THE STING

MELITTIN FORMS CHANNELS IN LIPID BILAYERS

MAGDALENA T. TOSTESON AND DANIEL C. TOSTESON, *Department of Physiology and Biophysics, Harvard Medical School, Boston, Massachusetts 02115*

ABSTRACT Melittin, a toxin of bee venom, is a cationic polypeptide composed of 26 amino acids. The six residues of the C-terminal end are polar and 19 of the 20 residues of the N-terminal end are hydrophobic. Exposure of a lecithin bilayer to melittin results in the formation of channels that are more permeable to anions than to cations. Unilateral addition of melittin produces a voltage-dependent increase in membrane conductance when the side where the polypeptide is present is made positive but not when it is made negative. At a fixed voltage, the conductance increases with the fourth power of the melittin concentration in the aqueous phase. At a fixed peptide concentration, the conductance increases approximately *e*-fold per 6-mV increase in the electrical potential difference across the membrane. These results suggest that four melittin monomers are needed to form a channel and, furthermore, that a minimum of four equivalent electronic charges need to be displaced by the electrical field to explain the voltage dependence of the conductance.

INTRODUCTION

The polypeptide melittin is, with respect to weight and activity, the main toxin of bee venom (1). The primary structure of melittin reveals its amphiphilic character (2):

(+)Gly-Ile-Gly-Ala-Val-Leu-Lys(+)-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-
10 15

Leu-Ile-Ser-Trp-Ile-Lys(+)-Arg(+)-Lys(+)-Arg(+)-Gln-Gln-NH_2
25

Melittin's ability to interact with membranes is demonstrated by its capacity to lyse cells (3) and to release marker ions from liposomes (4). Experiments using spin labels (5) and infrared spectra (6) indicate a distinctive interaction of melittin with phospholipids. The hydrophobic nature of this interaction has been demonstrated by fluorescence measurements (7) that show that the tryptophan residue, and presumably other hydrophobic regions of melittin, penetrate the apolar interior of the lipid bilayer. Futhermore, it has been recently shown, with the use of high resolution ^{1}H-NMR, that the structure of melittin bound to detergent micelles is very similar to the structure of the self-aggregated tetrameric form of melittin in aqueous solutions (8). In this form, the N-terminal region of melittin assumes a spatial folding that gives rise to an α-helical structure, as seen by circular dichroism, with the hydrophilic C-terminal end probably located at the surface of the aggregate (9).

To gain further insight into the molecular mechanism of melittin's action, we have studied the changes in the electrical properties of planar lipid bilayers induced by melittin. We show that addition of melittin to one of the aqueous phases of a lecithin bilayer results in the formation of channels that are more permeable to anions than to cations, and in the

appearance of a voltage-dependent conductance. At a fixed voltage, the conductance was found to increase with the fourth power of the melittin concentration in the aqueous solutions. These results suggest that when melittin incorporates into lipid bilayers, it forms tetrameric complexes that give rise to ion-conducting channels.

EXPERIMENTAL

Bilayers were formed from asolectin (20 mg/ml pentane) (Associated Concentrates, Woodside, N.Y.) by apposition of two monolayers spread at the air/solution interface (10). The hole in the teflon partition was pretreated with a saturated solution of veseline in pentane. The aqueous solutions contained unbuffered 1 M NaCl (pH ~ 6) or 1 M Na-acetate or Tris Cl with the pH adjusted to 6.0. The contents of both chambers were stirred with magnetic stirrers. Complete change of the solution in one or both compartments was accomplished with a pair of matched, mechanically coupled syringes. All experiments were conducted at room temperature (~20°C).

The steady-state membrane conductance was determined by measuring with silver-silver chloride electrodes the steady-state current flowing across the membrane in response to an applied potential difference. When the aqueous phases did not contain Cl, the electrodes were connected to the solutions via salt bridges. The sign of the electrical potential refers to the compartment to which melittin was added (*cis* compartment). Positive charge flowing from the *cis* to the *trans* compartment is plotted as positive (upward) current.

Melittin was purchased from Sigma Chemical Co. (St. Louis, Mo.) and checked for purity by sodium dodecyl sulfate gel electrophoresis (11).

RESULTS

The Current-voltage (I–V) Characteristics of Melittin-treated Bilayers

Fig. 1 shows that upon addition of melittin to one side of an asolectin bilayer, there is a steep rise of the current with voltage only when the *cis* compartment is positive. The inset in the

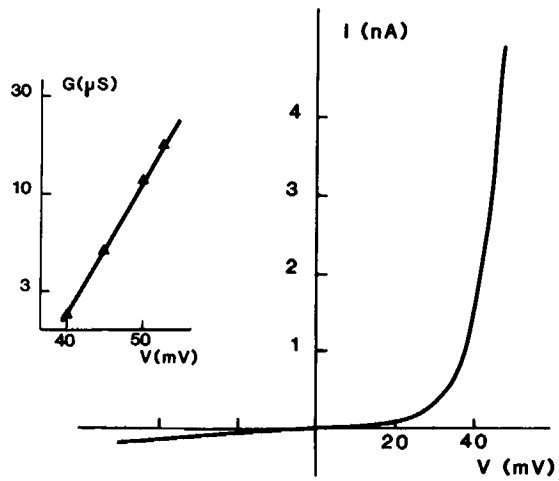

FIGURE 1 Steady-state *I–V* curve of a melittin-treated bilayer. Both aqueous compartments contained 1 M NaCl. Melittin was added to the *cis* compartment only, to a final concentration of 3.4×10^{-7} g/ml; area = 10^{-3} cm^2. The speed of the ramp, dV/dt = 0.1 mV/s, was sufficient to insure steady-state measurements of the current. The inset shows a semi-log plot of the steady-state membrane conductance as a function of transmembrane potential.

figure shows that the logarithm of the conductance is a linear function of membrane potential. From the slope of this line, it is possible to calculate that the conductance changes *e*-fold for every 6-mV change in the membrane potential.

Effect of Melittin Concentration on the Steady-state Conductance-voltage (G–V) Characteristics

Fig. 2 shows that increasing the melittin concentration generates a set of parallel lines in the log G vs. V plot. This result indicates that the dependence of the conductance on voltage (i.e., the slope of the lines) is not changed as the aqueous peptide concentration is changed. The magnitude of the conductance at a fixed voltage does change, increasing 16 times as the melittin concentration is doubled. Thus, the relation between the steady-state conductance at

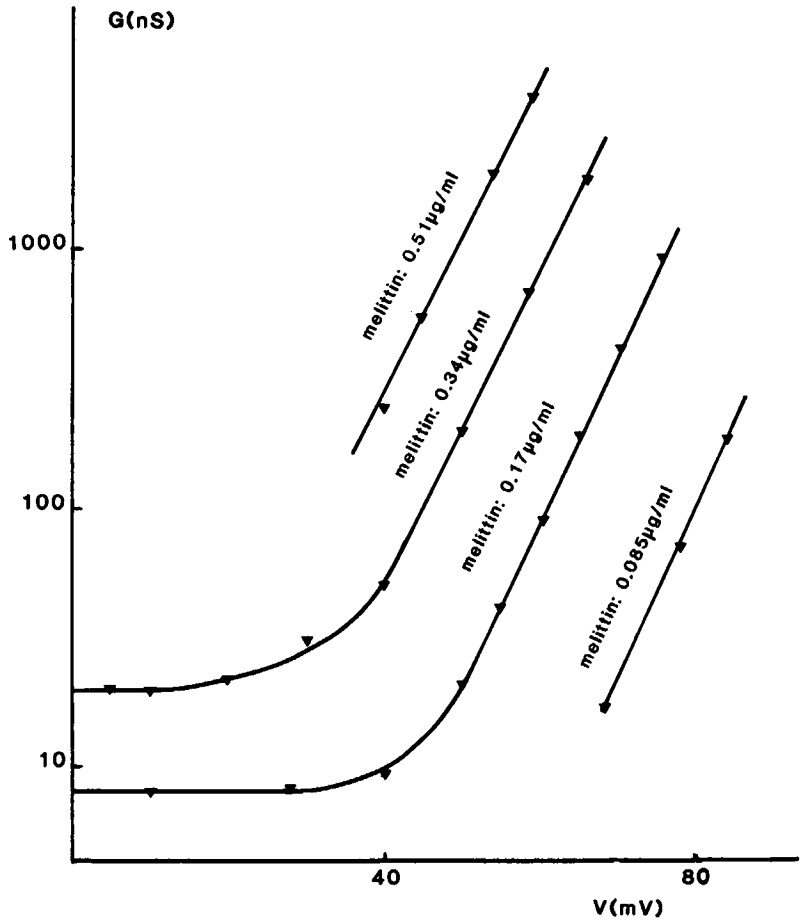

FIGURE 2 Effect of melittin concentration on the steady-state G–V characteristic of asolectin membranes. Membranes (area: 10^{-3} cm^2) formed in 1 M NaCl. Melittin was added to the *cis* compartment to achieve the concentrations indicated. After each increment in melittin concentration, 40–60 min were required for the membranes to achieve a stable G–V characteristic. Points are mean values obtained in four different membranes. Not all melittin concentrations were tested in all membranes. Slope of the lines: *e*-fold conductance change per 6 mV.

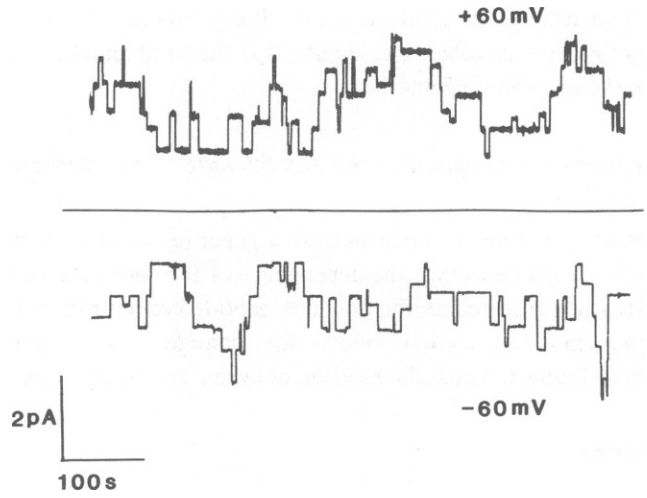

FIGURE 3 Current fluctuations of an asolectin membrane (area: 5×10^{-4} cm^2). Aqueous phases contained 1 M NaCl. Melittin added to *cis* compartment only to a final concentration of 3×10^{-9} g/ml. Solid line indicates zero current level. The lowest level corresponds to the current level of the unmodified membrane.

a voltage V (G_V) and the peptide concentration can be expressed as: $G_V \propto$ [Melittin]^4. This result suggests that there are four melittin molecules involved in the process which leads to the G–V characteristics shown in Figs. 1 and 2.

Kinetic Characteristics of Melittin-induced Channels

Fig. 3 is a record of the current fluctuations of an asolectin bilayer exposed to a low concentration of melittin (i.e., 3×10^{-9} g/ml). The discrete steps in current probably arise as a consequence of channel formation due to the interaction of melittin with the bilayer. The records in this figure further show that these channels remain in the various current levels for long periods of time (i.e., 30–60 s) and that the minimum current fluctuation corresponds to a conductance ~ 7–10 pS.

To determine if the macroscopic conductance observed in melittin-treated bilayers arises from the activity of channels such as those shown in Fig. 3, we compared the kinetic characteristics observed in bilayers with a high number of channels and those obtained in membranes containing only a few channels. Fig. 4*C* shows that when the voltage is changed from zero to a positive value, the current of a multi-channel membrane consists of an abrupt ohmic increase followed by a monotonical increase to a steady-state value. The half-time to reach steady-state is ~ 6 s. The current response to a negative pulse shows the ohmic and not the time-dependent conductance. The records shown in Fig. 4*B* were obtained by adding point by point the current obtained in response to pulses applied to a bilayer that contained only a few channels (i.e., Fig. 4*A*). This figure shows that the kinetic characteristics thus generated are similar to those obtained in a multi-channel membrane (i.e., Fig. 4*C*). This similarity supports the conclusion that the time-dependent conductance of a multi-channel membrane arises from the activity of independent channels induced by melittin.

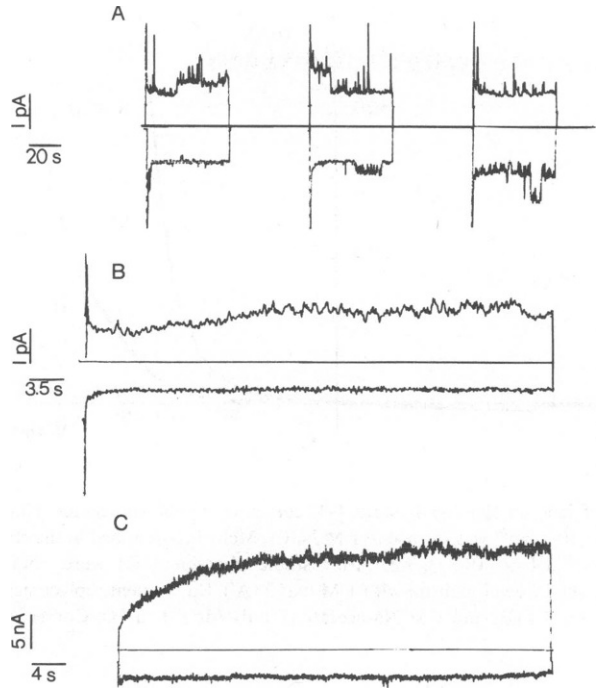

FIGURE 4 Melittin-induced current in an asolectin membrane in response to voltage steps. Membrane (area: 10^{-3} cm^2) was formed in 1 M NaCl. (*A, B*) melittin was added to the *cis* compartment only to a final concentration of 10^{-9} g/ml. 45 pulses of 50-s duration, amplitude +60 mV, were applied at a rate of 1×10^{-2} Hz and the current response was added in a signal averager. The same pattern was repeated for the negative pulses. (*A*) current responses to pulses No. 20, 21, and 22 recorded on a strip-chart recorder. (*B*) current after addition of all pulses. (*C*) melittin concentration in *cis* compartment: 3×10^{-7} g/ml. Current responses to one single positive and one single negative pulse (amplitude 60 mV, duration 52 s) obtained after a stable value of the steady-state current was obtained. Solid horizontal lines indicate zero current level. The current spikes due to the capacitative current at the end of the pulses were eliminated from the records (*A* and *B*).

Effect of Ions on the I–V Characteristics of Melittin-containing Bilayers

Fig. 5 shows the results of an experiment designed to determine the ionic selectivity (e.g., cations vs. anions) of melittin-treated bilayers. The bilayer, which had been formed in 1 M NaCl, was exposed unilaterally to melittin until the voltage-dependent conductance was established. Subsequently, the *cis* compartment was washed free of melittin. No change in the magnitude of the voltage-dependent conductance was observed, which indicates that the interaction of melittin with the bilayer is not reversible under these conditions (i.e., Fig. 5, curves A, A'). The solution in both compartments was next changed to 1 M Tris · Cl (curve B). Finally, the Tris solutions were replaced by Na-acetate in both compartments (curve C). It was also found that replacement of Na for K at constant Cl did not change the *I–V* curves. Moreover, when Cl was replaced by Hepes at constant Na, the *I–V* curves were identical to those of the untreated bilayer, even at melittin concentrations as high as 3.4×10^{-7} g/ml and for imposed voltages as high as 200 mV (not shown). Since the bilayer's electrical characteristics only changed when the anions were changed, these results suggest that melittin renders the bilayers more permeable to anions than to cations.

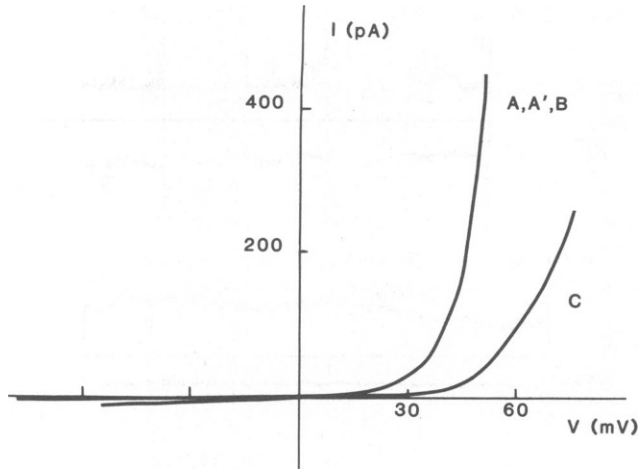

FIGURE 5 Effect of ions on the steady-state *I–V* curves of a melittin-treated bilayer. The asolectin membrane (area: 4×10^{-4} cm^2) was formed in 1 M NaCl. Melittin was added to the *cis* compartment to a final concentration of 3.4×10^{-7} g/ml. After stable characteristics were obtained (A), the *cis* compartment was washed free of melittin with 1 M NaCl (A'). Subsequent replacements in *cis* and *trans* compartments: 1 M Tris.Cl (B) and 1 M Na-acetate (C). dV/dt: 0.1 mV/s. Curves A, A', and B are all superimposed.

DISCUSSION

The data described in this paper establish that melittin interacts with lecithin bilayers in such a way as to form channels through which ions can move. Melittin shares this property with a few other polypeptides of known primary structure such as gramicidin, alamethicin, suzukacillin, and trichotoxin A-40 (12). While these compounds all contain amino acids not normally present in proteins; e.g., D-amino acids or α-amino isobutyric acid, all 26 of the residues in melittin are L-amino acids. When melittin is present on only one side (*cis* side) of the bilayer, the channels probably form only from that side, with the four positively charged residues from the C-terminal end lying at the interface between membrane and aqueous phase on the *cis* side. This hypothesis is consistent with recent evidence on the adsorption of melittin to planar bilayers that suggests that the charge-bearing residues from the C-terminal end of the compound do not penetrate through the membrane (13). The selectivity of the channels for anions over cations is presumably due to the positive charges located at their mouths or on their walls. From the data shown in Fig. 3, the size of the minimum current fluctuation corresponds to a conductance ~ 7–10 pS. It was found, however, that not all of the other levels present in this and other records are integral multiples of the lower level. This result suggests that the open state of a single melittin channel probably has more than one conductance level, like the channels of alamethicin and its analogues (12).

From the results shown in Fig. 2, it was concluded that the steady-state membrane conductance increases as the fourth power of melittin concentration. The simplest explanation for this result is that the channel is formed by four melittin monomers. This interpretation is consistent with the observation that melittin self-aggregates to form tetramers in aqueous solutions and when bound to detergent micelles (8, 9).

Fig. 1 shows that the conductance of lecithin bilayers exposed unilaterally to melittin

depends on the electrical potential gradient across the membrane. The conductance increases *e*-fold for each 6 mV by which the *cis* side becomes more positive to the *trans* side of the membrane. This result is consistent with the idea that there are at least four gating charges per channel (12). If one assumes a tetrameric structure for each channel, this would suggest one gating charge per melittin monomer. A good candidate for this role is the amino group on lysine 7. This residue is located in the relatively hydrophobic part of the molecule and might be expected to be located in the interior of the bilayer in a region where it would sense changes in the electric field across the membrane.

It is possible that the channel-forming properties of melittin may account for some of the physiological effects of the compound. In particular, the fact that melittin forms channels at aqueous concentrations well below those required to produce lysis (4) makes it attractive to speculate that this property of the molecule might also be important biologically, particularly in its capacity to stimulate neurons (1). Such stimulation could be the result of changes in the ionic permeability of nerve endings produced by melittin-induced channels.

In any case, melittin seems to offer an excellent opportunity to investigate the physicochemical basis of voltage-dependent conductances in bilayers. Its tertiary structure in crystals has been determined at the 6–Å resolution (14) and it is suitable for the full range of spectroscopic measurements (6–9). Furthermore, the fact that it is composed entirely of L-amino acids suggests that careful analysis of its capacity to form ion-conducting channels should lead to further insight into how other naturally occurring polypeptides and proteins perform this function in biological membranes.

We thank Dr. A. Pardee for introducing us to melittin, Dr. D. Benos for comments on the manuscript and Dr. R. Latorre for very helpful experimental suggestions and comments on the manuscript. The SDS gel electrophoresis was kindly performed by Dr. V. Sapirstein.

This work was supported by a grant from the National Institutes of Health (GM-25277).

Received for publication 17 March 1981 and in revised form 26 May 1981.

REFERENCES

1. Habermann, E. 1972. Bee and wasp venoms. *Science (Wash. D.C.)* 177:314–322.
2. Habermann, E., and Jentsch, J. 1967. Sequenzanalyse des Melittins aus den tryptischen und peptischen Spaltstücken. *Hope-Seyler's Z. Physiol. Chem.* 348:37–50.
3. Habermann, E., and H. Kowallek. 1970. Modifikationen der Amino-gruppen und des Tryptophans in Melittin als mittel zur Erkennung von Struktur-Wirkungs-Beziehungen. *Hoppe-Seyler's Z. Physiol. Chem.* 351:884–890.
4. Sessa, G., J. H. Freer, G. Colacicco, and G. Weissman. 1969. Interaction of a lytic polypeptide, melittin, with lipid membrane systems. *J. Biol. Chem.* 244:3575–3582.
5. Hegner, D., U. Schummer, and G. H. Schnepel. 1973. The interaction of a lytic peptide, melittin, with spin-labeled membranes. *Biochim. Biophys. Acta.* 291:15–22.
6. Verma, S. P., D. F. H. Wallach, and I. C. P. Smith. 1974. The action of melittin on phosphatide multibilayers as studied by infrared dichroism and spin labeling. A model approach to lipid-protein interactions. *Biochim. Biophys. Acta.* 345:129–140.
7. Mollay, C., and G. Kreil. 1973. Fluorometric measurements on the interaction of melittin with lecithin. *Biochim. Biophys. Acta.* 316:196–203.
8. Brown, L. R., J. Lauterwein, and K. Wüthrich. 1980. High-resolution ^{1}H-NMR studies of self-aggregation of melittin in aqueous solution. *Biochim. Biophys. Acta.* 622:231–244.
9. Lauterwein, J., C. Bösch, L. R. Brown, and K. Wüthrich. 1979. Physicochemical studies of the protein-lipid interactions in melittin-containing micelles. *Biochim. Biophys. Acta.* 556:244–264.

10. Montal, M., and P. Mueller. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci.* U.S.A. 69:3561–3566.
11. Knöppel, E., D. Eisenberg, and W. Wickner. 1979. Interactions of melittin, a preprotein model, with detergents. *Biochemistry* 18:4177–4181.
12. Latorre, R., and O. Alvarez. 1981. Voltage-dependent channels in planar lipid bilayer membranes. *Physiol. Rev.* 61:77–150.
13. Schoch, P., and D. F. Sargent. 1980. Quantitative analysis of the binding of melittin to planar lipid bilayers allowing for the discrete-charge effect. *Biochim. Biophys. Acta.* 602:234–247.
14. Eisenberg, D., T. C. Terwilliger, and F. Tsui. 1980. Structural studies of bee melittin. *Biophys. J.* 32:252–254.