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Reversible disc-micellization of dimyristoylphosphatidylcholine bilayers induced by melittin and [Ala-14]melittin

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The properties of melittin and a synthetic analogue, [Ala-14]melittin (P14A), in inducing reversible transitions between vesicles and micelles at the liquid-crystalline to gel phase transition temperature (T_m) in complexes with saturated phosphatidylcholines has been studied by deuterium NMR and freeze-fracture electron microscopy (EM). At concentrations between 3 and 5 mol% relative to lipid, each peptide causes reversible micellization of dimyristoylphosphatidylcholine (DMPC) bilayers when the temperature is lowered below T_m. At concentrations of 5 mol% relative to lipid, the peptides induce macroscopic magnetic orientation of DMPC bilayers at temperatures around the centre of the lipid phase transition; at temperatures a few degrees above T_m, magnetic orientation is lost. These effects suggest a progressive phase separation of peptide and lipid on cooling the complexes through the phase transition, resulting in increased vesicle deformability. The rates of gel phase micellization, and of bilayer reformation from micelles at temperatures above T_m, are decreased by 100-fold in P14A:DMPC complexes compared with melittin:DMPC complexes. Freeze-fracture EM indicates that P14A suppresses the formation of the gel phase in DMPC bilayers at temperatures below T_m. EM observations of the time-dependence of the reformation of bilayers from micelles after incubating P14A:DMPC micellar complexes at temperatures above T_m indicate that micelles fuse to form growing bilayer sheets from which multilamellar vesicles eventually form. The presence of intramembranous particles (IP) on the fracture faces of both melittin:DMPC complexes and P14A:DMPC complexes in the fluid phase indicates that under the conditions of the study (50 mM Tris-HCl (pH 7.5), 5 mM EDTA) the peptides are organized as discrete aggregates that penetrate deeply into the bilayer.

Introduction

Bee venom melittin [1–4] binds to membranes in an amphipathic helical conformation, inducing voltage-gated anion-selective ion permeability at nanomolar concentrations [5,6] and membrane lysis at micromolar concentrations [7–9]. Recently, a third membrane activity of melittin has been described [10–13]. At concentrations between about 3 and 6 mole% relative to lipid, melittin causes the micellization of bilayers composed of saturated phosphatidylcholines when the temperature is lowered below the gel to liquid crystalline phase transition temperature (T_m) of the lipid. This phase-dependent micellization is reversible. Stable vesicles containing melittin reform by fusion of the micellar particles when the temperature is raised above T_m. Electron microscopy of freeze-fractured melittin : lipid complexes quenched at temperatures below T_m indicates that the micellar particles existing below T_m are discs composed of a single bilayer with melittin molecules presumably lining the disc circumference, shielding the lipid acyl chains from contact with water [10,11]. These micellar discs are similar to the structures obtained with other peptides that adopt amphipathic helical conformations on interaction with lipids such as the apolipoproteins [14,15], glucagon [16] and detergents like lysophosphatidylcholine [17]. Of these amphiphiles, only melittin and lysophosphatidylcholine show phase-transition-de-

Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; EM, electron microscopy; HPLC, high-performance liquid chromatography; IP, intramembranous particle; P14A, [Ala-14]melittin; TFA, trifluoroacetic acid; T_m, gel to liquid-crystalline phase transition temperature of phospholipid bilayer.

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pendent reversible micellization of saturated phosphatidylcholine bilayers. The micellization of membranes in the form of bilayer discs has been proposed as a mechanism of melittin-induced hemolysis [10].

In a study of the relationship between the structure, dynamic properties and membrane actions of melittin we have prepared a synthetic analogue, [Ala-14]melittin (P14A), in which the proline residue (Pro-14) has been replaced by alanine [4]. This analogue has enhanced hemolytic activity, but is unable to form stable voltage-dependent ion channels in planar lipid membranes supporting the idea that discrete ion channel formation is not required for melittin-induced hemolysis. In this paper, we compare the properties of melittin and P14A in inducing reversible disc micellization of dimyristoylphosphatidylcholine bilayers, using deuterium NMR to follow the kinetics of the macroscopic changes between bilayers and micelles and freeze fracture electron microscopy to study the nature of the macroscopic structures formed on interaction of the peptides with DMPC. The deuterium NMR spectrum of *N*-methyl-deuterated DMPC in bilayers is a narrow powder pattern that can be accumulated with high sensitivity allowing kinetic processes involving changes in the NMR spectrum to be followed with a time resolution of a few seconds. We have found that the kinetics of the transitions induced by temperature in P14A : DMPC complexes are considerably suppressed compared to melittin : DMPC complexes allowing the time-dependence of these events to be followed by freeze-fracture electron microscopy.

Materials and Methods

Materials. Melittin was purified from bee venom as described [13]. DMPC was from Sigma, and DMPC deuterated in the choline methyls (DMPC-d_9) was prepared by methylation of dimyristoylphosphatidylethanolamine using CD_3I [18]. [Ala-14]Melittin (P14A) was synthesised by standard solid phase methods using *t*-Boc amino protection and was obtained as the crude peptide from Dr. J.E. Fox, Altabioscience, Birmingham University. The crude peptide was purified by gel-filtration on Sephadex G-25 (Pharmacia Fine Chemicals) in 2% acetic acid followed by reverse-phase HPLC using a Vydac C4 column in 0.1% trifluoroacetic acid with a gradient of acetonitrile. The peptide was more than 96% pure by analytical HPLC (Fig. 1) and had the correct amino acid sequence as determined by sequential assignment of the ^{1}H-NMR spectrum in methanol. The synthetic analogue, P14A, has reduced polarity compared to melittin, being more strongly retained on the reverse-phase HPLC column (Fig. 1).

Sample preparation. Complexes of DMPC containing melittin or P14A were prepared by swelling lipid in buffer (50 mM Tris-HCl (pH 7.5), 5 mM EDTA) containing peptide to give the desired peptide : lipid ratio. Quantitative amino acid analysis was used to determine an extinction coefficient for P14A which was 5600 $M^{-1} \cdot cm^{-1}$ at 280 nm (the same as for melittin), and this value was used to measure the concentration of stock solutions of each peptide. The lipid was dispersed at a concentration of 30–50 $mg \cdot ml^{-1}$ by vortexing at temperatures above T_m and the lipid : peptide mixture freeze-thawed six times before experiments. The EDTA was present to suppress residual phospholipase A_2 activity in melittin-containing lipid samples [19] and these were checked following experiments for the formation of lysophosphatidylcholine or fatty acid [13]. No detectable lipid hydrolysis occurred over the course of the experiments described in this paper.

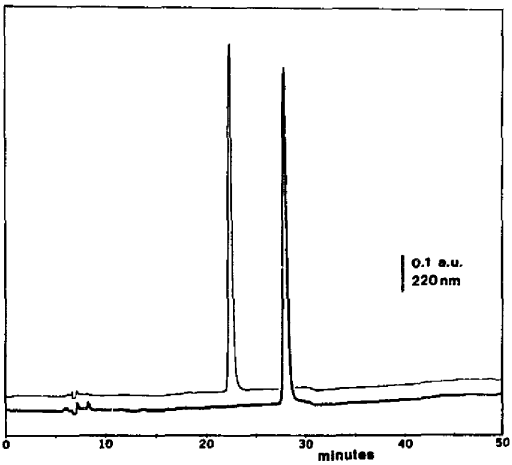

Fig. 1. Analytical HPLC of P14A (heavy trace) and melittin (light trace) on a Vydac C4 reverse-phase column using a gradient of 30% to 70% acetonitrile in water (0.1% TFA) between 10 and 40 min. 40 μg of each peptide was loaded.

Deuterium NMR. Deuterium NMR spectra were obtained at 55.3 MHz using a home-built spectrometer with an Oxford Instruments magnet operating at 360 MHz for protons and a solenoid probe tuned to the deuterium frequency. Spectra were obtained with single 90° pulses of 7 μs over a spectral width of 40 kHz into 2 K data points with a minimum preacquisition delay of 12 μs. The signal : noise ratio was improved by exponential multiplication equivalent to a line broadening of 20 Hz and the block zero-filled to 4 K before Fourier transformation to enhance the digital resolution of the spectra. Temperatures were controlled with a thermostatted air flow.

Freeze-fracture electron microscopy. Complexes of DMPC containing melittin or P14A were quenched for freeze fracture EM after incubation (for 30 min in the case of melittin and 24 h in the case of P14A, if not otherwise mentioned) at temperatures either well above or well below the main phase transition temperature

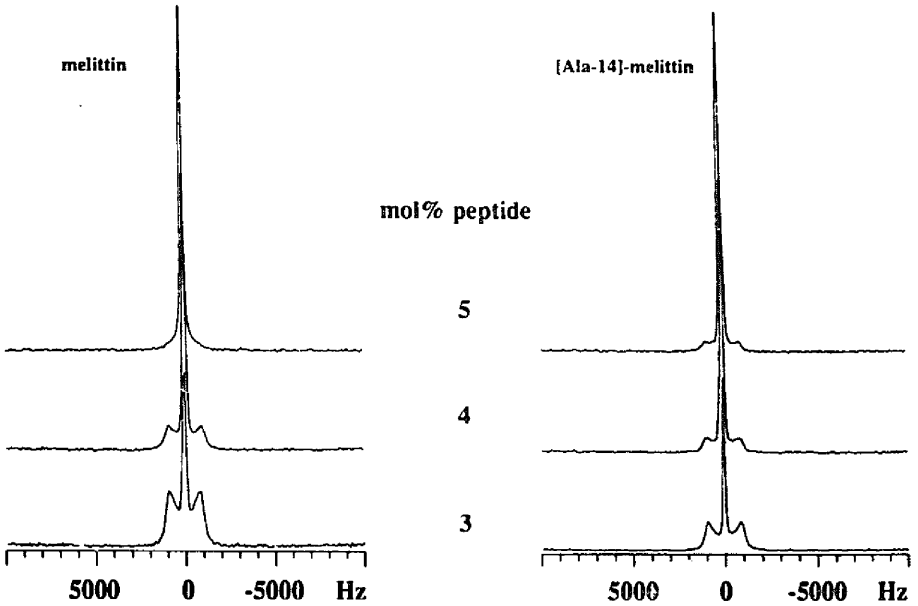

Fig. 2. Deuterium NMR spectra of DMPC-d_9 (50 mg·ml^{-1} in 50 mM Tris-HCl (pH 7.5), 5 mM EDTA) containing melittin or P14A at the mole percent indicated. The spectra were obtained at 14°C after lowering the temperature of the peptide: lipid complex from above the phase transition temperature of the lipid.

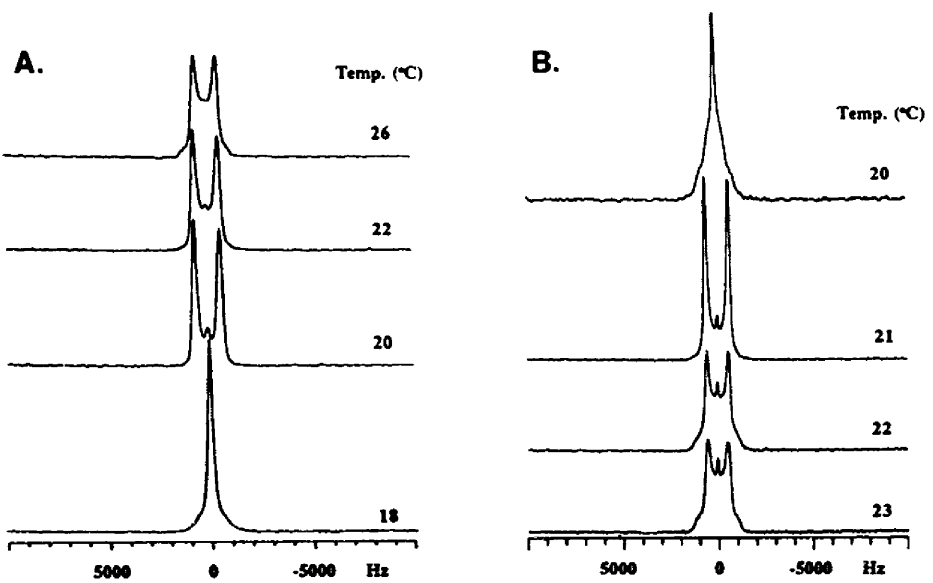

Fig. 3. Deuterium NMR spectra of DMPC-d_9 (buffer composition as for Fig. 2) containing 5 mol% melittin (A) or 5 mol% P14A (B). Spectra in (A) were obtained in a heating run (18–26°C) and in (B) in a cooling run (23–20°C). Samples were allowed to equilibrate at each temperature for 30 min before acquiring the spectra.

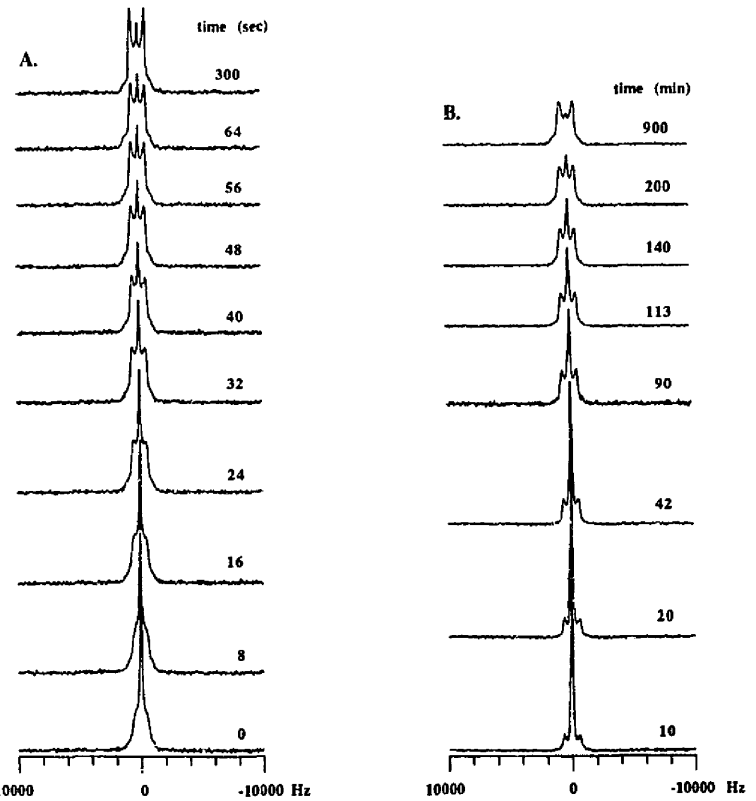

Fig. 4. Time-dependent variation of the deuterium NMR spectra from DMPC-d_9 (sample conditions as for Fig. 2) containing melittin (A) or P14A (B) at 4 mol% obtained after warming the peptide : lipid complexes from 14°C (below T_m) to 28°C (above T_m).

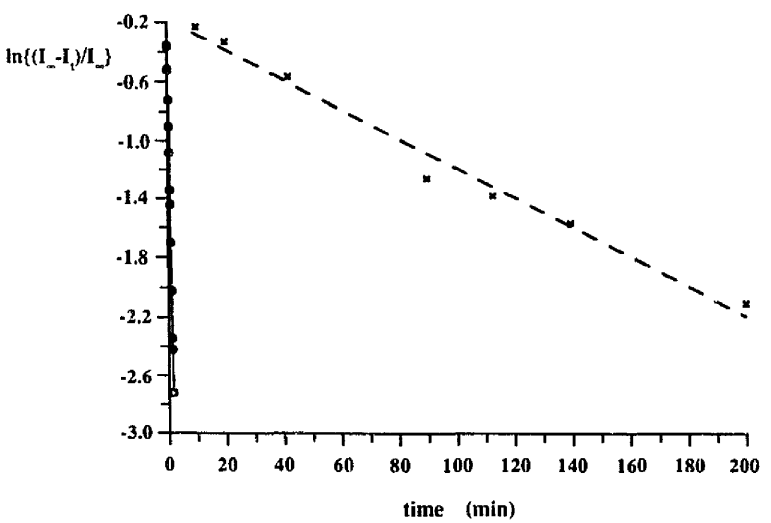

Fig. 5. Logarithmic plots of the time-dependence of the build up of the bilayer powder patterns in the deuterium NMR spectra shown in Fig. 4. I_t is the height of the 90° orientational component of the deuterium powder pattern at time = t and I_∞ is the height after complete reformation of the powder pattern. (○), melittin : DMPC-d_9; (×), P14A : DMPC-d_9.

(T_m) of the lipid in the complexes. Samples were quenched using the sandwich technique with nitrogen-cooled propane to give a quenching rate of approx. 10^5 $K \cdot s^{-1}$ [20]. The specimens were shadowed in a Balzers-400D freeze fracture device at −150°C. The cleaned replicas were examined in a Tesla BS-500 or Jeol Jem-100B electron microscope.

Results

Deuterium NMR

Both melittin and P14A cause the micellization of gel phase DMPC over a concentration range of around 3–5 mol% (peptide : lipid) when the temperature is lowered below T_m. At 2 mol% (peptide : lipid) and below, neither peptide disrupts the extended bilayer structure of DMPC in the gel phase (not shown), whereas at increasing concentrations of peptide the deuterium NMR powder pattern arising from extended bilayers of DMPC-d_9 is progressively converted into a narrow signal characteristic of small particles undergoing rapid isotropic motion on the timescale of the residual quadrupole splitting of about 1000 Hz (i.e., the correlation time for particle reorientation is shorter than a millisecond) (Fig. 2). Electron microscopy carried out on the samples used for NMR confirms that the particles giving rise to the narrow signal are micellar particles (see below).

The temperature dependence of the deuterium NMR spectra from complexes of DMPC-d_9 with melittin or P14A shows similar features whether the spectra are obtained in a heating or cooling run (Fig. 3). At 5 mol% peptide : lipid, much of the lipid is in a micellar phase below T_m, whereas at temperatures above T_m virtually all the lipid contributes to a powder pattern similar to that observed from extended bilayers of DMPC-d_9 in the absence of peptide (the quadrupole splitting is slightly reduced relative to peptide-free bilayers [13]). For both melittin or P14A complexes with DMPC-d_9 the spectra obtained at the phase transition temperature show features characteristic of membrane orientation (Fig. 3) [21]. Under these circumstances the 90° orientational component of the deuterium powder pattern is enhanced relative to the 180° components (the 'shoulders' of the deuterium powder pattern), indicating that there is a preferential orientation of the bilayers at the phase transition with the membrane plane parallel to the magnetic field (i.e., oriented vertically with respect to the laboratory frame). Similar orientation phenomena were previously observed with DMPC-d_9 samples containing micellizing amounts of melittin or lysophosphatidylcholine [13].

Figs. 4 and 5 show that the effects of melittin and P14A on the morphology of DMPC-d_9 bilayers differ significantly in their time-dependencies. When micellar complexes of melittin : DMPC-d_9, or P14A : DMPC-d_9 (4 mol% peptide : lipid) existing below T_m were warmed

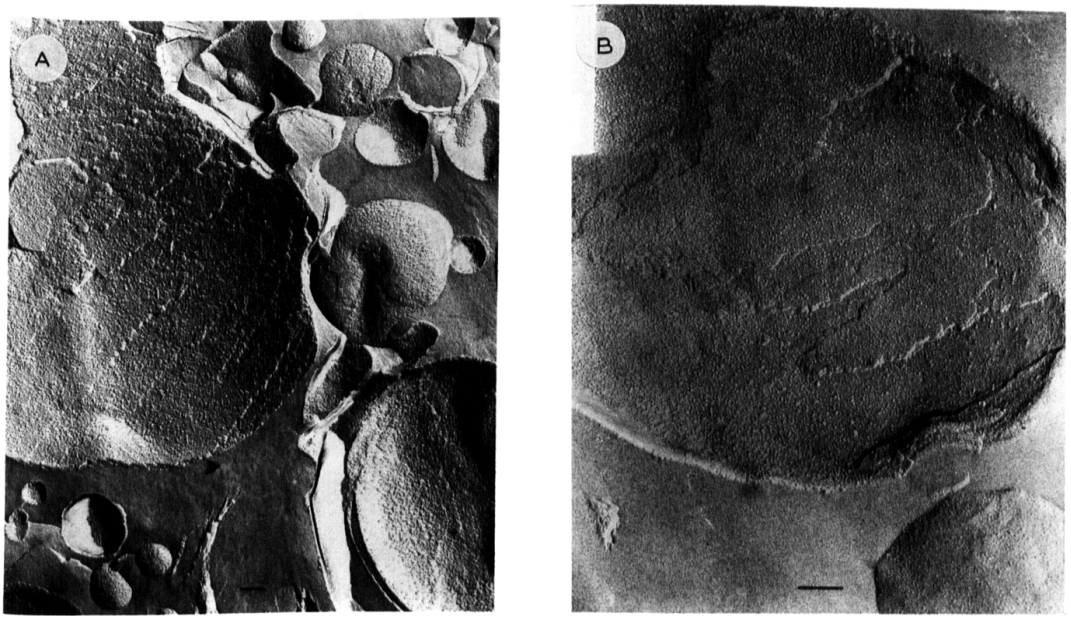

Fig. 6. Freeze-fracture electron micrographs of melittin : DMPC complexes (A) or P14A : DMPC complexes (B) at peptide concentrations of 5 mol% relative to lipid (buffer composition as for Fig. 2). The samples were quenched from 28°C. The scale bar represents 100 nm.

above T_m to 28°C, the conversion of the micelles to extended bilayers occurred much more quickly for melittin-containing complexes compared to P14A-containing complexes (Fig. 4). In each case, refusion of gel phase discs to extended bilayers follows first order kinetics (Fig. 5). The time constant for reformation of

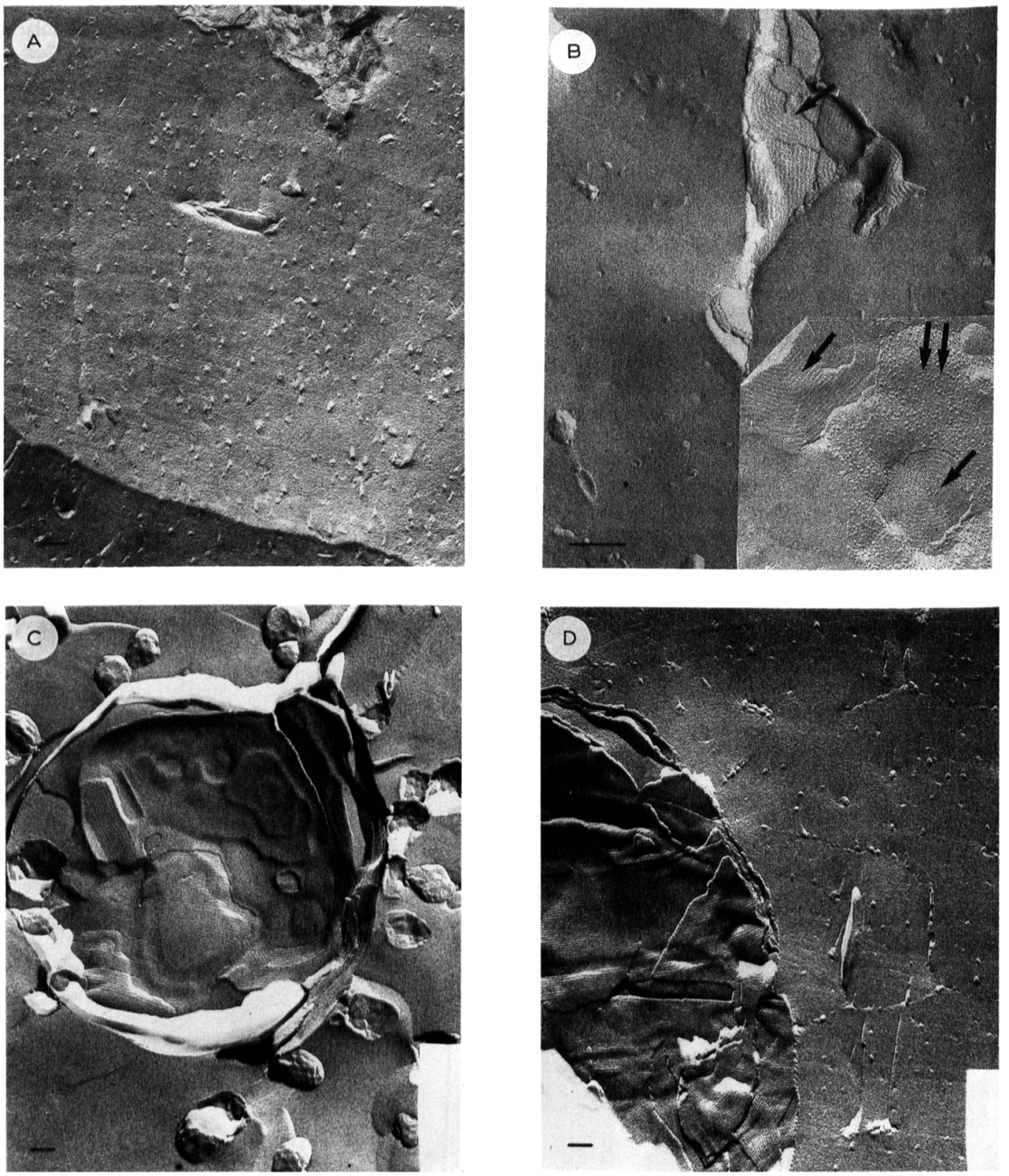

Fig. 7. Freeze-fracture electron micrographs of melittin : DMPC complexes (A) or P14A : DMPC complexes (B) at peptide concentrations of 5 mol% relative to lipid. The samples were quenched after overnight incubation at 11°C. Samples in (C), 0.5 mol% melittin : DMPC, and (D), 3 mol% melittin : DMPC, were quenched from 4°C. The scale bar represents 100 nm.

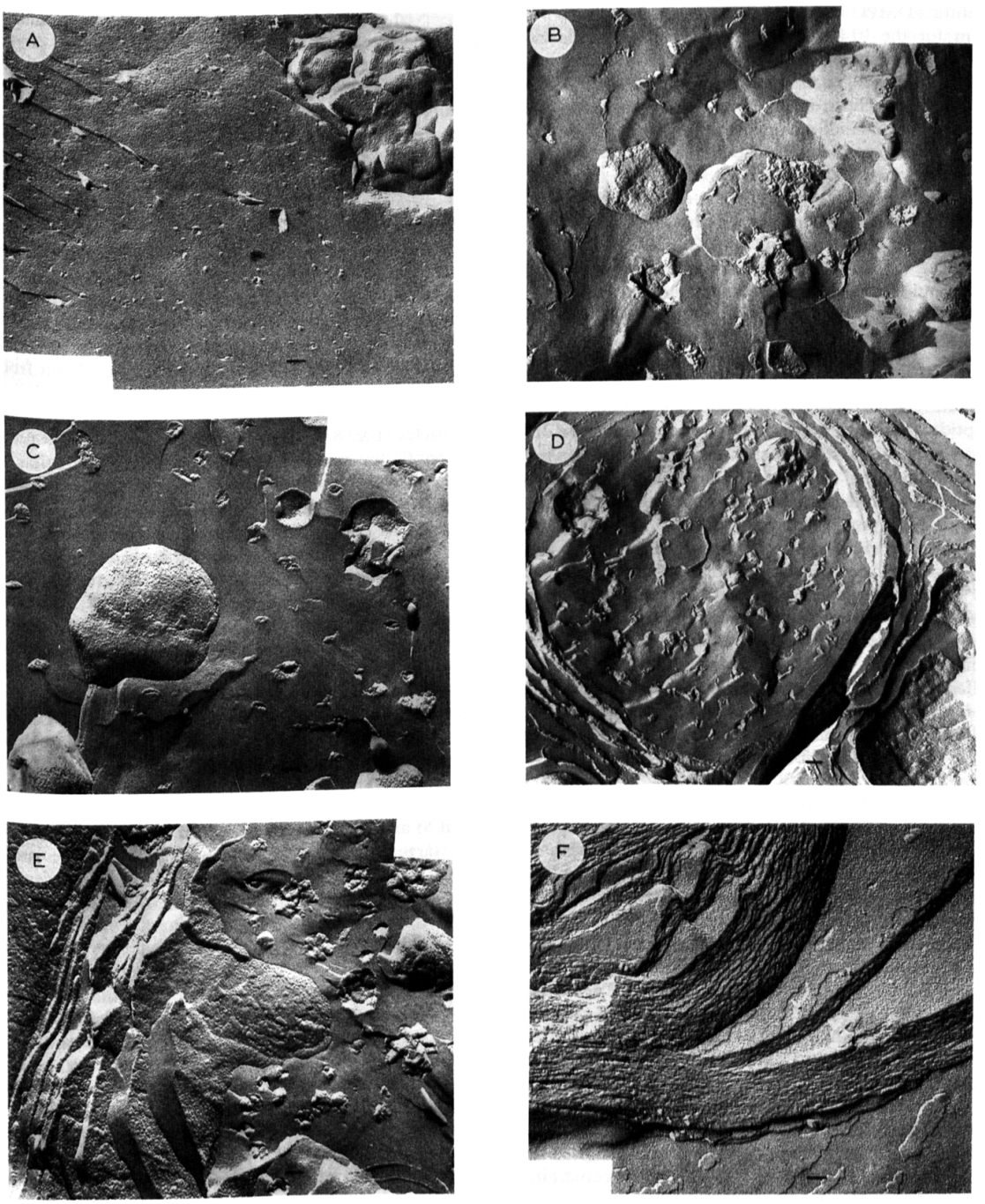

Fig. 8. Freeze-fracture electron micrographs of P14A : DMPC complexes (5 mol% peptide) quenched at increasing times after a temperature jump from 4°C to 55°C after overnight incubation at 4°C: zero time (A), 30 min (B), 120 min (C), 240 min (D), 360 min (E) and 720 min (F). The scale bar represents 100 nm.

melittin : DMPC complexes is about 100-times shorter than for the P14A : DMPC complexes when the two peptides are compared at equivalent peptide and lipid concentrations. The kinetics of micellization of DMPC bilayers by melittin is similarly about 100-times faster than for P14A-induced micellization (not shown, but see the electron microscopy results in Fig. 8 below).

Electron microscopy

When quenched from temperatures above T_m, (28°C) before fracturing, melittin : DMPC and P14A : DMPC complexes yield electron micrographs showing both large and small, unilamellar and multilamellar vesicles (Fig. 6). The fracture faces are decorated with intramembranous particles (IP) having diameters of around 4.5 and 8 nm that are distributed relatively uniformly across the fracture faces. The density of IP on the fracture face is roughly proportional to the peptide : lipid ratio (pure lipid does not show any IP), but the size of the IP is independent of the peptide : lipid ratio, suggesting that the IP correspond to discrete molecular species with no tendency for gross aggregation at high peptide concentrations (not shown). The size and distribution of IP in melittin : DMPC and P14A : DMPC complexes are similar (Fig. 6).

When samples were quenched after incubating overnight at 11°C (below the phase transition temperature of the peptide : lipid complexes), the melittin : DMPC complexes and the P14A : DMPC complexes showed different properties. At concentrations of 3 mol% or higher (peptide : lipid), each sample showed a massive collapse of liposomal structures into small particles (Fig. 7) consistent with the deuterium NMR data of Figs. 2 and 3. For both melittin and P14A, 5 mol% peptide : lipid was insufficient for complete micellization of all the lipid. In both samples some gel phase liposomes with ripple structure ($P_{\beta'}$) identical to peptide-free gel phase lipid are observed (Fig. 7A and 7B, single arrows). These liposomes do not exhibit IP on the fracture faces indicating that the peptide is disassociated and/or expelled from a bilayer-inserted location in gel phase lipid. In addition to the $P\beta'$ ripple structure, the P14A : DMPC complexes give fracture faces characteristic of fluid phase lipid (Fig. 7B, double arrows) indistinguishable from that observed for melittin : DMPC or P14A : DMPC complexes in the fluid phase at temperatures above T_m (cf. Fig. 6). The residual fluid phase fracture faces are decorated with randomly distributed IP of similar size to IP in fluid phase bilayers quenched from temperatures above T_m. These observations indicate that in DMPC samples containing 5 mol% P14A, the fluid to gel phase transition occurs very slowly and overnight incubation was insufficient to allow all of the lipid to convert completely into the gel phase. In accordance with the deuterium NMR data (Fig. 2; and Ref. 13) gel phase micellization is not observed at low peptide concentration (Fig. 7C), and at intermediate concentrations both $P\beta'$ rippled gel phases and micellar phases coexist (Fig. 7D). No IP are detectable in melittin-containing gel phase lipid at sub-micellizing peptide concentrations (Fig. 7C and D) indicating that the peptide is dissociated and/or moves to a surface location in gel phase lipid.

The considerable suppression of the rate of bilayer micellization in P14A : DMPC complexes after cooling below T_m and of the rate of reformation of extended bilayers by warming micellar particles above T_m allowed each process to be followed by quenching samples during the slow micelle fusion or bilayer micellization and examination of freeze-fracture electron micrographs. Fig. 8 shows a series of electron micrographs obtained at increasing times following a temperature jump from 11°C to 55°C. After 24–36 h at 4°C the membrane preparations exist completely in the form of micellar particles (Fig. 8A). Shortly after the temperature jump to 55°C, the micelles aggregate with the formation of small regions of bilayer with fracture faces decorated with IP characteristic of fluid phase bilayers containing peptide (Fig. 8B–E). These bilayer fragments, apparently produced by the fusion of the micellar particles of gel phase P14A : DMPC, do not all have the morphology of vesicles, but appear in much of the sample to be growing lipid sheets. After a period of between 4 to 24 h the lipid is largely in the form of multilamellar vesicles. The final form of the lipid obtained after leaving at temperatures above T_m for long periods is very similar to the melittin : DMPC and P14A : DMPC complexes obtained before cooling below T_m. The time-course for reformation of large lipid structures (bilayer sheets and single and multilamellar vesicles) is similar to the time-course observed by deuterium NMR (Figs. 4 and 5) and the EM results confirm that the reformation of large structures on incubating gel phase melittin : DMPC or P14A : DMPC micelles at temperatures above T_m occurs by fusion of micellar particles.

Discussion

Although a number of peptide and detergent amphiphiles micellize phospholipid membranes in the form of bilayer disc micelles, only melittin and lysophosphatidylcholine are known to form complexes with lipids that undergo reversible transitions between disc micelles and extended bilayers when the temperature is cycled through the gel to liquid crystalline phase transition temperature of the lipid [4]. Melittin-induced reversible micellization has been shown to occur only in bilayers of saturated phosphatidylcholines and only after the amphiphile has been incorporated into the bilayers in the fluid phase [10–13]. The molecular mechanism of disc micellization is not known, but is probably associated with an expulsion of the peptide from growing

domains of gel phase lipid with a resulting local increase in amphiphile concentration that results in bilayer fragmentation [11–13].

The macroscopic magnetic orientation of the DMPC bilayers observed in the deuterium NMR spectra of peptide : DMPC complexes at temperatures around T_m (Fig. 3) supports the interpretation of a progressive phase separation of the peptides and lipids as the temperature is lowered through T_m. The near complete orientation observed (Fig. 3A, 20°C spectrum; Fig. 3B, 21°C spectrum) requires the formation of highly asymmetric (non-spherical) membrane structures such as bilayer sheets or flattened vesicles. It was previously shown that macroscopic magnetic orientation is not favoured in bilayers composed of single lipids but only in mixed lipid systems [21], probably because partial phase separation in the fluid phase is necessary to allow the formation of domains of high membrane curvature associated with vesicle deformability required for macroscopic orientation. It is apparent from Fig. 3 that maximal orientation in DMPC bilayers containing melittin or P14A occurs only at temperatures close to the centre of the phase transition. At temperatures only one or two degrees higher, deuterium powder patterns characteristic of a near spherical distribution of orientations are observed. Under these conditions the peptide aggregates are randomly distributed in the bilayer as judged from the distribution of IP in the electron micrographs quenched from temperatures above T_m (Fig. 6). We conclude that on cooling the peptide-containing complexes through T_m, the phase separation that is ultimately required for disc micelle formation occurs progressively with an increase in vesicle deformability resulting in strong magnetic orientation. At some point the local amphiphile concentration becomes sufficiently high for the breakdown of the lipid structure into sheets or micelles in which the lipid domains are not large enough for magnetic orientation to compete with thermally-induced tumbling. The transition from unoriented vesicles with randomly distributed peptide (giving spherically averaged deuterium powder patterns) to structures having highly oriented gel phase lipid domains and finally to collapsed structures giving narrow deuterium NMR spectra takes place over only a few degrees and is reversible, occurring in both heating and cooling runs (Fig. 3).

The reverse process, the reformation of extended bilayers from disc micelles on warming the micellar particles above T_m presumably occurs by edge to edge fusion of bilayer discs [4]. This interpretation is supported by the electron micrographs of Fig. 8 which show that the micelles have fused to yield small fluid phase membrane patches shortly after raising the temperature of the complexes above the phase transition temperature.

The major difference between the effects of melittin and P14A on the reversible disc micellization of DMPC bilayers is the considerable suppression of the rate of micellization at temperatures below T_m, and of micelle fusion and vesicle reformation on warming P14A : DMPC complexes above T_m. The suppression of the rate of gel phase micellization in P14A : DMPC complexes results from suppression by the peptide of the phase transition itself. Whereas the deuterium NMR data of Fig. 3 indicate that T_m for DMPC containing 5 mol% melittin or P14A is reduced from 24°C (for pure DMPC) to around 20°C, the micrographs show that regions of fluid phase bilayer decorated with IP are present even after several hours at 11°C in P14A : DMPC complexes (Fig. 7B). The retention of the fluid phase for long periods after cooling to temperatures well below the phase transition temperature of the P14A : DMPC bilayers indicates that the redistribution of peptide and lipid to give peptide-free regions of gel phase bilayer occurs considerably more slowly for the P14A : DMPC complexes compared to the melittin : DMPC complexes.

The suppression of the rate of re-fusion of P14A : DMPC micelles to yield extended bilayers on incubating at temperatures above T_m (Figs. 4 and 5) is not due to the suppression of the phase transition because at short times after raising the temperature above T_m all the lipid in samples observed by EM (Fig. 8B) has reverted to a fluid phase indistinguishable from fluid phase lipid containing melittin or P14A observed before micellization. The suppression of the reformation of extended sheets or vesicles of fluid phase lipid is presumably due to the suppression of the rate of fusion of bilayer discs or sheets in P14A : DMPC samples.

The molecular basis for the altered properties of P14A compared with melittin in its interaction with DMPC bilayers is the altered structural properties of the peptide resulting from replacement of Pro-14 with alanine. NMR and amide exchange studies indicate that P14A forms a regular, stable α-helix in methanol (Ref. 4; C.E.D., Bazzo, R. and Campbell, I.D., unpublished data), whereas melittin adopts a 'hinged' α-helical conformation with considerable conformational flexibility in the centre of the molecule between the N- and C-terminal helical segments [22–24]. The structural characteristics associated with a central proline residue in melittin are not necessary for hemolysis because P14A (like a previous synthetic analogue of melittin lacking a proline [7]) has enhanced hemolytic activity compared with melittin [4]. On the other hand, the voltage-gated channel activity in P14A is suppressed compared with the activity of melittin, probably because the absence of a helix bend results in channel-destabilising electrostatic repulsion between the highly basic C-terminal segments when the peptide adopts the parallel transbilayer aggregates thought to underlie the channel structure. P14A, like melittin, seems, however,

to form discrete oligomers in fluid phase DMPC; if the formation of gel phase micelles requires dissociation of these oligomers then the slower micellization and the persistence of IP-decorated fluid phase bilayers for long periods at temperatures below T_m, indicates that P14A oligomers are less able to dissociate than melittin oligomers. We cannot rule out the possibility that the different kinetics of reversible micellization between the two peptides may also have a contribution from different partitioning between the aqueous and bilayer phases. At present we have no evidence of the nature of the associated state of the peptides in fluid phase DMPC bilayers except that the IP indicate a discrete sized oligomer which (as previously observed in DPPC bilayers [10]) penetrates into the centre of the bilayer. We have recently observed that the formation of IP in P14A : DMPC fluid phase bilayers is enhanced by the presence of EDTA (unpublished observations), a phenomenon which we are presently investigating.

Finally, we note that the enhancement of hemolytic activity of P14A relative to melittin [4], together with the suppressed micellizing activity of P14A observed here supports the conclusion that bilayer disc micellization does not underlie the hemolytic activity of melittin. This conclusion is reinforced by observations of the stabilization of DMPC bilayers to melittin-induced micellization on addition of negatively charged lipid (DMPS) to the bilayer [25], and the absence of detectable micellization of erythrocyte membranes by melittin at lytic concentrations [26].

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