Biochimica et Biophysica Acta, 426 (1976) 570–580

BBA 77224

VOLTAGE-INDUCED THICKNESS CHANGES OF LIPID BILAYER MEMBRANES AND THE EFFECT OF AN ELECTRIC FIELD ON GRAMICIDIN A CHANNEL FORMATION

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(Received August 27th, 1975)

SUMMARY

The thickness changes of black lipid membranes of different composition after a voltage jump were investigated. In a second series of electrical relaxation experiments the kinetics of channel formation by gramicidin A were measured. The time course of the membrane current was compared with the time course of the thickness change of the membranes. We found that the time course of the current as a consequence of channel formation by gramicidin A did not correlate with the thickness change of the lipid membranes. A possible direct influence of the electric field is discussed.

INTRODUCTION

Planar black lipid membranes formed by the Mueller-Rudin technique [1] are more or less compressible under the action of an externally applied voltage [2–4]. As shown by Bamberg and Läuger [5], channel formation kinetics of gramicidin A may be studied by electrical relaxation experiments. The results give some evidence that the cation-conducting gramicidin A channel consists of a dimer [6]. This hypothesis is supported by the finding that a covalent dimer formed by chemical linkage of two monomers at the formyl ends acts in a similar way to monomeric gramicidin [7]. Recently Veatch et al. [8] found a method of directly measuring the dimerization of gramicidin in a lipid bilayer using fluorescent-labeled gramicidin. For a survey of the literature on the action of gramicidin A in lipid membranes see refs. 9 and 10.

The aim of this paper is to investigate whether a direct correlation exists between the thickness change of the membrane induced by an externally applied voltage and the shift of the mononer/dimer equilibrium in the bilayer. In principle, there are two possibilities for the influence of voltage on channel formation. The first consists in a voltage-induced thickness change, i.e. a purely geometrical effect which disturbs the equilibrium between monomers and dimers of gramicidin A, caused by the thinning of the membrane. In accordance with the results of Hladky and Haydon [6], this process would tend to increase the concentration of dimers. A second possibility would be that the applied voltage acts directly on the gramicidin A molecule

in such a way that the electric field shifts the equilibrium between nonconducting monomers and dimers to the side of conducting dimers, as proposed by Urry [11]. In order to study this problem, we measured the time course of the thickness change of the membrane and the time course of the gramicidin A conductance after a voltage jump and tried to correlate the results of both measurements.

EXPERIMENTAL

Optically black lipid membranes were formed in the usual way from a 1–3 % (w/v) lipid solution in *n*-decane or *n*-hexadecane [12] in a thermostatted Teflon cell filled with an aqueous electrolyte solution. The area of the membranes was either $8 \cdot 10^{-2}$ cm^2 or $3 \cdot 10^{-2}$ cm^2 in the case of voltage jump measurements. The torus area was kept smaller than 5 % of the total area. For the measurement of the single channel conductance we used membranes with an area of about $4 \cdot 10^{-4}$ cm^2. Bilayer membranes from monolayers were formed as described by Montal and Mueller [13]. A Teflon cuvette was separated into two halves by a 12.5 μm thin Teflon septum. The diameter of the hole in the septum on which the membrane was formed was about 0.2–0.3 mm. In order to facilitate the membrane formation, the surroundings of the hole were coated with a thin layer of vaseline. In the presence of vaseline the capacitance of the membranes was found to be about 5–10 % smaller than without vaseline [4].

Dioleoyl phosphatidylcholine was synthesized and chromatographically purified in our laboratory by K. Janko. The monoglycerides monooleine ((18 : 1)-monoacylglycerol) and monoerucin ((22 : 1)-monoacylglycerol) were obtained from NuChek Prep, Elysian, Minnesota (USA).

Purified gramicidin A was a gift from Dr. E. Gross, Bethesda, Maryland. A stock solution of gramicidin A in methanol was kept at −20 °C. Small amounts of this solution were added to the electrolyte solution. In some experiments, especially with membranes of monooleine/*n*-hexadecane, we added the gramicidin A directly to the membrane-forming solution. Both methods gave essentially the same results.

Electrical relaxation experiments in presence of gramicidin A were carried out as described earlier [5] by applying a fast voltage step to the membrane through silver/silver chloride electrodes. The time course of the membrane current was measured as a voltage drop across an external resistor in series with the membrane and was recorded on a Tektronix 5115 storage oscilloscope. Values of the external resistance were choosen such that the decay time of the capacitive transient was much smaller than the relaxation time of the current resulting from the gramicidin A kinetics. For the measurement of the current fluctuations arising from the formation and disappearence of single channels [6] the same set-up was used as is described in an previous paper [14]. All experiments were carried out between 20 and 30 min after the blackening of the membrane.

The zero-voltage capacity of the membrane was obtained by two different methods as described earlier [2, 15]. In a first series of experiments a Wayne Kerr B 221 bridge which was operated with a sine-wave generator (General Radio 1210-C, 10 mV peak-topeak, 2 kHz) together with an external nulldetector (General Radio 1232-A) was used. In other experiments rectangular voltage pulses of 10 mV were applied to the membrane from a pulse generator. The voltage drop which was induced

by the capacitive current across an external resistance was measured with the Tektronix 5115 storage oscilloscope. The resistance used was between 10 and 50 kΩ depending on the required sensitivity and time resolution of the experiment. Because of the high membrane resistance ($J_0 \gg J_\infty$) the membrane capacity C_M was obtained using the relation $VC_M = \tau J_0$ with V being the applied voltage, τ the time constant of the exponential decay and J_0 the current extrapolated to zero time (see below). Both methods were checked with a dummy circuit replacing the membrane, and were also applied on the same membrane. The results obtained by both methods agreed within 1–2 %.

The area of the black films was determined with a calibrated eyepiece micrometer. The error of the area measurement was estimated to be less than 3 %. The area of the hole in the Teflon septum used in the experiments with bilayers formed from monolayers was measured in the same way [4].

The compressibility of the membranes was measured by the following method. Rectangular voltage pulses of 150 mV amplitude and variable duration t were applied to the membranes. The waiting time between the single pulses was 1–5 s. The exponential decay of the current after the rising and falling phase of the voltage pulse was recorded together on the screen of the storage-oscilloscope as shown in Fig. 1. The charging and discharging times were always small compared with the total pulse lengths. The voltage-induced increase ΔC_M of the membrane capacity after a time t can be evaluated by comparing the current-time curves for the on and the off phases of each pulse of length t. It is seen from Fig. 1 that the current at the off-phase of the pulse (upper part of the record) is always larger than the current at the on-phase, the difference increasing with pulse length t. An obvious explanation is that the membranes are reversibly compressed under the influence of the voltage with a characteristic time course [4, 16]. The capacity C_M was obtained by plotting the logarithm of the current J as a function of time. The slope of the straight line then gave the time constant τ and extrapolation to zero time yielded the initial current J_0 from which

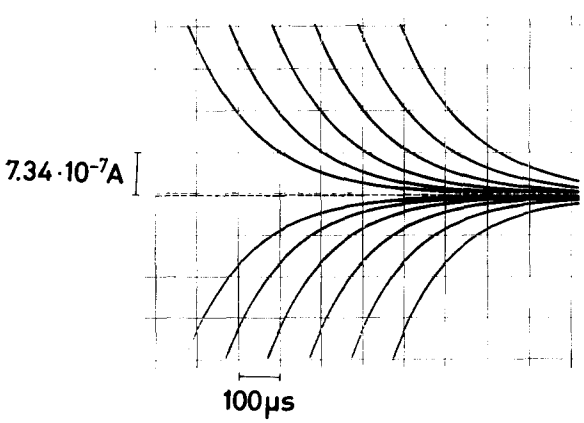

Fig. 1. Response of the membrane current to a voltage pulse of −150 mV. The lower curves correspond to the on process, the upper curves to the off process. The duration of the pulses was (from left to right) 20 ms, 50 ms, 100 ms, 200 ms, 500 ms and 1 s. The start of the first pulse is at the left border of the record; the trace of each subsequent pulse has been shifted by 100 µs to the right. The membrane has been formed from dioleoyl phosphatidylcholine dissolved in *n*-decane (1 % w/v); the area of the membrane was $1.26 \cdot 10^{-2}$ cm². 1 M NaCl, 25 °C.

C_M was calculated according to $C_M = \tau J_0/V$. In this way small differences ΔC_M between the rising and the falling phases of the voltage pulse could be obtained with high accuracy. Repeated measurements under identical conditions but with different membranes gave a reproducibility of $\Delta C_M/C_M$ of about 10 %. As the results are found to be independent of the ratio of the areas of torus and black film, it seems to be guaranteed that the early capacity change ($t < 1$ s) is due to a thickness change of the membrane and not to a change of the black area. The time course of the area change is found to be much slower (in the order of seconds) and correlates to an additional increase of the membrane current in experiments with gramicidin-doped membranes. In the case of membranes with a small torus ($\leqslant 5$ % of the total area) this effect was negligible.

RESULTS AND DISCUSSION

The capacity and maximal change of capacity of membranes made from different lipids and solvents are listed in Table I, which shows that the compressibility of a membrane is dependent on the lipid and the solvent composition. While membranes made from dioleoyl phosphatidylcholine in and from glycerol monooleate in *n*-decane give a maximal capacity change of 26–29 % and 18 %, respectively, the capacity change of membranes made from glycerolmonooleate and hexadecane is much smaller and approaches zero for solvent-free membranes [4].

The time course of the capacity change for different lipids after a voltage jump is represented in Fig. 2. It should be mentioned that the time course is independent of the ionic concentration in the electrolyte solution within the experimental error limits. This was shown in measurements over a salt concentration range between 1 and 10^{-2} M. Furthermore, we measured the temperature dependence of the time course for dioleoyl phosphatidylcholine/*n*-decane membranes, the results being also represented in Fig. 2. Both the time course of ΔC_M and the absolute values were found to be rather insensitive to temperature.

In a second series of experiments we measured the relaxation of the membrane

TABLE I

CAPACITY C_M AND MAXIMAL CAPACITY CHANGE $(\Delta C_M/C_M)_{max}$ OF MEMBRANES OF DIFFERENT LIPID AND SOLVENT COMPOSITION

$(\Delta C_M)_{max}$ is the change of C_M under the influence of a voltage of 150 mV for several seconds. C_M was measured at voltages smaller than 20 mV. The electrolyte in all experiments was 1 M NaCl.

Lipid	T(°C)	C_M(nF/cm²)	$(\Delta C_M/C_M)_{max}$
Di-(18:1)-phosphatidylcholine/*n*-decane, 1 % (w/v)	10	388±15	0.29
	25	378±18	0.27
Di(18:1)-phosphatidylcholine/*n*-decane, 3 % (w/v)	40	352±21	0.26
(18:1)-monoacylglycerol/*n*-decane, 3 % (w/v)	25	395±15	0.18
(18:1)-monoacylglycerol/*n*-hexadecane, 3 % (w/v)	25	587±18	0.04
(22:1)-monoacylglycerol/*n*-hexadecane, 2 % (w/v)	25	383±25	–
(22:1)-monoacylglycerol solvent-free membrane	25	590±35 [4]	0.01

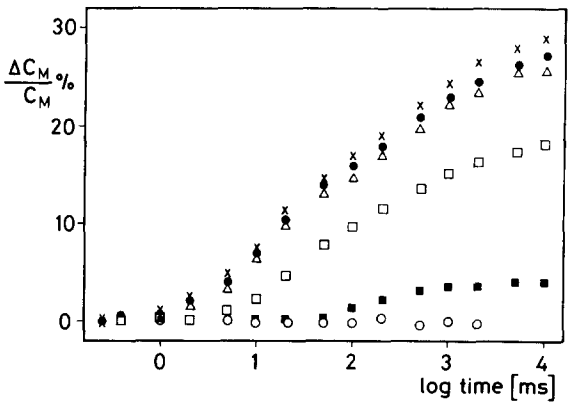

Fig. 2. Time course of the capacity change ΔC_M of membranes made from different lipids and different solvents after a voltage jump of 150 mV. C_M is the capacity in the limit of small voltage and is given in Table I. (×) dioleoyl phosphatidylcholine/*n*-decane, 1 % (w/v), $T = 40$ °C; (●) dioleoyl phosphatidylcholine/*n*-decane, 1 % (w/v), $T = 25$ °C; (△) dioleoyl phosphatidylcholine/*n*-decane, 1 % (w/v), $T = 10$ °C; (□) monoolein/*n*-decane, 2 % (w/v), $T = 25$ °C; (■) monoolein/*n*-hexadecane, 2 % (w/v), $T = 25$ °C; (○) monoerucin, solvent-free membrane, $T = 25$ °C.

current in the presence of gramicidin A after a voltage jump. In previous papers [5, 14] it was shown that a voltage jump disturbs the equilibrium between monomers and conducting dimers in the membrane.

The equilibrium in the membrane

$$A + A \underset{k_D}{\overset{k_R}{\rightleftharpoons}} A_2 \tag{1}$$

is shifted towards the side of the dimers at higher voltages. According to the dimer model of the channel, the current is proportional to the dimer concentration of gramicidin A in the membrane, and the current should relax to a higher stationary level after a voltage jump. It could be demonstrated [5, 14] for dioleoyl phosphatidylcholine membranes that the current $J(t)$ is governed by a single exponential for a wide range of gramicidin A-induced conductances:

$$J(t) = J_\infty + (J_0 - J_\infty)\, e^{-t/\tau} \tag{2}$$

where J_0 is the initial and J_∞ the steady-state current.

The relaxation time τ is given [5] by:

$$\frac{1}{\tau} = k_D + 4\sqrt{\frac{k_R \cdot k_D \lambda^\infty}{L\Lambda}} \tag{3}$$

where k_D is the dissociation rate constant, k_R the association rate constant for the dimerization process (Eqn. 1), L Avogadro's constant, and λ^∞ the steady-state conductance of the membrane, which is reached in the limit of long times t. Λ is the single channel conductance of gramicidin A.

Two examples of current relaxation are shown in Fig. 3. Fig. 3a represents the time course of the current in the case of a monoolein/*n*-hexadecane membrane,

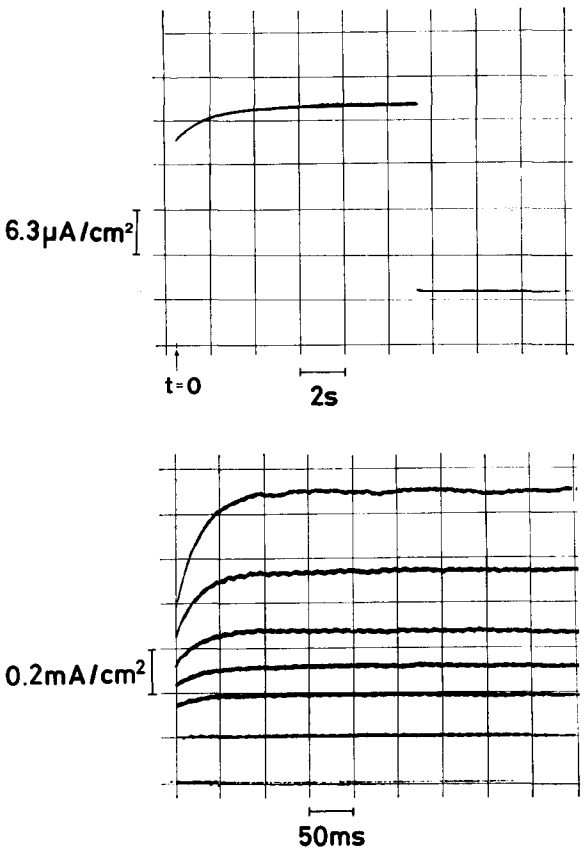

Fig. 3. Time course of the membrane current in the presence of gramicidin A after a voltage pulse of amplitude ΔV. (a) Monoolein/*n*-hexadecane membrane (area $3.14 \cdot 10^{-2}$ cm^2), $\Delta V = 150$ mV. (b) Solvent-free monoerucin membrane (area $2.5 \cdot 10^{-4}$ cm^2). The trace at the bottom of the record gives the zero current level. The pulse amplitudes ΔV were (from bottom to top): 50, 80, 100, 120, 180 mV. 1 M NaCl, 10^{-8} M gramicidin A, $t = 37$ °C.

whereas Fig. 3b shows the current relaxation after different voltage steps with a solvent-free monoerucin membrane. The analysis of these relaxation curves gave the same result as previously published. For both types of membrane the time course of the current may be represented by a single exponential. For monoolein/cholesterol/*n*-decane membranes S. B. Hladky recently reported [17] current relaxation curves with more than one time constant. One time constant was in agreement with the mean life-time of the conducting channel as calculated from single channel experiments, whereas the other relaxation had a much longer time constant. In some experiments where the torus surrounding the membrane had an area of more than 10 % of the area of the black film, we could also observe an additional nonexponential relaxation. Its reproducibility was very poor in contrast to the faster relaxation. This additional effect seems to originate from an increase in the area of the black film. This is further illustrated by Fig. 3b, which shows that solvent-free membranes which have presum-

TABLE II

RESULTS OF RELAXATION MEASUREMENTS AND SINGLE-CHANNEL EXPERIMENTS WITH GRAMICIDIN-DOPED LIPID BILAYER MEMBRANES

The abbrevations for the lipids are the same as in Table I. ΔV, amplitude of the voltage jump in the relaxation experiments; A, single-channel conductance at $V = 100$ mV, expressed in pS (1 pS $= 10^{-12}$ Siemens $= 10^{-12}\ \Omega^{-1}$); k_R, k_D, rate constants of association and dissociation (Eqn. 1); τ^*, mean lifetime of the single-channel. The data for dioleoy phosphatidylcholine were taken from previous papers [5, 13]. The value of τ^* for dioleoyl phosphatidylcholine at 25 °C which, because of a calibration error in the strip-chart recorder, had been erroneously given as 1.25 in the previous paper [12] has been corrected.

Lipid	Electrolyte (1 M)	T (°C)	V (mV)	A (pS)	k_R ($cm^2 \cdot mol^{-1} \cdot s^{-1}$)	k_D (s^{-1})	$1/k_D$ (s)	τ^* (s)
Di-(18:1)-phosphatidylcholine/*n*-decane (1 %)	NaCl	10	135	6.5	$2.3 \cdot 10^{13}$	0.25	4	3.8
Di-(18:1)-phosphatidylcholine/*n*-decane (1 %)	NaCl	25	135	12	$20 \cdot 10^{13}$	1.6	0.63	0.72
Di-(18:1)-phosphatidylcholine/*n*-decane (1 %)	NaCl	40	135	23	$68 \cdot 10^{13}$	4.5	0.22	0.15
(18:1)-monoacylglycerol/*n*-hexadecane (1 %)	NaCl	25	100	24	$1.2 \cdot 10^{12}$	0.45	2.2	2.0 [6]
(22:1)-monoacylglycerol, solvent-free	CsCl	35	150	80	–	8.7	0.114	0.27
(22:1)-monoacylglycerol, solvent-free	CsCl	27	150	62	–	2.02	0.49	0.72

ably no torus show a stable value of the stationary current J_∞ over an extended period of time.

Table II gives a survey of data obtained from relaxation and single channel experiments with membranes from different lipids in the presence of gramicidin A. For the measurements with monoerucin membranes we used 1 M CsCl as electrolyte. The reason was that the single-channel conductance of gramicidin A in the presence of Cs^+ is about 3 times higher than in the presence of Na^+. There was no detectable difference in the channel formation kinetics between 1 M NaCl and 1 M CsCl.

It is easy to see that the values of $1/k_D$ from the relaxation experiments are in reasonable agreement for all types of membranes with the values of the mean channel life-time τ^* obtained from the single channel experiments. The equality between τ^* and $1/k_D$ is required by the simple dimer model.

Previously it was shown from relaxation and single-channel measurements [14] that the value of k_D for dioleoyl phosphatidylcholine/*n*-decane membranes varies by a factor of 20 in the temperature range of 10–40 °C (these values are also inserted in Table II). From Fig. 2, however, one can see that the time course of the capacity change $\Delta C_M/C_M$, i.e. the thickness change of the dioleoyl phosphatidylcholine membrane after an applied voltage is almost independent of temperature. This finding suggests that the two processes, the compression of the membrane and the shift of the monomer dimer equilibrium, are not correlated.

A more detailed comparison is possible from the data given in Fig. 4, in which we compare the time course of the thickness change of the membrane with the current

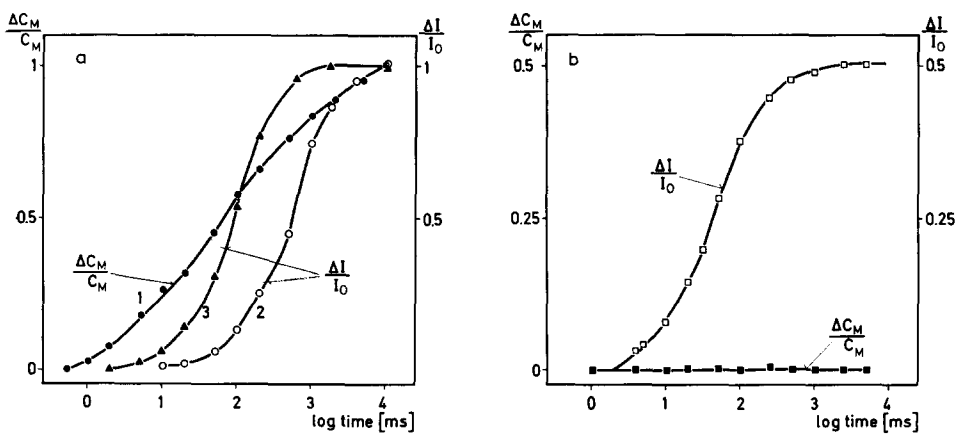

Fig. 4. (a) Time course of the relative change at the membrane capacity $\Delta C_M/C_M$ (curve 1 left hand ordinate) and time course of the relative change in gramicidin-dependent membrane current $\Delta J(t)/J_0$ (curves 2 and 3, right hand ordinate) after a voltage jump of $\Delta V = 150$ mV. The values at $\Delta C_M/C_M$ are obtained by dividing the normalized value by the normalization factors f given below. The membrane was formed from a dioleoyl phosphatidylcholine/*n*-decane solution, 1 % (w/v). (1) $\Delta C_M/C_M$ at $T = 10$, 25 and 40 °C ($f_{10} = 3.92$, $f_{25} = 3.70$, $f_{40} = 3.45$). The normalized values are virtually on the same curve. (2) $\Delta J(t)/J_0$ at $T = 25$ °C ($f = 0.33$). (3) $\Delta J(t)/J_0$ at $T = 40$ °C ($f = 0.38$). (b) Time course of the relative change of the membrane capacity $\Delta C_M/C_M$ and time course of the relative change in gramicidin dependent membrane current $\Delta J(t)/J_0$ after a voltage jump of $\Delta V = 150$ mV. A solvent free membrane was formed from 2 monolayers. The lipid was 22:1 monoacylglycerol. $T = 25$ °C, 1 M NaCl.

relaxation in the presence of gramicidin A. Fig. 4a clearly shows that the time course of the thickness change for different types of membrane does not correlate at all with the time course of the membrane current in the presence of gramicidin A. (Strictly speaking, the conditions of both sets of experiment were different in so far as no gramicidin was present in the compressibility measurements, but it has been checked by other experiments that gramicidin does not change the time course of ΔC_M. These experiments had been carried out in the presence of usual concentrations of gramicidin A and with $MgCl_2$ as a impermeable electrolyte.) The most striking example is given by the solvent-free monoerucin membrane which is virtually incompressible but, nevertheless, gives similar amplitudes of the current relaxation to those of the highly compressible *n*-decane membranes (Fig. 4b). Furthermore, the shape of the current relaxation curve is exponential, whereas the thinning curve ($\Delta C_M/C_M$) does not follow an exponential law. Another interesting result with respect to the solvent-free membranes is the value of $1/\tau^*$. At 27 °C we obtained from single channel experiments a value of $1/\tau^* = 1.4\ s^{-1}$, which agrees approximately with $k_D = 2.02$ from the relaxation experiments. The thickness of the solvent-free monoerucin membrane made from monolayers is approximately 32 Å [3]. For solvent-containing membranes of this thickness range Hladky and Haydon [6] found a mean life-time τ^* of about 2 s, corresponding to a value for $1/\tau^*$ of $0.5\ s^{-1}$. The lipid used was monoolein in *n*-hexadecane. A comparison of these results leads to the suggestion that not only the thickness of the membrane is responsible for the mean duration of the single channels, but also the nature of the hydrocarbon chains of the lipid. A second interesting phenomenon is that the single-channel conductance is reduced for monoerucin by about 30 % in comparison with monoolein membranes. These problems will be treated in more detail in a forthcoming publication.

The experimental results presented above strongly suggest that a geometrical effect, i.e. the thinning of the membrane, is not responsible for the relaxation phenomena in the presence of gramicidin A. This means that there is likely to be a direct influence of the electric field on the gramicidin A molecule in the membrane. A possible indication for the direct influence of the electric field is given by the experiment presented in Fig. 3b. The current voltage curve in Fig. 3b derived from an incompressible membrane from monoerucin was analyzed. We found, as to be seen in Table III, that the relation between relaxation current and voltage is approximately quadratic.

$$\Delta I = \alpha V^2$$
$$\Delta I = J_\infty - J_0 \qquad (4)$$

where α is a constant.

The slight increase of α at higher voltages is perhaps related with changes of the membrane structure at such high voltages.

A possibility for a direct influence of the electric field on the gramicidin A kinetics has already been suggested by Urry [18] who presented arguments for the existence of a conducting and a nonconducting form of the gramicidin A molecule. The conducting form is an anti-β_2^6-helix and the conducting form a $\pi_{L,D}^6$-helix. Both types of helix are in equilibrium

$$\text{anti-}\beta_2^6 \rightleftarrows \pi_{L,D}^6$$

TABLE III

ANALYSIS OF A CURRENT-VOLTAGE CURVE OF A SOLVENT-FREE MEMBRANE MADE FROM MONOERUCIN IN THE PRESENCE OF GRAMICIDIN A

V is the applied voltage, ΔI is the relaxation current and α a constant. Experiments performed at 37 °C in the presence of 1 M CsCl.

V(mV)	$\Delta I(\mu A)$	$\alpha(\Delta I/V^2)$
80	0.020	3.12
100	0.023	2.30
120	0.040	2.78
150	0.075	3.33
180	0.138	4.25

The $\pi^6_{L,D}$-helix forms a channel permeable for monovalent cations, whereas the β^6_2-helix does not have the structure of a pore. Furthermore, the nonconducting form cannot give stable head-to-head dimers. In Urry's paper it is shown that the β^6_2-helix has a lower net dipole moment than the $\pi^6_{L,D}$-helix. The action of an electric field in the membrane would then consist in shifting the equilibrium towards the side of the form with the higher dipole moment, which is the conducting form. With regard to a possible explanation of the field effect on channel formation we carried out an additional experiment. A voltage of +300 mV was applied to a membrane for a time sufficiently long to reach a steady current, and then the voltage was quickly switched to −300 mV. At the switching of the voltage the membrane current merely changed its polarity without any detectable relaxation. From this finding it may be excluded that the channel processes an intrinsic structural polarity with respect to the direction of the electric field. Otherwise, the switching of the voltage from $+V$ to $-V$ would cause a breakup of some of the channels followed by a formation of new channels with opposite polarity.

The result that gramicidin channels do not possess an intrinsic polarity is certainly what we would expect for a dimer that is linked head-to-head; on the other hand this finding leads to certain difficulties in the application of Urry's explanation of the field effect. A dimeric channel formed by head-to-head association of two monomers exhibits mirror symmetry with respect to the central phase of the membrane, and therefore a field change that is energetically favorable for the active form of the monomer in one half of the bilayer is unfavourable in the other half.

It is more likely that the right explanation involves a quadratic field-effect on the free energy of the channel. For instance, if the active $\pi^6_{L,D}$-helix has larger polarizibility than the inactive anti-β^6_2-helix, an increase of field strength would favour the former structure irrespective of the orientation of the helix axis with respect to the electric field. Another possibility for a field effect on channel formation, which is based on a proposal by Gordon [19], would be to assume that an inactive form of the gramicidin monomer exists which is bound at the membrane-solution interface. As the average dielectric constant of the monomer is larger than that of the hydrocarbon interior of the membrane, the monomer would be drawn into the membrane under the action of the electric field and thereby could be converted into the active form.

Recently, Veatch et al. [20] proposed a different class of dimeric helices con-

sisting of two gramicidin molecules which are wound around a common axis. As pointed out by Veatch et al., such a helix may assume either a parallel conformation with a large net dipole moment or an antiparallel conformation with zero dipole moment. From (+V)/(−V) pulse experiments we may conclude that the conformation with parallel orientation of the monomers is unlikely. For the dimer with two antiparallel peptide chains, which has the same symmetry as the head-to-head dimer, the same considerations apply as above.

ACKNOWLEDGEMENT

We wish to thank Dr. P. Läuger for his interest and many helpful suggestions and Mrs. M.-J. Quelle for the excellent technical assistance. This work has been supported by Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 138).

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