Interaction of a Lytic Polypeptide, Melittin, with Lipid Membrane Systems*

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SUMMARY

To study mechanisms whereby lytic proteins disrupt biomembranes, artificial phospholipid spherules (liposomes) were exposed to melittin, a cationic peptide in which sequences of hydrophobic (Positions 1 to 20) and hydrophilic (Positions 21 to 26) amino acids are unequally distributed. At concentrations above $10^{-8}$ M, melittin, which is the major toxin of bee venom, released marker anions ($\text{CrO}_4^{2-}$) or glucose from the model structures. Release of marker ions was equivalent whether liposomes were prepared with a net negative (dicetyl phosphate) or net positive (stearylamine) charge. Nor was the presence of cholesterol in the model membranes necessary for the action of melittin. Spherules with net negative charges competed approximately 10 times more effectively for melittin's lytic activity on erythrocytes than did spherules with positive charges. Studies with lipid monolayers indicated that melittin had a remarkable affinity for the air-water and more so for the lipid-water interface, for it spread as a film readily from water and penetrated lipid monolayers avidly, irrespective of the surface charge of the lipid film. Negatively stained preparations of liposomes in the electron microscope showed that melittin-treated spherules lost the integrity of their concentric lamellae, which became beaded, frayed, and finally fragmented. These studies indicate that the lytic effects of melittin upon biological membranes may be due to a similar reaction with structural phospholipids. Since ionic interactions between the cationic peptide and charged lamellae were not crucial for lysis, and since melittin has an extraordinary affinity for lipid membranes, it is suggested that the surface activity of melittin and convenient apolar associations between hydrophobic portions of melittin and the acyl chains of phospholipid could account for disruption of the spherules.

Mechanisms by which proteins lyse biomembranes remain unclear largely because neither the structures of the target membranes nor those of the lytic proteins have been rigorously defined. However, by the use of model lipid membranes and considerably purified bacterial hemolysins, it has been shown that proteins such as streptolysin S (1, 2) or staphylococcal α-toxin (3, 4) can directly disrupt the lamellar structure of phospholipids in aqueous solutions. It was therefore suggested that lytic proteins act on naturally occurring membranes in a similar fashion. Unfortunately, these larger, lytic proteins have not been isolated in sufficient purity to permit detailed study of primary amino acid sequences, which must precede any attribution of their unique action to a particular structure.

No such problem exists with melittin, which has been purified, resolved from related peptides, and analyzed structurally, by Habermann and co-workers (5–8). Melittin is the major component of bee venom, constituting over 50% of its dry weight. The cationic peptide is unusual, in that its amino acids are unequally distributed: Positions 1 to 20 are occupied by largely hydrophobic, and Positions 21 to 26 by hydrophilic, amino acids (Fig. 1). Since the peptide is too small to accommodate itself to a globular configuration, it appears to associate as tetramer micelles in aqueous media: this property accounts for the experimentally obtained molecular weight of approximately 12,000 as against the theoretical molecular weight of 3,400. Thus each melittin chain constitutes a large biological amphipath, the effects of which as a "surface active agent" have been held accountable for its biological properties (6). These include not only hemolysis, but disruption of isolated leukocytes, lysosomes, and mitochondria (9, 10).

If, then, a defined lytic peptide is available, so also are defined models of biomembranes. Phospholipids in the smectic mesophase can be induced to form spherules (liposomes), the permeability properties of which are somewhat similar to biological interfaces (review in Reference 11). The assemblies are, for example, capable of ion discrimination. Cations and divalent anions are retained, while monovalent anions and water diffuse freely: (12) the spherules swell in response to osmotic stimuli (13). In aqueous solution, liposomes exist as concentric lipid layers with intercalated water compartments in which marker ions or molecules are trapped. X-ray, electron microscopic, and polarized light studies indicate a lamellar substructure, the repeating unit of which is from 55 to 75 Å, depending on the

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Melittin

Habermann. Phosphatidylethanolamine from egg yolk was obtained
as chromatographically pure from General Biochemicals. Chol-
esterylamine from K and K Laboratories (Plainview, New York).

Preparation of Phospholipid Spherules (Liposomes)—Liposomes
were prepared by a slight modification of methods previously
described in detail (15). In brief, lipids (either lecithin-dicetyl
phosphate or stearylamine–cholesterol in molar ratios of 7:2:1,
or lecithin-dicetyl phosphate in the molar ratio of 8:2) were added
as a function of time.

In the film penetration studies, lipids (either lecithin-dicetyl
phosphate–cholesterol or lecithin-stearylamine–cholesterol, in
molar ratio of 7:2:1) were spread from a 0.5 μg per μl solution
in chloroform–methanol (85:15) until the desired initial film pres-
sure (p) was obtained. At this point, “zero time,” 50 μg of
melittin in 50 μl of water was injected into the subphase with a
microsyringe, and the increase in film pressure (Δp) was recorded
as a function of time.

In the determination of the surface isotherms, the lipid was
spread from 0.5 μg per μl solution in chloroform-methanol (85:15),
whereas melittin was applied to the surface from a 1.0 μg per μl
solution in distilled water.

Electron Microscopy—For electron microscopy, specimens were
negatively stained with 2% ammonium molybdate in 2% am-
onium acetate (pH 6.8) and applied to hydrophilic films of
carbon-coated Formvar supported on copper specimen mounts.
The preparations were examined in a Siemens Elmiskop 1A
electron microscope fitted with a decontamination device, with a
short focal length objective lens.

RESULTS

Release of CrO₄²⁻ from Negatively and Positively Charged
Spherules by Melittin

It is possible to prepare spherules with either a negative or
a positive net surface charge by incorporating into the lamellar
structures either anionic dicetyl phosphate or cationic stearyl-
amine (12). Spherules carrying a net negative charge were
exposed to melittin at final concentrations varying from 10⁻⁴ M
to 10⁻² M. Triton X-100 was also studied, as a relative standard,
since, at a final concentration of 0.2%, v/v, the detergent induces
maximum release of markers from the spherules; its effect upon
release of anions from liposomes has previously been exten-
sively described (2, 20). At concentrations of 10⁻⁴ M (Fig. 2),
melittin released significant amounts of divalent anions into
Release of CrO\textsuperscript{4−} from Liposomes by Increasing Amounts of Melittin

The release of CrO\textsuperscript{4−} from liposomes by increasing amounts of melittin was observed. Spherules prepared with lecithin-dicetyl phosphate-cholesterol in molar ratio of 7:2:1 (16.6 μmoles of lipid per ml). Results are expressed as the percentage of CrO\textsuperscript{4−} appearing in the dialysate after 30 min of incubation at 37°C with Triton X-100 (0.2%). To control samples, 0.2 ml of NaCl-KCl (0.145 M) was added. Mean of three experiments.

Release of CrO\textsuperscript{4−} from Spherules Prepared in Absence of Cholesterol

At least some amphipaths, e.g. amphotericin B, nystatin (15) filipins I and III (20), preferentially disrupt liposomes which contain sterol as part of their lamellar composition. To determine whether lytic polypeptides also require sterol for optimum membrane action, liposomes were prepared without cholesterol. Such spherules (Fig. 4) did not respond to melittin with augmented anion release; indeed, the results are quite comparable to those in Figs. 2 and 3. From these experiments, it appeared that the reaction of melittin with liposomes was independent of sterol receptors, resembling in this property those of steroid hormones (2), staphylococcal α-toxin (3, 4), and filipin II (20).

Release of Glucose from Phospholipid-Cholesterol Spherules by Melittin

Since a portion of the melittin molecule contains polar, cationic groups, the possibility remained that these nonpermeant polyions in the medium surrounding the spherules could be responsible for an increased efflux of marker anions. Diffusion of anions across individual lamellae would be due to the incapacity of melittin in the medium to permeate the liposomes and the requirements for both counter- and psi-bound anions would lead to anion efflux (22). To exclude such a possibility, studies were performed with spherules containing glucose, a nonelectrolyte (Fig. 5). However, glucose was released from liposomes at the same concentrations of melittin which induced the surrounding medium, while at concentrations of 10\textsuperscript{−4} M, the disruption of the spherules, as determined by anion release, approached that obtained with Triton X-100.

Were the action of melittin, which is a basic protein with an isoelectric point above 8 (6), due to saltlike interactions between its cationic groups and the negative groups on the lipid lamellae, one would expect negatively charged spherules to release far more markers than the positively charged ones. Indeed, mutually repulsive forces between the cationic melittin micelles and target membranes prepared with stearylamine might block lytic interactions.\textsuperscript{1}

Therefore, spherules were prepared with stearylamine. Essentially the same findings were observed as with negative spherules (Fig. 3). Again, melittin caused considerable disruption of the spherules at a concentration as low as 10\textsuperscript{−6} M, while at higher concentration leakage approximated that obtained with Triton X-100. Minor differences between results in Figs. 2 and 3 can readily be accounted for by differences in control preparations. These findings suggest that ionic interactions do not play a critical role in the effect of melittin on the spherules.

\textsuperscript{1} It will be appreciated that in aqueous media of electrolytes direct ionic interactions between charged lipids and peptides are not the only possible ones (21). Indeed, interactions between these and small inorganic solvent ions can readily lead to indirect charge-induced interactions. For convenience, both of these associations will be referred to as "saltlike interactions."
anion release. These data suggest that the release of markers, after exposure of spherules to melittin, is dependent upon gross alterations in the lipid lamellae rather than upon the nature of the marker present within the spherules.

**Hemolysis of Human Erythrocytes by Melittin**

The foregoing experiments did not clarify whether melittin became tightly associated with the lipid layers of the spherules or whether, in detergent-like fashion, its activity remained undiminished in bulk solution after disruption of the target structures. Therefore, we determined the residual hemolytic activity of solutions containing melittin, before and after incubation with lipid spherules. If melittin had interacted with the lipids to form an irreversible complex, its hemolytic activity remaining in the supernatant would be expected to diminish; this type of association has previously been shown with the filipins (20).

Fifty per cent hemolysis (a value obtained from data in Fig. 6) of a 0.7% suspension of human erythrocytes (7 min at 37°) was brought about by 3 μg of melittin. However, when melittin solutions were incubated with 0.4 μmole of positively charged spherules per ml, more than 16 μg were necessary to achieve the same degree of hemolysis (Fig. 6). At the same lipid concentration of negatively charged spherules (0.4 μg per ml), no hemolysis was detectable even at a melittin concentration of 30 μg. Only if the lipid concentration was decreased to 0.01

**Fig. 4.** Release of CrO₄²⁻ from liposomes by increasing amounts of melittin. Spherules prepared with lecithin and dioleoyl lecithin in molar ratio of 8:2 (16.6 μmoles of lipid per ml). Results are expressed as the percentage of CrO₄²⁻ appearing in the dialysate after 30 min of incubation at 37° with Triton X-100 (0.2%). To control samples, 0.2 ml of NaCl-KCl (0.145 M) was added. Mean of three experiments.

**Fig. 5.** Release of glucose from liposomes by increasing amount of melittin. Spherules prepared with lecithin-dioleoyl phosphatidylcholine in molar ratio of 7:21 (16.6 μmoles of lipid per ml). Results are expressed as the percentage of glucose appearing in the dialysate after 30 min of incubation at 37° with Triton X-100 (0.2%). To control samples, 0.2 ml of NaCl-KCl (0.145 M) was added. Mean of two experiments.

**Fig. 6.** Release of hemoglobin from human erythrocytes produced by increasing amounts of melittin (micrograms per ml) in the presence or absence of liposomes. Liposomes, prepared either with lecithin-dioleoyl phosphatidylcholine, or with lecithin-stearoyl amine-cholesterol in ratio of 7:2:1, were allowed to swell in phosphate buffer, 0.067 M, pH 7.0. After 1 hour liposomes (0.1 ml) were incubated with indicated amounts of melittin for 20 min at room temperature. Thereafter, 0.5 ml of human erythrocytes (0.7%, v/v, suspension in phosphate buffer, 0.067 M, pH 7.0) was added. After incubation at 37° for 7 min, the suspension was centrifuged for 20 min at 10,000 x g. Hemolysis was calculated as the percentage of total hemolysis induced by saponin or hemolysis in controls or (ΔA₆₅₀ saponin - ΔA₆₅₀ solvent + phospholipid)/(ΔA₆₅₀ melittin - ΔA₆₅₀ solvent + phospholipid) x 100.
μmole per ml could 50% hemolysis be obtained with 11 μg of the peptide. These experiments indicated that negatively charged spherules competed more effectively with erythrocytes (by a factor of 10) for melittin than did positively charged spherules. This suggested not only that ionic interactions of cationic polypeptide and anionic liposomes could be detected, but that these were not crucial for lysis of the target spherules, since liposomes were equally disrupted whether positively or negatively charged. If this increased requirement for melittin (in hemolytic systems with negatively charged spherules) does indeed represent nonlytic interaction of melittin with liposomes, then it appeared possible that the crucial step in polypeptide lysis was the insertion of melittin's hydrophobic residues into lipid layers, although the initial attraction might be saltlike.

**Surface Activity of Melittin**

In order to test the "insertional" hypothesis, namely, whether melittin could penetrate natural or artificial lipid layers, we studied the surface activity of melittin at the air-water interface.

**Surface Isotherm of Melittin**—Melittin spread readily from water and formed a stable film which had the relatively high collapse pressure of 24.5 dynes per cm (Fig. 7); on a weight basis, the water-soluble melittin was markedly more surface-active than the lipid. Indeed, melittin surfaced from a concentration of 1 μg per ml in the aqueous subphase in the absence of lipid and formed a film, the saturation pressure of which (24.5 dynes per cm) was the same as the collapse pressure of the melittin film obtained from spreading. The curves of pressure with respect to concentration and the collapse pressure (about 36 dynes per cm) of the lipid film containing lecithin, cholesterol, and either dicetyl phosphate or stearylamine in the molar ratio of 7:2:1 were similar to those of egg lecithin. In line with a recent observation (18), the lipid did not form a film by spreading from aqueous dispersion, whereas melittin did so readily.

**Penetration of Lipid Monolayers by Melittin**—As observed with other proteins (19), a water surface covered by lipid films (Fig. 8) was penetrated by melittin much more readily than a clean air-water interface. The lipid films were prepared by spreading a mixture of lecithin-dicetyl phosphate-cholesterol, in the same molar ratio as that used for the spherules (7:2:1), on a liquid phase-phosphate buffer. The initial pressure ($\pi_i$) of the lipid film was varied from 2 to 30 dyne per cm, and it was found (Fig. 8) that at an initial pressure of 2 dyne per cm the injection of melittin produced an increase in the film pressure ($\Delta \pi$) of 17 dyne per cm within 10 min, which reached a plateau ($\Delta \pi$) at 25 dyne per cm in 30 min. At an initial film pressure of 10 or 20 dyne per cm there was also a considerable increase in pressure. An increase could indeed be detected at an initial pressure of 20 dyne per cm, which is clearly above the collapse pressure of melittin. When stearylamine was substituted for dicetyl phosphate in identical experiments, curves were obtained which were in every way comparable to those shown in Fig. 8; these are omitted for the sake of brevity. Finally, when lecithin alone was used, essentially similar results were again found.
Fig. 9.
Spherules were prepared with lecithin-dicetyl phosphate-cholesterol in the usual ratio, at a total lipid concentration of 4 μmoles per ml (which was one-fourth of that used for the leakage experiments), and allowed to swell in water. In Fig. 9 a preparation is shown of liposomes negatively stained with ammonium molybdate. Lipid lamellae intercalated by electrondense aqueous compartments as described by Bangham, Standish, and Watkins (12) are clearly visible. After addition of melittin to the liposomes, the characteristic concentric lamellar arrangement of lipids was no longer evident. Long strands and coalesced sheetlike structures appeared, which seemed to arise from the disruption of the lamellae. Large areas of such sheets appeared as nets of material, possibly due to molecular rearrangements brought about by the presence of melittin (Fig. 9B). At higher magnifications (Fig. 9C), different regions of the preparation showed what appeared to be different degrees of disruption, ranging from lamellae, through flat sheets of material showing many small holes, to the netlike areas made up of threadlike structures of uniform dimensions. Under the same conditions of examination, melittin alone could not be clearly resolved and appeared amorphous. Furthermore, negatively stained preparations of positively charged spherules treated with melittin could not be distinguished from negatively charged liposomes so treated.

**Discussion**

The experiments presented above document the capacity of melittin to disrupt artificial phospholipid arrays (liposomes and monolayers). If the integrity of biomembranes, at least in part, is governed by lipid-lipid interactions analogous to those of the models, then it is quite likely that the effects of melittin on cellular and intracellular membranes are due to the peptide's interruption of such forces. The structure of liposomes has been discussed elsewhere in detail (11, 23), but their over-all integrity is governed both by intermolecular cohesive forces (polar, apolar, hydrogen bonding) and by forces arising from the solvent per se, either set of which could be affected by melittin.

Since melittin in aqueous solutions can be considered a cationic detergent which forms micelles (6), the individual peptide chains will be in constant equilibrium between these and the bulk solution. Thus melittin can disrupt liposomes by one of at least four mechanisms (21, 24, 25). The peptide could (a) overcome polar adhesive forces between phospholipids; (b) disrupt apolar (hydrophobic) cohesive forces between the acyl chains; (c) break cohesive forces imposed by water (as ice) (21) in the region of the charged head groups; or (d) act in classical detergent fashion to form mixed micelles of phospholipid with melittin, in which the individual acyl chains of the liposomes would associate with melittin's hydrophobic portion, the polar groups of phospholipids and melittin facing the bulk solution. The last mechanism would clearly be a more extreme consequence of (b). Whatever cohesive forces may arise from staggered structures of the polar head group of natural membranes (26, 27) recent X-ray studies with pure lamellar bilayers (as in liposomes) indicate that the head groups of phospholipids are fully extended in the presence of water (28). Thus the surfactants should provide little cohesion to the liposome structure, especially in the presence of salt (Na-KCl). Furthermore, the presence of net repulsive forces between long chain anions or cations (stearlylamine or dicetyl phosphate) would overcome any polar cohesive forces. Similarly, although the presence of structured water in the vicinity of the polar head groups has been held responsible for cohesion of closely packed lamellae (29), there is little evidence that the head groups of pure lecithins are restricted in their movement (or structured) in the presence of over 16% water (30). Indeed, since the aqueous channels within liposomes are separated by at least 45 A (13), the free water area is very large relative to any structured “ice” layer.

The major cohesive forces which hold liposomes together are therefore likely to be apolar. It is then also likely that melittin can overcome these forces in the manner of an ionic detergent, after penetrating the outer layers of liposomes. Penetration of monolayers was indeed shown to be avid, proceeding also at high initial values of the film pressure (πl). Indeed, melittin penetrated lipid films at initial pressures which exceeded the collapse pressure of the peptide. Depending upon the local concentration of reactants, the final structures would consist of micelles containing varying proportions of phospholipid to long chain union or cation to melittin. If micelles of unlike charge (e.g. mixed micelles of cationic melittin with dicetyl phosphate) were more stable and removed more structural acyl chains from liposomes, then liposomes prepared with dicetyl phosphate should have been more susceptible to melittin disruption. Since this was not found, it is likely that only a few melittin molecules need to insert into the lipid layers, and that transitions from lamellar to micellar forms could arise locally. Since hydrogen bonding is stronger in apolar environments (31), and since proteins can participate in such bonds to a greater extent than simple acyl chains, the single peptide chains may form stronger intra- and intermolecular bonds in the phospholipid milieu than the bonds which the hydrophobic chains of these lipids can form with each other. Indeed, melittin inserted into monolayers below the critical pressure of 34 dynes per cm which was used to separate “detergency” from “penetration” by Pethica and Schulman (32).

In the foregoing, melittin has been considered as an ionic detergent of biological origin. Some of these compounds have previously been shown to interact preferentially with cholesterol in model lipid systems (33, 34). Since the presence or absence of cholesterol made little difference in the disruption of liposomes, melittin clearly does not fall into this group. Nor were charge-induced, ionic interactions between the cationic peptide and the target structures crucial for lysis. Our observation that negatively charged spherules removed more of the hemolytic activity from solutions of melittin can be accounted for by at least two mechanisms. Both anionic and cationic liposomes were disrupted at equal concentrations of melittin. Therefore, amounts of melittin sufficient for lysis entered the lamellae of spherules
bearing either charge, but negative spherules took up more of the excess molecules which did not participate in lysis. This may be a further demonstration that the hemolytic system is more sensitive in detecting "prolytic" removal of amphipathic surface from suspension that is the spherule system. Such an observation has already been made with filipin (20) and reflects the high lipid to solvent ratio of liposomes relative to erythrocytes. Alternatively, as Lucy has suggested (24), much of the added diacyl phospholipase in liposome suspensions may exist as a population of anionic micelles which are not necessarily part of the general liposome population. Were this the case, saltlike interactions would be detected of melittin with diacyl phospholipid micelles, but these would have no influence upon the amount of melittin needed for lysis of the formed liposomes.

The interpretation of electron micrography of lipids is difficult. Although Lucy and Glauert (35) have presented evidence that lipids dry in salts like ammonium molybdate as if imbedded in a glass, there is contrary evidence that their exact configuration varies with water content (36). Fine structural details of control spherule preparations examined in the above manner do, however, correlate well with results of both optical and X-ray diffraction studies. Ultrastructural evidence indicates that melittin induces not only functional, but morphological changes in spherules of pure lipids. Beading, fraying, coalescence, and the appearance of netlike areas were observed when the peptide disrupted lipid layers. However, these do not permit conclusions as to whether the new structures were due to composites of melittin with phospholipids or of phospholipids alone in a solution containing a cationic detergent such as melittin. It must be remarked that no such discrete structures were observed when liposomes were treated with a nonionic detergent such as Triton X-100 (2), but that both streptolysin S (1) and staphylococcal α-toxin (4) produced new morphological structures which were characteristic of each lysin.

Therefore, although much uncertainty still exists as to the mechanisms of lipid film penetration by proteins (18) or detergents (32), some results of our monolayer experiments cannot be overlooked. Namely, melittin is extremely surface-active, as (a) it spreads readily as a film from water, (b) it forms a film from its aqueous solutions at a high rate, and (c) it penetrates lecithin and mixed lipid films (Fig. 8) at a rate and to an extent which exceed any heretofore described in the literature of biological surfactants (18, 19). These physical properties of melittin may well account for its ability to penetrate and disrupt natural and artificial lipid assemblies, and its capacity equally well to penetrate cationic, anionic, and uncharged (lecithin) lipid monolayers. Whether melittin interacts with the zwitterionic structure of lecithin may be relevant, but this was not established. Whatever the exact mechanism involved in disruption of lamellar lipids by melittin, it is clear that a single explanation for the effects of this peptide upon biomembranes is its capacity to disrupt the three-dimensional structure of phospholipid arrays.

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