilamellar vesicles are investigated by studying the acoustic properties of the system. The temperature dependencies of sound velocity and absorption have been measured at 7.2 MHz in the temperature range of 20–55°C, for different peptide/lipid molar ratios, R. The most pronounced effects were observed at $R = 5 \times 10^{-3}$, in the vicinity of the pretransition, with a simultaneous increase in sound absorption and velocity. This indicates that melittin affects the polar head group region of the bilayer resulting in a decrease in mobility of the polar head groups. A nonmonotonic dependence of the main transition temperature, with an initial decrease followed by an increase as melittin is added, is interpreted as a consequence of a destabilizing action of the interfaces between melittin-affected clusters and the unaffected phase.

INTRODUCTION

The study of the acoustic properties of model membranes provides a powerful technique to investigate the kinetics of lipid phase transitions (Mitaku et al., 1978; Mitaku and Dale, 1982). In a recent work (Kharakoz et al., 1993), it has been shown how this method, together with a simple model based on Frenkel's theory of heterophase fluctuations in the vicinity of first order phase transitions (Frenkel, 1947; Ubbelohde, 1978), can be used to estimate important thermodynamic and kinetic quantities; most importantly, the interphase line tension between a new phase and the parent phase can be estimated for bilayer systems.

Contrary to some widely used thermodynamic techniques like differential scanning calorimetry (Papahadjopoulos et al., 1976; Jain and Wu, 1977), ultrasonic methods have not been extensively applied to study the effects of biological active molecules on natural or model membranes (Sakanishi et al., 1979; Hianik et al., 1992).

In this work, we apply this technique for the investigation of the effects of melittin on 1,2-dipalmitoylphosphatidylcholine (DPPC) multilamellar systems. Melittin is a small peptide composed of 26 amino acids, for which the sequence is shown below, and is the main component (50% weight) of bee venom (Habermann and Jentsch, 1967; Habermann, 1972).

NH_3^+-**Gly**-Ile-**Gly**-Ala-Val-Leu-**Lys**$^+$-Val-Leu-

Thr-**Thr**-**Gly**-Leu-Pro-Ala-Leu-Ile-**Ser**-Trp-Ile-

Lys$^+$-**Arg**$^+$-**Lys**$^+$-**Arg**$^+$-**Gln**-**Gln**-$CONH_2$

In solution, melittin may exist either as a monomer or tetramer, depending on the concentration and solvent properties such as ionic strength and pH (Quay and Condie, 1983). Both structures have been determined to high resolution by x-ray crystallography and nuclear magnetic resonance techniques (Terwilliger and Eisenberg, 1982; Bazzo et al., 1988). Because of its amphiphilic nature, which is manifested in both the primary and secondary structure (amphipathic α-helix; Kaiser and Kezdy, 1987), melittin binds readily to membranes. Upon binding to bilayers, melittin seems to be mostly in the monomeric form (Hermetter and Lakowicz, 1986; Lauterwein et al., 1979; Lavialle et al., 1982). Only at very special conditions (high salt concentrations and melittin/lipid molar ratios above 1/200) has aggregation of the peptide been observed in membranes (John and Jähnig, 1991). The way melittin inserts into the bilayer, i.e., (i) parallel to the membrane plane (Brauner et al., 1987), (ii) parallel to the membrane normal (Vogel, 1987), or (iii) bound to the membrane surface in a wedge-like form, without spanning the bilayer (Maurer et al., 1991), seems to depend on specific characteristics of the system, such as lipid composition and aggregation state of the lipids, i.e., multilamellar or unilamellar and monolayer or bilayer, respectively. It has been reported by Frey and Tamm (1991) that the orientation of melittin in model membranes also depends on the degree of hydration of the system.

The biological, i.e., cytolytic, effects of melittin are observable already at very low peptide/lipid ratios (Sessa et al., 1969; Habermann, 1972). The physicochemical basis for these effects, however, is still not sufficiently clear. Concerning the nature of melittin at these low concentrations, the studies by Talbot et al. (1979, 1982) and Lauterwein et al. (1979) show that the peptide acts most probably as a monomer. At such peptide/lipid ratios, calorimetric (Mollay, 1976), densitometric (Posch et al., 1983), and x-ray diffraction studies (Jähnig et al., 1982) of saturated diacylphosphatidylcholine multilamellar systems indicate that the pretransition gradually disappears when the peptide is added. In 1,2-dihexadecylphosphatidylcholine systems (ether analog of DPPC) a nonlamellar ordered structure is induced at peptide/lipid molar ratios $R = 10^{-3}$ (Colotto et al., 1991).

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In the present ultrasonic study, these low dose effects are also observed. The changes in the acoustic parameters indicate that the peptide effects on the bilayers are also related to relaxational processes connected to dynamic properties of the system. Particularly significant are the changes in the temperature dependence of the ultrasound absorption and velocity in the range of the gel phases, $L_{\beta'}$ and $P_{\beta'}$. This is in agreement with observations made through other techniques (see above). Two possible interpretations of the characteristics of the acoustic properties measured are discussed. The first one refers to acoustic relaxation only and can be understood as an increase in the relaxation time of the head group rotation. The second one is based on the appearance of an additional new state in this temperature range.

MATERIALS AND METHODS

DPPC, synthetic, puriss. (99%), was purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Melittin, from bee venom, was obtained commercially from Serva (lot 24029; Heidelberg, Germany) and also used without further purification. Purity of the peptide regarding phospholipase A_2 contamination was assayed by incubation of melittin with 1-palmitoyl-2-oleoyl-phosphatidylcholine at a peptide/lipid molar ratio of $R = 0.01$, at 45°C for 24 h. Aliquots of these samples where taken and analyzed by thin layer chromatography using $CHCl_3/CH_3OH/NH_{3conc}$ (65:35:5, v/v) as solvent. Only a single spot could be detected, indicating integrity of the samples. Thin layer chromatography has also been used to check the purity of DPPC before and after experiments to confirm the absence of hydrolytic products.

Sample preparation

Multilamellar lipid-peptide samples were prepared by dissolving the lipids in $CHCl_3/CH_3OH$ (2:1, v/v) and by adding aliquots of melittin dissolved in the same solvent to obtain a homogeneous solution, resulting in the desired peptide/lipid molar ratio, R. The solutions were then dried under a stream of N_2 and finally in a vacuum overnight. Subsequently, deionized water was added to the dry samples, which were then incubated at 45°C for about 4 h. In order to obtain homogeneous hydration, the suspensions were vortexed periodically during this time. The final lipid concentration was 2.3 wt.%; because of experimental limitations, it was not possible to measure samples with higher lipid concentration. Samples were degassed under a water pump vacuum for about 1 min before measurement to prevent bubble formation during the experiment. The multilamellar nature of the samples under all conditions covered in the present investigations has been verified by x-ray diffraction (Colotto, 1993).

Ultrasonic measurements

Ultrasonic velocity and absorption were measured simultaneously in the same sample with a differential fixed-path interferometer using the acoustic resonators described by Sarvazyan (1982). The ultrasonic frequency ranged between 7.0 and 7.4 MHz (data have been interpolated to correspond to a single reference frequency, $f = 7.2$ MHz). The resonator cells used required a minimum sample volume of 0.8 ml. The distance between the transducers was 0.8 cm, and their diameter was 0.7 cm. The fundamental frequency of the piezotransducers was 10 MHz.

The frequency, f, and the half-power width, δ, of a resonance peak of the acoustic resonator are related, respectively, to sound velocity, u, and absorption per wavelength, $\alpha\lambda$, by (Eggers and Funck, 1973):

$$\Delta u/u = (\Delta f/f) \times (1 + d) \tag{1}$$

and

$$\Delta\alpha\lambda = \Omega \times (\Delta\delta/f) \tag{2}$$

where Δu and Δf are changes in sound velocity and resonance frequency, respectively, caused by a change in the measured liquid, d is a correction term which is negligible in the resonators used in this work ($d < 0.003$) (Sarvazyan and Chalikian, 1991), and Ω is the calibration coefficient for sound absorption. For an ideal resonator $\Omega = \pi$, if the peak width is measured at the half-power level of the peak. For a real system the value Ω has to be obtained by calibration. The calibration was performed by using solutions of known ultrasonic absorption, $MnSO_4$ and $Zn(CH_3COO)_2$ (data taken from Stuehr and Yeager, 1965). The maximum error for the absorption measurements was about 7% (Kharakoz et al., 1993).

The precision of sound velocity measurements depends on the experimental conditions. When sound velocity is measured in a liquid with low sound absorption (like water) and at a single temperature, the relative precision is about $\pm 2 \times 10^{-4}$%. In the case of a temperature-dependent measurement, the reproducibility of the baseline (when both cells are filled with water) is within $\pm 1 \times 10^{-3}$% and can serve as an estimate of the precision of the experiment. Reproducibility of the baseline for absorption is the same as that at a single temperature, about 1%.

Temperature dependencies of sound velocity and absorption were measured by changing the temperature step-by-step: after setting a temperature, the sample was equilibrated (controlled by the variation in sound velocity), and data were taken. The procedure was repeated for each new temperature. The total time for a single step was about 5 min.

The results are given in terms of the velocity number, $[u]$, and absorption number, $[\alpha\lambda]$, defined as:

$$[u] = (u - u_o)/u_o c \tag{3}$$

and

$$[\alpha\lambda] = (\alpha\lambda - \alpha\lambda_o)/c \tag{4}$$

where c is the concentration of the solution expressed in [g/cm^3] and the index o refers to pure water.

Characteristics of sound velocity and absorption numbers

The sound velocity number consists of two parts: an instantaneous, $[u]_\infty$, which is mainly defined by the stiffness of the intermolecular interaction potentials, and a relaxational part, $[u]_r$, caused by pressure-induced changes in the structure of the system (e.g., in size distribution of heterophase fluctuations):

$$[u] = [u]_\infty + [u]_r \tag{5}$$

Similarly, the sound absorption number consists of classic absorption, $[\alpha\mu]_{class}$, which is due to shear viscosity (negligible for dilute lipid suspensions in the MHz frequency range used in this work (Sarvazyan and Chalikian, 1991) and relaxational absorption, $[\alpha\lambda]_r$, caused by energy dissipation in relaxation processes taking place in the system:

$$[\alpha\lambda] = [\alpha\lambda]_r + [\alpha\lambda]_{class} \approx [\alpha\lambda]_r \tag{6}$$

Moreover, for the ith relaxation process, the following relation between $[\alpha\lambda]_{r,i}$ and $[u]_{r,i}$ gives an expression for the relaxation time:

$$\tau_i = \frac{-1}{2\pi\omega} \cdot \frac{[\alpha\lambda]_{r,i}}{[u]_{r,i}} \tag{7}$$

which in the case of more than one relaxation processes

occurring in the system, becomes an effective quantity, τ_{eff}, that is, an average characteristic of the whole set of relaxation processes being detected at a single frequency (Kharakoz et al., 1993). It should be noted that this value can significantly differ from the apparent relaxation time, which is determined usually from the frequency of the maximum absorption in a frequency-dependent spectrum.

RESULTS AND DISCUSSION

The temperature dependence of sound velocity and absorption has been studied for the DPPC/H_2O/melittin system, at the following peptide/lipid molar ratios: $R = 0$, $R = 5 \times 10^{-4}$, and $R = 5 \times 10^{-3}$. The results are shown in Fig. 1 for sound velocity and absorption numbers, respectively. A most striking feature observed is the appearance of a huge absorption peak at about 33°C, for $R = 5 \times 10^{-3}$, which is accompanied by an increase in sound velocity. Another feature of the curves obtained, both for sound velocity and absorption, is the nonmonotonic dependence of these two parameters on the peptide content. This is best discussed in the framework of the following four aspects:

1. Below the pretransition, which for pure DPPC lies around 34°C, the sound velocity is higher for $R = 5 \times 10^{-4}$ as compared to both $R = 0$ and $R = 5 \times 10^{-3}$; the same is observed for the absorption curves below 30°C.

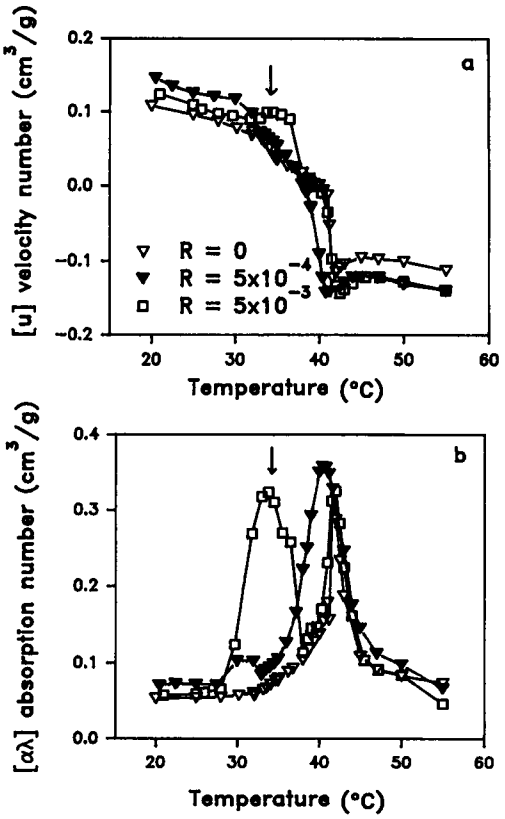

FIGURE 1 Temperature dependence of (*a*) sound velocity number, [*u*], and (*b*) sound absorption number, [αλ], of hydrated lipid multilamellar systems at three different melittin/lipid molar ratios: ▽, $R = 0$; ▼, $R = 5 \times 10^{-4}$; and □, $R = 5\ 10^{-3}$. $f_m = 7.2$ MHz.

2. Above the main transition, i.e., above 41°C, the sound velocity is approximately the same for the two melittin-containing systems and lower as compared to that of the pure lipid system. At the same time, the absorption changes only insignificantly in the presence of melittin. This means (see Eqs. 5 and 6), that melittin affects only the instantaneous, nonrelaxational, part of the fluid state compressibility.

3. At the low temperature side of the main transition, an increase of absorption together with a decrease in sound velocity is seen at $R = 5 \times 10^{-4}$. This is probably due to an increase of the relaxation strength caused by an increase of heterophase fluctuations; the acoustic effects of these heterophase fluctuations are described by Kharakoz et al. (1993). At $R = 5 \times 10^{-3}$, no significant difference from the pure lipid is observed.

4. A nonmonotonic behavior of the chain melting (main transition) temperature is observed: the temperature decreases by about 1°C at $R = 5 \times 10^{-4}$ and re-increases at $R = 5 \times 10^{-3}$.

In the following, the discussion will be focused on two main points: (*a*) the simultaneous increase of sound velocity and absorption, and (*b*) the nonmonotonic change in the transition temperature.

a. A simultaneous melittin-induced increase in sound velocity and absorption, between the pretransition and main transition temperatures at $R = 5 \times 10^{-3}$

As the temperature reaches about 33°C, an increase of absorption is seen together with an increase of sound velocity (*arrows* in Fig. 1). This is the most striking result of this study and can be understood either simply in terms of acoustic relaxation (I) or by the appearance of a new state (II). If this were to reflect a change of the relaxation properties of the system, then the relaxation time of the process involved must be extremely short ($\tau < (2\pi \cdot 7.2)^{-1}$ MHz ≈ 22 ns). The only process that fulfills this condition in the gel state is the head group rotation. The other possible explanation implies the appearance of a new state kinetically differentiated from the parent one. These two possibilities are discussed below.

I. A change in the relaxational contributions to the sound velocity and absorption may be explained either in terms of a *change in the relaxation strength* or as being associated to an *alteration of the relaxation time* of the existing processes (Stuehr and Yeager, 1965). In the first case, an increase of relaxation strength results in an increase of sound absorption and decrease of sound velocity and vice versa (Fig. 2 *A*). In the second case, the situation can be visualized as shown in Fig. 2 *B*, where the sound velocity and absorption are plotted against frequency. The illustration corresponds to a single relaxation process, in which the absorption frequency (peak position) coincides with the inflection point of the sound velocity curve. There are two possible situations: (i) The relaxation time of the existing process decreases (sound velocity and absorption curves are shifted to higher frequen-

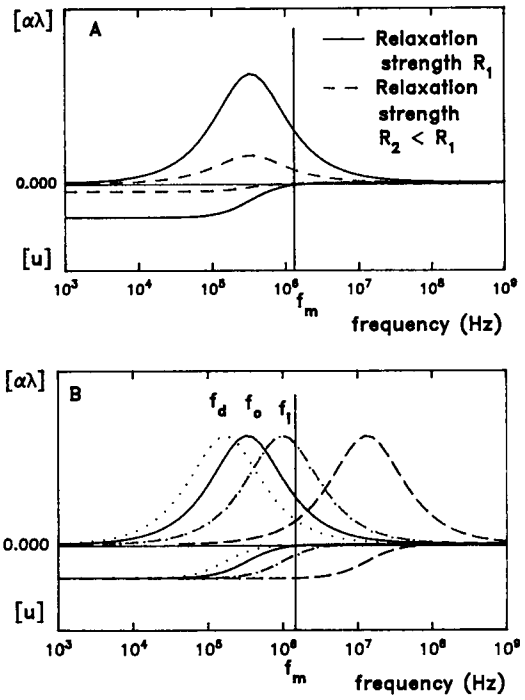

FIGURE 2 Acoustic sound velocity and absorption numbers as a function of frequency. Dependence of acoustic properties on (*A*) the relaxation strength of the process and (*B*) the relaxation time of the process. f_0 is the absorption frequency in an initial condition, f_i shows the absorption frequency when the relaxation time of the process decreases, and f_d shows the absorption frequency when the relaxation time of the process increases. f_m, measurement frequency (see text).

cies; *dotted-dashed line*); or (ii) The relaxation time of the existing process increases (sound velocity and absorption curves are shifted to lower frequencies; *dotted line*). The only situation where a simultaneous increase of sound velocity and absorption, as seen in our results, would be possible is that the relaxation frequency of the process involved is higher than the experimental frequency (*dashed lines* in Fig. 2 *B*), i.e., for fast processes. One example of a fast process is the *trans-gauche* transformation, with relaxation times on the order of 1 ns (Holzwarth, 1989; Bloom et al., 1991 and references therein). Another example of a fast process is the heterophase fluctuations observed in the liquid crystalline phase, with relaxation times between 20 and 50 ns (Mitaku and Date, 1982; Kharakoz et al., 1993), comparable to the characteristic time of our ultrasonic measurement, 20 ns. Apparently the only processes that would satisfy the condition of a simultaneous increase in sound velocity and absorption, in the frequency used, are those occurring in the same time scale as the *trans-gauche* transformation. However, these are processes characteristic of the liquid crystalline phase. Our x-ray results (Colotto, 1993; Colotto et al., manuscript submitted) have clearly shown that at $R = 10^{-3}$ and at $T < 41°C$, the system corresponds to a corrugated gel phase with hexagonal chain packing; under these conditions the chain rotation frequency is on the order of 10^{-7} s (Cevc and Marsh, 1987). In the gel phase, the only process that would be fast enough to cause the changes in the acoustic properties seen here is the head group rotation, which between the pretransition and main transition temperatures has a maximum relaxation time $\tau = 4$ ns (Shepherd and Büldt, 1978; reviews by Cevc and Marsh, 1987; Laggner and Kriechbaum, 1991). If changes in this process are considered to be the cause of the effects observed, then (Fig. 2 *B*) melittin must be inducing an increase in the relaxation time of the head group rotation. On the basis of this interpretation, the temperature dependence of the changes induced by melittin in the acoustic properties and the interval of temperatures at which these changes are observed have to be considered more closely.

Fig. 3 shows the change in sound absorption, $\Delta[\alpha\lambda]$, and velocity, $\Delta[u]$, respectively, induced by melittin at $R = 5 \times 10^{-3}$, plotted against temperature. $\Delta[\alpha\lambda]$ and $\Delta[u]$ are given by

$$\Delta[\alpha\lambda] = [\alpha\lambda_m] - [\alpha\lambda_l] \tag{8}$$

and

$$\Delta[u] = [u_m] - [u_l] \tag{9}$$

where the subscripts m and l stand for the melittin-containing system and for the pure lipid system, respectively. Above 40°C, the sound absorption does not significantly change, while the sound velocity decreases. This means that melittin affects only the instantaneous part of the fluid state compressibility, which increases. Below 30°C, there are too few points available to allow any definite statement; nevertheless, it seems that in this temperature range, again only the instantaneous part of the compressibility is affected, but in an opposite way from that seen in the fluid state, and with only a slight increase in sound velocity (decrease in compressibility). Between 30 and 40°C, the simultaneous in-

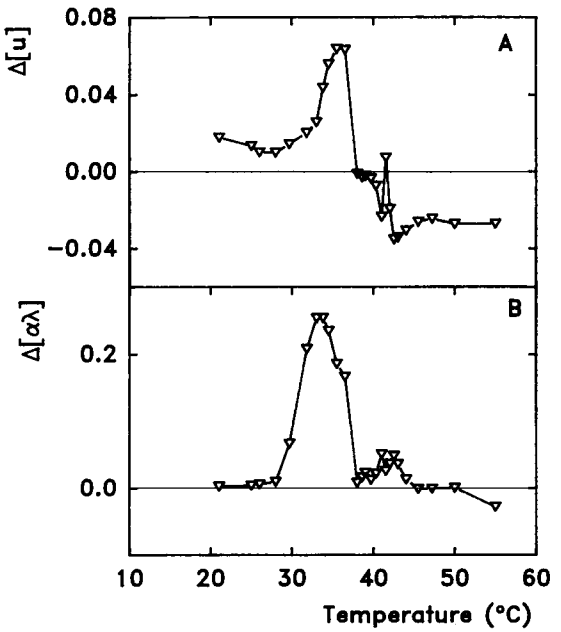

FIGURE 3 Temperature dependence of the changes induced by melittin ($R = 5 \times 10^{-3}$) in (*A*) Sound velocity number ($\Delta[u] = [u_m] - [u_l]$); and (*B*) sound absorption number ($\Delta[\alpha\lambda] = [\alpha\lambda_m] - [\alpha\lambda_l]$). See text.

crease in sound velocity and absorption indicates that the relaxation time of the head group rotation is increasing. It is known from measurements of the dieletric properties of DPPC/H_2O systems that the head group rotation relaxation is temperature dependent (Shepherd and Büldt, 1978). Unfortunately, due to experimental limitation to a single measurement frequency, the temperature dependence of the relaxation strength and relaxation time of the process involved cannot be determined from the acoustic properties. It is, therefore, not possible to make a precise analysis of the shape of the curves in Fig. 3. It is known, however, from measurements of the dieletric properties, that the relaxation time of the head group rotation, varies from 4 to 3.4 ns as temperature is increased from 35 to 42°C, i.e., between the pretransition and main transition. Since the acoustic properties depend on the relaxation time of the process involved, the strong temperature dependence of the change induced by melittin in the sound velocity and absorption, as observed, is rather reasonable. The fact that this temperature dependence is already seen at temperatures as low as 30°C is consistent with the calorimetric result of a decrease in the pretransition temperature down to 29.8°C at $R = 3 \times 10^{-3}$ (Colotto, 1993; Colotto et al., manuscript submitted). It is interesting to note that an increase in relaxation time (decrease of relaxation frequency) of DPPC/H_2O systems has also been induced by adding ethylene glycol to the system (Shepherd and Büldt, 1978), which increases the viscosity of the bilayer. An increase in viscosity induced by melittin is in agreement with the macroscopic changes observed in the melittin-containing samples studied.

A quantitative discussion of the phenomena in terms of different, individual relaxation processes (with characteristic relaxation times and strength) can only be performed on basis of data from a multifrequency, spectroscopic type of experiment. Nevertheless, the pronounced alterations of the sound absorption, $[\alpha\lambda]$, and sound velocity, $[u]$, due to the incorporation of melittin observed here warrant the attempt for a qualitative analysis.

II. The only other way to explain an increase of sound velocity simultaneously with an increase in sound absorption is to assume that melittin induces the appearance of a new state at temperatures between the pretransition and main transition. Below, a qualitative analysis is presented to explain the shape of the curves obtained, in this temperature range, for the melittin-containing system with $R = 5 \times 10^{-3}$.

This analysis is based on the hypothesis of the appearance of a new state, and on the consideration of three main contributions to the sound absorption and velocity (as discussed in "Materials and Methods") in a lipid system (Fig. 4):

1. In each state, the *instantaneous* part of sound velocity is an approximately linear function of temperature with a characteristic magnitude and slope. For the absorption, there is no instantaneous contribution, but only a *relaxational* one. When no relaxation is present, the sound absorption is negligible. At the transitions, the curves for sound velocity have a sigmoidal shape which depends on the properties of the state.

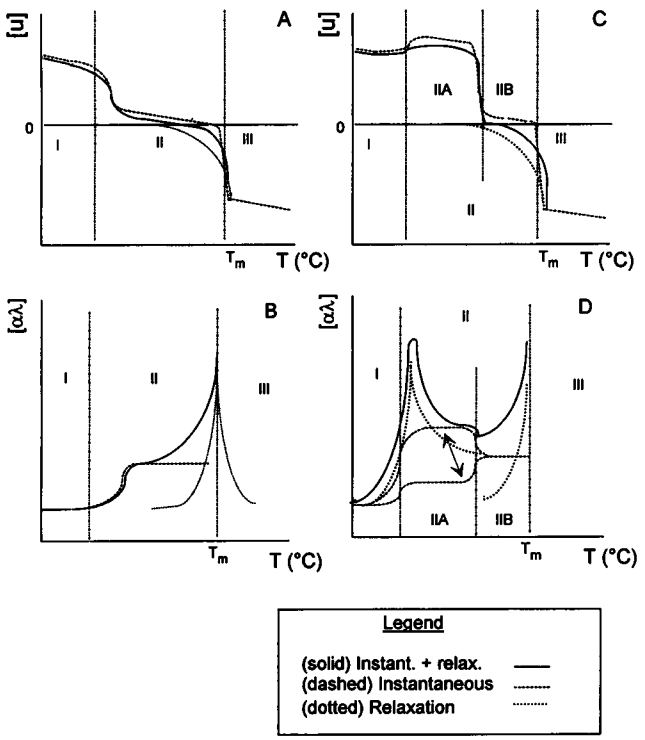

FIGURE 4 Qualitative reconstruction of the experimental data on the acoustic properties: sound velocity number (A) and absorption number (B), respectively, of the pure lipid system and sound velocity number (C) and absorption number (D), respectively, of the melittin-containing system ($R = 5 \times 10^{-3}$). Dashed lines represent the instantaneous contributions, dotted lines represent the temperature-dependent contribution, and solid lines represent the sum of all contributions and should reassemble the experimental curves. For clarity, the curves are not superimposed. Note that for $R = 5 \times 10^{-3}$, there are two possible pathways for the instantaneous contribution of sound absorption.

2. Furthermore, in each state, there is a *temperature-independent relaxation* contribution for both the sound velocity and absorption numbers which, sufficiently far from any transition, is a constant characteristic of each state. At the transitions, this contribution has a sigmoidal shape for both parameters.

Since both the above mentioned contributions are characteristic of the state, for this treatment they are combined in one (*dashed lines* in Fig. 4) and called, from now on, "instantaneous" to distinguish it from the temperature-dependent relaxation contribution.

3. A highly *temperature-dependent relaxation* contribution for both parameters reflects the existence of heterophase fluctuations. This kind of relaxation process is a direct consequence of a phase transition and only takes place in the vicinity of the transition temperature. It typically has an exponential shape and reflects characteristics of the phase transition (Kharakoz et al., 1993).

The hypothetical reconstruction of the experimental data using the above assumptions for sound velocity and absorption numbers is shown in Fig. 4, *A-D*, respectively. These calculations serve only to summarize qualitatively the contributions as discussed above and do not represent a quantitative functional analysis. They show, nevertheless, that, indeed, our experimental results can be reconstructed in the above discussed way. For DPPC/H_2O phases, there are three well defined states in the temperature range investigated, which are specified in the figure by roman numbers (I–III): the $L_{\beta'}$ gel state (I), the rippled $P_{\beta'}$ state (II), and the liquid crystalline L_α state (III) (Small, 1986). For the melittin-containing system, state II divides into two different states: IIA and IIB. The relatively high magnitude of the instantaneous part of sound velocity at state IIA means that this state is less compressible. Nothing can be said about the instantaneous part of absorption in this state, and in Fig. 4 *D*, two possible pathways are illustrated. The contributions to the acoustic parameters of state IIB are the same as those to state II. As regards the transitions between these states, each of them, between I and IIA and IIA and IIB, takes place in a rather narrow interval of temperature. This would suggest that the processes are highly cooperative. However, the fact that such processes are not observed through differential scanning calorimetry leads to the conclusion that they are accompanied only by a *negligible change in entropy*.

The shape of the absorption curve around the transition between states I and IIA resembles that around the transition between II and III ($P_{\beta'}$ to L_α, main transition). The behavior of sound absorption in the high temperature edge of the main transition has been interpreted, for the DPPC/H_2O system, as being due to heterophase fluctuations which are also reflected in the temperature dependence of sound velocity (Kharakoz et al., 1993). If in the vicinity of the transition I to IIA, heterophase fluctuations were also taking place, then a strong deviation of the sound velocity curve from the sigmoidal one should be, in analogy, expected, which is not the case. Still, heterophase fluctuations may be taking place. According to Eq. 7, the relaxation time of these processes would have to be very long or else the new state should have a high relaxation strength.

The existence of such a new state has not been observed under the same conditions either by differential scanning calorimetry or x-ray diffraction studies (Colotto, 1993). These techniques show the disappearance of the pretransition at about $R = 5 \times 10^{-3}$, but neither of them indicates the existence of two transitions. This could suggest that the states involved can be differentiated neither structurally nor thermodynamically but kinetically. It is noteworthy, however, that the specific volume has been found to increase significantly at low doses of melittin (Posch et al., 1983).

In examining the two different mechanisms outlined above, it seems easier to accept the first one as the more plausible interpretation, based on the increase in relaxation time of the head group rotation. This is in rather good agreement with other results found in the literature and avoids the assumption of a new state for which there exists no other experimental support.

b. The nonmonotonic change in the main transition temperature with melittin content

As pointed out already, a decrease in the transition temperature at a low peptide content ($R = 5 \times 10^{-4}$) is followed by a re-increase at higher doses of melittin ($R = 5 \times 10^{-3}$). This is in contrast to previous studies by densitometry (Posch et al., 1983). There the dependence of the transition temperature on the melittin content was characterized by an initial increase (up to $R = \times 10^{-3}$) followed by a decrease as more peptide was added. The reason for this discrepancy is not clear at present and requires further systematic studies. It is nevertheless possible to explain the present results by assuming that the addition of melittin at low concentrations leads to the accumulation of defects in the system. These defects result from the fact that each group of lipids within the range of action of a peptide molecule will constitute a domain with different structural and thermodynamic properties from the nonaffected lipids. Defects would be created at the interfaces between the affected domains and the nonaffected phase, resulting in the destabilization of the whole gel phase. At higher concentrations, a coalescence of the clusters should take place, thus leading to the disappearance of the boundary defects and a stabilization of the gel state. The structural nature of such defects is the subject of x-ray studies (Colotto, 1993; Colotto et al., manuscript submitted).

CONCLUSIONS

The present approach by ultrasonic methods to elucidate the physical effects of the toxic peptide melittin on lipid membranes has led to interesting new insights, not paralleled by structural (x-ray) or thermodynamic (calorimetry, densitometry) results, which are not directly sensitive to dynamic properties of supramolecular systems. This further manifests the value of ultrasonic methods, which are sensitive to dynamics in the time scale of tens of nanoseconds and hence highly informative in relation to lipid molecular motions. As the most remarkable result, the study has shown that the acoustic properties of the lipid system are changed drastically by low doses of melittin in the temperature range of the pretransition. This is known to correspond to a state of dynamic heterogeneity both with regard to the lipid and associated water moieties (Falkovitz et al., 1982; Wittebort et al., 1981; Strenk et al., 1985; Hawton and Doane, 1987; Tsuchida and Hatta, 1988). The present results indicate, therefore, that low doses ($R \leq 10^{-2}$) of melittin are strongly capable of shifting the proportions in the equilibrium of these dynamic states. They further suggest that this effect corresponds mainly to a reduction in head group rotational mobility, opposite to the action by, e.g., cholesterol, which also decreases the pretransition temperature and enthalpy. Although this result from a model lipid system cannot be directly applied to the situation in natural membranes, it further

supports our view (Posch et al., 1983) that melittin may perform its primary toxic function by shifting the percolation balance (Shimshick and McConnell, 1973; Almeida et al., 1993) of gel- and liquid-like states that may coexist. Since a reduced mobility in the monolayer domains affected by melittin is most likely to also influence the lateral packing density and in turn the curvature of the bilayer, this agrees with the observation of a curvature modulatory effect of melittin (Batenburg and DeKruijff, 1988), which was indeed verified by our x-ray diffraction studies (Colotto et al., 1991).

Before concluding, it is worthwhile to note that the defect-like action of melittin proposed to explain the nonmonotonic dependence of the main transition temperature on the peptide/lipid molar ratio is consistent with the thermodynamic description by Biltonen (1990), of situations where two states coexist in a vesicle. The approach allows an explanation for the ability of anesthetics to stabilize interfacial regions between gel- and liquid-crystalline clusters in terms of a reduction of the absolute magnitude of the interaction energy between unlike nearest neighbors. In the present work, the magnitude of the interaction energy is not reduced itself, but a reduction of the extension of the boundaries between the different clusters takes place upon coalescence of like ones.

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