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CHARGE-PULSE RELAXATION STUDIES WITH LIPID BILAYER MEMBRANES MODIFIED BY ALAMETHICIN

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Summary

Charge-pulse relaxation studies with the alamethicin-lipid membrane system reveal a triphasic decay of membrane voltage. At short times (resolution time 2 μs), where a voltage decay due to the orientation of alamethicin dipoles from the interface into the membrane interior ("gating current") could possibly be expected, only a slow decrease with a time constant determined by the bare membrane conductance occurs. After approximately 1 ms (depending on the experimental conditions) the formation of alamethicin pores starts, leading to an increase in the voltage decay rate. When the characteristic voltage V_c^{cp} is approached, pores close and after passing V_c^{cp} the voltage decreases slowly again according to the bare membrane conductance.

V_c^{cp} is determined as a function of the initially applied voltage V_o, alamethicin and KCl concentration. Since the membrane voltage decreases continuously, the system does not reach the equilibrium states obtained at constant voltages. Taking the presented experimental results into account the estimate of the electrical potential at the functional membrane of photosynthesis induced by a saturating single turnover flash of $\Delta\Phi_o \approx 105$–135 mV (Zickler, Witt and Boheim (1976) FEBS Lett. 66, 142–148) is changed to $\Delta\Phi_o \approx 200$ mV.

Introduction

The polypeptide antibiotic alamethicin forms voltage-dependent pores of multi-state behaviour in lipid bilayer membranes [1–7]. Evidence has been given that several molecules are inserted into the membrane by the voltage to form an ion-conducting pore. This orientation process seems to be caused by the interaction of the electric field with the dipole moment of single ala-

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methicin molecules [7,8]. Pore formation probability is influenced by voltage, V, and alamethicin concentration C_{AL} in such a way that a given number of pores is created by a V_1, $(C_{AL})_1$ pair as well as by a lower voltage, $V_2 < V_1$, but correspondingly higher concentration $(C_{AL})_2 > (C_{AL})_1$ [7].

This correlation between voltage and alamethicin concentration was used to estimate the light-induced electrical potential, $\Delta\Phi_o$, at the functional membrane of photosynthesis [9]. A saturating single-turnover flash induced an electrical potential difference across the thylakoid membrane within a few ns which was, under the given experimental conditions, above the characteristic voltage, V_c^{cp}, of pore formation. Consequently, alamethicin pores were formed in the membrane and due to the resulting increase of conductance, the electric field decreased more rapidly. When V_c^{cp} was reached, the pores disappeared and thereafter the field decayed more slowly. The addition of alamethicin therefore changed the monophasic exponential signal into a biphasic decay with a fast phase of $\tau \approx 10$ ms and a slow one of $\tau \approx 150$ ms (at 23°C and 10^{-2} M ionic strength, approximately). Recent experiments [7] indicate that the correlation factor ρ_i (see Eqn. 3) for voltage and alamethicin concentration-dependence of a measurable variable, i, (which was used for the estimate of $\Delta\Phi_o$) strongly depends on the experimental procedure of ρ_i-determination. The results are different whether the system is analyzed at constant or continuously decreasing voltage. Furthermore, a different behaviour is observed whether the applied voltage is changed with the alamethicin concentration in order to compare different sets of these variables at equal pore concentration, or whether the initially applied voltage is kept constant at varying alamethicin concentrations. The thylakoid membrane was charged up by a saturating single-turnover flash of half lifetime approx. 20 μs [9]. The membrane voltage then decreased continuously due to charge movement across the membrane. The equivalent method for the lipid bilayer system, therefore, is not the method of current-voltage curves [3,7] or that of voltage-jump current-relaxations [7] but the charge-pulse technique [10,11], where a distinct voltage is applied across the membrane after approx. 50 ns and its discharging is directly recorded. This technique, which enables a higher time resolution than the voltage-jump method, was used to study the electrical properties of lipid bilayer membranes in the presence of hydrophobic ions [10] and ion carriers [11].

In this paper we report on charge-pulse relaxation studies with alamethicin-modified lipid membranes. Contrary to the situation with the thylakoid membrane, with the artificial system the membrane voltage and its change with time can be recorded directly. Thus the voltage-alamethicin concentration relation factor ρV_c^{cp} for the characteristic voltage, V_c^{cp}, corresponding to this peculiar experimental situation (cp means charge pulse) can be determined from both experimental quantities. Another question which arises in the case of alamethicin pore formation is the current-pulse after a voltage-jump caused by voltage-dependent dipole orientation of alamethicin molecules. Such non-faradaic charge movements (displacement current) have been observed in nerve membranes for the opening and closing reaction of the Na-channel [12,13]. The relaxation time of these gating currents in nerve was found in the 100 μs range. Up to a time resolution of approx. 2 μs we looked for a gating current of alamethicin pore formation.

Materials and Methods

Bilayer lipid membranes were formed from 1,2-dioleoyl-*sn*-3-glycerophosphocholine (di-(18 : 1)-phosphatidylcholine) synthesized by the method of K. Janko [14]. Membrane-forming solutions were made of 1% (w/v) lipid in *n*-decane.

Our experiments were carried out with the pure R_F30 fraction of alamethicin checked by thin layer chromatography. It was purchased from Microbiological Research Establishment, Porton Down, Salisbury [15]. Alamethicin was added from a stock solution of 100 $\mu g \cdot cm^{-3}$ in ethanol/water (1 : 9, v/v). The antiobiotic was present in identical amounts in both aqueous compartments prior to membrane formation. KCl salt solutions were 1 M, 0.1 M and 0.01 M, unbuffered, pH 5.5—6. KCl was pure analytical grade from Merck.

The experiments were performed as described earlier [10] using a voltage source (10 mV—6V) in series with a fast battery operated FET-switch (2N5653, Pan Elektronik, Taufkirchen, G.F.R.). The impedance of the switch in the "open" position was larger than 10^{12} Ω. The membrane capacity was charged up to a voltage between 50 and 300 mV by a brief current pulse of 50 ns duration. The switch was triggered by a separate battery-operated pulse generator. The voltage transient across the membrane was measured with a voltage follower (Analog Devices 42 K, input impedance $>10^{12}$ Ω) and recorded with a storage oscilloscope (Tektronix 549/1A7A of Tektronix 7633/7A22).

Test experiments were carried out with dummy circuits replacing the cell with the membrane. No distortion of the exponential decay of the voltage was observed up to 2 μs with the 7633 Tektronix oscilloscope, whereas the 549 Tektronix oscilloscope showed overload effects in the 10 μs range. In a second series of experiments undoped membranes from di-(18 : 1)-phosphatidylcholine/*n*-decane were investigated. In case of 1 M KCl and voltages $\leqslant$50 mV a pure exponential decay with a time constant τ_m according to $\tau_m = R_m \cdot C_m$ (R_m is membrane resistance, C_m = 374 nF cm^{-2} [14]: membrane capacitance) was observed starting at 2 μs. In the range of 100—150 mV a relaxation was found in the ms time scale which presumably originated from a change of membrane thickness and area [14]. The corresponding relaxation time becomes smaller at higher voltages (200—300 mV). This relaxation is also visible in the presence of alamethicin. In case of 0.01 M KCl solutions the resolution of the system is reduced to approx. 20 μs because of electrode polarisation and diffusion polarisation effects. Therefore, the initial decay visible in Fig. 2 which is observed down to 10 mV and lower is not caused by alamethicin.

Two Ag/AgCl electrodes were used. Voltage will be designated positive if the more positive potential is applied on the front compartment side. Temperature was 25 ± 1°C.

Results

The typical triphasic decay of membrane voltage after application of a charge-pulse to an alamethicin-modified lipid membrane system is shown in Figs. 1a, b and 2a. Besides the initial spike which is seen also without alamethicin, no charge transfer process (except that of slow discharging across the

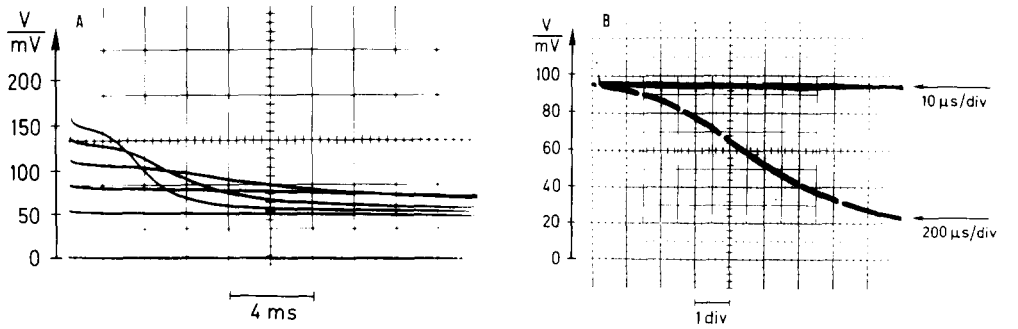

Fig. 1. Decay of membrane voltage, V, after a charge pulse in the presence of 1 M KCl in the aqueous solutions. a. At time $t = 0$, the membrane capacity was charged up to various voltages, V_0, by current pulses of 50 ns duration. The initial spike is observed also without alamethicin. After a voltage-dependent induction period alamethicin pores are formed and the voltage decay rate increases. If the characteristic voltage, V_c^{cp}, of pore formation is approached, pores are turned off and after passing V_c^{cp} the relaxation time is determined only by the bare membrane resistance. It is seen that V_c depends on V_0. For determination procedure of V_c^{cp} see text. Oscilloscope, Tektronix 549. b. The initial decay period of V was recorded with two different sweep times as indicated on the right side of the oscillogram. Up to the experimental time resolution of 2 μs no charge movement due to the orientation of dipole molecules is observed. Oscilloscope, Tektronix 7633. Membrane solution, 1% di-(18 : 1)-phosphatidylcholine in n-decane; salt solution, 1 M KCl, unbuffered; antibiotic concentration, (a) 0.15 $\mu g \cdot cm^{-3}$, (b) 0.40 $\mu g \cdot cm^{-3}$ alamethicin R_F 30; temperature, 25°C.

bare membrane) is visible up to the ms time range. For a clearer presentation of this fact a series of experiments was done up to a time resolution of 2 μs with a Tektronix 7633 oscilloscope (Fig. 1b) at a relatively high alamethicin concentration and correspondingly lower membrane voltage. Fig. 1b demonstrates the absence of any process of measurable amplitude in the 2—200 μs range.

After a delay period which strongly depends on the initial voltage, V_0, an increase in the decay rate of the membrane voltage, V, is seen in Figs. 1a and 2a. Then, within a distinct voltage range, the decay rate becomes slower and switches over again to that of the bare membrane. It is now useful to introduce

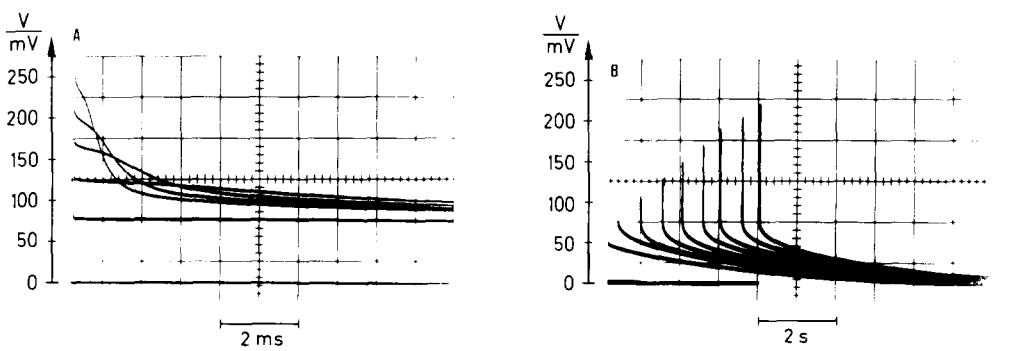

Fig. 2. Decay of the membrane voltage, V, after a charge pulse in the presence of 10 mM KCl in the aqueous solutions. Charging and discharging of the membrane capacity occurs in the same way as described in Fig. 1a. The initial spikes are observed also without alamethicin. The decay of V was recorded with two different sweep times (a) 1 ms/div and (b) 1 s/div. The characteristic voltage, V_c^{cp} is nearly independent of the initially applied voltage, V_0. Membrane solution, 1% di-(18 : 1)-phosphatidylcholine in n-decane; salt solution, 0.01 M KCl unbuffered; antibiotic concentration, (a) 1.0 $\mu g \cdot cm^{-3}$, (b) 2.0 $\mu g \cdot cm^{-3}$ alamethicin R_F 30; temperature, 25°C; oscilloscope, Tektronix 549.

a characteristic voltage, V_c^{cp}, as has been done by Zickler et al. [9] for their experiments at the thylakoid membrane, at which virtually all alamethicin pores are switched off again. Since the two decay times (with and without alamethicin pores) are sufficiently different, V_c^{cp} will be defined by extrapolating the slow exponential decay process to zero time (according to Fig. 2b) or by the intersection of the tangent through the inflexion point of the fast process with the straight line obtained by an approximate extrapolation of the final slow process (see Figs. 1a and 2a), respectively. Within the experimental limits of reproducibility the two procedures of determining V_c^{cp} give comparable results. It should be noted at this point that the characteristic voltage V_c defined by Eisenberg et al. [3] was measured under conditions different from those of V_c^{cp} determination with the charge-pulse method. In the first case a continuously increasing voltage starts below V_c, whereas in the other case the voltage decreases continuously from above V_c^{cp}. Since no pores are formed below V_c, the method of Eisenberg et al. [3] yields V_c values independent of the starting voltage $V_o < V_c$. This is different with our method in the presence of 1 M KCl (Fig. 1a) where $V_o > V_c^{cp}$ (see below).

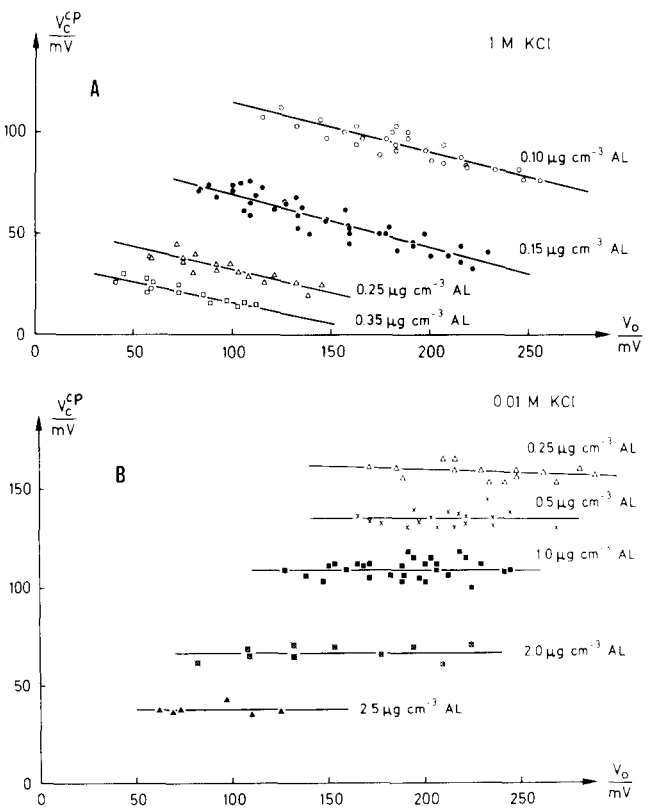

Fig. 3. Dependence of the characteristic voltage, V_c^{cp}, on the initially applied voltage, V_o. The experiments were carried out at 1 M KCl (a) and 10 mM KCl (b) salt solutions, unbuffered. For details see text. Membrane solution, 1% di(18 : 1)-phosphatidylcholine in *n*-decane; temperature, 25°C; antibiotic concentration, a : 0.10 $\mu g \cdot cm^{-3}$ (○), 0.15 $\mu g \cdot cm^{-3}$ (●), 0.25 $\mu g \cdot cm^{-3}$ (△) and 0.35 $\mu g \cdot cm^{-3}$ (□) alamethicin R_F 30; b: 0.25 $\mu g \cdot cm^{-3}$ (△), 0.50 $\mu g \cdot cm^{-3}$ (X), 1.0 $\mu g \cdot cm^{-3}$ (■), 2.0 $\mu g \cdot cm^{-3}$ (⊗) and 2.5 $\mu g \cdot cm^{-3}$ (▲) alamethicin R_F 30.

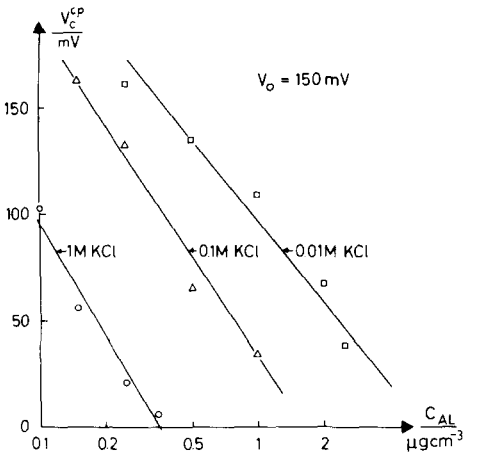

Fig. 4. Dependence of the characteristic voltage, V_c^{cp}, on alamethicin concentration, C_{AL}, at constant initially-applied voltage, V_o = 150 mV for different KCl salt concentrations: 1 M KCl (○), 0.1 M KCl (△) and 0.01 M KCl (□), unbuffered. The drawn points have partly been obtained by extrapolation (see Fig. 3). For details see text. Membrane solution, 1% di-(18 : 1)-phosphatidylcholine in *n*-decane; temperature, 25°C.

The experimental data of Figs. 3 and 4 have been obtained in the following way. The membrane was formed when alamethicin was already present in the aqueous solution, and maintained at zero voltage for 1 h. Then several charge-pulses of varying magnitude were applied and the voltage decay recorded. The procedure was repeated with several others, newly-formed membranes. The membranes are quite stable during the course of charge pulse experiments compared to the situation under constant voltage conditions. The higher V_o, i.e. the larger the number of pores, the faster the membrane voltage decays thus reducing automatically membrane stress at high conductance and voltage.

Fig. 3 demonstrates the dependence of V_c^{cp} on V_o. Whereas in 1 M KCl solution V_c^{cp} shows a pronounced dependence on V_o, V_c^{cp} is virtually constant at 0.01 M KCl. With 0.1 M KCl solution the dependency is intermediate.

In order to analyze the alamethicin concentration dependence of V_c^{cp} one has to define under which conditions the comparison has to be carried out at the different alamethicin concentrations. For instance, the systems might be compared (i) at constant V_o or (ii) by keeping the difference $V_o - V_c^{cp}$ constant. In the case of the thylakoid membrane [9] a constant initial voltage, V_o, is induced by a saturating single-turnover flash. According to situation (i), therefore, we plotted the characteristic voltage V_c^{cp} obtained at V_o = 150 mV (partly by extrapolation) versus the logarithm of the alamethicin concentration C_{AL} in Fig. 4. A straight line has been drawn through the experimental points in order to characterize the dependency by a single parameter (see Discussion). However, the V_c^{cp}/log C_{AL} relation seems not to be linear in general.

Discussion

The experimental results (Figs. 1 and 2) show that the interpretation of charge-pulse experiments in the presence of the complex alamethicin system is

rather complicated. This situation is different with hydrophobic ions and carriers [10,11]. In principle, three kinds of information might be accessible in the case of alamethicin: (a) a fast voltage decay due to charge displacement across the membrane, e.g. dipole orientation, (b) the delay time for the switching-on process of alamethicin pore formation (which reflects the pore formation probability, however, under conditions of decreasing voltage), (c) the characteristic voltage at which virtually all pores have been switched-off.

Regarding (a), we did not observe a fast voltage decay in the 2—200 μs range which seems to indicate that no orientation of an appreciable amount of dipole molecules occurs before pore formation. We will consider the following estimate. As it was shown by Benz et al. [10] in the case of the hydrophobic ion dipicrylamine, a minimum charge transfer of 10^{-14} mol · cm^{-2} corresponding to $6 \cdot 10^9$ molecules/cm^2 is resolvable by the charge-pulse method. Following the estimate of Mueller [6] one would obtain a collision rate of about 300 s^{-1} for alamethicin monomers inserted into the membrane assuming a lateral diffusion constant of 10^{-8} cm^2 · s^{-1} and a concentration of $6 \cdot 10^9$ molecules · cm^2. Since, under comparable experimental conditions, a mean life-time of the pore states of approx. 3—10 ms is observed [7], the above estimated value means the minimum collision rate for a diffusion-controlled reaction without considering a sterical factor which could account for the experimentally-observed pore state transition frequency. Therefore, since we observed no charge displacement, we conclude that the activation energy for insertion of single monomers, which then may diffuse laterally in the membrane to form the conducting pore, turns out to be too high. This is consistent with the concept of alamethicin preaggregate formation at the membrane interface prior to pore formation [3,5—7]. The pore nucleus is created then by simultaneous insertion of two or three molecules. Consequently, alamethicin molecules are inserted into the membrane only if a pore is formed or if a pore changes its state. Then the observation of an alamethicin displacement (insertion) current seems not to be possible without blocking the pores. But blockage has not been accomplished up to now, since such large ions as Tris · H$^+$ and HEPES$^-$ (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulphonic acid) are able to pass through the pore [Boheim, G., to be published]. The gating current of the Na-channel in nerve was observed in the 100 μs time range by blocking both the Na- and the K-channels [12,13].

Regarding (b), an evaluation of the delay time was not carried out in detail, since it seems to be more convenient to perform the analysis under constant voltage conditions. Nevertheless, Figs. 1a and 2a show qualitatively that the delay time decreases with increasing voltage, which is equivalent to an increase in the pore formation probability. This behaviour is known for the alamethicin system [1,3,5—7].

Regarding (c), the third quantity, the characteristic voltage, V_c^{cp}, has been investigated in detail by us in order to compare the results obtained at the thylakoid membrane with those of the artificial lipid bilayer under identical conditions of voltage application. Figs. 1a and 3a demonstrate that V_c^{cp} depends on V_o in 1 M KCl, whereas according to Figs. 2a and 3b, this effect nearly vanishes in 0.01 M KCl. An explanation for the V_o dependency may be based on the finite mean lifetime of the pore. The more pores are formed at

$V > V_c^{cp}$, the higher is the probability to find long-living pores near V_c^{cp}. Then the extent of the hysteresis under continuously changing voltage conditions is a function of the mean lifetime of the pore. The vanishing of the hysteresis in 0.01 M KCl is consistent with the experimental fact that the mean lifetime of the alamethicin pore in the case of equal pore concentrations in the membrane decreases with decreasing ionic strength (ref. 16, see also Figs. 1a and 2a).

In order to compare experiments with thylakoid membranes and with artificial lipid bilayers under comparable conditions, the alamethicin concentration dependence of V_c^{cp} was studied at constant V_o. Fig. 4 shows that the experimental points can only approximately be described by a straight line in a V_c^{cp} versus log C_{AL} plot. Taking this approximation into account, the following description of the conductance, λ, of the alamethicin-modified membrane is useful [3,7]:

$$\lambda = C \cdot \exp\{\alpha u\} \cdot C_{AL}^{\delta} \cdot C_{KCl}^{\epsilon} \tag{1}$$

with λ, conductance of the alamethicin-modified membrane system; C, constant; u, $= FV/RT$: reduced voltage (F, Faraday constant; R, gas constant; T, absolute temperature); α, δ, ϵ, experimentally determined parameters of voltage (V), alamethicin concentration (C_{AL}) and KCl concentration (C_{KCl}) dependence, respectively.

The characteristic voltage V_c^{cp} ($\propto u_c$) corresponds to a distinct pore formation probability which in turn yields a characteristic conductance $\lambda_c = \lambda\,(V_c^{cp})$ in a given system. According to Eqn. 1 λ_c can be achieved by several sets of related V_c^{cp} and C_{AL} values

$$\begin{aligned}\lambda_c &= C \cdot \exp\{\alpha \cdot (u_c)_1\} \cdot (C_{AL}^{\delta})_1 \cdot C_{KCl}^{\epsilon} \\ &= C \cdot \exp\{\alpha \cdot (u_c)_2\} \cdot (C_{AL}^{\delta})_2 \cdot C_{KCl}^{\epsilon} \,.\end{aligned} \tag{2}$$

A voltage-alamethicin concentration relation factor ρ is given by

$$\rho_{V_c^{cp}} = \frac{\alpha}{\delta} = \frac{\ln\{(C_{AL})_2/(C_{AL})_1\}}{(u_c)_1 - (u_c)_2} \,. \tag{3}$$

The charge-pulse experiments (Fig. 4) yield 1 M KCl, $\rho_{V_c^{cp}} = 0.34$; 0.1 M KCl, $\rho_{V_c^{cp}} = 0.38$; 0.01 M KCl, $\rho_{V_c^{cp}} = 0.47$.

Different values of ρ have been obtained for different experimental variables with different experimental methods. Eisenberg et al. [3] obtained for their V_c at 23°C, $\rho_{V_c} = 6.5/9 = 0.72$; Mueller and Rudin [1] for the steady-state conductance λ at 35°C, $\rho_\lambda = 5.5/6 = 0.92$; Boheim and Kolb [7] for the steady-state conductance λ at 25°C, $\rho_\lambda = 6.3/9.4 = 0.67$, for the slow relaxation time, τ_S, after a voltage jump [7] at 25°C, $\rho_{\tau_S} = 2.6/2.6 = 1$ and for the pore state distributions, p_ν [3,7] $\rho_{p_\nu} = 0.93/1 = 0.93$. It has been shown by Boheim [3,7] that $\rho \approx 0.93$ seems to reflect an intrinsic dipole moment of the alamethicin molecules which corresponds to two opposite charges separated by a distance of the length of the pore. On the other hand, $\rho_\lambda \approx 0.7$ is the value (at 25°C) for the multi-pore conductance under steady-state conditions. Smaller values $\rho_{V_c^{cp}} \approx 0.3$—$0.5$ are obtained with the charge-pulse method, since a steady-state in λ is not reached at the continuously changing membrane voltage. The $\lambda(V)$

dependency is not as pronounced under these dynamic conditions as it is under steady-state conditions. Zickler et al. [9] used values of $\rho \approx 0.7$—0.9 to estimate the light-induced electrical potential at the functional membrane of photosynthesis. If our bilayer experiments are used as a basis for the interpretation of the thylakoid studies, i.e., if under the given conditions of approximately 0.01 M ionic strength a value of $\rho_{V^{c}p} \approx 0.5$ is assumed, a maximum potential difference, $\Delta\phi_o$ induced by a single-turnover flash of $\Delta\phi_o \approx 200$ mV is estimated. This value differs by a factor of 4 from $\Delta\phi_o \approx 50$ mV obtained by another method [17]. Thus, the agreement is quite unsatisfactory. Besides the arguments given elsewhere [9] we wish to draw attention to the following point. Whereas the properties of alamethicin-induced pores are well known for planar bilayers, it is, however, not the case for small cells. Since probably not more than one pore per thylakoid is formed, its properties are difficult to predict. Therefore, the usefulness of alamethicin as a probe for voltage-dependent effects in biological cells has to be tested by calibrating experiments with adequate small systems. Furthermore, a change in lipid composition can shift the characteristic voltage under otherwise identical conditions as much as 100 mV and also the mean life-times of the pores and pore states depend appreciably on the type of lipid [3,5,7]. Since it is not known how lipids are organized in the thylakoid, it is difficult to estimate its influence. However, a comparison of the time scale of the fast voltage decay shows that the decay rate is only slightly slower in case of the thylakoid membrane [9].

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