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Kinetics of melittin-induced fusion of dipalmitoylphosphatidylcholine small unilamellar vesicles

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We have studied the kinetics of fusion of dipalmitoylphosphatidylcholine small unilamellar vesicles at 51°C which is induced by bee venom melittin at a protein-to-lipid molar ratio of 1/60. This was done by following with a stopped-flow fluorometer the reduction in the ratio of the excimer to monomer fluorescence intensities of 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-phosphorylcholine that accompanies fusion. At a low melittin concentration and low ionic strength, for which case the protein is monomeric, the value of the rate constant for fusion is 0.006 s^{-1}. This is much smaller than that of 0.06 s^{-1} obtained for a high melittin concentration at low ionic strength, i.e. for the protein in the tetrameric form which is not induced by a high salt concentration. The value of the rate constant for fusion for a low melittin concentration in the presence of 2 M NaCl, i.e. for the protein in the tetrameric form which is induced by a high salt concentration, is 0.12 s^{-1}. This is twice as large as that for fusion induced by the tetramer in a low ionic strength solution. These findings show that the state of aggregation of the protein in solution and, to a lesser extent, electrostatic interactions play an important role in the kinetics of melittin-induced fusion of vesicles.

The peptide melittin, which constitutes approx. 50% of the dry weight of honey bee venom, has for some time received considerable attention in the study of model protein–phospholipid interactions. It has been reported that the protein lyses model and natural membranes [1–3] and fuses small unilamellar acidic [4] and large unilamellar zwitterionic vesicles [5]. The fusogenic properties of melittin are of considerable interest because of their relevance with respect to the protein's physiological effects, and because a knowledge of the mechanism involved will enhance our understanding of this important biological process.

Here, we report the results of our studies on the kinetics of melittin-induced fusion of dipalmitoylphosphatidylcholine (DPPC) small unilamellar vesicles (SUVs). The fusion assay which we used exploits the change that takes place in the ratio of the excimer to monomer (E/M) fluorescence intensities of pyrene, covalently attached to a phosphatidylcholine, when labeled vesicles are induced to fuse with nonlabeled vesicles and the local pyrene concentration is thus caused to decrease.

We find that, for a protein-to-lipid molar ratio of 1/60, the rate constant for the initial fusion

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; PS, phosphatidylserine; SUVs, small unilamellar vesicles; EDTA, ethylenediaminetetraacetic acid; pyrene-PC, 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-phosphorylcholine; E/M, ratio of the excimer to monomer fluorescence intensities.

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process is affected by the state of aggregation of melittin in solution. Electrostatic interactions also appear to play a role.

Materials and Methods. Analytical grade Tris was obtained from Fisher. Ethylenediaminetetraacetic acid (EDTA), analytical grade, and dipalmitoylphosphatidylcholine (99 + %) were obtained from Sigma or Avanti. Sodium chloride, analytical grade, was from Mallinckrodt and melittin was from ICN or Sigma. We included in the buffer 1 mM EDTA for the low melittin concentration and 5 mM EDTA for the high melittin concentration in order to inactive any phospholipase A_2 present in the melittin sample. No detectable lipid degradation takes place under these conditions for the time span of our measurements (Ref. 6 and Bradrick and Georghiou, unpublished observations). Three buffers were used, 50 mM Tris, 1 mM EDTA (pH 7.6); 50 mM Tris, 5 mM EDTA (pH 7.6); and 50 mM Tris, 1 mM EDTA, 2 M NaCl (pH 7.6). All were prepared in triply distilled water. 1-Palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-phosphorylcholine (pyrene-PC) was obtained from KSV-Chemicals Oy.

As has been shown [7,8], the ratio of the excimer E to monomer M fluorescence intensities of pyrene is given by

$$\frac{\mathrm{E}}{\mathrm{M}} \propto \frac{\tau T}{\eta}[\text{pyrene}] \tag{1}$$

where τ is the fluorescence decay time of the excimer, T is the absolute temperature, and η is the viscosity. The square brackets denote concentration. Equation 1 indicates that, at constant temperature, the excimer to monomer (E/M) ratio is proportional to the local pyrene concentration. This property has been utilized to follow the kinetics of vesicle fusion by serum albumin [9] and the kinetics of fusion of Sendai virus particles with vesicles [10]. As can be seen from Eqn. 1, however, the E/M ratio is also proportional to the fluidity ($1/\eta$) of the environment of pyrene and to the decay time of the excimer. Therefore, in general, a correction must be made to the data in order to obtain a true measure of the rate of the pyrene-PC dilution which results from fusion. This correction is determined by measuring the E/M ratio for the fusion of labeled vesicles with labeled vesicles at the same lipid concentration as that for the labeled plus nonlabeled mixture. When the E/M ratios are normalized to their initial values, one obtains the pyrene concentration (also normalized to its initial value) as a function of time, t

$$\frac{\left[\frac{E}{M}\right]'_0}{\left[\frac{E}{M}\right]'_t} \cdot \frac{\left[\frac{E}{M}\right]_t}{\left[\frac{E}{M}\right]_0} = \frac{[\text{pyrene}]_t}{[\text{pyrene}]_0} \tag{2}$$

In this equation the primed quantities refer to labeled-fusing-with-labeled vesicles, and the unprimed quantities refer to labeled-fusing-with-nonlabeled vesicles. It was this corrected and normalized pyrene concentration as a function of time that was used to examine the kinetics of vesicle fusion. We note that, for the melittin-to-lipid molar ratio of 1/60 which we have used in this study, we found this correction to be virtually negligible for the cases in which a low concentration of lipid was used. (For the case of high lipid concentration, see below). We found, however, that, for a molar ratio of 1/30, the correction became significant. We have also tested the phosphatidylserine (PS)-Ca^{2+} system, which has been extensively studied by other techniques; we found that, for 2 mM Ca^{2+} and 0.5 mM PS SUVs, the E/M ratio for labeled-with-labeled vesicle fusion (for which the local pyrene concentration does not change) decreased by about 15% in 4 s. This implies that a correction to the fusion data is also necessary in that case.

For the maximum pyrene-PC concentration used, the absorbance of the solution in the stopped-flow sample cell was about 0.05 at the excitation wavelength of 319 nm. We verified that the E/M ratio for pyrene-PC in DPPC SUVs varied linearly with its concentration over the range encountered in these experiments. By mixing labeled with nonlabeled vesicles and monitoring the E/M ratio as a function of time, we found that no detectable spontaneous lipid transfer takes place over the time span of our measurements. This is in agreement with a previous report [11].

Results and Discussion. We have analyzed our data using the mass-action kinetics model of vesicle fusion as developed by Bentz et al. [12]. At short times, where higher-order processes can be

ignored, the reaction can be modeled by

$$V + V \underset{D}{\overset{C}{\rightleftarrows}} A \xrightarrow{f} F \tag{3}$$

Here, V denotes one of the original, nonfused, vesicles, A denotes an aggregate of two such vesicles, and F denotes this aggregate's fusion product. C is the rate constant for aggregation, D is the rate constant for deaggregation, and f is the rate constant for fusion. The complexity of the analysis of the data is significantly reduced if the deaggregation step is omitted. In that case, the concentration of fused vesicles as a function of time depends on C and f through [12]

$$\frac{[F]}{X_0} = \frac{f}{2}\exp(-ft)\int_0^t \exp(ft')\frac{2CX_0t'}{1+2CX_0t'}\,dt' \tag{4}$$

Here, X_0 is the concentration of single vesicles, V, at zero time.

We also measured the excimer to monomer fluorescence intensity ratios of solutions which contained mixtures of 4 mol% and 2 mol% labeled vesicles, for various ratios of the two populations, to determine how the E/M ratios of the mixtures depend on the relative concentrations of the two populations. On this basis, we have determined that the E/M ratio is related to the concentration of fused vesicles through

$$\frac{\left[\frac{E}{M}\right]_t}{\left[\frac{E}{M}\right]_0} = 1 - Q\frac{[F]}{X_0} \tag{5}$$

For the low lipid concentrations used in this study, which contained one part labeled and ten parts nonlabeled vesicles, Q is 10/11. For the high lipid concentration, which contained one part labeled and 967 parts nonlabeled vesicles, Q is 967/968. A nonlinear least-squares program was used to vary C and f and fit Eqn. 4 to the experimental values of $[F]/X_0$ as obtained from Eqn. 5.

Results from our study of the melittin-induced fusion of DPPC SUVs, at 51°C, for a melittin-to-lipid molar ratio of 1/60, are presented in Figs. 1 and 2. Fig. 1 shows how the E/M ratio changes with time for a low-salt solution. As can be seen, at long times the E/M ratio approaches approximately one-third its initial value. This indi-

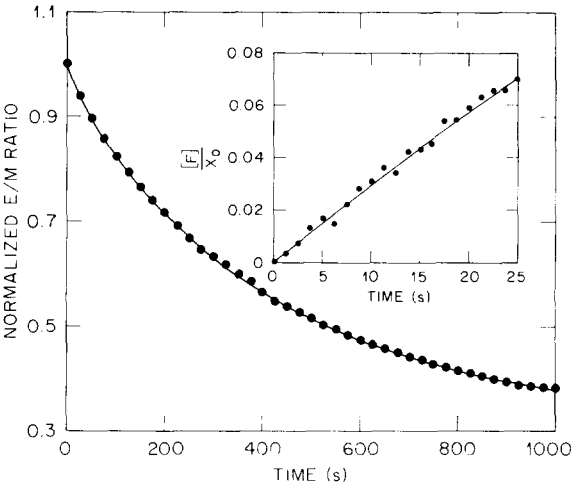

Fig. 1. Excimer to monomer fluorescence intensity ratio E/M of pyrene-PC as a function of time for 0.68 mM DPPC SUVs and for a melittin-to-lipid molar ratio of 1/60, in 50 mM Tris, 1 mM EDTA (pH 7.6) at 51°C. Small unilamellar vesicles were prepared in the following way. A 4 mol% solution of pyrene-PC with DPPC was formed in chloroform, dried under nitrogen, and vacuum desiccated for 24 h. The dried lipids were hydrated above their transition temperature to obtain a concentration of 1 mg/ml, and then vortexed and sonicated to clearness in a Laboratory Supplies Co. bath sonicator. Finally, they were centrifuged at 65000 × g for 30 min. All steps which involved the labeled vesicles were carried out under low-intensity red light in order to avoid photodecomposition of the fluorescently labeled lipid. Vesicle fusion kinetics were measured on an Aminco DW-2 UV/VIS spectrophotometer equipped with a stopped-flow accessory which we modified to allow us to view the fluorescence at right angles to excitation. In one chamber one part labeled DPPC vesicles was combined with ten parts nonlabeled vesicles. The total lipid concentration was 1.36 mM. The other chamber was filled with a 22.5 μM solution of melittin. Equal volumes from the two chambers were mixed. Separate trials were performed to measure the time-dependent excimer and monomer intensities; these signals were corrected for light scattering by repeating the trials using nonlabeled vesicles only, at a concentration equal to that of the labeled plus nonlabeled mixture. The excitation monochromator was set at 319 nm, with a bandpass of 20 nm. Pyrene monomer emission was viewed by placing Corning filters 0-52 and 7-51 in the emission path. Pyrene excimer emission was viewed through a Corning 3-72 filter. The output of the photomultiplier (EMI 6256S) was recorded on a Houston Instruments model 100 X-Y recorder. For clarity of presentation, the number of points shown in the main plot has been reduced. The insert shows the experimental $[F]/X_0$ data as obtained via Eqn. 5 from the values (at early times) of the normalized E/M ratio. The data were analyzed on an IBM-PC using a nonlinear least-squares program. The best fit is shown by the full line in the insert.

cates that, on the average, fusion stops after the addition of a third vesicle to the original fused doublet. The insert shows the experimental values for $[F]/X_0$ at early times and the least-squares fit to the data. This yields a value of $f = 0.006 \pm 0.001$ s^{-1} for the rate constant of fusion. Fig. 2 shows the data in the presence of 2 M NaCl. Here, the best fit gives $f = 0.12 \pm 0.01$ s^{-1}. Structural and ionic effects may be responsible for this observed increase. Melittin is known to be tetrameric in high ionic strength solutions and monomeric in low ionic strength solutions [13,14]. Also, shielding of charges on the protein and on the lipid by the salt may alter the mechanism by which fusion takes place. In order to delineate these two effects, we determined the rate constant for fusion for a high concentration of melittin in a low salt solution, for which case melittin is known to be tetrameric [6,15]. We used a protein concentration of 1 mM and a lipid concentration of 60 mM. (Because of the greatly increased lipid concentration, the fusion process took place quite rapidly due to the enhanced rate of aggregation and the data had to be recorded on a storage oscilloscope, Tektronix model 564.) A value of $f = 0.06 \pm 0.006$ s^{-1} was then obtained from the least-squares fit to the data (not shown) *. This value for tetrameric melittin is seen to be 10-times larger than that of 0.006 s^{-1} which we obtained for monomeric melittin (in a low-salt solution). This finding is of particular importance as melittin appears to be tetrameric in bee venom. (This may be inferred from the concentration of the protein in venom [16,17].) The moderate increase in the value of f in 2 M NaCl (0.12 s^{-1}) relative to that for a high melittin concentration at low salt (0.06 s^{-1}) may have its origin in a destabilizing effect on the bilayer which results from charge shielding. This suggests that electrostatic interactions play a role

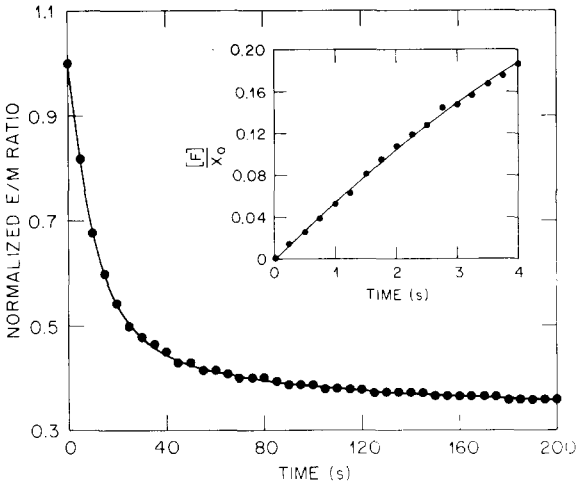

Fig. 2. Excimer to monomer fluorescence intensity ratio E/M of pyrene-PC as a function of time for 0.68 mM DPPC SUVs and for a melittin-to-lipid molar ratio of 1/60, in 50 mM Tris, 2 M NaCl, 1 mM EDTA (pH 7.6) at 51° C. See caption to Fig. 1 for more details.

in the fusion process. Work in progress in our laboratory explores further this effect by employing negatively charged lipids.

We note that because of the relatively high lipid concentrations used here, the values of the rate constant for aggregation could not be accurately determined; this would require much lower lipid concentrations in order to reduce the rate at which aggregation takes place. We are currently making improvements to our instrumentation in order to achieve the higher sensitivity which is required for such measurements.

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* We note that in this case the correction of the E/M data along the lines described above could not be made, because in such a labeled-fusing-with-labeled system the absorbance of the fluorescent probe in the stopped-flow cell would have been too great (more than 4). This is most probably not a source of serious error, as can be inferred from our observation that for the melittin-lipid system in a 2 M NaCl solution, in which melittin is also tetrameric, the correction is negligible.

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