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Melittin induced voltage-dependent conductance in DOPC lipid bilayers

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Melittin-induced conductance was measured on planar bilayers made from dioleoylphosphatidylcholine. Upon application of a fixed voltage, the current response was monophasic and remained so even after prolonged observation times. The conductance of melittin-doped bilayers increased exponentially with voltage. In addition, an ohmic contribution appeared after some current had passed. The voltage-dependent conductance increased e-fold every 22 mV and was proportional to the fourth power of the aqueous monomeric peptide concentration, for all salt concentrations investigated (0.4–1.8 M NaCl). Discrete conductance steps could be resolved at all these salt concentrations. The amplitudes of these steps were highly variable. In each experiment, conductance was initially only observed for potentials which were positive on the side of peptide addition. As more and more current passed across the bilayer, the current-voltage curves became symmetric. The system needed some time to reach stationary current-voltage characteristics: about 50 min at pH 7 but only about 15 min at pH 8, suggesting involvement of the N-terminus (p*K* around 7.5) of melittin in the slow formation of a 'prepore'.

Introduction

Melittin, the main constituent of bee venom, is a peptide composed of 26 amino acids [1]:

$Gly^{(+)}$-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-$CONH_2$

The sequence exhibits a pronounced amphiphilic nature. Its twenty N-terminal residues are largely hydrophobic whereas the six residues on the C-terminal side are decidedly polar. Most strikingly, the molecule is well soluble in water but also associates strongly with lipid membranes [2,3]. These peculiar characteristics have aroused considerable interest in the underlying physical chemical behavior, triggering a wealth of structural and functional studies (see Ref. 4 for a recent review). In aqueous solution the formation of tetrameric aggregates due to added salt or high pH has been investigated intensively [5–8]. On the other hand, interaction with membranes was found to cause lysis [9–11]. According to spectroscopic data the membrane associated state of melittin forms to a large extent a helical secondary structure, presumably comprising the predominantly hydrophobic residues 1–20 [12,13]. A 'helical wheel' diagram of this part of the molecule shows segregation of polar and apolar residues to opposite faces of the α-helix [4]. In this respect, melittin resembles the signal peptides which mediate protein translocation across membranes [14]. Such amphipathic helices can form membrane-spanning bundles [15] or else accumulate in the surface region of the bilayer [16]. The latter tendency would be predicted for melittin as judged from a hydrophobic moment/hydrophobicity plot [16]. Experimental evidence favors a surface location of this peptide, some parts of it intruding into the hydrophobic core [10,17,18].

This does not exclude that a minor fraction of the membrane-associated melittin may form a bilayer-spanning aggregate. Such a notion can be inferred from the voltage-dependent conductance which has been shown to occur at sub-lytic concentrations of the peptide [20–25]. Apparently this phenomenon reflects the formation of porous peptide aggregates, as indicated by single-channel conductance states observed at low melittin conductance [20,25]. In the most extensive study, done with asolectin bilayers, a quite complex kinetic response

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to voltage steps was observed, composed of two different activation phases and one inactivation process [21,22]. The underlying mechanisms are so far not understood.

In view of the complexities which might arise from interaction of the cationic groups of melittin with a partially charged bilayer, it is of interest to study the interaction of this peptide with purely zwitterionic lipid. The present article is a report on conductance measurements carried out with planar membranes made of dioleoylphosphatidylcholine (DOPC) to which highly purified melittin is added. This system proved to respond to voltage steps in a most simple monophasic way. We have the further advantage that pertinent thermodynamic as well as kinetic data are available for the association of melittin with the bilayer of DOPC vesicles [26]. (For technical reasons the more extensively studied DMPC (see also Refs. 4 and 27, and references therein, is less appropriate for forming planar bilayers). We find a relatively simple behavior in our system. Quantitative evaluation under various conditions give some new clues to the molecular mechanisms which are involved in the membrane interaction and pore formation of this peptide.

Materials and Methods

Black lipid membranes (BLM) were formed in a circular hole in a teflon septum, which separates two aqueous compartments of either 1 ml or 4 ml of volume. The cells were made from teflon. The membrane area was $4 \cdot 10^{-3}$ cm^2 (diameter 0.6 mm) for the macroscopic conductance measurements and $8 \cdot 10^{-3}$ cm^2 (diameter 0.1 mm) for the single pore experiments.

Membrane bathing solutions contained 0.4 M, 1 M or 1.8 M NaCl. They were buffered in 10 mM Tris-HCl at pH 7.0, if not stated otherwise. Salts and buffers were analytical grade, obtained from Merck (Darmstadt, F.R.G.). Water was used from a Barnstead Nanopure system with a specific resistance of 18 MΩ cm. Solutions could be stirred on both sides by magnetic teflon stirrers, which rest in shallow wells sunk into the bottom of the chamber. This geometry prevents stress on the membrane. All experiments were carried out at room temperature 22° ± 2°C.

Membranes were painted from a 10 mg/ml solution of DOPC (Avanti Polar Lipids) in decane. The hole was pretreated with a 5 mg/ml solution of DOPC in hexane. Formation of the membrane was monitored by measurements of the capacitance signal. Alternatively we attempted to use DOPC bilayers formed by apposition of hexane-spread monolayers according to the technique of Montal-Müller. The cell was precoated with the lipid-in-hexane solution in order to stabilize the monolayers. In the presence of melittin these virtually solvent-free bilayers turned out to be much less stable than the solvent-containing painted membranes. We therefore settled on the latter type of bilayers and did only a few control experiments with the monolayer technique.

Voltage from a function generator (Exact model 128) or a battery-driven potentiometer was applied by using a pair of Ag/AgCl electrodes, inserted into the aqueous solutions. Membrane current was amplified with an operational amplifier (OPA 104) in a selfmade feedback circuit or with a Keithley amplifier 427 which had variable amplification and better time resolution. The I-V curves were recorded by a X-Y recorder (Watanabe WX 451) and a storage oscilloscope (Tektronix T912), the current-time curves by a video tape-recorder (Hitachi PCM-V300E) and plotted with a fast linear recorder (Watanabe).

Melittin was a gift from Horst Vogel and was purified as described by Vogel and Jähnig [13]. It showed only one band after electrophoresis on cellogel strips and was free of phospholipase and formylated melittin.

In a typical experiment, an appropriate small volume of a 0.2 mg/ml stock solution of the peptide in water was added with an adjustable Hamilton syringe to one side of the chamber (called cis) which was then stirred 3–5 min. The voltage, V, is defined positive relative to the trans side (virtual ground). The current, I, measures positive charges flowing from cis to trans. After stirring was switched off, I-V curves could be recorded and became stationary after a certain time.

We accepted data only if a membrane showed reproducible capacitance and resistance after formation of the bilayer and no abrupt changes after addition of the melittin.

Results

1. Melittin-induced conductance

After having added melittin to a preformed DOPC bilayer and allowing for sufficient stirring, the current across the bilayer was recorded under applied voltage steps or continuous voltage ramp (sweep rate: 100–300 mV/min). It took about 10–50 min until the current-voltage curves became stationary (see more details below). The I-V curves always had an exponential contribution. The very first current-voltage response rose steeply from pure membrane conductance after a certain threshold voltage was reached, but once a current had passed across the membrane, i.e., after the first voltage ramp was applied, an ohmic contribution appeared. Its magnitude increased as more and more voltage ramps were applied to the membrane (see Fig. 1a). Such a voltage-independent conductance is well known from other membrane active peptides, e.g., alamethicin [28,29]. It has also been documented for melittin conductance in asolectin bilayers [20–22]. In order to evaluate the voltage-dependent part of the

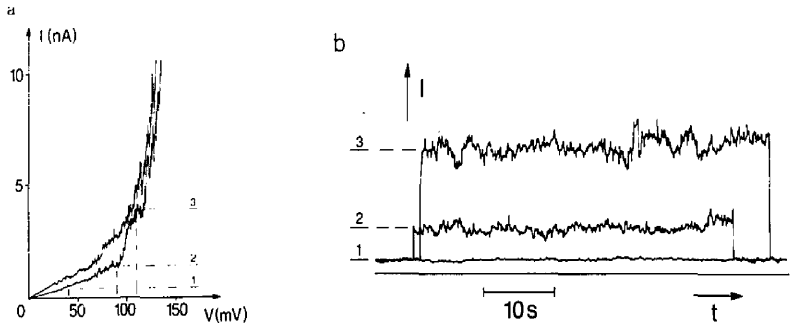

Fig. 1. (a) Typical *I-V* curve of a melittin-doped bilayer at stationary state. Solutions were 1.8 M NaCl (10 mM Tris, pH 7); melittin concentration was 0.4 μg/ml (cis). The exponential *I-V* curve contains always an ohmic contribution which increases with further voltage ramps applied. (b) Applying constant voltage pulses of 40 mV (1), 90 mV (2) and 110 mV (3), the current signals were monophasic and remained constant even after approx. 50 s of voltage applied. Signal height corresponds to the *I-V* curve in (a).

conductance these ohmic (linear) contributions to the *I-V* curve were subtracted from the signal. In the following we shall be only concerned with the voltage-dependent conductance.

In our melittin/DOPC system, the measured increase of current displayed a simple monophasic time course when a voltage step was applied from zero voltage to a particular value (see Fig. 1b). The current rise was very fast, in the millisecond range or below. This applied to the ohmic as well as to the exponential part of the *I-V* curve. Even when the voltage was kept on for up to 50 s, we could not detect any additional activation or inactivation responses like those reported for the melittin/asolectin system [21–23]. This also applies to control experiments done with virtually solvent-free DOPC bilayers prepared according to the technique of Montal-Müller. Consequently *I-V* curves evaluated from the signals at a series of constant voltage pulses corresponded to the curves obtained with voltage ramps. They were consistently found to be independent of the ramp speed applied in the range of 100 mV/min to 100 mV/s. Any increase of current at constant voltage was only due to an increase of the ohmic part, favored by applying higher voltages at prolonged exposure times. This was controlled by applying voltage pulses of 1–2 s duration, switched between low and high voltage, where the low voltage was in the range of purely ohmic contributions.

The probability for membrane rupture owing to prolonged exposure to voltage increased at higher melittin concentration. We never got stable membranes at stationary state conditions with concentrations higher than 1.2 μg/ml.

2. Discrete events

Using a small hole (diameter 0.1 mm) and a low melittin concentration, discrete stepwise conductance fluctuations could be observed routinely at 1.8 M NaCl as well as 0.4 M NaCl. This apparently suggests the existence of defined single pores. However, the amplitudes of the various steps were highly variable. There was a tendency of individual pore states to stabilize after some time, especially if melittin had been added from both the cis and trans sides. Fig. 2 shows such a pore state which remained stable enough to be observed for a long time at different voltage settings (50–100 mV). In control experiments with Montal-Müller DOPC bilayers the single pore events were of shorter duration.

The pore conductance turned out to be ohmic; it was about 110 pS (at 1.8 M NaCl). The frequency of the openings increased with voltage as did the mean life-

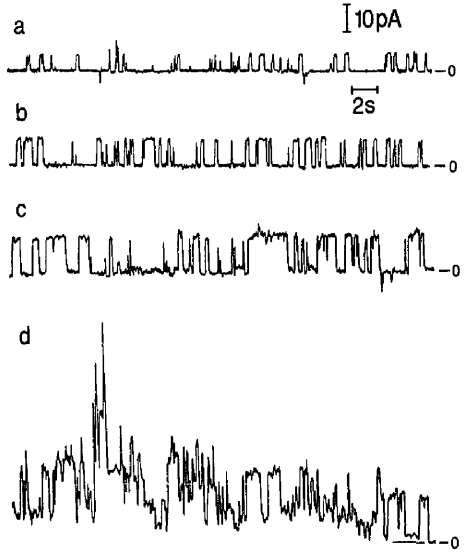

Fig. 2. Single-pore steps at stationary state conditions at 1.8 M NaCl (10 mM Tris, pH 7). The traces show the effect of voltage on the open probability of a single pore at (a) +50 mV (b) +70 mV (c) +90 mV (d) +100 mV. The pore is ohmic with a conductance of about 110 pS.

time of the pore. Thus the exponential increase of the conductance seen in the current-voltage relation is due to an increase of the probability to find open pores and not to a voltage-dependent change of the single pore conductance. In addition, the observed single pore state was asymmetric, appearing at positive potentials only. Raising the voltage further lead to the induction of more, possibly superimposed, conductance levels, making a quantitative analysis of the trace rather difficult.

For a detailed evaluation of conductance parameters of melittin we shall concentrate in the following on macroscopic conductance measurements, where a larger number of pores contribute to the signal.

3. *Voltage and concentration dependence of the conductance*

The voltage-dependent conductance G_{vd} could be described by an exponential relation:

$$G_{vd} = g_t \cdot \exp(V/V_e) \quad (1)$$

The parameter V_e is listed in Table I for a number of different conditions. It was determined from a logarithmic plot of the conductance and turned out to be $V_e = 22 \pm 2$ mV independent of the melittin and salt concentrations of the solution. A slightly steeper voltage-dependence was sometimes observed when measuring the first *I-V* curve shortly after addition of melittin to the membrane bathing solution long before the stationary state had been established.

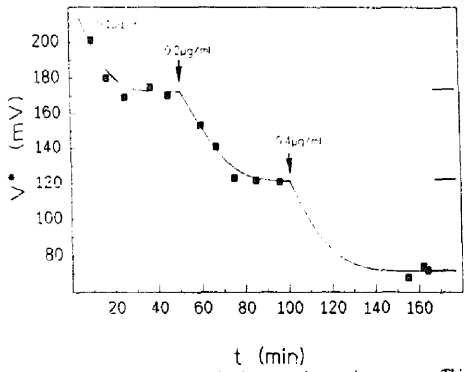

Fig. 3. *I-V* curves need about 50 min to reach a stationary state. This holds after each addition of new peptide to the aqueous compartment. The diagram shows the time course of the critical voltage V^* ($G = 30$ nS) to the stationary state on doubling twice the melittin concentration on the cis-side. Solutions were 1 M NaCl (10 mM Tris, pH 7).

After having obtained stationary *I-V* curves at a certain melittin concentration in solution, further additions of peptide could be made. Then we observed a parallel shift of the *I-V* curve to lower voltages, until a new stationary state was eventually attained (see Fig. 3). At the same time the ohmic part increased.

TABLE I

Parameters of the concentration- and voltage-dependent I-V curves at different [NaCl] (pH7)

c_0: total peptide concentration in solution; c_{mono}: monomeric peptide concentration in solution; K_4: tetramerization constant of melittin in solution; V_e: e-fold increase of the *I-V* curve; α: equivalent gating charge number (calculated as kT/eV_e, where kT is thermal energy and e elementary charge); $\overline{\Delta V^*}$: shift of the critical voltage at stationary state $\overline{V^*}$ ($G = 30$ nS) on changing c_0 in the cis compartment.

[NaCl] 10 mM Tris (pH 7)	c_0 (μg/ml)	c_0 (μM)	$\overline{\Delta V^*}$ (mV)	V_e (mV)	α	K_4 (μM^{-3})	c_{mono}/c_0	V_0 (mV)	m
1.8 M	0.2	0.07		26			0.99		
			54					84	
	0.4	0.14		23			0.94		
			45			6.5 ± 1.5		89	
	0.8	0.28		20			0.78		
			21					94	
	1.2	0.42		18			0.65		
Average				22 ± 2	1.1			89 ± 3	4.0 ± 0.2
1.0 M	0.1	0.04		21					
			51			6.5 ± 1.5	no	74	
	0.2	0.07		20		$\cdot 10^{-3}$	tetra-		
			50				mers	72	
	0.4	0.14		18					
Average				20 ± 1	1.3			73 ± 6	3.7 ± 0.4
0.4 M	0.2	0.07		24					
			50				no	72	
	0.4	0.14		20		n.d.	tetra-		
			28				meters	69	
	0.8	0.28		25					
Average				23 ± 2	1.1			71 ± 6	3.1 ± 0.5

Quantification of the concentration dependence was difficult, especially at lower salt concentrations, because large fluctuations occurred from one bilayer to another. We could nevertheless achieve a satisfactory degree of certainty by averaging over a large number of measurements and comparing with experiments where up to three additions of peptide were done sequentially with a single bilayer. These were the most reliable. In general, the melittin concentration dependence could be described by a power law of the form:

$$G_{vd} = \hat{g}_0 \cdot c^m \cdot \exp(V/V_e) \qquad (2)$$

as found by others with different bilayer systems [22–24]. We have, however, actually taken c as the concentration of monomeric melittin, not the total peptide concentration. At 1.8 M NaCl, melittin tetramerization in solution must be taken into account in order to calculate c from the total concentration of added melittin. For the other salt concentrations, tetramer formation in the aqueous phase was negligible. Tetramerization constants were measured observing the shift in the wavelength of the tryptophan fluorescence emission maximum (see Ref. 8 for the relevant evaluation procedure and Table I for numerical values). Fig. 4 shows the concentration dependence of the I-V curves at 1.8 M NaCl. For the evaluation of m the critical values V^* were compared, at which a constant reference conductance of $G^*_{vd} = 30$ nS was reached. The voltage shift due to a change of c at the stationary state is given by

$$\overline{\Delta V}^* = -V_0 \cdot \Delta \ln c \quad \text{with} \quad V_0 = m \cdot V_e \qquad (3)$$

Relevant data are shown in Table I. The number m is between 3 and 4, in agreement with previous work. It slightly decreases from 4 to 3 going to lower salt concentrations, but the difference is at the limit of our experimental uncertainty (about 0.5 units in m). Divalent salts, as well as higher pH, are known to favor melittin tetramerization in solution [30]. The effect of enhanced tetramerization on the conductance could be observed by the addition of Na_2SO_4 to a final concentration of 100 mM to both compartments. The I-V curves, resp. V^* at stationary state conditions ($\overline{V}^*$), were shifted to higher values of voltage corresponding to a smaller fraction of monomers in solution.

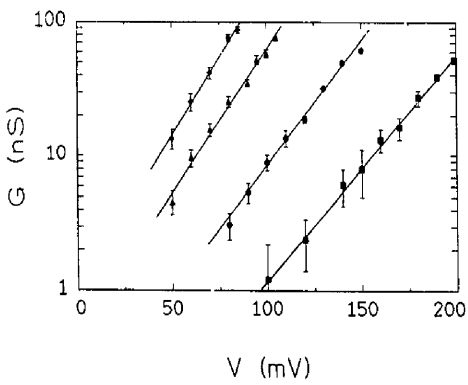

Fig. 4. Logarithmic plot of the effect of melittin concentration on conductance-voltage curves at 1.8 M NaCl (10 mM Tris, pH 7). Melittin was added on the cis-side: 0.2 μg/ml (■), 0.4 μg/ml (●), 0.8 μg/ml (▲) and 1, 2 μg/ml (◆). The curves were taken at stationary state conditions and increased e-fold every 22 mV.

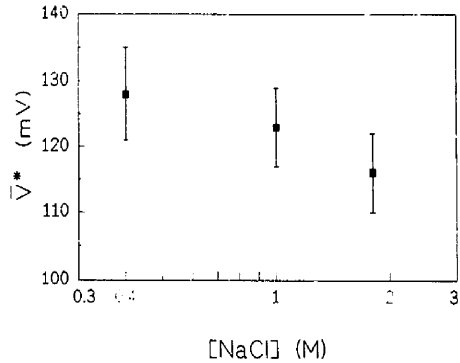

Fig. 5. Salt dependence of melittin conductance at a concentration of 0.2 μg/ml (pH 7). The critical voltage $\overline{V}^*$ is propotional to $[NaCl]^n$; $n \approx 0.5$.

The measured I-V curves depended on the amount of added NaCl. Because of difficulties encountered when the salt concentration was changed using a single membrane, we used a different membrane for each salt concentration. Although reproducibility in such melittin experiments is elusive, it was feasible to quantify the salt dependence at a low peptide concentration (0.2 μg/ml). Generally, measurement at the higher salt concentration yielded better reproducibility than at lower salt. Salt dependence can typically be described as some nth power of the concentration

$$\hat{g}_0 = \hat{g}'_0 \cdot [NaCl]^n \qquad (4)$$

as indicated in Fig. 5. Roughly $n \approx 0.5$, i.e., there is no strong effect of salt in generating conductance pathways through the membrane.

4. Slow kinetics towards the stationary state

If we recorded I-V curves shortly after addition of melittin to the preformed bilayer and stirring, the critical voltage V^* needed to induce conductance was very high. In the course of time, the curves shifted slowly to lower values of voltage until the stationary state was reached, typically after 50 min. This effect was also observed after each addition of peptide to the same membrane, as Fig. 3 shows. Changing the pH of the

aqueous solutions from 7 to 8.8 shortened the time to reach the steady state down to 10 min (see Fig. 6). Concomitantly $\bar{V}^*$ was higher at pH 8.8 as expected due to increased tetramerization and depletion of the monomer pool.

We emphasize that the observed rate of conductance increase after having added the melittin remains much slower than found with alamethicin, another pore forming peptide [29]. When this agent is used under otherwise like conditions we have already seen strong effects after less than 30 s.

5. Evolution of current-voltage symmetry

We measured the stationary-state response of the system to positive and negative voltage ramps, when melittin was added only on one side. In the first ramps applied, the current-voltage curves were markedly asymmetric ($\overline{\Delta V}^*_{\pm} \approx 50$ mV), i.e., voltages higher in magnitude by about 50 mV were needed to induce the reference conductance if the potential was negative on the side of peptide addition than if it was positive. V_c was 22 mV, independent of voltage polarity. By repeatedly applying positive voltage, this asymmetry decreased and tended to vanish after several *I-V* curves (Fig. 7). The loss of asymmetry depended only on the time of voltage application, independent of whether it was started shortly after adding the peptide or after waiting for the stationary state conditions. In one type of experiment, after symmetric conditions were attained, we allowed the system to relax without any voltage across the membrane. Even after 70 min of waiting the resulting *I-V* curves remained virtually invariant. In a further experiment we added melittin on the trans side, after the system reached a stationary state with the peptide on the cis side. This led to a further increase of conductance on the cis as well as on the trans side. Eventually a fully symmetrical conductance-voltage curve was obtained with $\bar{V}^*$ about the same as expected if all the peptide had been added from one side only.

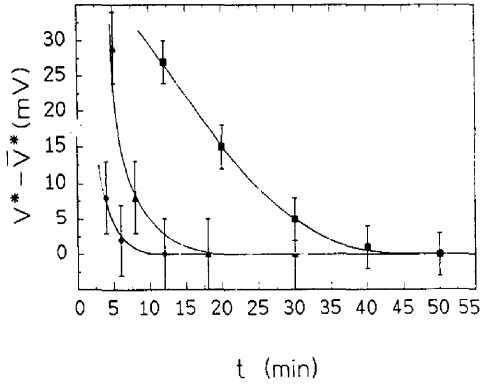

Fig. 6. Time course of the *I-V* curves (critical voltage V^*) to the stationary state ($\bar{V}^*$) at pH7 (■), pH8 (▲) and pH 8.8 (◆). The time to reach the stationary state decreases from 50 min at pH 7 to 15 min at pH 8. Melittin concentration was 0.4 μg/ml (cis) at 1.8 M NaCl.

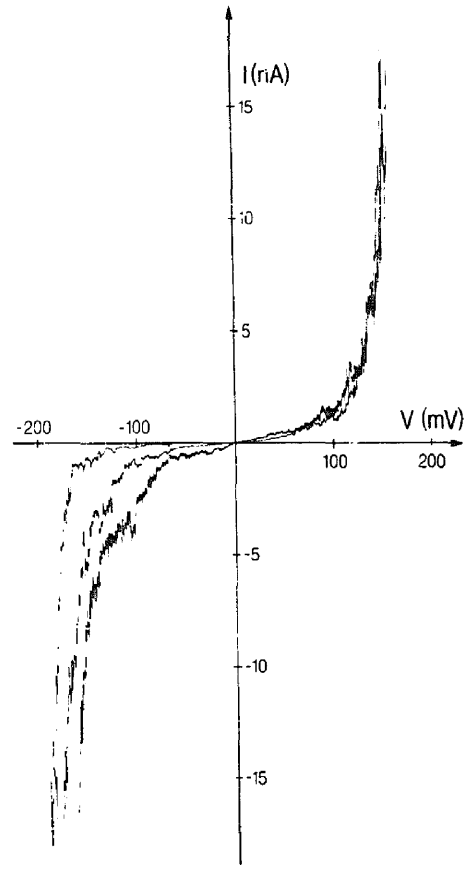

Fig. 7. *I-V* curves at stationary state, applying positive and negative voltage ramps. An initial asymmetry of about 50 mV decreases with applying more positive voltage ramps; curves at positive voltages remain constant, those at negative voltages shift to lower voltages. $V_c = 22$ mV at positive and negative voltages. Melittin was added to the cis side only (0.4 μg/ml). Solutions were 1.8 M NaCl (10 mM Tris, pH 7).

Discussion

Melittin has previously been shown to induce voltage-dependent conductance of rather complex kinetics in asolectin bilayers [21–23], including two different activation phases and one inactivation phase. In contrast, we find a simple monophasic activation behaviour with application of voltage across DOPC membranes no matter wether these are painted or virtually solvent-free. We cannot definitely rule out from these experiments that the second activation as well as inactivation do not

exist in our system (they may be kinetically retarded); in any case there was no sign of deviation from monophasic behavior even with prolonged exposure to the voltage step of nearly 1 min. Obviously then, evaluation and interpretation of data appears comparatively simpler with the DOPC system.

The parameters m and V_e, describing the dependence of the conductance on melittin concentration and voltage, respectively, match closely those found for the first activating phase with asolectin bilayers [22] (cf. Table I). Bathing the membrane in salt solutions of 0.4 M to 1.8 M NaCl yields nearly the same dependence of the membrane conductance on the fourth power of peptide concentration. On the assumption that the observed pores are eventually in a true equilibrium state of the system, this figure would indicate that the pores are formed from tetrameric aggregates. However, the stationary state of the system may actually be off thermodynamic equilibrium resulting from steady state conditions of the kinetic scheme. Then the observable order of the rate law would be that of the rate determining step in the underlying mechanism, and the actual pore size could be larger than tetrameric. In fact there is independent evidence from efflux experiments using carboxyfluorescein-loaded vesicles that melittin may form pores large enough to pass this dye (Zong, Robert, Stankowski and Schwarz, unpublished). In addition, the large variation in single pore amplitudes observed would be compatible with the assumption of multiple pore sizes.

Establishment of stable pore events as well as of reproducible macroscopic current-voltage curves took nearly one hour at pH 7 after addition of peptide. Similar waiting times have been reported for reaching stationary conditions in the case of other pore-forming peptides, e.g. alamethicin [29,31,32]. In the latter case, however, pore activity is appearing at a rather fast rate, as was indicated above, and then declines towards a stationary state (Ref. 33, and S. Stankowski, unpublished). For melittin, the activity was first very low, needing very high voltages for observing any current, but increased steadily with time until the stationary state was reached. Such a behaviour indicates the existence of a slow intermediate step in the pathway to pore formation. We exclude that this stems from the diffusion of melittin across the unstirred layer between the aqueous solution and the membrane. The thickness of that layer can be estimated as $l \approx 5 \cdot 10^{-3}$ cm [34]. With an approximate diffusion coefficient of the melittin molecule, $D \approx 2.5 \cdot 10^{-6}$ cm^2/s, a diffusional time constant of about $l^2/2D \approx 5$ s is obtained. This is corroborated by the already mentioned experimental fact that alamethicin (which is of a comparable molecular size) exhibits strong pore activity within a fraction of a minute. Furthermore, melittin association with DOPC bilayers is very fast [26]. Accordingly, we conclude that the rate limiting slow step is likely to involve some rearrangement (aggregation, conformational change) of membrane-associated peptide.

In fact, that this step is accelerated upon raising the pH to 8.8 suggests that the N-terminus of melittin is somehow involved since this is the charged group with a pK in this range of pH [19]. Whereas the slow step occurs in the absence of applied voltage, kinetics are very rapid after application of a voltage step (below milliseconds). Furthermore, the voltage-dependence of pore formation is rather low: $V_e = 22$ mV, equivalent to a 'gating charge' of 1.1 elementary charge units (gating charge number $\alpha = 1.1$), is close to the value expected for moving one elementary charge across the full width of the bilayer in the process of gating ($V_e = 25$ mV, $\alpha = 1$). However, this tells not very much about the physical events involved. Instead of one charge moving the whole way, several charges could be displaced a correspondingly smaller distance. Alternatively, dipole moments could also be involved as is thought to be the case for alamethicin and related peptides [35,36]. For instance, inserting a dipole of 120 Debye units (the value obtained for melittin in organic solvents [37]) across a DOPC bilayer would yield an energy contribution closely corresponding to a gating charge number of unity.

For an aggregate of the size of a tetramer or larger, the formal gating charge (or equivalent dipole contribution) that we found would require not more than a quarter of an elementary charge per monomer. Similar figures have been reported for melittin, as well as values twice as large ($V_e \cong 12$ mV, $\alpha \cong 2$) [24] or even four times as large for the second activating phase in asolectin [22]. Both the rapid kinetics and the small value for the gating charge per monomer makes it unlikely that the voltage-dependent gating step in our system is the crossing of the bilayer by the charged amino-terminal end of the peptide, as was suggested from an analysis of melittin action on bilayers made from oxidized cholesterol [24]. At pH 7, such a step should involve the transfer of one charge (the amino terminus) across the full width of the bilayer and a second charge (lysine 7) at least half way that distance. Additional contributions might even come from the dipole moment; they would roughly correspond to a gating charge number of 0.5. The experimental value is indeed very small in comparison with this expectation. In summary, data from our melittin/DOPC system suggest a voltage-independent slow formation of a 'prepore' and a fast voltage-dependent formation of the conducting pore.

Once pores have been opened and current passed across the membrane, a second slow process is observed: the decay of the asymmetry of the current-voltage response. In the first current-voltage curves recorded on a bilayer, 50 mV higher trans-positive potentials were needed than cis-positive in order to induce

pore activity (if peptide had been added on the cis side). Upon application of repeated voltage ramps, the trans-positive activity increased until symmetry was obtained. Surprisingly, the symmetry persisted after more than one hour waiting time without applying any voltage. The most natural explanation might be to assume flipping over of melittin molecules to the trans side of the bilayer, mediated by open pores. In principle, one would expect such flipped molecules to dissociate into the solution of the trans compartment so that asymmetry should be reestablished after some time, in the absence of open pores. On the other hand, if irreversible steps were involved, pore activity would be expected to increase indefinitely without reaching a stationary state. Perhaps the symmetry puzzle can be explained by taking into consideration the ohmic conductance contribution which is inevitably induced upon repeated voltage application. If these voltage-independent pathways catalyze flipping of melittin molecules from the cis to the trans side in a way similar to that of the voltage-dependent pores, they may be able to maintain the melittin flux to the trans side even in the absence of voltage.

Slow flipping of peptide molecules leading to decay of asymmetry has already been reported for alamethicin in DOPC [29,38]. There is one interesting difference, however, concerning the asymmetry properties of the two peptides, melittin and alamethicin. After addition of melittin to the cis side of the bilayer and establishment of the stationary state, further addition of peptide at the trans side increases the current for both trans and cis voltages. In contrast, the same procedure does not affect the cis-side current in the case of alamethicin (Refs. 38 and 39, and S. Stankowski, unpublished).

The parameters m and V_e characterising concentration and voltage dependence of melittin were found not to depend on salt concentration in the range of 0.4 to 1.8 M NaCl. I-V curves were less noisy, however, at the higher salt concentrations. At 1.8 M salt concentration, some of the aqueous peptide is tetramerized; the conductance induced in DOPC bilayers was found to depend on the aqueous monomer concentration in the same simple exponential form as at lower ionic strength. Of course the monomer (and not the total) aqueous concentration is the thermodynamically relevant variable, to which all other concentrations are directly related via mass action law and partition equilibrium. Membranes tended to break at melittin concentrations above 1.2 μg/ml (420 nM). Consistently, spontaneous permeabilization of vesicles without any applied voltage has been demonstrated to occur in this concentration range, as indicated by the release of self-quenching dye out of vesicles (Zong, Robert, Stankowski and Schwarz, unpublished).

There has been a long-standing debate in the literature about whether the melittin conductance is produced by a perturbation of the lipid phase or by a proteinaceous pore [20,24]. Our preparations show clearly resolved steplike single events which suggests defined protein pores. The large variations of conductance values observed for these events, however, remain puzzling. At present, evidence is not firm enough to prove the existence of proteinaceous pores in an unambiguous way. Nevertheless, there may be some reasons why pores formed by melittin may show a range of different conductance values. As mentioned above, consideration of the current-voltage curve asymmetry indicates that channels can be assembled by monomers having their carboxyl termini at different sides of the bilayer. Since melittin forms an anion specific pore [20], peptides having the C-terminus at the side of the negative potential would accumulate higher local concentrations of charge carriers at the mouth of the pore, thereby increasing the electrochemical gradient and thus the conductance. The converse would hold for peptides with the opposite orientation, having their C-terminus at the side of the positive potential. Therefore the ratio of cis- and trans-oriented peptides within a pore aggregate should have a marked influence on the conductance of the pore. Furthermore, as discussed above, the fourth power concentration dependence does not necessarily imply that the pore aggregates are all tetramers; if the experimental $m = 4$ referred to the rate determining step leading to a non-equilibrium steady state, this would be consistent with the existence of larger sized aggregates. Taken together, these arguments show that a wide variability of pore size can actually be rationalized for proteinaceous channels formed from bundles of α-helices.

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