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MELITTIN INDUCES FUSION OF UNILAMELLAR PHOSPHOLIPID VESICLES

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Melittin, the soluble lipophilic peptide of bee venom, causes fusion of phospholipid vesicles when vesicle suspensions are heated or cooled through their thermal phase transition. Fusion was detected using a new photochemical method (Morgan, C.G., Hudson, B. and Wolber, P. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 26–30) which monitors lipid mixing. Electron microscopy and gel filtration confirmed that most of the lipid formed large vesicular structures. Fluorescence experiments with a water-soluble, membrane-impermeable complex of terbium (Wilschut, J. and Papahadjopoulos, D. (1979) Nature 281, 690–692) demonstrate that these ionic contents are released during fusion. The large structures formed by melittin-induced fusion are impermeable to these ions and are resistant to further fusion. This is in contrast to the behavior observed for the cationic detergent cetyltrimethylammonium bromide (CETAB). The large size of the vesicles formed, the extreme speed of the fusion event and the appearance of electron microscope images of the vesicles prior to fusion suggest that the mechanism of the fusion process includes a preaggregation step.

Introduction

Melittin, the principle toxic peptide of bee venom, lyses cells and disrupts the structure of phospholipid liposomes [3]. Its amphiphilic nature is evident in its structure [4]:

(+)H_3N-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 aqueous solutions of low ionic strength, this small peptide exists in a monomeric form, while at high concentrations or in high ionic strength solutions, melittin aggregates as a tetramer [5]. The single tryptophan (Trp_{19}) exhibits a blue shift (352–337 nm) in its fluorescence emission upon aggregation [5,6] and when added to phospholipid preparations [6,7]. This shift results from the movement of the tryptophan into a less polar environment, and has been attributed to either penetration of the tryptophan into the hydrophobic core of the bilayer, or to shielding of the tryptophan inside a tetramer [8,9]. Recent conductance studies suggest that melittin forms tetrameric channels in black lipid membranes which are more permeable to anions than cations [10]. Melittin is an interesting model for lipid/protein interaction studies because it exists in both soluble and membrane-bound forms and because of its known three-dimensional structure [11].

Melittin forms micellar complexes with detergents and phospholipids. Recent high resolution ^{1}H-NMR and CD experiments indicate that melit-

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Abbreviations: CETAB, cetyltrimethylammonium bromide; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DLPC, dilauroylphosphatidylcholine; DEPC, dielaidoylphosphatidylcholine; NBD, nitrobenzodioxazole.

tin adopts a similar conformation when aggregated as a tetramer and when bound to detergent or phospholipid micelles [12–16]. In order to understand the structural basis for its biological function, studies of the peptide in aqueous solutions and with model membranes are important. We present results which show melittin is capable of acting as a fusogen of model membrane vesicles resulting in very large structures. (We define fusion as the process in which vesicular lipid bilayers merge to form larger vesicular bilayer structures which are closed with respect to impermeable reagents and in which the lipids of the original structures are co-mingled to form a larger contiguous bilayer.) This activity of the melittin appears to be independent of its state of aggregation but requires temperature cycling through the thermal transition of the lipid.

The effect of melittin on vesicles was monitored by studying the kinetics of the irreversible photochemical dimerization reaction of phospholipids containing one *trans*-parinaroyl chain [1]. The essential feature of this reaction in the present application is that its rate depends on the local two-dimensional concentration of parinaroyl-labeled lipids in the bilayer. Fusion of labeled vesicles with unlabeled vesicles results in dilution of the label and therefore a decreased photochemical rate. This method for detecting bilayer fusion depends on the intimate mixing of the lipid components so that, in contrast to energy transfer [17–19] or light scattering [19,20] methods, it is not affected by aggregation without fusion.

This assay of fusion requires the dilution of parinaroyl-labeled lipids by unlabeled lipids. If the fusogenic procedure of heating and cooling in the presence of melittin is carried out for labeled lipids in the absence of unlabeled lipids there is no change in the photochemical rate. A significant observation is that this pretreatment procedure results in large structures which are not subject to additional fusion as judged by lipid mixing. This observation, plus the fact that the final fused structures are closed with respect to impermeable reagents, clearly distinguishes this fusion process from that expected and observed for detergent action.

Materials and Methods

Melittin, the gift of Dr. William Wickner, was purified by descending paper chromatography (4:2:1 v:v:v *n*-butanol/acetic acid/water) to remove residual phospholipase A_2 activity. Melittin purchased from Sigma was teated to remove phospholipase A_2 by the method of Mollay et al. [22]. Aqueous solutions of melittin (0.1 mg/ml), were stored at 4°C. Terbium chloride and dipicolinic acid were purchased from Aldrich, CETAB was from Sigma, and NBD-diocytlamine was from Molecular Probes.

DPPC, DSPC, DLPC, DEPC, and egg yolk phosphatidylcholine (egg PC) were obtained from Sigma and Calbiochem and were used without further purification. Stock solutions (40 mM) of phospholipids were prepared in ethanol and stored under argon at −20°C. The fluorescent analogue with palmitic acid at the 1 position was also synthesized and gave identical results. The labeled phospholipid was stored as an ethanolic solution at −20°C, under argon, in the dark.

The ethanolic stock solutions of phospholipids were mixed in the desired ratios to prepare samples containing parinaroylphospholipids. These mixtures were slowly injected into rapidly stirred buffer at a temperature above the phase transition of the lipid [23] to produce moderate size (ca. 800 Å diameter) predominantly unilamellar vesicles. The final lipid concentration in the aqueous suspensions was 0.3 mg/ml (0.4 mM) in all samples, and the ethanol concentration was 1% (v/v). To prepare vesicles containing terbium (Tb^{3+}), and dipicolinic acid trapped within, phospholipids were injected into 10 mM Tris buffer (pH 8.0) containing 35 mM dipicolinic acid or 12 mM Tb^{3+}, applied to Sephadex columns (2.5 × 50 cm) and eluted with pure buffer to remove external ions. The Tb^{3+}/dipicolinic acid complex was also trapped in vesicles, and external ions were removed with a similar column.

Samples for electron microscopy were applied directly to air glow discharged carbon films. After absorption of the specimen to the film for 60 s, the grid was washed by floating on a droplet of glass-distilled water for 30 s, and then stained by floating on a drop of 1% (w/v) sodium phosphotungstate (pH 7.0) for 5 s. This procedure does not

appear to select for vesicles of a particular size. Electron microscopy was performed using a Phillips EM300 operated at 80 kV with a liquid nitrogen cooled anticontamination device. Micrographs used for measurement of vesicle diameter were taken at magnifications between 6000 and 12000. The magnification was calibrated using images of negatively stained catalase crystals taken under identical conditions.

Vesicle size was also estimated by gel filtration. Samples were chromatographed on a Bio-Gel A-150m column (2.5 × 50 cm). To locate phospholipid fractions, a 2 μl aliquot of a 5 mM solution of NBD-diocytlamine was added to each tube collected. The final NBD concentration was $5 \cdot 10^{-6}$ M. The quantum yield of this fluorescent molecule is low in water but is enhanced when it is bound to lipids. Fluorescence was excited with 470 nm radiation; emission was detected at 550 nm using a Spex fluorometer.

Parinaric acid fluorescence was excited at 295 nm and emission was monitored at 420 nm. Excitation at longer wavelengths introduces a small amount of a first order component in the photochemical decay. Fusion was monitored by determining the rate constant of the second-order photochemical reaction of phospholipids containing one *trans*-parinaroyl chain [1]. This reaction results in an irreversible loss of parinaroyl fluorescence. The second-order rate constant is the slope of a plot of $1/I(t) - 1/I(0)$ versus time.

Results

The melittin-induced fusion of DPPC, DSPC, DEPC, DLPC, and egg PC vesicles was studied using 1-myristoyl-2-*trans*-parinaroylphosphatidylcholine as the fluorescent probe. In a typical experiment, vesicles were prepared from unlabeled lipid, or with lipid to probe molar ratios of 50 : 1 or less. Fig. 1 shows the second-order rate plots obtained from DPPC vesicles containing the parinaroyl-labeled lipid. Curve 1′ is a sample with a phospholipid-to-probe ratio of 50 : 1; curve 4 is a 200 : 1 sample. Curve 1 results when 3 vol. of unlabeled vesicles are added to 1 vol. of labeled vesicles. The initial fluorescence intensity is identical to that of the 200 : 1 sample, since the total concentration of fluorophore is the same. The photochemical rate, however, depends on the local two-dimensional concentration of fluorophore, and therefore is unchanged by the addition of unlabeled vesicles. Curve 2 results when this sample is heated to 55°C, above T_m, the phase transition temperature of DPPC (42°C) for 5 min and cooled to 20°C. The addition of melittin to a final concentration of $2 \cdot 10^{-6}$ M (lipid/protein ratio

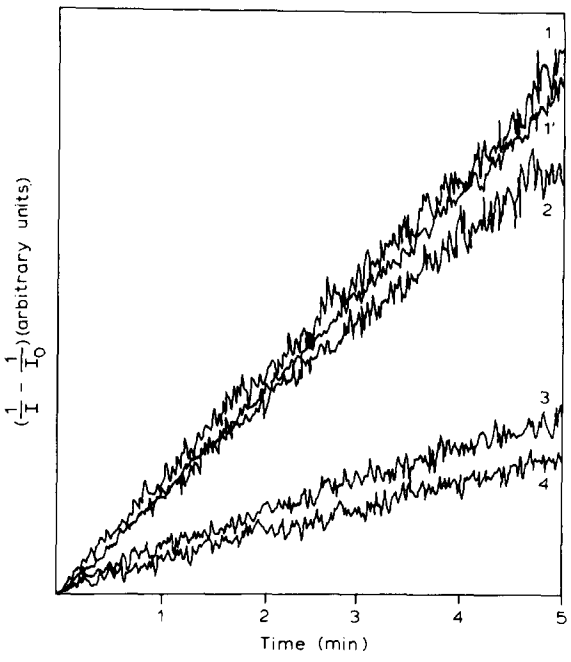

Fig. 1. The melittin-induced fusion of DPPC ethanol injection vesicles was determined by measurement of the photochemical reaction of 1-myristoyl-2-*t*-parinaroyl-3-*sn*-glycerolphosphatidylcholine. Vesicles were prepared by injection of 40 mM (30 mg/ml) ethanolic solutions of lipid and probe, in the desired concentration ratios, into 50 mM phosphate buffer (pH 7.4) which contained EDTA to inhibit phospholipase. The final lipid concentration was 0.4 mM (0.3 mg/ml) in all samples. Samples were kept from oxygen and light and were used within 24 h. Fluorescence spectra were obtained with a Spex Fluorolog fluorometer with excitation at 295 nm, and emission at 420 nm. The data are shown as second-order rate plots as described in Ref. 1. Curve 1′ is the reaction for the original DPPC vesicles labeled with 1 *t*-parinaroylphosphatidylcholine per 50 unlabeled lipids. Curve 1 is the mixture of these vesicles with a three-fold excess of unlabeled vesicles at 20°C. Curve 2 resulted when this mixture was heated to 55°C for 5 min, and cooled to 20°C. Curve 3 is the same as 2, except that melittin was added at a lipid : protein ratio of 200 : 1 before heating. Curve 4 is the reaction for a mixture of labeled and unlabeled lipids at a ratio of 1 : 200, the final concentration if mixing of lipids resulted in a uniform label distribution.

200 : 1) before heating and cooling results in curve 3. The equivalence of this rate with that observed for the more dilute sample indicates that the local concentration of parinaroylphosphatidylcholine was approx. 200 : 1. The observed change in the photochemical rate shows that the mixing of the constituents of labeled vesicles with those of unlabeled vesicles establishes a random distribution of label. This indicates that mixing is extensive, either involving the formation of large structures from many small vesicles or because melittin results in exchange of phospholipids between vesicular structures. Neutral phosphatidylcholines exchange on a time scale of hours [27], much slower than the process being observed here. The elimination of an exchange mechanism as an explanation for these results is presented below.

Similar results were obtained for DSPC, DLPC, DEPC, and egg PC vesicles when 1-palmitoyl-2-*t*-parinaroylphosphatidylcholine was used as the fluorescent probe. For the photochemical rate decrease to occur in egg PC, DEPC, and DLPC, these samples were cooled to 0°C in order to pass through the phase transition of these lipids. The rate of fusion was much lower for the lipids which were cooled than for the heated lipids. Fusion occurred in less than 5 min for DPPC, but DLPC had to be cooled for 50 h to observe a decreased photochemical rate at 20°C. DEPC and DLPC samples without melittin stored at 4°C for 50 h also had a decreased photochemical rate. This indicated that phospholipid exchange had probably occurred.

The experiments described above were performed in 50 mM dibasic sodium phosphate (pH 7.4) buffer. Further experiments were performed in other buffers to determine whether the aggregation state of the peptide affected the fusion process. Identical samples, 50 : 1, 200 : 1, and unlabeled, were prepared in 10 mM Tris (pH 8.0) and 100 mM and 500 mM dibasic sodium phosphate (pH 7.4). Melittin exists in a monomeric structure in the former system, but aggregates as a tetramer in the latter two buffers [5]. The addition of melittin to each sample had the same effect on the photochemical rate as that described above. This shows that the binding of melittin to membranes, and subsequent fusion, is independent of its solution structure or any other interactions affected by this ionic strength change.

In order to compare the action of melittin with that of a simple detergent, experiments were performed with the cationic detergent, CETAB. The critical micelle concentration of CETAB in water, at 20°C, is 0.9 mM [30]. This is equivalent to a lipid/detergent ratio of 0.4 : 1. DPPC vesicles were labeled 50 : 1 with *t*-parinaroylphosphatidylcholine and mixed with unlabeled vesicles as above. A solution of CETAB, added at a 200 : 1 lipid/detergent ratio (1.8 μM), at room temperature, had no effect on the photochemical rate, even after heating and cooling. However, at higher detergent concentrations (4 : 1 lipid/detergent ratio, 90 μM) the photochemical rate was decreased upon heating above T_m and cooling. No change in rate was observed without heating and cooling. Thus, at concentrations of CETAB 50-times higher than the melittin concentrations, the action of CETAB in terms of lipid mixing would appear to be similar to that of melittin. That this is not the case will now be demonstrated.

When CETAB is used to induce lipid mixing, addition of more unlabeled vesicles followed by heating and cooling results in a further reduction of the photochemical rate. In contrast, the melittin induced fusion process results in DPPC vesicles that are resistant to further fusion. This was determined as follows. Melittin was added to a 50 : 1 DPPC : parinaroylphosphatidylcholine sample which was heated to 55°C and cooled to 20°C. The photochemical rate remained constant as expected. Unlabeled vesicles were added, and the sample was again heated and cooled. The photochemical rate remained unchanged. Similarly, melittin was added to unlabeled vesicles which were heated and cooled and then added to 50 : 1 labeled vesicles. Heating and cooling a second time did not affect the photochemical rate of the 50 : 1 sample. These experiments demonstrate that melittin does not, by itself, influence the photochemical dimerization rate. More importantly, this behavior excludes an exchange or detergent-like mechanism for melittin-induced lipid mixing. Heating and cooling in the presence of melittin must produce structures resistant to further lipid mixing. This is not observed with CETAB.

Electron microscopy using negative staining [26] confirmed that the treatment described above re-

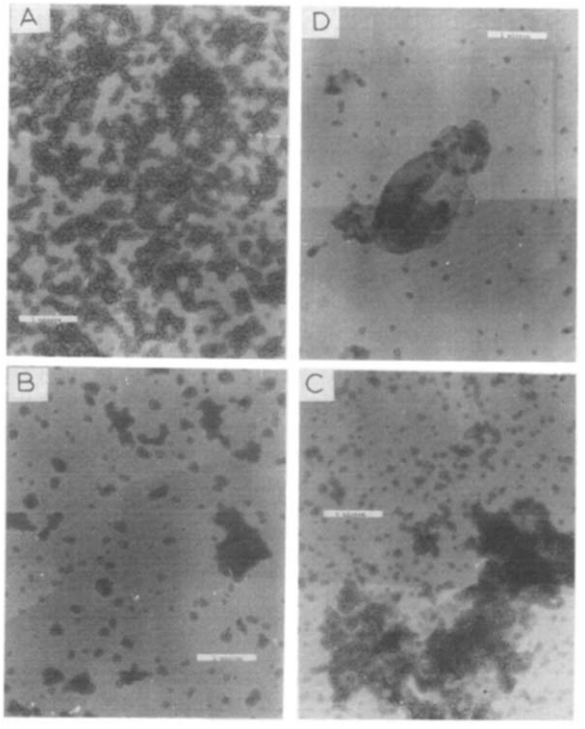

Fig. 2. Electron micrographs illustrating the fusion of lipid vesicles induced by melittin. The white bar represents 1 μm in each case. The ethanol injection vesicles shown in (A) are roughly 800 Å in diameter. After a heating and cooling cycle the vesicles appear to aggregate but they do not fuse (B). The addition of melittin also causes aggregation as in (C). A heating and cooling cycle at a lipid/melittin ratio of 200:1 results in vesicles in the 1–2 μm size range (D).

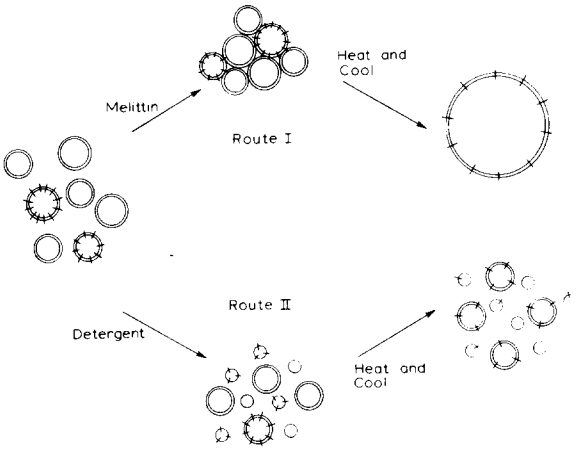

Fig. 3. A schematic representation of the proposed mechanisms of melittin-induced fusion and detergent-induced probe randomization. The parinaroyl-labeled phospholipid is represented by short straight line segments. The original lipid vesicle population shown at the left appears to aggregate in the presence of melittin; heating and cooling results in very large structures with a probe concentration corresponding to the average of that of the original sample (route I). The structures produced by this procedure are resistant to further mixing with additional lipids. Addition of CTAB at 'high' concentrations (1 per every four lipids, 50-times that of melittin) also results in lipid mixing after heating and cooling. Presumably, this proceeds through the formation of micelles which are in dynamic equilibrium with the remaining vesicular structures (route II).

sults in DPPC and DSPC vesicles which are much larger than the initial species. The results for DPPC are shown in Fig. 2. The preparation of vesicles by ethanol injection results in fairly uniform 600–1000 Å diameter unilamellar structure [25] (Fig. 2a). Heating and cooling the sample in the absence of melittin may result in aggregation (Fig. 2b), but not fusion since the outlines of the 'small' vesicles are apparent in the aggregate. The addition of melittin without heating also apparently produced aggregation as in Fig. 2c. Large (20000 Å) single-walled structures are formed when the melittin-containing sample is heated and cooled through the phase transition as shown in Fig. 2d. The estimated increase in the surface area of vesicles in Fig. 2d relative to those in 2a, indicates that at least 600 small vesicles fused to form the larger structures. The large number of vesicles which merge to form these final structures is consistent with the photochemical rate which indicates a final uniform composition. The phospholipid to protein ratio used in these studies was roughly 200:1. Since the original vesicles of 800 Å diameter consist of about 50000 lipid molecules, this corresponds to roughly 250 melittin monomers per vesicle.

Gel filtration experiments using a Bio-Gel A-150m column also indicated that melittin-treated DPPC vesicles were larger than the original vesicles. This filtration medium has an exclusion radius of about 2500 Å. A 2 ml sample of melittin-fused DPPC vesicles was eluted with 0.1 M dibasic sodium phosphate buffer. The lipid was detected by the fluorescence intensity of added NBD-diocytlamine, and a single peak was observed directly after the column void volume. A mixture of melittin-fused DPPC vesicles and untreated vesicles was applied to the column, and

there were two distinct peaks of equal magnitude. The first peak was at the same volume as the melittin/DPPC sample; the second peak 50 ml later. These experiments demonstrate that the melittin fusion procedure converts most, if not all, of the lipid to large structures.

Experiments were performed to determine if the melittin fusion procedure results in retention of vesicle contents. Terbium ions experience a 10 000-fold enhancement in fluorescence when complexed with dipicolinic acid [2,27]. Both species and their complex are membrane impermeable. The dipicolinate ligand can be displaced by EDTA to form an impermeable complex that does not show appreciable fluorescence. Contents retention during fusion of DPPC vesicles was studied using these aqueous fluorescent species (Fig. 3). Addition of melittin to a mixture of vesicles containing entrapped terbium ions with similar vesicles containing dipicolinic acid did not affect the fluorescence intensity appreciably. When these samples were heated to 60°C and cooled, the fluorescence increased. Addition of an EDTA solution to a final concentration of $7.5 \cdot 10^{-5}$ M quenched the fluorescence. This indicates that vesicular contents were not retained during fusion. Similar results were obtained with DSPC, DLPC, and egg PC samples. DLPC and egg PC samples stored at 4°C for 50 h leaked their contents without melittin. Heating and cooling the DPPC sample without melittin did not cause leakage.

The above experiments demonstrate that vesicle contents are lost at some stage of the melittin fusion procedure but do not address the question of whether the large vesicles produced by this procedure are themselves leaky. In order to test this point vesicles were prepared containing the entrapped terbium complex, heated and cooled in the presence of melittin, and then external ions were removed by gel filtration. These fused structures did not leak as evidenced by the inability of added EDTA to quench the fluorescence of the terbium complex. Thus, the large vesicular structures produced by melittin treatment are not leaky.

Discussion

The experiments described above demonstrate that heating and cooling 800 Å diameter vesicles in the presence of low concentrations of melittin results in conversion of most of these structures to very large impermeable vesicles. These final structures are resistant to further fusion. This could be due either to their decreased radius of curvature or to their very slow diffusion and, therefore, collision rate.

The original vesicle contents are not retained in this fusion process. The fact that the product vesicles are impermeable and that the contents are not exposed except during a heating and cooling cycle in the presence of melittin, indicates that the loss of contents occurs in the fusion process itself. We have only demonstrated loss of specific small ionic species; it is possible that larger species will be retained during fusion.

The size of the large vesicles formed by fusion and the decrease of the photochemical rate to the value observed for fully random mixing demonstrates that a large number of initial vesicles are involved in the fusion event. The rapid rate of the process for DPPC and DSPC implies that a pre-equilibration process may be involved rather than a rate limiting step involving diffusion. The electron microscope images suggest that this initial step is aggregation of a large number of vesicles. We therefore propose that the fusion event which occurs on passage through the thermal phase transition consists of the merger of this aggregate to form a very large vesicle. (See Fig. 3, route I.) Melittin may either increase the probability of formation of these large aggregates or enhance their fusion or both. The details of this mechanism may be related to the lack of fusion of the large vesicles. The effect of melittin can be contrasted with the effect of a typical detergent on these structures. Detergent treatment results in lipid mixing which continues upon addition of a second population of distinguishable vesicles. Presumably this process proceeds through an exchange mechanism, probably involving mixed micelles (Fig. 3, route II).

The action of melittin described here is of interest from the point of view of the nature of the interaction of amphipathic peptides with vesicular structures [30]. Under the conditions used here, it has been shown that the 'lytic' (contents releasing) action of melittin occurs in parallel or concomitant with a 'fusion' (vesicle expansion) process, at least

for DPPC and DSPC. Further development of this procedure may lead to useful methods for cell surface modification or component delivery.

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