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STRUCTURAL AND MEMBRANE MODIFYING PROPERTIES OF SUZUKACILLIN, A PEPTIDE ANTIBIOTIC RELATED TO ALAMETHICIN

PART B. PORE FORMATION IN BLACK LIPID FILMS

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SUMMARY

Suzukacillin, a polypeptide consisting of presumably 23 amino acids and 1 phenylalaninol, is produced by a *Trichoderma viride* strain No. 1037 and it can be isolated from the culture medium. It shows membrane-modifying properties similar to those of alamethicin. Discrete conductance fluctuations indicate the formation of oligomer pores of varying diameter. On the basis of voltage jump relaxation experiments evidence is given that the dimer is the nucleation state from which pore formation starts and the oligomer disappears. According to the voltage-current characteristics, voltage-dependent and voltage-independent conductances are observed. A slow process is involved, which can be interpreted as a change in the equilibrium distribution between different conformations of the suzukacillin monomer at the membrane interphase. This change results from its interaction with the lipid matrix. Differences in experimental observations between suzukacillin and alamethicin are attributed to the relatively larger α-helix and higher number of aliphatic side chains of the suzukacillin monomer and to a more intense interaction with the lipid membrane. This leads to a higher probability of forming dimers from monomers and to the occurrence of "inactivation".

INTRODUCTION

The phenomena induced by alamethicin in black lipid membranes created a lot of interest since the original report of Mueller and Rudin [1]. More insight into its way of action has been given by recent investigations, especially those on single-channel fluctuations [2–9].

Melling and McMullen [10] showed the natural alamethicin (antibiotic U-22.324; Upjohn Co., Kalamazoo), which was discovered in the culture broth of the fungus *Trichoderma viride* by Meyer and Reusser [11] to be a mixture of at least

two components. According to their R_F-value on a thin-layer chromatography plate with the solvent system chloroform/methanol/water (65 : 24 : 4, by vol.) they were labeled AL 30 (main) and AL 50 (minor) fractions. Both fractions show similar behavior in lipid bilayer membranes [6].

Different from these compounds is suzukacillin produced by strain No.1037 of *T. viride* belonging to the same genus as No. 63 Cl [12, 13]. The thin-layer chromatography test shows suzukacillin to differ from alamethicin in that it has an R_F value of about 40 under similar conditions. In the preceding part A of our investigation we described sequential and conformational studies on suzukacillin [14]. In this part B of the paper we will demonstrate two facts: first that in lipid membranes suzukacillin acts in a way similar to alamethicin, second that the data obtained from voltage jump-current relaxation experiments are described by the oligomeric pore model without any additional assumption.

OLIGOMER PORE THEORY

From the statistical analysis of single-pore fluctuations of alamethicin-doped black lipid membranes and from model calculations which explain excitability phenomena observed with the alamethicin system, an oligomer pore model has been proposed by Boheim [6] and Baumann and Mueller [5], respectively, where transitions to the next higher or lower pore state occur by uptake or release of one monomer. The main voltage-dependent step is the insertion of this monomer into the membrane after complexation with a cation. After orientation of the molecule perpendicular to the membrane interface the cation is released to the opposite membrane side and the uncomplexed monomer may oligomerize within the membrane before returning to the initial interface [6].

At least two alternative models have been taken into consideration by others. Gordon and Haydon [7, 8] proposed the active aggregate to be a group of parallel channels, which open and close in a somehow statistical way. On the other hand Hall [9] claims alamethicin to form stable two-dimensional micelles, which are pulled into the membrane by the electric field and then fluctuate among various conformations.

A discussion about these different models will be presented in a forthcoming paper (Boheim, G., in preparation). The intention of the paper will be to demonstrate that data obtained from single channel fluctuation measurements and many channel relaxation measurements can be described by the same set of parameters on the basis of the oligomer pore model. It should be noted that the efficiency of the other proposed models concerning quantitative descriptions has not yet been tested.

In the following we will show that the experimentally observed time course of a current relaxation after a voltage jump is exhibited by an oligomer pore system under assumptions consistent with single channel data. The equilibrium concentrations of different oligomer pore states are given [6] by

$$N = N_s \cdot \exp\left\{\frac{\alpha_m FV - \Delta G_m}{RT}\right\} \tag{1}$$

$$N_\nu = N \cdot \Gamma^{\nu+1} \cdot \exp\left\{(\nu+1)\frac{\alpha \cdot FV - \nu \cdot \Delta G_R/2}{RT}\right\} \tag{2}$$

$\nu = 0, 1, 2, \ldots$

with

$$\Gamma = \frac{N_s}{N^*} \cdot \exp\left\{-\frac{\Delta G_0 + \Delta G_m}{RT}\right\} \tag{3}$$

$$\alpha = \alpha_m + \alpha_p \approx \alpha_m \tag{4}$$

$$\Delta G_\nu = \Delta G_0 + \nu \cdot \Delta G_R \tag{5}$$

The time dependence is given by the following set of differential equations:

$$\frac{dN}{dt} = k_{sm} N_s - k_{ms} N - k_{m0} N^2 + 2k_{0m} N_0 + \sum_{\nu=1}^{\infty} (-k_{\nu-1,\nu} N N_{\nu-1} + k_{\nu,\nu-1} N_\nu) \tag{6}$$

$$\frac{dN_0}{dt} = k_{m0} N^2 - k_{0m} N_0 - k_{01} N N_0 + k_{10} N_1 \tag{7}$$

$$\frac{dN_\nu}{dt} = k_{\nu-1,\nu} N N_{\nu-1} - k_{\nu,\nu-1} N_\nu - k_{\nu,\nu+1} N N_\nu + k_{\nu+1,\nu} N_{\nu+1} \tag{8}$$

$\nu = 1, 2, 3, \ldots$

N_s: concentration per cm^2 of monomers at the membrane interface; N: concentration per cm^2 of monomers in the membrane; N_0: concentration per cm^2 of nonconducting dimers; N_ν: concentration per cm^2 of conducting oligomers in state ν; N^*: standard concentration in $mol \cdot cm^{-2}$; F: Faraday constant; R: gas constant; T: absolute temperature; V: applied voltage. The voltage is designated positive, if the more positive potential is applied on the side where the antibiotic has been added; $\alpha_m \cdot FV$: energy (per mol) due to the transfer of one elementary charge across the fraction α_m of the membrane thickness (dipole orientation); $\alpha_p \cdot FV$: voltage dependent part of the energy (per mol) associated with the uptake and release of an uncomplexed monomer by the pore; ΔG_m: change in Gibbs free energy per mol associated with insertion of monomers from the interface into the membrane interior; ΔG_0: change in Gibbs free energy per mol associated with dimerization within the membrane phase; ΔG_ν: change in Gibbs free energy per mol associated with uptake and release of a monomer by an oligomer of state ν; ΔG_R: energy per mol which is required to enlarge the channel in the membrane against compressive forces; k_{sm}, k_{ms}: rate constants of monomer insertion from interphase into membrane interior; k_{m0}, k_{0m}: rate constants of dimerization process (nucleation); $k_{\nu-1,\nu}$, $k_{\nu,\nu-1}$: rate constants of oligomerization processes (growth); $\nu = 1, 2, 3, \ldots$

The relation between the measured current density I $[A \cdot cm^{-2}]$ and the pore density N_p in the membrane of a many pore system can be written as

$$I = V \cdot \bar{\Lambda}(V) \cdot N_p \tag{9}$$

with

$$\bar{\Lambda}(V) = \sum_{\nu=0}^{\infty} p_\nu \Lambda_\nu \text{ : mean conductance of a single pore} \tag{10}$$

$$N_p = \sum_{\nu=0}^{\infty} N_\nu : \text{total concentration of pores per cm}^{-2} \tag{11}$$

$$p_\nu = \frac{N_\nu}{N_p} : \text{probability of finding a pore in state } \nu \tag{12}$$

Λ_ν: conductance of pore state ν

Due to the voltage dependence of p_ν, which results from a shift of the most probable state to higher oligomeric states with increasing voltage, $\bar{\Lambda}$ becomes a function of voltage. The time dependence of $N_p(t)$ during the course of a relaxation experiment is given by

$$\frac{dN_p}{dt} = \sum_{\nu=0}^{\infty} \frac{dN_\nu}{dt} = k_{m0} N^2 - k_{0m} N_0 \tag{13}$$

With $N_0 = p_0 N_p$ we obtain

$$\frac{dN_p(t)}{dt} = k_{m0} N^2 - k_{0m} p_0 \cdot N_p(t). \tag{14}$$

Considering the experimental results given below two cases will be analyzed in more detail. (1) A relaxation curve corresponding to a first order reaction is observed. With

$$\mu = k_{m0} N^2 = \text{constant} \tag{15}$$

$$\sigma = k_{0m} p_0 = \text{constant} \tag{16}$$

Eqn. 14 becomes identical with the equation for a first order reaction which has been used by Eisenberg et al. [4]. Eqn. 15 implies the monomer concentration N to be constant during the observed first order relaxation process. This is the case if the insertion of monomers into the membrane is very fast compared to the dimerization process and, furthermore, if either N or the interfacial concentration N_s is large compared to the quantity of monomers of which the oligomer pores consist. The alternative possibility of rapid molecule exchange between water phase and membrane interphase seems to be unlikely because of the very amphiphatic nature of the antibiotic molecule. Eqn. 16 is valid, if p_0 is constant. This means that during the observed first order relaxation the pore state distributions are in equilibrium which they have attained in a fast process. If we assume k_{m0} and k_{0m} to be approximately voltage independent, μ should reflect the voltage dependence of N^2 and σ that of p_0. It should be mentioned that the observed first order process cannot be the consequence of a slow insertion of the monomers into the membrane, which is followed by fast dimerization and oligomerization processes. In this case because of the coupled oligomerization processes an S-shaped time course had to be expected.

To obtain a rough estimate for the voltage dependence of N_p which is necessary to compare our evaluation with that one of Mueller and Rudin [1] and Eisenberg et al. [4] obtained with alamethicin we consider a voltage range, where the index ν of the most probable pore state does not change by more than 1 (with alamethicin this

interval works out to be 40–50 mV [6]). There we introduce a mean value $\bar{\nu}$ (which is not necessarily an integral number) by Eqns. 1, 2 and 4

$$N_p = \sum_{\nu=0}^{\infty} N_\nu = \sum_{\nu=0}^{\infty} N_s \cdot \Gamma^{\nu+1} \cdot \exp\left\{(\nu+2)\frac{\alpha FV}{RT} - \frac{\Delta G_m + (\nu+1)\cdot\nu\cdot\Delta G_R/2}{RT}\right\}$$

$$\approx N_s \cdot \Gamma^{\bar{\nu}+1} \cdot \exp\left\{(\bar{\nu}+2)\frac{\alpha FV}{RT} - \frac{\Delta G_m + (\bar{\nu}+1)\cdot\bar{\nu}\cdot\Delta G_R/2}{RT}\right\} = N_{\bar{\nu}} \tag{17}$$

If N_{p0} ($V = 0$) is the initial pore density corresponding to a current I_0 and $N_{p\infty}$ (V) the pore density corresponding to the final steady-state current I_∞ after a voltage jump from 0 voltage to V, we may write for the voltage dependence

$$N_{p\infty} - N_{p0} \approx N_{\bar{\nu}\infty} - N_{\bar{\nu}0} \sim \exp\left\{(\bar{\nu}+2)\alpha \cdot \frac{FV}{RT}\right\} \tag{18}$$

According to Eqn. 9, as a consequence of the voltage dependence of $\bar{A}$ (V) the ratio $(I_\infty - I_0)/V$ does not reflect the voltage dependence of the pore density N_p. In the case of alamethicin, to a good approximation $\bar{A}$ (V) is a linear function of voltage [6]. For an estimate of the voltage dependence of $\bar{A}$ (V) we shall use an alternative procedure. As mentioned above, the pore state distributions p_ν reach their equilibrium values in a process fast compared to the first order relaxation. If the initial pore density N_{p0} does not change during this short time the ratio I_0/V yields the voltage dependence of $\bar{A}(V) \cdot I_0$ is the current value obtained by extrapolation to $t = 0$ from the plot of $\ln(I_\infty - I(t))$ versus t.

Following Mueller and Rudin [1] and Eisenberg et al. [4] we describe the voltage dependence of the experimental data by exponential functions

$$\frac{I_\infty - I_0}{V} \sim \exp\left\{\alpha_\infty \cdot \frac{FV}{RT}\right\} \tag{19}$$

$$\bar{A}(V) \sim \frac{I_0}{V} \sim \exp\left\{\alpha_0 \cdot \frac{FV}{RT}\right\} \tag{20}$$

From Eqns. 9, 17, 19 and 20 we thus obtain

$$N_{\bar{\nu}\infty} - N_{\bar{\nu}0} \approx \frac{1}{\bar{A}(V)} \cdot \frac{I_\infty - I_0}{V} \sim \frac{I_\infty - I_0}{I_0} \sim \exp\left\{(\alpha_\infty - \alpha_0)\cdot\frac{FV}{RT}\right\} \tag{21}$$

The comparison with Eqn. 18 yields

$$\alpha_\infty - \alpha_0 = (\bar{\nu}+2)\cdot\alpha \tag{22}$$

$\bar{\nu}$ is an estimate for the most probable pore state.

According to Eqns. 14, 15 and 16 the pore formation rate μ is given by

$$\mu = \sigma \cdot N_{p\infty} \tag{23}$$

With the assumption that k_{m0} is approximately voltage independent we obtain from Eqns. 1 and 4:

$$\mu = k_{m0} N^2 \sim \exp\left\{2\alpha \cdot \frac{FV}{RT}\right\} \tag{24}$$

If we express μ by (with Eqn. 20)

$$\mu = \sigma \cdot \frac{I_\infty}{V} \cdot \frac{1}{\bar{A}(V)} \sim \exp\left\{(\alpha_\mu - \alpha_0) \cdot \frac{FV}{RT}\right\}, \tag{25}$$

we are able to determine α from Eqns. 24 and 25 with the relation

$$\alpha_\mu - \alpha_0 = 2 \cdot \alpha \tag{26}$$

(2) The occurrence of "inactivation" is observed. The inactivation phenomenon describes the decrease in membrane current following a rapid increase, after the voltage had been changed abruptly. This effect has been observed with the current through the sodium channels in the nerve axon membrane [15]. Contrary to case 1, where the experimental conditions have been assumed such that the monomer concentration N in the membrane is constant, possibly because of the presence of a large pool of molecules at the interface, we now consider a different set of experimental conditions where the interfacial concentration changes but the allover number N_s^0 of antibiotic molecules at the membrane interface and within the membrane remains constant.

$$N_s^0 = N_s + N + \sum_{\nu=0}^{\infty} (\nu+2) \cdot N_\nu = \text{constant} \tag{27}$$

Introducing this into Eqns. 6, 7, 8 and 14 we obtain a complicated set of coupled differential equations. An inactivation curve obtained by numerical calculations on the basis of the oligomer model has been published by Baumann and Mueller [5]. Here we want to give only a qualitative picture for its understanding with simplifying assumptions which are in accordance with those of case 1. We assume that the oligomerization (growth) is a fast process compared to the dimerization (nucleation), but a slow process compared to the insertion of monomers into the membrane. We consider the situation where the total number of molecules present in the form of oligomers is comparable to the number of monomers in the membrane.

After a voltage jump a few pores which are present at zero time would grow to higher oligomeric states as a consequence of the increase in N. Following the slow formation of new dimers a redistribution of the antibiotic molecules occurs. Large pores have to become smaller due to the formation and growth of small pores. Experimentally we first expect a fast increase in membrane current and then an additional slower increase or even a decrease. This depends on whether the resulting conductance of the few pores in high oligomeric states is smaller or larger than the conductance of the many pores in low oligomeric states.

MATERIALS AND METHODS

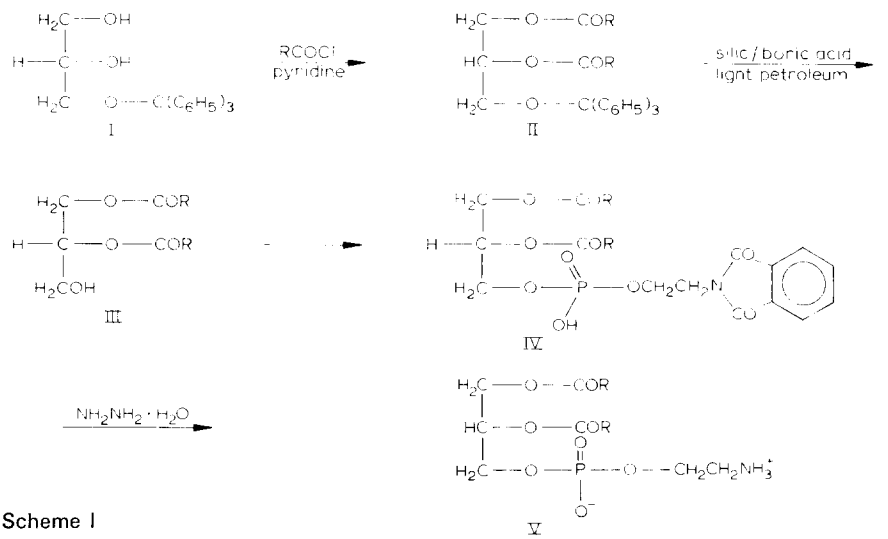
Black lipid membranes were formed from L-α-dioleoyl phosphatidylcholine, L-α-dierucoyl phosphatidylcholine [13] and from D,L-α-dioleoyl phosphatidylethanolamine. Membrane-forming solutions were made of about 1 % (w/v) lipid in *n*-decane.

Synthesis of D,L-α-*dioleoyl phosphatidylethanolamine*

We have carried out the synthesis of D,L-α-dioleoylcephalin starting with D,L-α,β-diolein prepared from D,L-α-tritylglycerol (I) [17] via steps I to III.

Acylation of compound (I) with oleoyl chloride resulted in D,L-α,β-dioleoyl-α′-tritylglycerol (II), whose trityl group was removed by passing through a silicic acid/boric acid chromatograph column with light petroleum. The detritylation as well as the chromatographic separation of diacylglycerol from triphenylcarbinol is a continuous operation and proceeds with little or no acyl migration [18, 19].

D,L-α-Dioleoylcephalin (V) was obtained by phosphorylation of D,L-α,β-dioleoylglycerol (III) with phthalimidoethylphosphoryl dichloride [20] in presence of triethylamine, saponification of the corresponding chloro derivatives and removal of the phthaloyl blocking group of D,L-*N*-phthaloylcephalin (IV) by hydrazinolysis [21]. The purity of cephalin (V) was established by thin-layer chromatography. Compounds (III, IV, V) were purified by chromatography on silica gel (Merck Art. 7734).



Scheme I

The structure elucidation and conformational studies of suzukacillin are described in the preceding paper [14]. Pure suzukacillin A checked by thin-layer chromatography was added from ethanolic stock solutions of $2 \cdot 10^{-4}$ or $2 \cdot 10^{-3}$ g/ml, respectively, in amounts of 10–35 μl to 10 ml aqueous solution. KCl salt solutions were 1 M, unbuffered (pH approx. 6).

The measuring assembly is described elsewhere [6]. Single channel experiments were made with a Teflon cell containing a hole of 0.4 mm diameter, whereas relaxation curves and current-voltage characteristics were obtained with experimental cells containing a hole of 1 mm diameter. The area of the black membrane was determined via a calibrated scale in the ocular of the microscope. Aqueous solutions were stirred by Teflon-coated steel bars. Stirring was stopped after 15 min and time allowed for the system to reach a steady state.

Current and voltage were measured separately with four Ag/AgCl electrodes.

In asymmetric systems voltage will be designated positive, if the more positive potential is applied on the side of suzukacillin addition. Current direction is defined as positive for cation transfer from the suzukacillin containing compartment to the opposite one. Temperature was measured by a Pt-resistance thermometer inserted into one aqueous compartment. The values were accurate within ± 1 °C.

RESULTS

(*a*) *Single channel experiments*

Single channel fluctuations of suzukacillin have been observed with di-(18 : 1)-phosphatidylethanolamine in 1 M KCl. Fig. 1 shows the typical sequence of stepwise current changes under two different conditions. The fluctuations were obtained with the same membrane, whereby the cooling-down procedure took about 30 min. The conductances Λ_ν of pore states ν and their ratios are listed in Table I. These two sets of conductance values which have been obtained at 7 and 19 °C differ by a factor of about 1.75. Analogously to the situation with alamethicin, pore states have been identified from the conductance ratios of neighboring pore states assuming that these ratios do not change much with temperature. In both cases a voltage of 130–150 mV had to be applied to create pores. Then voltage was rapidly reduced until only one pore remained active. With suzukacillin this procedure to observe single pore fluctuations was less successfull than with alamethicin. Either the pore closed after a short

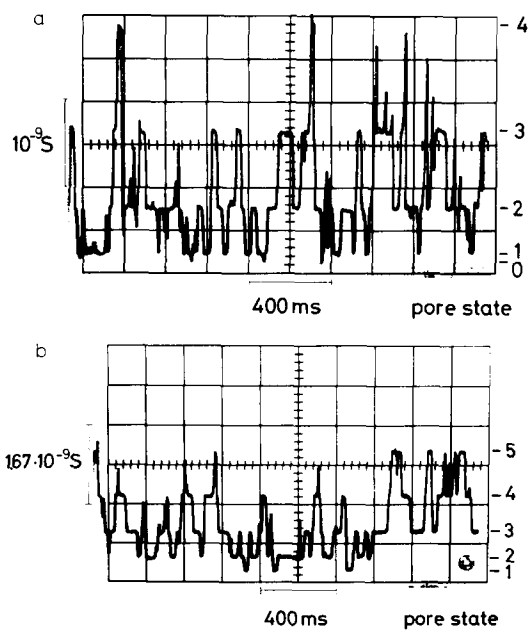


Fig. 1. Current fluctuations of a suzukacillin-doped black lipid membrane as a function of time in the presence of a single conducting channel. Membrane solution: 1.25 % di-(18 : 1)-phosphatidylethanolamine in *n*-decane; salt solution: 1 M KCl; antibiotic concentration: $2 \cdot 10^{-7}$ g/ml suzukacillin in one compartment only; temperature: (a) 19 °C, (b) 7 °C; applied voltage; (a)+100 mV, (b)+30 mV.

TABLE I

PORE-STATE CONDUCTANCES Λ_ν AS CALCULATED FROM THE CURRENT FLUCTUATIONS (Fig. 1)

Pore state ν	19 °C, 100 mV		7 °C, 30 mV	
	$\Lambda_\nu[10^{-10}S]$	$\Lambda_\nu : 1/\Lambda_\nu$	$\Lambda_\nu[10^{-10}S]$	$\Lambda_\nu : 1/\Lambda_\nu$
1	1.0	6.2	(0.5)	(6.4)
2	6.2	2.4	3.2	2.7
3	15.0	1.81	8.7	1.85
4	27.1	–	16.1	1.59
5	–	–	25.6	–

fluctuation period or, in most cases, additional pores appeared which made the analysis impossible. Therefore, a complete statistical analysis of suzukacillin pore fluctuations has not yet been made.

(b) Voltage-current characteristics

Two different voltage-current characteristics have been obtained at different times after suzukacillin addition to a black membrane system. Constant voltages had been applied in steps across the membrane and the corresponding current was recorded (Fig. 2). The length of the bars represents the largest steady-state current noise amplitude. A large increase in membrane conductance with increasing voltage of both signs can be seen. Corresponding to the time course first a voltage dependent conductance appears. After several minutes an increase in zero voltage conductance

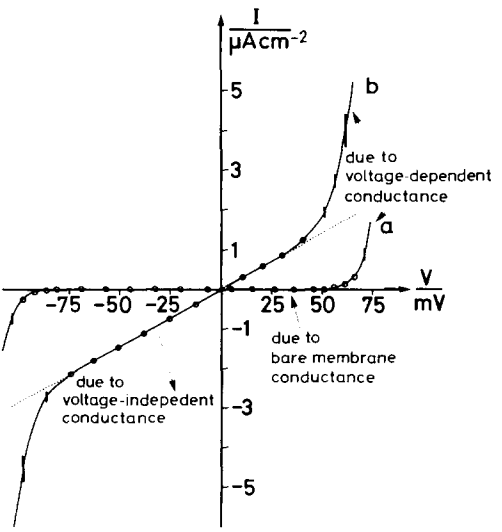


Fig. 2. Voltage-current characteristic of a suzukacillin-doped black lipid membrane at different times after suzukacillin addition: (a) after 5 min; (b) after 60 min. Membrane solution: 1 % di-(18 : 1)-phosphatidylcholine in *n*-decane; salt solution: 1 M KCl; antibiotic concentration: $3 \cdot 10^{-7}$ g/ml suzukacillin in one compartment only; temperature: 25 °C.

of up to two orders of magnitude is observed. The same occurs with alamethicin-doped lipid membranes. There these two parts have been labeled voltage-dependent and voltage-independent conductance [3, 22]. In case (a) of Fig. 2 the current was chosen to be not higher than 1 $\mu A \cdot cm^{-2}$ to prevent hysteresis effects. In case (b) the hysteresis was within the deviations of the noise bars.

(*c*) *Current relaxation experiments*

Current relaxation curves after an abrupt voltage jump [4, 23, 24] have been recorded and analysed. Typical relaxation curves with suzukacillin modified lipid membranes are represented in Figs. 3 and 4. Relaxation experiments have been carried out under these two different conditions demonstrated in Fig. 2a and 2b.

Under conditions of low voltage-independent conductance the properties of the voltage-dependent conductance have been studied. Curves corresponding to a first order relaxation process are observed (Fig. 3a). Series of relaxation experiments have been carried out in the following way. At the highest voltage measured, the membrane conductance has been observed until a steady state was attained with respect to the final conductance I_∞/V at this voltage and to the course of relaxation curves for jumps from 0 mV to this voltage, respectively. Then at distinct intervals relaxation experiments have been recorded with voltage steps of decreasing amplitude, starting from zero voltage. Data obtained in this way showed a reproducibility of the

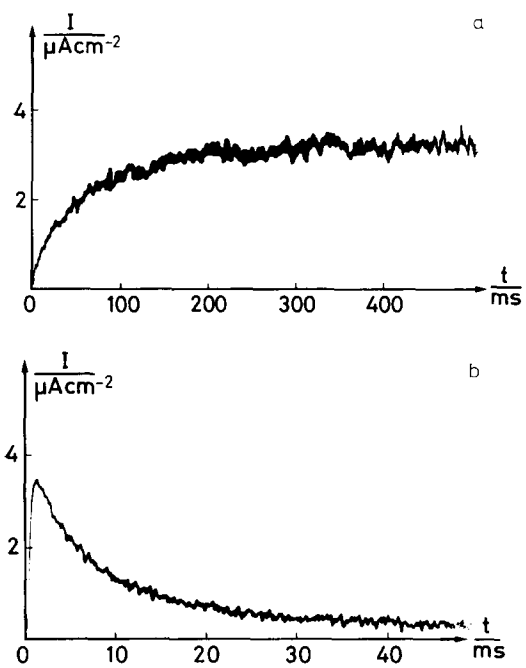


Fig. 3. Current relaxation after a voltage jump in the presence of a low voltage-independent conductance. Membrane solution: 1 % di-(18 : 1)-phosphatidylcholine in *n*-decane; salt solution: 1 M KCl; antibiotic concentration: (a) $7 \cdot 10^{-7}$ g/ml suzukacillin on both sides, (b) $3 \cdot 10^{-7}$ g/ml suzukacillin in one compartment only; temperature: (a) 25 °C, (b) 11 °C; applied voltage: (a) 0 mV to 40 mV, (b) 0 mV to 140 mV.

TABLE II

CONDUCTANCES AND RECIPROCAL TIME CONSTANTS FROM RELAXATION EXPERIMENTS WITH SUZUKACILLIN-MODIFIED LIPID MEMBRANES

I_0 is the extrapolated initial and I_∞ the final current of a relaxation curve. Membrane solution: 1 % di-(18 : 1)-phosphatidylcholine in *n*-decane; salt solution: 1 M KCl; antibotic concentration: (a) $5 \cdot 10^{-7}$ g/ml suzukacillin on both sides; (b) $3 \cdot 10^{-7}$ g/ml suzukacillin in one compartment only.

V[mV]	(a) 25 °C			(b) 11 °C		
	$\frac{I_\infty}{V}\left[\frac{\mu S}{cm^2}\right]$	$\sigma = \frac{1}{\tau}\,[s^{-1}]$	$\frac{I_0}{V}\left[\frac{\mu S}{cm^2}\right]$	$\frac{I_\infty}{V}\left[\frac{\mu S}{cm^2}\right]$	$\sigma = \frac{1}{\tau}\,[s^{-1}]$	$\frac{I_0}{V}\left[\frac{\mu S}{cm^2}\right]$
20	–	–	–	5.55	1.7	1.6
35	13.0	14.1	7.9	78.0	0.44	2.4
40	24.4	11.3	9.0	–	–	–
45	47.8	8.6	10.3	–	–	–
50	107.0	6.0	11.6	1860.0	0.092	–

slope of I_∞/V versus V and of σ versus V within ± 10 %, whereas the absolute values could differ by a factor of 5–10 for different membranes, depending on the pretreatment of the membrane.

In Table II two series of relaxation experiments are listed to demonstrate that the same voltage dependence is observed under two different sets of conditions. In case (a), starting with a jump from 0 to 50 mV, the final voltage was stepwise reduced by 5 mV at 1 min intervals. The duration of the voltage impulse was 2 s, i.e. at least 10 times the relaxation time. In case (b) it was started with the same voltage jump, but the final voltage was reduced by 15 mV at 5 min intervals. The duration of the voltage impuls was 50 s, which is about 5 times the longest relaxation time. Both, the final conductances I_∞/V and the reciprocal relaxation times $\sigma = 1/\tau$ show a pronounced voltage dependence, whereas the voltage dependence of the initial conductance I_0/V is relatively weak.

The exact analysis of the current course at short times shows at least one further relaxation process with a much shorter relaxation time.

At low suzukacillin concentrations, where the voltage-dependent conductance appears only at very high voltages, the occurrence of inactivation can be observed under the condition of low voltage-independent conductance. Fig. 3b shows that the membrane conductance increases after a voltage jump from 0 to 140 mV according to a fast process, but then decays following again the time course of a first order reaction.

Under the condition of a large voltage-independent conductance relaxations of the membrane current have also been observed. If the molecular mechanism for the voltage-dependent and voltage-independent conductance were completely different, we would expect the instantaneous appearance of the current that results from the voltage-independent conductance and starting from this level the slow first order relaxation process which is characteristic of the voltage-dependent conductance. Fig. 4 demonstrates the existence of a fast relaxation process, which is followed by a much slower one. Depending on the applied voltage the current still continues to increase (Fig. 4a) or passes through a maximum value (Fig. 4b). Thus the experi-

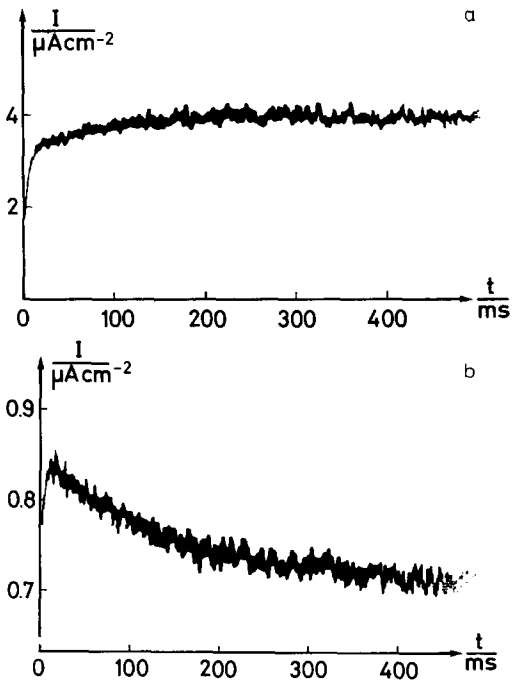


Fig. 4. Current relaxation after a voltage jump in the presence of a large voltage-independent conductance. Membrane solution: 1 % di-(18 : 1)-phosphatidylcholine in *n*-decane; salt solution: 1 M KCl; antibiotic concentration: $7 \cdot 10^{-7}$ g/ml suzukacillin on both sides; temperature: 25 °C; applied voltage: (a) 0 –25 mV, (b) 0–12 mV.

mental data indicate that the qualitative observations are the same despite different levels of voltage-independent conductance.

The additional effect which complicates the analysis of pore formation with suzukacillin and also with alamethicin [22] is a slow interfacial process. Following the induction of a high conductance with an appropriate voltage, the steady-state conductance I_∞/V observed at a lower voltage decreases with time. Table III shows an example for the time dependence of the quantity $(I_\infty - I_0)/V$. The membrane had

TABLE III

DECREASE OF VOLTAGE-DEPENDENT CONDUCTANCE WITH TIME

At intervals of 10 min, 30-mV impulses of 0.2 min duration have been applied, starting from zero voltage. $I_0/V = 7.6\ \mu S \cdot cm^{-2}$ (see text for details of the experiment). Membrane solution: 1 % di-(22 : 1)-phosphatidylcholine in *n*-decane; salt solution: 1 M KCl; antibiotic concentration: $2 \cdot 10^{-6}$ g/ml suzukacillin on both sides; temperature: 25 °C.

t [min]	$I_\infty - I_0/V$ [$\mu S \cdot cm^{-2}$]
10	7.8
20	5.0
30	3.8
40	2.3

attained a steady state at 60 mV. Then the voltage was reduced to 0 mV. At intervals of 10 min, 30 mV impulses of 0.2 min duration have been applied. The time constant for an e-fold decrease of the final minus initial conductance at 30 mV is calculated to be about 26 min, whereas the initial conductance did not change during the observation time.

DISCUSSION

The single pore fluctuations show that suzukacillin acts in a similar way to alamethicin. A pore is created if the positive potential is applied on the side of suzukacillin addition. Furthermore, the conductance ratios of neighbouring pore states are the same for both antibiotics. In Table I the two conductance sets differ by a factor of about 1.75. If this is accounted for by the temperature difference (assuming that the single channel conductances are ohmic), an activation energy of the mobility of the transported ions in the pore of 7.4 kcal/mol is calculated. A similar value has been found for gramicidin A in di-(18 : 1)-phosphatidylcholine membranes by Bamberg and Läuger [25]. The temperature dependence of the conductivity of a 1 M KCl solution in water corresponds to an activation energy of 4.3 kcal/mol within the temperature range considered. The main difference to alamethicin is the failure in observing one single pore for a sufficiently long time which makes it difficult to carry out a statistical analysis with suzukacillin.

The voltage-current characteristics of Fig. 2 demonstrate that suzukacillin induces voltage-dependent as well as voltage-independent conductances. They appear in the same time sequence as with alamethicin [22]. The occurrence of a voltage-independent conductance seems to be the consequence of the ability of suzukacillin to create pores without an applied voltage. Evidence is given by Fig. 4 that they are of the same structure as the pores in the voltage-dependent range. If a large number of monomers is present at the interface a distinct part may be inserted into the membrane, possibly without a complexed cation, and form a pore. As there is no evidence for the presence of a strong cation binding site in the molecule we think that in case of complexed monomers the cation is released to the opposite membrane side (more negative potential side), before the uncomplexed monomer may oligomerize within the membrane. This is supported by the experimental observation that suzukacillin acts in 1 M Tris · Cl (pH 7) just in the same way as in 1 M KCl (Boheim, G., unpublished results). The slow development of the voltage-independent conductance reflects the time course of an interfacial reaction which precedes the actual pore formation process. With increasing voltage more (cation complexed) monomers are inserted into the membrane leading to much higher pore densities. We will discuss the interfacial effect more extensively later.

The results of relaxation experiments with suzukacillin in the range of the voltage-dependent conductance are equivalent to those of alamethicin [26]. A similar dependence of the steady-state current and the relaxation time on voltage is observed with alamethicin and with suzukacillin. If we evaluate the data of Table II by plotting $\ln [(I_\infty - I_0)/V)]$ versus V (Eqn. 19), we obtain from (a) $\alpha_\infty = 4.7$ and from (b) $\alpha_\infty = 5.0$. From the plot of $\ln (I_0/V)$ versus V (Eqn. 20) for (a) and (b) $\alpha_0 = 0.7$ results. If we plot $\ln (\sigma \cdot I_\infty/V)$ versus V according to Eqn. 25 the result is $\alpha_\mu = 2.6$ (a) and $\alpha_\mu = 2.4$ (b). Introducing these values into Eqn. 26 yields $\alpha = 0.95$ (a) and

$\alpha = 0.85$ (b), which may be compared with $\alpha \approx 0.9$ in case of alamethicin [6, 26]. With the α values estimated above we obtain from Eqn. 22 (a) $\bar{\nu} = 2.2$ and (b) $\bar{\nu} = 3.0$. This means that the most probable pore states found in the voltage range of the relaxation experiments are the second (tetramer) and the third (pentamer). With alamethicin under similar conditions the most probable pore states are the third (pentamer) and the fourth (hexamer) (Boheim, G., unpublished results).

Thus in cases where we observe a relaxation curve describable by a single first order reaction (Fig. 3a) we may conclude that the rate limiting step is the dimerization of monomers inserted into the membrane. This is the nucleation process of pore formation. From μ = constant it is likely to assume that the monomer concentration N remains nearly constant during the time course of the observed first order relaxation process (Eqn. 15), i.e. only a small part aggregates to oligomers. In addition, because N changes with voltage, the insertion reaction has to be fast compared to the dimerization process. From σ = constant we may conclude that in these relaxation experiments the pore-state probabilities p_ν, $\nu = 0, 1, 2, \ldots$ (Eqn. 16) quickly reach their final values. This means that the oligomerization (growth) processes are fast compared with the nucleation reaction. Under these conditions it is allowed to assume a time-independent mean pore conductance.

Fig. 4a demonstrates that in the presence of a voltage-independent conductance at least two relaxation processes exist. The slow relaxation corresponds to the above discussed pore nucleation process. The fast one which might be a series of relaxation processes is supposed to originate from the different oligomerization steps. This conclusion is supported by the experimental observation (Boheim, G., unpublished results) that in the presence of a voltage-dependent conductance at a distinct applied voltage the relaxation curve after an additional voltage jump also shows these two different relaxation processes, as with a voltage-independent conductance. The voltage dependence of the extrapolated current I_0 in Table II also reflects this fast oligomerization reaction.

The occurrence of "inactivation" with suzukacillin is shown in Figs. 3b and 4b. This phenomenon has been demonstrated for monazomycin and investigated on the basis of numerical calculations for an oligomer pore model by Baumann and Mueller [5]. Pores which already exist prior to the application of the voltage rapidly grow to higher oligomers after the monomer concentration N has been changed by the voltage. But then, as a consequence of the formation of new dimers, the mean pore state decreases again, which results in the observed conductance decrease. Figs. 4a and 4b demonstrate that a modification of the applied voltage may shift the relaxation behaviour of the system from a monotonous increasing to an inactivating conductance. In addition, all data presented are compatible with the assumption that the voltage-dependent and the voltage-independent conductance result from the same pore structures.

The analysis in terms of the oligomer pore model shows all experimental data to be explained without any further assumption. The occurrence of fast and slow relaxation processes had to be expected from single channel experiments. The voltage dependence of the slow relaxation time is consistent with the voltage dependence of the p_0 distribution in the case of alamethicin [6]. In addition it has been demonstrated that the slow relaxation time increases with the alamethicin concentration as it is expected for p_0 as a consequence of the voltage-alamethicin concentration correlation [26].

The difference in time scale of Figs. 3b and 4b is explained by a difference in the p_0 value. In the case of low voltage-independent conductance, p_0 is larger than under conditions of large voltage-independent conductance. The mean pore state is higher in the latter case. This effect overcompensates the temperature dependence of k_{0m} (compare Table II).

Finally we will discuss the slow interfacial reaction, which is associated with the development of the voltage-independent conductance (Fig. 2) and with the decay of the voltage-dependent conductance from a high conductance level, which has been previously induced by prolonged application of a high voltage (Table III). The investigation of the structural properties of suzukacillin by Jung et al. [14] has demonstrated a marked α-helical content which has also been shown to be extremely stable for alamethicin [27]. The α-helical part of the molecule is increased from about 20 % to 40 % by a change from a hydrophilic (e.g. hexafluoroacetone · $3H_2O$) to more hydrophobic (*n*-butanol) environment. We may therefore assume that suzukacillin can exist in at least two interfacial conformations, namely, the conformation in water with a relatively low α-helix content of about 20 % and, on the other hand, the more hydrophobic conformation with an α-helix content of about 40 %. In the latter case the interaction of the helical part of the molecule with the lipid matrix may be quite intense [27] and is very temperature dependent. The non-helical part of the antibiotic molecule is likely to be situated at the membrane interphase or may form an integral part of the hydrophilic channel crossing the membrane. The concept of a partly helical pore subunit has been proposed already by Baumann and Mueller [5] for alamethicin.

On the basis of the linear structure of suzukacillin [14] (and of alamethicin [27, 28]) we propose a probable conformation of the monomer unit of the suzukacillin pore within the lipid membrane, which is represented in Fig. 5. The Corey-Pauling-Koltun (CPK) model shows an α-helical range which might be situated apart from the conducting channel and a curved part in which β-turns are stabilized by hydrogen bonds. The length of the latter part is estimated to be about 25 Å. Oligomers might be stabilized by =C= O · · · H–N= hydrogen bonds at the phenylalaninol-glutamine end. As a consequence of this structure the difference between alamethicin and suzukacillin is mainly given by a different length of the α-helical part, where the additional amino acids of suzukacillin are located [14]. Experiments with 1 : 1 mixtures of suzukacillin and alamethicin (AL 30) show similar single pore fluctuations and relaxation curves as seen with suzukacillin or alamethicin alone. The concentration dependence of the steady-state current is nearly the same, if alamethicin is partly replaced by suzukacillin. The same is observed with mixtures of the AL 30 and AL 50 alamethicin components (Boheim, G., unpublished results). This indicates that pore formation is not hindered by the interaction of these different antibiotics and that possibly formation of mixed (hybrid) pores occurs.

A crucial problem is the stabilization of the oligomer pore by interactions between the monomer subunits. According to the subunit model of Fig. 5 no hydrogen bridge can be formed except at the phenylalaninol-glutamine end. This would imply that the dissociation of monomers from the oligomer pore most probably starts near the helical part. This is consistent with the observation that after pore formation the molecule usually returns to the initial membrane interface as pointed out by Eisenberg et al. for alamethicin [4]. Thus the stabilization of the oligomer is achieved to a great

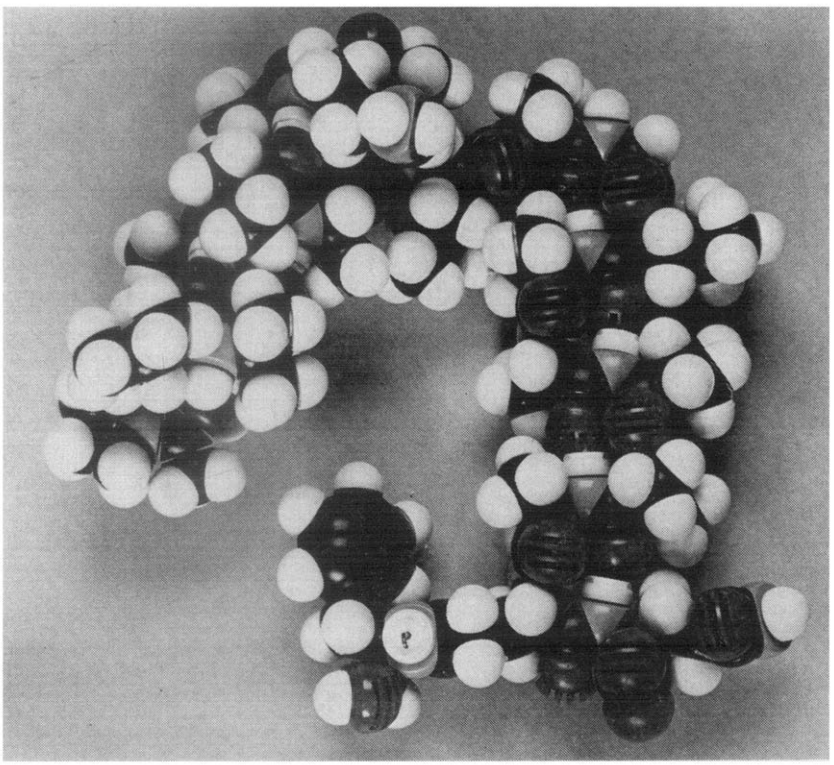


Fig. 5. Proposed conformation of the monomer unit of the suzukacillin pore within the lipid membrane. The Corey-Pauling-Koltun model shows an α-helical range which might be situated apart from the conducting channel and a curved part in which β-turns are stabilized by hydrogen bonds. Oligomers might be stabilized by $=C=O \cdots H-N=$ hydrogen bonds at the phenylalaninol-glutamine end.

deal by the electrostatic interaction of hydrophilic groups in a hydrophobic environment. The weak temperature dependence of the mean life time of the pore states as estimated from Figs. 1a and 1b may give additional support for this interpretation which implies only a small difference in Gibbs free energy between the different pore states.

One may speculate that the slow interfacial process mentioned above may consist in a change of the equilibrium distribution between different suzukacillin monomer conformations of varying α-helix content at the membrane interface. Whereas the growth of the α-helix of a single molecule is a fast process, the transition of an ensemble from the adsorbed state at the water-membrane interface to a state where the monomers interact more intensely with the membrane lipid might be slow. In case of alamethicin such a relatively slow process has been observed in water/ethanol mixtures after the addition of ethanol to the water solution and vice versa [27]. This effect was attributed to an aggregation-dissociation phenomenon.

With the increase of the α-helical content a change in the difference of Gibbs free energy for the interfacial and inserted monomer state is likely to be associated. A large α-helix leads to an intense antibiotic-lipid interaction, to an increased prob-

ability for monomer insertion, and thus to the voltage independent conductance. The slow decrease in the voltage dependent conductance according to Table III could also be associated with changes in the equilibrium distribution between conformations of different α-helix contents.

According to the primary structure of suzukacillin [14] an α-helix could be built up to the second proline. But in this case the remaining amino acids would not be able to bridge the membrane. On the other hand, the second proline and the glycine may act as nuclei for β-bends which would limit the α-helix.

The proposed monomer structure provides a basis for the understanding of the observed differences between suzukacillin and alamethicin. The relatively large lipophilic part of suzukacillin may lead to a more intense antibiotic-lipid interaction and to longer life times for the membrane-inserted monomer state. This in turn increases the nucleation (dimerization) rate. Thus the failure to observe one single pore for a relatively long time may be due to the facilitated dimerization process, i.e. to the enhanced formation of new pores. On the other hand the same effect leads to the inactivation process observed with suzukacillin which can be explained by a shift of the equilibrium between monomers and dimers to the formation of dimers, as discussed above.

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