

Stopped-Flow Fluorometric Study of the Interaction of Melittin with Phospholipid Bilayers: Importance of the Physical State of the Bilayer and the Acyl Chain Length

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ABSTRACT Stopped-flow fluorometry has been employed to study the effects of melittin, the major protein component of bee venom, on dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) small unilamellar vesicles (SUVs) on the millisecond time scale, before melittin-induced vesicle fusion takes place. Use is made of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), which is an oriented fluorescent probe that anchors itself to the bilayer-water interface and is aligned parallel to the normal to the bilayer surface; its fluorescence anisotropy reports on the "fluidity" of the bilayer. For DMPC bilayers, melittin is found to decrease their fluidity only at their melting transition temperature. This perturbation appears to be exerted almost instantaneously on the millisecond time scale of the measurements, as deduced from the fact that its rate is comparable to that obtained by following the change in the fluorescence of the single tryptophan residue of melittin upon inserting itself into the bilayer. The perturbation is felt in the bilayer over a distance of at least 50 Å, with measurements of transfer of electronic energy indicating that the protein is not sequestered in the neighborhood of TMA-DPH. The length of the acyl chains is found to be an important physical parameter in the melittin-membrane interaction: unlike the case of DMPC SUVs, melittin does not alter the fluidity of DPPC SUVs and has a considerably greater affinity for them. These results are discussed in terms of the concept of elastic distortion of the lipids, which results from a mismatch between the protein and the acyl chains that are attempting to accommodate it. Melittin is also found to cause a small (~10%) enhancement in the total fluorescence intensity of TMA-DPH, which is interpreted as indicating a reduction in the degree of hydration of the bilayer.

INTRODUCTION

The conformation of the protein melittin from bee venom is known to be a random coil when free in solution and an α -helix when bound to membranes (Strom et al., 1978; Drake and Hider, 1979; Vogel, 1981). In recent years its interaction with phospholipid vesicles has attracted considerable attention for the following reasons: 1) this association is linked with the physiological effects of the protein, and 2) the results of experiments are of broader importance, because the amphiphilic α -helical conformation of this cytolytic toxin when in membranes resembles those of apolipoproteins and peptide hormones (Degrado et al., 1982; Kaiser and Kezdy, 1983) as well as signal peptides (Knöppel et al., 1979; Garnier et al., 1980). Its state of aggregation when membrane bound (Hermetter and Lakowicz, 1986; Talbot et al., 1987; Altenbach and Hubbell, 1988; Bradrick et al., 1989; John and Jähnig, 1991) and depth of penetration into the hydrophobic core of the bilayer, as well as its orientation relative to the plane of the bilayer (Dawson et al., 1978; Terwilliger et al., 1982; Vogel et al., 1983; Stanislawski and Rüterjans, 1987; Altenbach et al., 1989; Frey and Tamm, 1991; Dempsey and Butler, 1992), have been the subject of considerable research. Recent relevant reviews

have appeared (Dempsey, 1990; Sansom, 1991; Cornut et al., 1993).

A promising technique for elucidating some of the aspects of the melittin-membrane association is stopped-flow fluorometry through the use of intrinsic and/or extrinsic fluorescent probes. It has been established that this association takes place on the millisecond time scale (Schwarz and Beschiaschvili, 1989; Sekharam et al., 1991). On a longer time scale (seconds to minutes) melittin has been reported to fuse phospholipid vesicles (Eytan and Almary, 1983; Morgan et al., 1983; Bradrick and Georghiou, 1987). Of special interest are the primary events of the interaction before more complex vesicle structures are formed. Such studies are capable of yielding information regarding i) protein effects on the order and dynamics of bilayers, ii) the distance over which the perturbation induced by the protein is felt and the time scale over which it propagates, and iii) the depth of penetration of the protein into the hydrophobic core of the bilayer.

In the present study we have used 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) as an extrinsic fluorescent probe of small unilamellar saturated phospholipid vesicles (SUVs) to elucidate the aforementioned aspects i) and ii) of the melittin-phospholipid interaction on the millisecond time scale through polarized stopped-flow fluorometry. TMA-DPH is a cationic analog of DPH which, unlike the latter, is an oriented probe that anchors itself to the bilayer-water interface and is aligned parallel to the normal to the bilayer (Prendergast et al., 1981; Lentz, 1989; Sijs et al., 1993). The fluorescence

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anisotropy of this probe reports on the "fluidity" (a term that encompasses both order and dynamics; see, e.g., Lentz, 1989) of the glycerol backbone and the uppermost region of the bilayer. Moreover, because its fluorescence quantum yield in water is negligible (Stubbs et al., 1984; Lentz, 1989), it can report on the accessibility of water to the interior of the bilayer. We should add that a very recent Monte Carlo dynamics simulation study (van der Heide and Levine, 1994) has reported that TMA-DPH perturbs the bilayer only marginally and reports faithfully on its dynamics. In addition to TMA-DPH, we have employed the single tryptophan residue at position 19 of melittin as an intrinsic fluorescent probe to monitor the interaction of the protein with the bilayer by using the sensitivity of its fluorescence to the polarity of its environment. The results show that the protein decreases the fluidity of the dimyristoylphosphatidylcholine (DMPC) bilayer only at its melting transition temperature T_m . The perturbation is transmitted beyond the lipids that are in the immediate vicinity of the protein, over a range of at least 50 Å. The protein exhibits acyl chain selectivity in its interaction with bilayers, in that it does not affect the fluidity of dipalmitoylphosphatidylcholine (DPPC) SUV bilayers and has a considerably greater affinity for DPPC than for DMPC bilayers. The findings are discussed in the context of a mismatch between the acyl chains and the hydrophobic part of the protein as described by the mattress model for protein-membrane interactions (Mouritsen and Bloom, 1984; Sperotto and Mouritsen, 1991).

MATERIALS AND METHODS

DMPC and DPPC of a purity greater than 99% were obtained from Avanti Polar Lipids (Alabaster, AL). 1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluenesulfonate (TMA-DPH) was a product of Molecular Probes (Eugene, OR). Tris and EDTA-disodium salt, both analytical grade, were obtained from Fisher (Pittsburgh, PA) and Sigma Chemical Co. (St. Louis, MO), respectively. *N,N*-dimethylformamide, spectroquality grade, was from Mallinckrodt (Paris, KY). The buffer used was 50 mM Tris, 1 mM EDTA, pH 7.6 prepared in triply-distilled water. Melittin was obtained from Sigma. For the melittin concentrations used in these experiments, EDTA at a concentration of 1 mM is sufficient to eliminate the activity of any residual phospholipase A_2 in the sample (Dasseux et al., 1984; Bradrick and Georgiou, unpublished observations).

Each day's experiments were performed with a freshly prepared sample of DMPC or DPPC small unilamellar vesicles (SUVs), which was made in the following way. For those experiments in which the change in the fluorescence anisotropy of TMA-DPH was monitored during melittin binding to the vesicles, a stock lipid dispersion with a concentration of 5.9 mM was formed by hydrating the appropriate amount of lipid in 10 ml of buffer at a temperature 10°C above the phase transition temperature T_m of the particular multilamellar vesicles (i.e., hydration was done at 33°C in the case of DMPC and at 51°C in the case of DPPC.) To this dispersion was added 120 µl of a 1 mM solution of TMA-DPH in *N,N*-dimethylformamide. The resulting lipid:fluorescent probe molar ratio was about 500:1; this high ratio was chosen so as to minimize any perturbation of the bilayer by the probe. The lipid dispersion was then stirred for 30 min at 10°C above T_m to allow incorporation of the fluorescent probe into the lipid bilayers. (The formation of the TMA-DPH stock solution in *N,N*-dimethylformamide, as well as all subsequent steps that involved the formation and use of the TMA-DPH-labeled vesicles, was carried out under cover of

dim red light so as to avoid photobleaching this fluorescent probe.) The vesicles were then sonicated in a Laboratory Supplies Co. (Hicksville, NY) bath sonicator at either 33°C (for DMPC) or 47°C (for DPPC) for two 20-min periods separated by a 5-min rest. This was followed by another 30-min rest, during which the resulting small unilamellar vesicles (SUVs) were allowed to anneal. Finally, the sample was centrifuged at $65,000 \times g$ for 30 min at either 33°C or 47°C for 25 min to pellet any remaining multilamellar vesicles. After centrifugation, the supernatant was removed (under red light) and stored in a screwcap polystyrene tube. As needed, aliquots of this lipid sample were diluted by a factor of 2 and then loaded into the thermostated stopped-flow reservoir. Upon mixing of a portion of this with an equal volume of melittin solution, the final lipid concentration in the stopped-flow cell was 1.48 mM. The resulting concentration of TMA-DPH in the cell was then such that it had an absorbance of about 0.06 at the excitation wavelength of 365 nm. For this absorbance, fluorescence intensity is proportional to probe concentration.

Lipid samples for the baseline to TMA-DPH fluorescence anisotropy measurements, or for the measurement of the kinetics of melittin binding (based on the resulting change in the melittin tryptophan fluorescence), were prepared as described above with the exception that they were not labeled with TMA-DPH. Some vesicle samples were made, however, with the addition of the appropriate amount of the TMA-DPH solvent *N,N*-dimethylformamide to determine whether its presence had a measurable effect on their characteristics and the kinetics of melittin binding to them. We have found that there was no difference in sonication time, amount of scattered light, or the kinetics of melittin binding between solutions that contained *N,N*-dimethylformamide and those that did not. Therefore, all subsequent measurements that involved nonlabeled vesicles were carried out on samples that did not contain the TMA-DPH solvent.

The baseline contributions of scattered light to polarized measurements of TMA-DPH fluorescence were determined by using a nonlabeled lipid solution as described above. The ratio of scattered light (baseline) to fluorescence signal has been found to be about 3% in the case of DMPC SUVs and 12% in the case of DPPC SUVs.

The concentration of the melittin stock solution was determined spectrophotometrically on a Shimadzu (Columbia, MD) UV-265 spectrophotometer by using a value of $5570 \text{ M}^{-1} \text{ cm}^{-1}$ for the molar extinction coefficient at 280 nm (Quay and Condie, 1983). Stock solutions were refrigerated and used within 5 days after their formation. For all the measurements described here, the final melittin concentration after stopped-flow mixing was less than 0.03 mM, for which melittin is known to be monomeric in solution (Faucon et al., 1979).

Stopped-flow fluorometric measurements were carried out on an Aminco DW-2 UV/Vis spectrophotometer equipped with a stopped-flow module, which we have modified to allow fluorescence to be measured at right angles to excitation. Sample temperature was controlled with a circulating water bath. The exciting light was provided by a Spectral Energy (Westwood, NJ) 600 W Xe-Hg arc lamp, the intensity of which was attenuated with quartz neutral density filters. In the case of measurements made of the polarized fluorescence of TMA-DPH, the intensity was further attenuated to avoid photobleaching of the fluorophore. The extent to which the beam needed to be attenuated was determined by observing the time course of the fluorescence intensity of TMA-DPH-labeled vesicles mixed with buffer. Because this signal should be flat, the intensity of the exciting light was reduced until this fluorescence signal remained constant over the same time scale during which kinetics were observed when labeled vesicles were mixed with melittin. The exciting beam then passed through a Corion (Holliston, MA) 365-nm (7-nm bandwidth) interference filter to select the excitation wavelength, and a Karl Lambrecht Corp (Chicago, IL) SQWO depolarizer. The presence of the latter, as well as that of a Karl Lambrecht Corp SQLO depolarizer on the emission side, removed any polarization bias that the optical system of the stopped-flow fluorometer might otherwise have had (see below). Finally, the light passed through a DaLite (Cincinnati, OH) 105UV polarizer, which was used to select polarized light for exciting the sample. The sample fluorescence was viewed by a Thorn EMI (Fairfield, NJ) 6256S photomultiplier through a Schott (Duryea, PA) KV418 cut-off filter (with 50% transmission at 418 nm) and a DaLite 105UV polarizer. The photomultiplier output was digitized by an IBM PC

equipped with an Analog Devices (Norwood, MA) RTI-815 A/D converter.

For the measurements made of the kinetics of melittin binding to DMPC and DPPC SUVs, in which the fluorescence of the single tryptophan residue of melittin was measured, lipid samples were prepared as described above, with the exception that they were not labeled with TMA-DPH. Measurements were made at the same temperature and protein and lipid concentrations as for the TMA-DPH fluorescence anisotropy measurements. The aforementioned depolarizers on the excitation and the emission sides were left in place, but the two polarizers were removed. Excitation was at 280 nm by using a Melles Griot (Irvine, CA) interference filter of 10 nm bandwidth; the maximum sample absorbance at this wavelength was about 0.03. Fluorescence was viewed through a 0–51 Corning cut-off glass filter (obtained from ESCO, Oak Ridge, NJ), which has a 50% transmission at 385 nm. It is known that as melittin binds to vesicles its fluorescence spectrum shifts from about 352 nm to 337 nm (Georghiou et al., 1982); consequently, the fluorescence intensity as viewed through the 0–51 filter decreases during this process. The values for k listed in Table 2 represent averages taken from five separately prepared samples; for each of these samples, an average of 12 stopped-flow shots were taken.

Experiments were also carried out to determine whether energy transfer takes place between the excited single tryptophan residue of membrane-bound melittin (the donor) and TMA-DPH (the acceptor). TMA-DPH-labeled vesicles were prepared as described above, as were melittin stock solutions. Stopped-flow kinetic measurements were also made as described above, except that the two polarizers were removed; the two depolarizers, however, were left in place. Melittin was excited by using a 280-nm interference filter, and the fluorescence was viewed through a 7–60 Corning filter that selectively transmits the melittin fluorescence. (For a lipid-to-melittin molar ratio R of 60, the fraction of light absorbed by TMA-DPH at 280 nm is very small, ~5%.) We found that the presence of TMA-DPH had a negligible effect on the fluorescence of the protein at both 21°C and 33°C. This implies that transfer of electronic energy from the tryptophan to TMA-DPH is very inefficient. The critical transfer distance R_0 (at which the transfer is 50% efficient) for this system was calculated from the following equation (Förster, 1965):

$$R_0^6 = \frac{8.79 \times 10^{-25}}{n^4} k^2 q_0 \int_0^\infty \frac{F(\bar{\nu}) \epsilon(\bar{\nu}) d\bar{\nu}}{\bar{\nu}^4} \quad (1)$$

Here, n is the refractive index of the medium, which is assumed to be equal to 1.4 for lipid membranes (Vogel and Jähnig, 1986), q_0 is the fluorescence quantum yield of the single tryptophan residue of membrane-bound melittin in the absence of transfer of energy to TMA-DPH, which is equal to 0.36 (Vogel and Jähnig, 1986), $F(\bar{\nu})$ is the fluorescence intensity of the tryptophan at $\bar{\nu}$ for melittin bound to membranes, which was corrected for the change in the instrument sensitivity with emission wavelength (Georghiou et al., 1982) (with the fluorescence spectrum normalized to unity on a wavenumber scale), and $\epsilon(\bar{\nu})$ is the molar extinction coefficient of TMA-DPH at $\bar{\nu}$ (Cundall et al., 1979; Prendergast et al., 1981). In Eq. 1, k is the orientation factor, the square of which attains a value of $\frac{2}{3}$ for randomly distributed dipoles (Förster, 1965). In these calculations, we have used this value as an approximation. From polarized fluorescence measurements at 33°C with DMPC vesicles (excitation at 297 nm, emission observed through a Corning 0–51 filter), we find that for $R = 60$ the anisotropy r_0 of bound melittin is about 0.09 (Bradrick and Georghiou, to be published). Also, for TMA-DPH (excitation at 365 nm) we find $r = 0.180$. As these anisotropy values are relatively low, we may consider the transition dipole moments of the tryptophan of melittin and TMA-DPH to undergo dynamic averaging to a good extent during the lifetimes of their excited states, so that $k^2 \approx \frac{2}{3}$. At 21°C and for $R = 60$, for melittin we find r_0 to be about 0.13 (Bradrick and Georghiou, to be published), and for TMA-DPH, $r = 0.275$ (Table 1). Thus, the transition dipole moment of the tryptophan may be considered to undergo dynamic averaging to a good extent, whereas that of TMA-DPH appears to be more restricted in its motion. If θ is the angle made by the separation vector (which connects the

two dipoles) and the dipole of TMA-DPH (which we consider to be approximately immobile), then $k^2 = (1 + 3 \cos^2 \theta)/3$ (Dale and Eisinger, 1975). Thus, the lowest value k^2 can attain in such a case is 0.33, which corresponds to $\theta = 90^\circ$. This would reduce the value of R_0 calculated from Eq. 1 by a factor of $(0.33/0.67)^{1/6} = 0.89$ relative to that for $k^2 = \frac{2}{3}$. Thus, the uncertainty in the value of k^2 does not introduce a serious error in the calculation of R_0 . Returning to Eq. 1, with the aforementioned values of the parameters, using $k^2 = \frac{2}{3}$, and calculating the spectral integrals, we obtain a value of 39 Å for the tryptophan–TMA-DPH system. This is the distance at which energy transfer is 50% efficient. Consequently, the finding that, despite this high value of R_0 , energy transfer is inefficient indicates that the protein–TMA-DPH average distance is large, i.e., greater than 39 Å. This is further elaborated upon in the Discussion.

The fluorescence anisotropy r for each data file was calculated using the equation

$$r = \frac{I_{VV} - \left(\frac{I_{HV}}{I_{HH}} \right) I_{VH}}{I_{VV} + 2 \left(\frac{I_{HV}}{I_{HH}} \right) I_{VH}} \quad (2)$$

Here, I denotes the fluorescence intensity, and the subscripts V and H denote vertical and horizontal polarization orientations, respectively; the first subscript pertains to the orientation of the polarization of the exciting light and the second pertains to the orientation of the fluorescence component. The ratio I_{HV}/I_{HH} accounts for any polarization bias that the detection system might have. The presence of depolarizers in the excitation and emission sides removed any such bias; the ratio I_{HV}/I_{HH} has been measured and found to be equal to unity. After the time-dependent fluorescence anisotropies were calculated for all the fluorescence data files, they were simultaneously fitted with the equation

$$r(t) = (r_0 - r_\infty) \exp(-k_1 t) + r_\infty \quad (3)$$

Here, r_0 is the fluorescence anisotropy of TMA-DPH at zero time, r_∞ is the fluorescence anisotropy at long times, and k_1 is the effective rate constant for the observed change. In all cases it was found that the anisotropies were well described by a decaying exponential plus a constant, as judged from the random shape of the residuals for the fits. The values for r_0 , r_∞ , and k_1 represent averages from five separately prepared samples; on average, five stopped-flow shots were taken for each polarized fluorescence component per sample.

The total TMA-DPH fluorescence intensities $F(t) = I_{VV} + 2I_{VH}$ were calculated for each of the individual files and were simultaneously fitted with the equation

$$F(t) = A \exp(-k_2 t) + B \quad (4)$$

As was the case for the fluorescence anisotropy, the results for k_2 from five separate experiments were averaged.

We also made stopped-flow spectrophotometric measurements to determine whether the observed increase in F upon binding of melittin is the result of an increase in the absorbance of TMA-DPH at the excitation wavelength of 365 nm, which was caused by this binding. These were made at 21°C for DMPC vesicles at 365 nm. For this mode of operation the optical path length of the cuvette is equal to 1 cm. (For the fluorescence measurements the optical path length is equal to 2 mm.) To enhance the sensitivity of the measurements, the TMA-DPH and lipid concentrations were twice those used in the fluorescence measurements; therefore, the TMA-DPH absorbance at 365 nm was equal to 0.6. For a 10% increase in the absorbance, the fraction of transmitted light, which is equal to $10^{-\epsilon c d}$, would then decrease by about 13%. (Here, ϵ , c , and d are the molar extinction coefficient, the molar concentration, and the optical path length of the cuvette, respectively.) This decrease should be detectable by this method. We did not, however, detect any change in the intensity of the transmitted light upon melittin binding. This result discounts the likelihood of any significant melittin-induced change in the absorbance of TMA-DPH at 365 nm. (We should add that, as the lipid concentration was twice that

used in the fluorometric measurements, the amount of added melittin was adjusted on the basis of the association constant for the melittin-DMPC binding (Payongayong et al., unpublished observations; see Appendix) to preserve the same relative concentration of bound melittin as that for the fluorescence measurements for $R = 60$.)

RESULTS

We have carried out fluorescence anisotropy measurements to determine the effects of melittin on what may be called the "fluidity" of saturated phospholipid bilayers. This term encompasses both the order and the dynamics of the acyl chains (see, e.g., Lentz, 1989) on the millisecond time scale. We will first present the results for 21°C, the melting transition temperature T_m of DMPC small unilamellar vesicles (SUVs). A typical plot is shown in Fig. 1. The upper panel shows the fluorescence anisotropy r of TMA-DPH as a function of time for a lipid-to-melittin molar ratio R of 90. The values of r have been calculated from the vertical I_{VV} and horizontal I_{VH} fluorescence components using Eq. 2. The smooth line is the best fit of Eq. 3 to the data; the fit has yielded the values of 0.224, 0.278, and 6.9 s^{-1} for the initial r_o and the final r_∞ anisotropies and the rate of change k_1 of r , respectively. The anisotropy, therefore, is seen to increase by about 25% upon melittin binding. Such an increase has also been observed for the other values of R . The results are summarized in Table 1. Thus, melittin induces a significant reduction in the fluidity of the bilayer in the vicinity of the fluorescent probe, i.e., at the glycerol backbone and the uppermost region of the bilayer where the probe is located (Prendergast et al., 1981; Lentz, 1989; Sijs et al., 1993). We should note here that we previously reported (Bradrick et al., 1987) by nanosecond time-resolved fluorometry with DPH as the fluorescent probe that melittin also decreases the fluidity of DMPC SUVs at 21°C, which had been incubated with the protein and therefore were fused by it (Eytan and Almary, 1983; Morgan et al., 1983; Bradrick and Georgiou, 1987).

Another quantity of interest is the total fluorescence intensity $I_{VV} + 2I_{VH}$ of the probe. As melittin interacts with the bilayer, this quantity is found at 21°C for DMPC SUVs to increase with time with a rate k_2 , which for all lipid-to-melittin molar ratios R used is within experimental error identical to the rate k_1 obtained from the increase in the anisotropy r with time. A small overall increase in $I_{VV} + 2I_{VH}$ has been observed, about 8%, 7%, 7%, and 6% for $R = 60, 90, 135$, and 180 , respectively. Fig. 2 shows the results for $R = 90$. In view of the fact that the fluorescence of TMA-DPH in water is negligible (Stubbs et al., 1984; Lentz, 1989), its enhancement, which is induced by the binding of melittin, may be taken to suggest that melittin reduces somewhat the degree of hydration of the bilayer. (Further discussion of this point is given below.) It should be noted that the initial values of $I_{VV} + 2I_{VH}$ and of r for these experiments were found to be the same as those determined in the absence of melittin for all four values of R . This observation implies that no changes are induced in

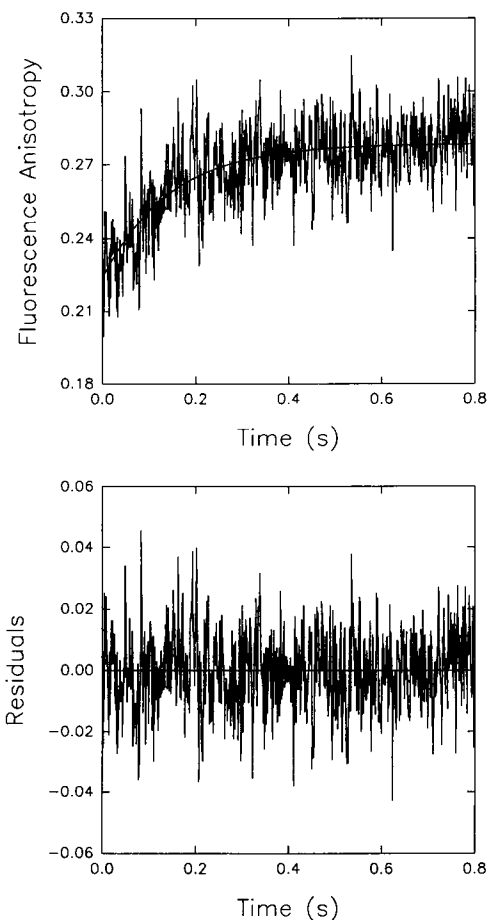


FIGURE 1 Effect of melittin binding as a function of time on the fluorescence anisotropy r of TMA-DPH incorporated into DMPC small unilamellar vesicles. The buffer was 50 mM Tris, 1 mM EDTA, pH 7.6, prepared in triply-distilled water. The lipid concentration was 1.48 mM, the lipid-to-melittin molar ratio was 90, and the sample temperature was 21°C. (Top) Fluorescence anisotropy r , calculated from the individual vertical I_{VV} and horizontal I_{VH} fluorescence components, respectively, using Eq. 2, for vertically polarized exciting light; the smooth line is the fit of Eq. 3 to the data. This analysis yielded values of 0.224, 0.278, and 6.9 s^{-1} for the initial (r_o) and final (r_∞) values of the anisotropy and its rate of change, k_1 , respectively. (Bottom) Residuals for the fit.

TABLE 1 Effects of melittin on the kinetics of the fluorescence anisotropy r and the total fluorescence intensity $I_{VV} + 2I_{VH}$ of TMA-DPH in DMPC small unilamellar vesicles

Lipid/melittin molar ratio R	21°C*			33°C k_2 (s ⁻¹) [§]
	k_1 (s ⁻¹)*	r_o	r_∞	
60	9.4 ± 3.5	0.225 ± 0.006	0.275 ± 0.012	43 ± 2
90	6.9 ± 1.4	0.224 ± 0.006	0.278 ± 0.007	33 ± 3
135	4.7 ± 0.7	0.220 ± 0.004	0.279 ± 0.008	14 ± 3
180	3.3 ± 0.7	0.220 ± 0.005	0.274 ± 0.008	6 ± 2

*Rate of increase of the fluorescence anisotropy r , which was found within experimental error to be identical to the rate k_2 of increase of the total fluorescence intensity. r_o and r_∞ are the initial and final values of r , respectively.

†Melting transition temperature T_m of DMPC SUVs.

§Rate of increase of the total fluorescence intensity. The fluorescence anisotropy at 33°C was found to be invariant with time on the millisecond time scale.

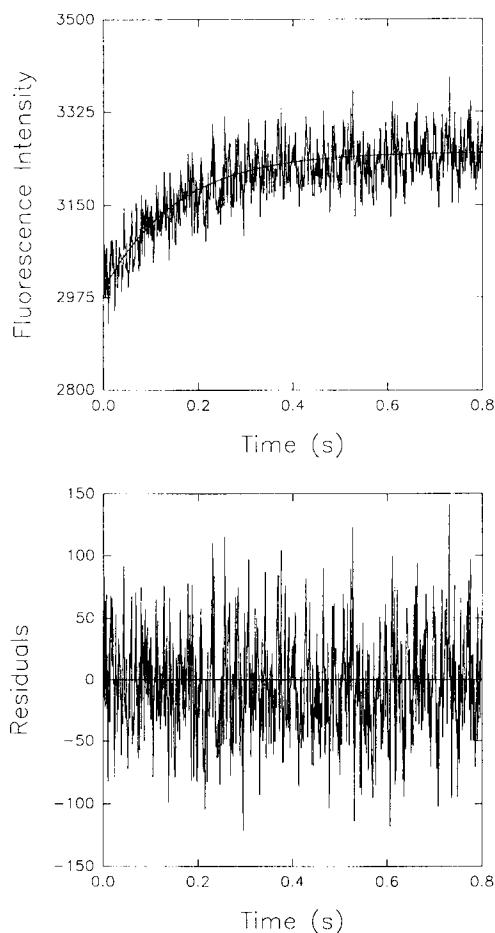


FIGURE 2 Effect of melittin binding as a function of time on the total fluorescence intensity F of TMA-DPH incorporated into DMPC small unilamellar vesicles for a lipid-to-melittin molar ratio of 90. The buffer was 50 mM Tris, 1 mM EDTA, pH 7.6, and the sample temperature was 21°C. (Top) Total fluorescence intensity $F = I_{VV} + I_{VH}$, where I_{VV} and I_{VH} are the vertical and horizontal fluorescence components, respectively, for vertically polarized exciting light; the smooth line depicts the best fit of Eq. 4 to the data. This analysis yielded a value for the rate of change k_2 of $I_{VV} + 2I_{VH}$, which is identical within experimental error to that of 6.9 s^{-1} obtained from the analysis of the TMA-DPH fluorescence anisotropy data for this system under the same experimental conditions (see Fig. 1). (Bottom) Residuals for the fit.

the bilayer by the protein within the deadtime of the stopped-flow fluorometer (about 3 ms).

We have found that, for DMPC SUVs at 33°C and for all four values of R , the TMA-DPH fluorescence anisotropy r was practically independent of time. Also, the initial value of r was found to be invariant upon addition of melittin. These observations imply that melittin does not affect the fluidity of these bilayers above their T_m . The total fluorescence intensity $I_{VV} + 2I_{VH}$ of the probe exhibits an increase with time, however. This increase was found to be about 7%, 6%, 5%, and 3% for $R = 60, 90, 135$, and 180, respectively. The values of the rate k_2 of the increase in the fluorescence are listed in Table 1.

We have also carried out measurements of the apparent rate of binding k of melittin to the DMPC SUVs by following the decrease in the fluorescence intensity of its single tryptophan residue, which is at position 19. This decrease in the fluorescence transmitted through the 0–51 Corning filter is the result of the shift of the fluorescence spectrum of the tryptophan to shorter wavelengths because of the change in its environment as melittin inserts itself into the bilayer (Georgiou et al., 1982). Fig. 3 shows a plot for $R = 90$ at 21°C and the fit of the functional form $A \exp(-kt) + B$ to the data. Table 2 summarizes the values of k so obtained for all values of R at 16°C, 21°C, and 33°C; the absolute protein and lipid concentrations were the same as those used in the measurements in which TMA-DPH was the extrinsic fluorescent probe (Table 1). As can be seen from Tables 1 and 2, at 21°C the rate k_1 of the increase in the TMA-DPH

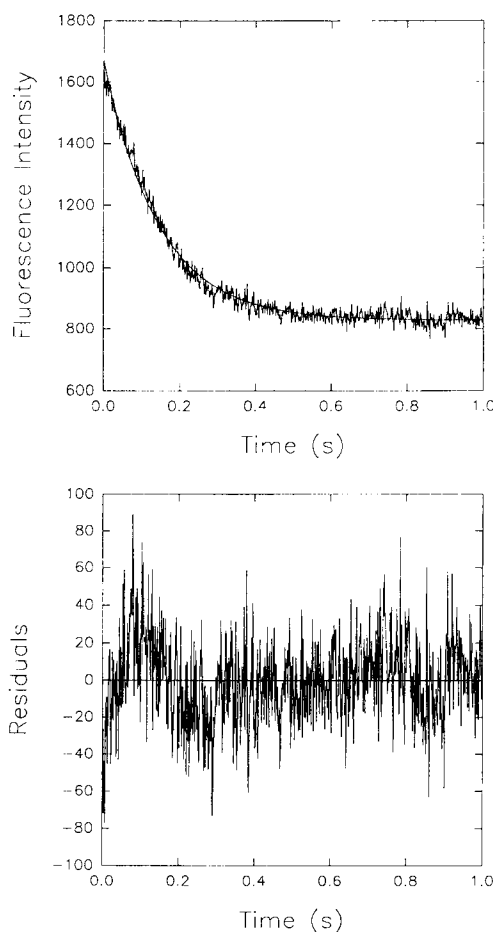


FIGURE 3 Plot of the fluorescence intensity of the single tryptophan residue of melittin as it binds to DMPC small unilamellar vesicles at 21°C for a lipid-to-melittin molar ratio of 90 as a function of time (top). The buffer used was 50 mM Tris, 1 mM EDTA, pH 7.6. The smooth line in the upper panel shows the fit of the function $A \exp(-kt) + B$ to the data, where k is the apparent rate of melittin binding to the vesicles. The lower panel shows the residuals for the fit. The value of k so obtained was found to be identical within experimental error to those obtained from the analysis of the data for the fluorescence anisotropy and the total fluorescence intensity of TMA-DPH (Figs. 1 and 2, respectively).

TABLE 2 Rate k (s^{-1}) of melittin binding to DMPC and DPPC small unilamellar vesicles as determined by measuring the rate of change in the fluorescence of the single tryptophan residue of melittin while binding takes place

Lipid/melittin molar ratio R	DMPC			DPPC		
	16°C	21°C (T_m)	33°C	25°C	37°C (T_m)	51°C
60	2.7 ± 0.2	8.1 ± 1.4	44 ± 4	27 ± 4	42 ± 7	26 ± 4
90	3.3 ± 0.3	7.2 ± 1.0	37 ± 4	28 ± 6	43 ± 6	23 ± 7
135	5.1 ± 0.3	7.5 ± 1.0	32 ± 4	50 ± 8	68 ± 14	117 ± 32
180	8.0 ± 1.0	8.5 ± 1.0	30 ± 3	99 ± 11	99 ± 25	143 ± 30

anisotropy is comparable to the rate k of the decrease in the fluorescence of melittin. This suggests that the perturbations of the bilayer induced by the protein arrive at the probe practically instantaneously on the millisecond time scale of the measurements. This is discussed further in the next section.

For DMPC SUVs at 16°C, below their T_m , no changes in either the anisotropy r or the total fluorescence $I_{VV} + 2I_{VH}$ were detected within the time scale of 4 s. (This is a shorter time scale than that over which more complex vesicle structures are formed as a result of melittin-induced fusion (Bradrick and Georgiou, 1987).) The values of the apparent rate of binding k are listed in Table 2.

We have also carried out measurements for DPPC SUVs, similar to those described above for DMPC SUVs, to investigate the importance of the acyl chain length with regard to the effects of melittin on the bilayer. The values for the rate k of change of the melittin fluorescence at 25°C, 37°C, and 51°C are shown in Table 2. In this case it was found that there was virtually no change in the values of the fluorescence anisotropy or the total fluorescence intensity of TMA-DPH at these temperatures for the four lipid-to-melittin molar ratios R of 60, 90, 135, and 180. Only for $I_{VV} + 2I_{VH}$ at 51°C for the two lowest R values of 60 and 90 was any change observed with melittin binding, for which rates of $20 \pm 4 s^{-1}$ and $11 \pm 2 s^{-1}$ and overall increases of about 15% and 10% were obtained, respectively. The corresponding values of k are $26 \pm 4 s^{-1}$ and $23 \pm 7 s^{-1}$ (Table 2).

To investigate the possibility that the protein is sequestered into the vicinity of TMA-DPH, we have carried out measurements of transfer of electronic energy. As was discussed under Materials and Methods, we have found that TMA-DPH had no effect on the fluorescence of melittin. This indicates that the average donor (the single tryptophan residue of melittin)-acceptor (TMA-DPH) distance is greater than the value of 39 Å, which we have calculated for R_0 , the distance for which transfer is 50% efficient (see Materials and Methods).

DISCUSSION

As was pointed out above, melittin has its greatest effect on DMPC SUVs at 21°C, the lipid phase transition temperature T_m . At T_m , both the fluorescence anisotropy and the total fluorescence intensity of TMA-DPH increase with time upon binding of melittin (Figs. 1 and 2). The former indi-

cates that the protein has caused the membrane to become less fluid; this is further discussed below. First, however, we comment on the observed small, but measurable, fluorescence enhancement of TMA-DPH (which is about 8% for a lipid-to-melittin molar ratio of 60) for which we consider the following explanations:

1. A somewhat increased exclusion of water from the bilayer. This is based on the observation that the fluorescence quantum yield of TMA-DPH is negligible in water (Stubbs et al., 1984; Lentz, 1989). (Because the photophysical origin of this fluorescence quenching is not known, the extent of water exclusion from the bilayer cannot be quantified.) The fluorescent probe is anchored at the bilayer-water interface and extends down past the glycerol backbone and as far as about the C_{10} position of the lipid acyl chains (Prendergast et al., 1981); thus, most likely this reduction in the hydration takes place mainly at the glycerol backbone. In this context, there is evidence that water penetrates to some extent up to this region (Wiener and White, 1992; Zhou and Schulten, 1995), which is the most tightly packed region of the bilayer (Metcalf, 1971; Hubbell and McConnell, 1971).

2. The result of a more rigid TMA-DPH. This appears unlikely because we have also observed an enhancement of the TMA-DPH fluorescence by melittin above the T_m of the bilayer, where its fluidity is found not to be affected by the protein. In this regard it is reported that the fluorescence of TMA-DPH is not sensitive to the viscosity of its environment (Prendergast et al., 1981).

3. An increase in the absorbance of TMA-DPH at the excitation wavelength of 365 nm upon melittin binding. This possibility has been discounted through stopped-flow spectrophotometric measurements in which we have found that the intensity of transmitted light at 365 nm (and therefore the absorbance of TMA-DPH) does not change upon melittin binding, either during the deadtime of the stopped-flow spectrometer or over the time range of 800 ms for which the fluorometric measurements reported in the present study were made (see Materials and Methods).

Thus, we consider a reduction in the degree of hydration of the bilayer to be the most likely explanation for the observed increase in the total fluorescence intensity of TMA-DPH upon melittin binding.

The long-time (equilibrium) value of the fluorescence anisotropy, r_∞ , is seen from Table 1 to be practically

constant at T_m as the added concentration of melittin is varied by a factor of 3. Actually, the observed enhancement of the total fluorescence intensity at the higher melittin concentrations (lower R values) of $\sim 10\%$ implies that the lifetime of the excited state of TMA-DPH in the presence of melittin is somewhat longer; this in turn implies that a greater depolarization of the fluorescence of TMA-DPH has actually taken place. Thus, for the higher melittin concentrations, the fluidity of the bilayer, as expressed by r_∞ , is somewhat lower. For low R values, $R = 60$ and $R = 90$, the rate k_1 of the change of the fluorescence anisotropy of TMA-DPH (Table 1) is very similar to the rate k of the change of the melittin fluorescence (Table 2). For $R = 135$, there is a difference between k_1 and k that is outside the experimental error by $\sim 20\%$. This difference increases to $\sim 60\%$ for $R = 180$ and is perhaps the result of a reduction in k_1 that stems from the increase in the average nearest-neighbor distance from the point of melittin insertion to TMA-DPH. (We note that this difference would increase somewhat if the rate of the change of the fluorescence anisotropy of melittin were measured instead of the rate of the change of its total fluorescence intensity (Otto et al., 1994) because of the increase by $\sim 60\%$ of its fluorescence quantum yield upon binding to the bilayer (Vogel and Jähnig, 1986).) Overall, then, the rate at which melittin induces a reduction in the fluidity of the bilayer is seen to be comparable to the rate at which melittin binds to the bilayer. This implies that the perturbation of the bilayer by the protein is felt almost instantaneously by the fluorescent probe TMA-DPH on the millisecond time scale of the measurements.

To discount the possibility that melittin is sequestered in the neighborhood of TMA-DPH, we carried out measurements of energy transfer from the tryptophan of melittin to TMA-DPH, with negative results. Here, we calculate the average distance between a donor (melittin) molecule and its nearest-neighbor acceptor (TMA-DPH) molecule and find it to be indeed quite large; this finding implies that the transfer is very inefficient, in agreement with our experimental observations. We consider the average SUV to have a diameter of 250 \AA , with approximately 2700 lipid molecules per vesicle, two-thirds of which are in the outer leaflet of the bilayer (Huang, 1969; Szoka and Papahadjopoulos, 1980). For a random distribution and for the lipid-to-TMA-DPH molar ratio of 500 used here, this leads to a surface probability density $\sigma_{\text{TMA-DPH}}$ of approximately $1.8 \times 10^{-5} \text{ \AA}^{-2}$ in the outer leaflet. To calculate the average nearest-neighbor distance, we follow the methodology of Chandrasekhar (1943), which was developed for the case of an infinite three-dimensional space. Here, for a spherical shell of radius r with a surface probability density σ and the "origin" located on the positive z axis at the shell's north pole, the probability that the nearest neighbor is located at the azimuthal angle θ is given by

$$w(\theta)d\theta = 2\pi r^2 \sigma \sin\theta \exp[2\pi r^2 \sigma (\cos\theta - 1)]d\theta \quad (5)$$

The average nearest-neighbor distance $\langle D \rangle$ through the spherical shell is then

$$\langle D \rangle = \frac{\int_0^\pi r \theta w(\theta) d\theta}{\int_0^\pi w(\theta) d\theta} \quad (6)$$

For the above value of $\sigma_{\text{TMA-DPH}}$, Eqs. 5 and 6 yield a value of $\langle D \rangle \approx 120 \text{ \AA}$, which corresponds to a through-space distance of about 115 \AA . (The integral in the numerator of Eq. 6 was evaluated numerically using the Qtrap and Trapzd subroutines from Numerical Recipes (Press et al., 1992).) With respect to the probability of energy transfer from melittin to TMA-DPH, we note that because at any one time the fraction of melittin molecules in the excited state is very low, we may assume that there are no other excited donor (melittin) molecules within this range; therefore, 120 \AA may be taken to be the average donor-acceptor distance. (For a lipid-to-melittin molar ratio of 60, the surface probability density σ_{melittin} would be approximately $1.5 \times 10^{-4} \text{ \AA}^{-2}$, so that there would be on the average an additional seven melittin molecules within the nearest-neighbor distance of 120 \AA . The probability that any of these additional seven melittin molecules would be in the excited state and serve as an energy transfer donor is considered to be negligible.) The efficiency f of transfer is given by (Förster, 1965)

$$f = \frac{R_0^6}{R_0^6 + d^6} \quad (7)$$

where d is the average donor-acceptor distance. For the critical transfer distance of 39 \AA and $d \approx 115 \text{ \AA}$, Eq. 7 yields $f \approx 0.2\%$ for this system. The result of this calculation is consistent with the absence of any detected transfer of energy from the tryptophan of melittin to TMA-DPH, which discounts the likelihood that melittin is sequestered close to TMA-DPH.

An approximate calculation of the extent of the melittin-induced perturbation of the bilayer, as reported by TMA-DPH, can be made in a manner similar to the above. The question asked is, what is the average distance between an excited probe (TMA-DPH) and the nearest-neighbor melittin? For a lipid-to-melittin molar ratio R of 90, σ_{melittin} is approximately equal to $1.0 \times 10^{-4} \text{ \AA}^{-2}$, which yields for the average nearest-neighbor distance $\langle D \rangle$ a value of about 50 \AA in this case. (Within this distance there is a probability of about 15% of finding another TMA-DPH molecule, which we will ignore.) Fifty angstroms is then the range over which effects of the binding of melittin are felt in the membrane for a lipid-to-melittin molar ratio of 90. Thus, the melittin-induced perturbation of the bilayer is seen to propagate over a large distance. We should add that a similar finding was reported by Rehorek et al. (1985) for the

bacteriorhodopsin-DMPC system. We also note that values for $\langle D \rangle$ similar to those we determined above are obtained if the bilayer is treated as an infinite two-dimensional plane, in which case the calculation yields the analytic solution of $\langle D \rangle = 0.5\sigma^{-1/2}$.

The observation that at 33°C, above the DMPC phase transition, the TMA-DPH fluorescence anisotropy is not altered by melittin implies that the protein does not affect appreciably the fluidity of the bilayer in its liquid-crystalline phase. This is also the case for DPPC bilayers above their lipid phase transition (see below). We should note in this regard that ^2H NMR studies reached a similar conclusion for a number of proteins in the lipid fluid phase (reviewed by Bloom, 1979; Seelig and Seelig, 1980; Jacobs and Oldfield, 1981; Mouritsen and Bloom, 1984). Bloom (1979) has proposed that the outer region of proteins is fluid-like and provides a mechanical match with the fluid bilayer; consequently, the perturbation of the fluidity of the bilayer is minimized.

As was discussed above, the binding of melittin reduces somewhat the degree of hydration of the DMPC bilayer at its T_m of 21°C, an inference that was drawn from the observed enhancement of the total fluorescence intensity of TMA-DPH (Fig. 2). Melittin also induces such an enhancement at 33°C at a rate k_2 that is larger than that at 21°C (Table 1). (In making this comparison, it should be noted that the values of k_2 at 21°C have been found to be identical, within experimental error, to those of k_1 listed in Table 1.) For DPPC, actually no fluorescence enhancement is observed at its T_m of 37°C, whereas such an enhancement is observed at 51°C, above its T_m . We hypothesize that such a reduction in the degree of hydration of the DMPC and DPPC bilayers above their T_m in the absence of any observed significant reduction in their fluidity is the consequence of the effects of melittin on the headgroups of the lipid. In this regard, melittin has been reported to strongly affect the headgroups through electrostatic interactions (Kuchinka et al., 1989; Dempsey et al., 1989; Beshiaschvili and Seelig, 1990). It is then conceivable that such effects may perturb the packing density of the lipid headgroups, which in turn could alter the degree of hydration of the glycerol backbone. With regard to the dependence of the rate of total fluorescence increase k_2 on the lipid-to-melittin molar ratio R for DMPC at 21°C and 33°C, it is seen from Table 1 that it exhibits an increase at the low R values of 90 and 60 that is more pronounced at 33°C; for these two ratios for which the concentrations of melittin are relatively high, the values of k_2 are seen to be very similar to those for the apparent rate of melittin binding k (Tables 1 and 2). This suggests that the perturbations of the bilayer responsible for the fluorescence enhancement are communicated to TMA-DPH almost simultaneously with the binding of melittin. As was discussed above, a similar inference was also reached with regard to the melittin-induced decrease of the fluidity of the bilayer. The smaller values of k_2 relative to those of k for the lower concentrations of added melittin (Tables 1

and 2) are perhaps the result of the increase in the average nearest-neighbor distance from melittin to TMA-DPH.

The invariability of the fluorescence anisotropy of TMA-DPH and its total fluorescence intensity with time at 16°C for DMPC SUVs indicates that melittin binding does not substantially affect the bilayer in that case. Little is known about the binding of melittin to lipid vesicles that are in the gel phase. The greater packing density of the bilayer may not allow melittin to penetrate into the bilayer to a depth that is significant enough to perturb it. The observation that melittin does not activate phospholipase A_2 in the gel phase of the bilayer (Mollay and Kreil, 1974) is consistent with this mode of interaction; also, the vesicles have an unusual faceted or polygonal structure (Blaurock and Gamble, 1979; Lichtenberg and Schmidt, 1981).

The interaction of melittin with DPPC SUVs is found to have no effect on the TMA-DPH fluorescence anisotropy at their T_m . This is in contrast to the interaction of melittin with DMPC SUVs at their T_m , which results in an increase in the anisotropy as was discussed above (Table 1). The questions then arise as to why only DMPC is perturbed and only at its T_m . In attempting to answer these questions, we resort to theoretical descriptions of protein-membrane interactions. In the presence of a protein, a mismatch between its hydrophobic length and the length of the acyl chains is predicted to induce a perturbation of the bilayer, which peaks dramatically at T_m (Marcelja, 1976; Owicki et al., 1978; Jähnig, 1981; Sperotto and Mouritsen, 1991). The distance over which the perturbation propagates, called the coherence length, is equal to a number of molecular layers at T_m and is less than one layer far from the thermal transition region (Sperotto and Mouritsen, 1991; Bloom et al., 1991; Mouritsen and Biltonen, 1993). These considerations are in harmony with the findings of the present study: i) a decrease in the fluidity of the DMPC bilayer is induced by melittin only at T_m , and ii) the decrease in the fluidity is felt by the lipid over a large distance. In the absence of protein, the density fluctuations at T_m are much stronger and have a larger coherence length in DMPC than in DPPC (Ipsen et al., 1990). On the basis of the predicted strong correlation between the magnitude of the density fluctuations and the strength of the protein-induced perturbation, i.e., the coupling of the protein to the fluctuations (Marcelja, 1976; Owicki et al., 1978; Jähnig, 1981; Sperotto and Mouritsen, 1991; Mouritsen and Biltonen, 1993), one would then expect melittin to perturb the DMPC bilayer to a greater extent. This provides a potential framework for rationalizing the present finding that the fluidity of DMPC, but not that of DPPC, is affected by melittin at the corresponding T_m .

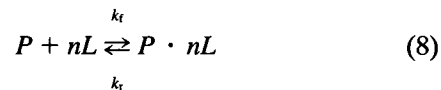
Another factor that may play a role in this selectivity of the protein in disturbing the bilayer is the stability of the protein-bilayer molecular complex. Preliminary isothermal titration calorimetric measurements (Bradrick et al., unpublished observations) find that the association constant of the melittin-DPPC complex is considerably greater than that for the melittin-DMPC complex. Consistent with this is the fact

that analysis of the present data for the observed rate k of melittin binding finds that the rate constants for association k_f with these two lipid bilayers exhibit this same trend, with melittin having a considerably greater affinity for DPPC than DMPC bilayers (see below and the Appendix). (The determination of the association constants from the results of this analysis, however, is not currently possible because of a considerable uncertainty associated with the rate constant for dissociation k_r .) The apparent greater stability of the molecular complex formed by DPPC with melittin would suggest a better matching between the acyl chains of this lipid and the membrane-embedded part of the protein as compared to the corresponding matching for the complex formed by DMPC with melittin. Consequently, on the basis of the concept of protein-acyl chain mismatch (Mouritsen and Bloom, 1984; Sperotto and Mouritsen, 1991), the perturbation of the DPPC bilayer would be expected to be much smaller, as has been observed in the present study (see above). In terms of the cluster model (Papahadjopoulos et al., 1973; Marsh et al., 1976; Freire and Biltonen, 1978; Kanehisa and Tsong, 1978), melittin may be considered to have a preference for the mismatches between gel and liquid domains, which are more numerous for the less cooperative chains of DPPC (Mabrey and Sturtevant, 1976). In this regard, the depth of penetration of melittin into the bilayer may be different for DPPC than for DMPC; this is currently being explored in our laboratory.

We should add here that the concept of mismatch was put forward for integral proteins that span the bilayer (Mouritsen and Bloom, 1984; Sperotto and Mouritsen, 1991). It has not been established whether melittin spans the bilayer, although evidence for it has been presented (Vogel et al., 1983). Two other models have been proposed, one in which melittin forms a wedge (Dawson et al., 1978) and another in which the helical axis of melittin lies approximately parallel to the plane of the membrane (Terwilliger et al., 1982); in both of these cases melittin penetrates into only one leaflet of the bilayer. The results of the present study do not necessarily support a particular model. In any case, we consider it likely that a mismatch between the acyl chains and the hydrophobic part of the protein, which could be only partially embedded in the membrane, would be sufficient to cause an elastic perturbation. (This consideration is in line with the qualitative discussion of protein-membrane interactions by Israelachvili (1977) with regard to the distortion of the acyl chains as a function of the depth of penetration of the protein.) Such a perturbation may produce a compressional wave that travels outward from the point of protein insertion. As the energy necessary for lateral compression of lipid bilayers is very small, $\sim kT$ (Lis et al., 1982), the generation of a compressional wave is not an energetically costly process. Thus, this is a possible mechanism for the propagation of the structural and dynamic modifications of the bilayer that are induced by melittin.

The rate k of melittin binding to DPPC SUVs is seen from Table 2 to decrease considerably as the concentration of added melittin is increased at the T_m of the lipid. By con-

trast, k for the DMPC SUVs does not exhibit any concentration dependence (Table 2). To rationalize these experimental results, the binding of melittin to lipid bilayers may be modeled by the following kinetic scheme:



Here, P is a free melittin molecule, nL may be considered to be a protein "binding site" that is composed of n^{-1} lipid molecules, $P \cdot nL$ is the resulting protein-lipid complex, and k_f and k_r are the rate constants for association and dissociation, respectively. (We note that this model has been successfully used to describe the interaction of other amphiphilic α -helices and lipid bilayers (see, e.g., Myers et al., 1987).) The observation (see Results) that for all the melittin fluorescence measurements the decay profile is described by the form $A \exp(-k_{\text{eff}}t) + B$, where k_{eff} is an "effective" rate constant that is identified with k , is seen in the Appendix to be consistent with this model; k_{eff} is given by

$$k_{\text{eff}}^2 = \{k_r + k_f([nL]_0 + [P]_0)\}^2 - 4k_f^2[P]_0[nL]_0 \quad (9)$$

Here, the subscript 0 denotes initial (i.e., at zero time) concentrations. A nonlinear least-squares analysis of the data of Table 2 for k in terms of Eq. 9 for DPPC and DMPC has yielded the following values i) for DPPC at its $T_m = 37^\circ\text{C}$, $k_f \approx 8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $k_r \approx 1 \text{ s}^{-1}$, and number of lipids per binding site $n^{-1} \approx 74$, and ii) for DMPC at its $T_m = 21^\circ\text{C}$, $k_f \approx 5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, $k_r \approx 1 \text{ s}^{-1}$ and $n^{-1} \approx 77$. Thus, melittin is seen to have a significantly greater affinity for DPPC than for DMPC bilayers. We defer to a subsequent study the systematic investigation of this aspect of the melittin-bilayer interaction, which will be carried out in terms of the fluorescence anisotropy of the tryptophan of melittin (see Appendix). In any case, the above model is able to account for the disparate dependencies of k on melittin concentration for these two lipids (see Table 2).

The findings of the present study may be of significance with regard to the physiological effects of melittin. Melittin activates the enzyme phospholipase A_2 , which catalyzes the hydrolysis of fatty acid ester bonds at the *sn*-2 position of phospholipids. There is evidence that this activation is brought about through a modification of the physical properties of the membrane rather than through the formation of a molecular complex between the protein and the enzyme (Mollay and Kreil, 1974; Yunes et al., 1977). The present study finds that melittin renders the bilayer stiffer and somewhat less hydrated. It is conceivable that these altered characteristics are part of the modified lipid conformation, which the enzyme recognizes and complexes with. Furthermore, the bilayer may become functionally compromised to some extent as a result of the modifications of its conformation; this may play a role in the melittin-induced enhancement of the permeability of bilayers to ions and solutes (Sessa et al., 1969; Ohki et al., 1994) with which ion channels appear to be associated (Tosteson et al., 1990;

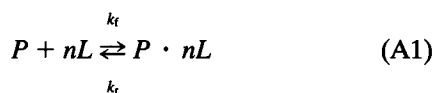
Schwarz et al., 1992) and, ultimately at high melittin concentrations, in the formation of small structures and the lysis of the bilayer (Dufourc et al., 1986; Dempsey and Watts, 1987).

In summary, the results of the present study show that the reduction in the fluidity of saturated phospholipid bilayers i) is maximal at the transition temperature of the bilayer, ii) extends to at least 50 Å, and iii) depends strongly on the acyl chain length, being considerable for DMPC and negligibly small for DPPC. The chain length selectivity of melittin is also manifested in its having a significantly greater affinity for DPPC than for DMPC bilayers. These observations may be understood in terms of the concept of elastic perturbations that arise from a mismatch between the protein and the chains of the lipid that are attempting to accommodate it (Mouritsen and Bloom, 1984; Sperotto and Mouritsen, 1991).

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APPENDIX

One possible model for the binding of a protein to phospholipid vesicles is given by



Here, P is a free protein (in this case, melittin) molecule, nL is a protein "binding site" composed of n^{-1} lipid molecules, $P \cdot nL$ is the bound protein-lipid complex, and k_f and k_r are the rate constants for association and dissociation, respectively. This model assumes that melittin, which is monomeric in solution (as was the case for this study), binds as a monomer and does not undergo self-aggregation in the membrane. In this regard, early studies reported conflicting results, but more recent studies have presented evidence that supports the validity of this model (Bradrick et al., 1989; John and Jähnig, 1991, and discussion therein).

In the context of mass-action kinetics, the rate of change in the concentration of the protein-lipid complex is given by

$$\frac{d[P \cdot nL]}{dt} = -k_r[P \cdot nL] + k_f[P][nL] \quad (\text{A2})$$

where the square brackets denote concentration. Given the stoichiometry, from which $[P]_t = [P]_o + [P \cdot nL](t)$ and $[nL]_t = [nL]_o = [nL](t) + [P \cdot nL](t)$, where the t and o subscripts denote total and initial concentrations, respectively, and the initial condition that $[P \cdot nL]_o = 0$, it follows from Eq. A2 that

$$[P \cdot nL](t) = \frac{b - k_{\text{eff}}}{2k_f} \frac{1 - \exp(-k_{\text{eff}}t)}{1 - \left(\frac{b - k_{\text{eff}}}{b + k_{\text{eff}}}\right)\exp(-k_{\text{eff}}t)} \quad (\text{A3})$$

where

$$b = k_r + k_f([nL]_o + [P]_o) \quad (\text{A4})$$

and

$$k_{\text{eff}}^2 = \{k_r + k_f([nL]_o + [P]_o)\}^2 - 4k_f^2[P]_o[nL]_o \quad (\text{A5})$$

For all of the experiments described in this paper, the total melittin concentration $[P]_o$ was kept low enough so that the maximum sample absorbance at the excitation wavelength of 280 nm was about 0.03 over the 2-mm cuvette path length from which fluorescence was collected. In that case, the fluorescence intensity $F(t)$ is proportional to the fluorophore (melittin) concentration and, in the context of the kinetic model being used (Eq. A1), is given by

$$F(t) = I\gamma_f \epsilon_f q_f [P](t) + I\gamma_b \epsilon_b q_b [P \cdot nL](t) \quad (\text{A6})$$

Here, γ is the transmittance of the emission filter, ϵ is the molar extinction coefficient at the wavelength of excitation, q is the fluorescence quantum yield for a particular melittin species (free or bound as denoted by the subscripts f and b , respectively), and I denotes the (relative) intensity of the exciting light. To simplify the fitting of Eq. A6 to the data, Eq. A3 may be expressed as a series expansion in powers of $\exp(-k_{\text{eff}}t)$:

$$[P \cdot nL](t) = \frac{b + k_{\text{eff}}}{2k_f} - \frac{k_{\text{eff}}}{k_f} \sum_{m=0}^{\infty} \left(\frac{b - k_{\text{eff}}}{b + k_{\text{eff}}}\right)^m \exp(-mk_{\text{eff}}t) \quad (\text{A7})$$

(Note: $[(b - k_{\text{eff}})/(b + k_{\text{eff}})]\exp(-k_{\text{eff}}t) < 1$.) If terms in $m > 1$ make a much smaller contribution than the first-order term, a simplified single-exponential expression for $[P \cdot nL](t)$ may be substituted into Eq. A6. The latter is then reduced to the form

$$F(t) = A \exp(-k_{\text{eff}}t) + B \quad (\text{A8})$$

As this is the same function that was found to fit all the melittin fluorescence data (see Results), we identify k_{eff} with the values for the observed rate of melittin binding k listed in Table 2 and expect that a graph of k^2 vs. $[P]_o$ will give plots (not shown) to which nonlinear least-squares fits of Eq. A5 should yield best-fitting values for the parameters k_f , k_r , and n . The results of the analysis are, for DPPC at 37°C, $k_f = (8 \pm 3) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $n^{-1} = 74 \pm 6$, and for DMPC at 21°C, $k_f = (5 \pm 3) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, $n^{-1} = 77 \pm 12$. Depending on the magnitudes of the activation energies, some contribution to the difference in the values of k_f for DPPC and DMPC can, of course, be made by the difference in the temperatures of 21°C and 37°C at which the measurements were carried out. Nevertheless, these values for k_f suggest that melittin has a greater affinity for DPPC than for DMPC bilayers. For both lipids, the estimated value for k_r was found to be about 1 s^{-1} ; there was, however, considerable uncertainty associated with this value, which precludes a calculation of the association constants for the binding of melittin to these two lipid bilayers.

As was stated in the Discussion, the purpose of this analysis was to rationalize the observed dependence of k on melittin concentration for the DMPC and DPPC bilayers at their respective melting transition temperatures (Table 2). We should add that, although the main objective with regard to the measurement of k was to compare its values with those of the rate k_1 , analysis of the data for k has yielded useful information concerning k_r , as has been discussed in this section. It is not unexpected, however, that this type of analysis, in which an approximate expression was fitted to a plot of the observed rate constant vs. ligand concentration, should result in values having considerable uncertainty. A more accurate experimental approach would be to globally fit the exact expression for the fluorescence intensity (Eq. A6) or the corresponding expressions for the polarized fluorescence intensities to the appropriate decays obtained for several different protein and lipid concentrations; the latter is currently being employed in our laboratory, and the preliminary results (Payongayong et al., unpublished observations) suggest that for the case of the DMPC-melittin complex at 21°C, $k_f \approx 1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, $k_r \approx 1 \text{ s}^{-1}$, and $n^{-1} \approx 35$. We therefore deduce that the values for these parameters, which were obtained from the analysis presented here, are in fair agreement with those obtained with the more rigorous approach.

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