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TRICHOTOXIN A-40, A NEW MEMBRANE-EXCITING PEPTIDE *

PART B. VOLTAGE-DEPENDENT PORE FORMATION IN BILAYER LIPID MEMBRANES AND COMPARISON WITH OTHER ALAMETHICIN ANALOGUES

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Summary

Trichotoxin A-40 induces voltage-dependent pores in bilayer lipid membranes comparable to those formed by alamethicin and suzukacillin. The conductance values of the trichotoxin A-40 pores are of similar magnitude and show the same characteristic pattern sequence of non-integral multiples of a unit-conductance step as alamethicin and suzukacillin.

However, voltage-jump current-relaxation experiments show significant differences between trichotoxin A-40 and alamethicin and suzukacillin. With trichotoxin A-40 three different relaxation processes could be observed, whereas with alamethicin and suzukacillin only two processes had been distinguished. The fast process in each case is related to pore state transitions and the slower (medium) process to the decay rate of pores. The third very slow process, which is not found with alamethicin and suzukacillin, could not clearly be assigned to a molecular mechanism. Whereas in the case of alamethicin and suzukacillin the relaxation amplitude of the slow process is considerably larger than the relaxation amplitude of the fast process, the reverse is true for trichotoxin A-40, where the largest relaxation amplitude is that of the fast process.

Contrary to alamethicin and suzukacillin, trichotoxin A-40 is soluble in the lipid/decane membrane-forming solution, when added from an ethanolic stock solution. Its bilayer-modifying properties are not changed, whether the antibiotic is added to the aqueous salt solution or to the membrane-forming solution.

Several different analogues of alamethicin, suzukacillin and trichotoxin A-40 have been investigated and compared with respect to the induced current-volt-

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age characteristics in lipid bilayers. A suzukacillin A-derivative where phenylalaninol had been split off is active as well as trichotoxin A-40 which lacks the phenylalaninol group by nature. Different C-terminal groups like -COOH, $-CONH_2$, $-COOCH_3$ and -CO-Ala-Ala-OCH_3 cause qualitative changes but not the loss of the pore-formation property.

Introduction

The formation of voltage-dependent pores of multistate behaviour and the occurrence of excitability phenomena [1] in lipid bilayer membranes by the polypeptide antibiotics alamethicin [1—16] and suzukacillin [17,18], which are produced by different strains of the fungus *Trichoderma viride*, have created considerable interest. In order to elucidate relations between structure and function for this class of antibiotics experiments with natural and semisynthetic analogues are of great importance. Several analogues have been investigated in this paper.

The antibiotic trichotoxin A-40 is produced by the *Trichoderma viride* strain NRRL 5242. Its isolation and characterization is described in part A of this paper [19]. Trichotoxin A-40 differs from alamethicin and suzukacillin in that it consists of only one proline amino acid and that it lacks the phenylalaninol residue. The membrane-modifying properties of trichotoxin A-40 are described in this paper.

Materials and Methods

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

Trichotoxin A-40 has been characterized in the preceding paper [19]. Pure trichotoxin A-40, checked by thin-layer chromatography, was added from stock solutions of 0.1 mg · cm^{-3} in ethanol/water (1 : 9, v/v) or of 1.0 mg · cm^{-3} in ethanol, respectively, in amounts of 10—25 μl to 10 ml aqueous solution. 10 μl from the 1 mg · cm^{-3} stock solution was also added to 100 μl of lipid/decane solution. In contrast to alamethicin and suzukacillin, trichotoxin A-40 did not precipitate in decane. The chemical procedures to obtain the methyl and the Ala-Ala-methyl analogues of trichotoxin A-40 are described in part A of this paper [19].

Suzukacillin A and suzukacillin B, isolated from the *Trichoderma viride* strain NRRL 1037 and separated by thin-layer chromatography, were a gift of Dr. Tadaaki Ooka, Asahi Chemical Industry Co., Ltd., 2-1 Samejima, Fuji-Shi, Shizuoka (Japan). The hydrolysis procedure applied to obtain a phenylalaninol-free analogue of suzukacillin A, which is called suzukacillin A'20, was described by Irmscher and Jung [20]. According to an amino acid-analysis suzukacillin A'20 seems to lack the C-terminal glutamine and phenylalaninol groups and also some residues at the N-terminus including the proline amino-acid. Thus, an α-helix can still be built up with a length comparable to that of alamethicin at the N-terminal end.

Alamethicin R_F 30 and alamethicin R_F 50 were purchased from Microbiological Research Establishment, Porton Down, Salisbury, U.K. and were checked for purity by thin-layer chromatography.

The alamethicin F 50 compound was isolated and purified by Irmscher and Jung [20] from the *Trichoderma viride* strain NRRL 3199. Alamethicin R_F 50 and alamethicin F 50 seem to be identical. According to Martin and Williams [21] alamethicin R_F 30 and alamethicin R_F 50 only differ in the C-terminal -COOH or -$CONH_2$ end groups, respectively.

Salt solutions were 1 M or 0.1 M and were unbuffered. LiCl, NaCl, KCl, RbCl, CsCl, NH_4Cl, Tris · HCl, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, K_2SO_4, Na_2SO_4, $NaNO_3$, $CH_3COONa \cdot 3H_2O$ and $(CH_3COO)_2UO_2 \cdot 2H_2O$ were pure analytical grade from Merck. NH_3CH_3Cl, $NH_2(CH_3)_2Cl$, $NH(CH_3)_3Cl$ and $N(CH_3)_4Cl$ were "zur Synthese"-grade from Merck. $N(CH_3)_4Cl$ was purum and D(+)glucosamin · HCl purissimum from Fluka. CH_3COOTl was LAB-grade from Merck and $LaCl_3$, 99.99% pure, from Schuchardt.

The measuring assembly has been described elsewhere [6]. Single-channel experiments were performed with Teflon cells which had a hole of approx 0.1 mm diameter, whereas relaxation curves and current-voltage characteristics were obtained with experimental cells with a hole of 1 mm diameter. The area of the bilayer membrane was determined via a calibrated scale in the ocular of the microscope. Aqueous solutions were stirred by Teflon-coated steel bars.

Current and voltage were measured by two Ag/AgCl electrodes or by two platinized platinum electrodes. There was no difference if two additional electrodes were used to determine the membrane voltage separately by a differential voltmeter. Voltage will be designated positive if the more positive potential is applied on the front compartment side. Current direction is defined as positive for cation transfer from the front compartment to the rear compartment. Temperature was measured by a Pt-resistance thermometer inserted into one aqueous compartment. The values were accurate within ±1°C.

Results

Single-pore experiments

The single-pore fluctuation pattern of a trichotoxin A-40-modified lipid membrane is shown in Fig. 1. When the record is obtained with a 10 kHz resolution, many short-lived spikes are superimposed on the conductance of a long-lived pore (Fig. 1a). However, at a resolution of 100 Hz the conductance sequence characteristic of a multi-state pore can be seen (Fig. 1b). This multi-state behaviour is comparable to that of alamethicin [2,4,6,11] and suzukacillin [18]. A quantitative comparison of the data obtained with trichotoxin A-40 and alamethicin R_F 30 is presented in Tables I and II. Table I shows that the single-pore state conductances, Λ_ν, are of comparable magnitude, Λ_ν being insignificantly larger in the case of alamethicin R_F 30. The ratios of adjacent pore-state conductances exhibit, therefore, the same typical sequence of a pore whose size varies by uptake or release of identical units. The activation energies of the pore-state conductances $E_A(\Lambda_\nu)$ are estimated to be approx. 20 kJ · mol^{-1} (5 kcal · mol^{-1}) for both antibiotics.

In Table II the data obtained by a statistical analysis of single-pore state

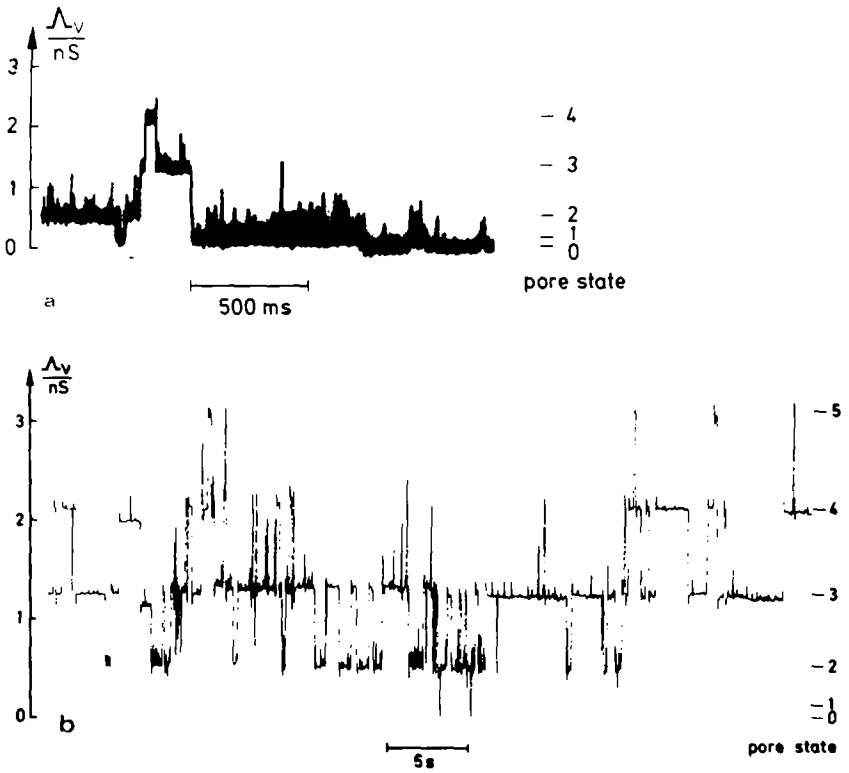


Fig. 1. Single-pore fluctuation pattern of a trichotoxin A-40-modified lipid membrane. (a) The record was obtained at a 10 kHz resolution. A large number of short-lived spikes is superimposed on the conductance of a long-lived pore. (b) At a resolution of 100 Hz the characteristic conductance sequence of a multi-state pore is seen. Membrane solution, 1% di-(18 : 1)-phosphatidylcholine in decane; salt solution, 1 M KCl, unbuffered; antibiotic concentration, nominally 200 ng · cm^{-3} trichotoxin A-40 in both compartments; membrane voltage, (a) 120 mV, (b) 100 mV; temperature, −1°C.

fluctuations [6,13] are listed. The most probable pore state which is observed under the experimental conditions of 1 M KCl, −1°C and 100 mV is $\nu^* = 3$. The ratio $K_\nu/K_{\nu+1}$, which varies around a mean value of about 8, is comparable to that found with alamethicin R_F 30 at the same temperature. At higher temperatures $K_\nu/K_{\nu+1}$ is decreased for alamethicin R_F 30 [6,14]. The most probable pore state at −1°C in the case of alamethicin is $\nu^* \approx 4$ [13,14], i.e., one pore state above ν^* found for trichotoxin A-40. Since the mean lifetimes, τ_ν, of the pore states, ν, depend to some extent on ν^*, the τ_ν values of fluctuating alamethicin pores of similar p_ν-distributions were considered for comparison. The τ_ν of trichotoxin A-40 was twice as large as the corresponding values for alamethicin R_F 30.

Multi-pore experiments

A typical current-voltage characteristic of a trichotoxin A-40-modified phosphatidylcholine membrane is presented in Fig. 2a. It shows the same occurrence of steep conductance increase starting from a distinct voltage as observed with alamethicin [1,4,8,10,13] and suzukacillin [18]. The I/V curve is nearly

TABLE I

SEQUENCE OF SINGLE PORE-STATE CONDUCTANCES, Λ_ν, OF TRICHOTOXIN A-40- AND ALAMETHICIN R_F 30-MODIFIED LIPID MEMBRANES AT DIFFERENT VOLTAGES AND TEMPERATURES

Membrane solution, 1% di-(18 : 1)-phosphatidylcholine in *n*-decane; salt solution, 1 M KCl, unbuffered; antibiotic concentration, nominally 200 ng · cm^{-3} trichotoxin A-40 or 100 ng · cm^{-3} alamethicin R_F 30. The activation energies of pore-state conductances E_A (Λ_ν) are estimated to be approx. 20 kJ · mol^{-1} (5 kcal · mol^{-1}).

Pore-state	Trichotoxin A-40							Alamethicin R_F 30	
	Λ_ν (nS), −1°C				Λ_ν (100 mV)	Λ_ν (nS), 4°C		Λ_ν (nS), 100 mV	
	50 mV	80 mV	100 mV	120 mV	$\Lambda_{\nu-1}$ (100 mV)	80 mV	120 mV	−1°C	4°C
1	0.12	0.11	0.12	0.14		0.13	0.14	0.11	0.12
2	0.48	0.49	0.53	0.54	4.4	0.60	0.67	0.53	0.68
3	1.08	1.12	1.25	1.30	2.4	1.33	1.47	1.35	1.53
4		2.08	2.17	2.18	1.74		2.48	2.22	2.56
5			3.10	3.14	1.43			3.16	3.63
6				4.16				4.19	

TABLE II

PORE-STATE PROBABILITIES (p_ν), FORWARD AND BACKWARD RATE CONSTANTS ($k_{\nu-1,\,\nu}$, $k_{\nu,\nu-1}$) OF TRANSITIONS BETWEEN PORE STATES ($\nu-1$, ν) AND MEAN LIFETIMES (τ_ν) OF SINGLE PORE-STATE FLUCTUATIONS IN THE PRESENCE OF TRICHOTOXIN A-40 AT 100 mV, -1°C

For comparison, τ_ν values of a fluctuating alamethicin pore of similar p_ν-distributions are listed. Conditions of membrane solution, salt solution and antibiotic concentrations are the same as for Table I. Observation time, 128 s (trichotoxin A-40) and 15 s (alamethicin R_F 30); number of events, 404 (trichotoxin A-40) and 98 (alamethicin R_F 30).

Pore-state	Trichotoxin A-40						Alamethicin R_F 30
	p_ν (%)	$K^*_\nu = \frac{p_\nu}{p_{\nu-1}}$	$\frac{K_\nu}{K_{\nu+1}}$	$k^*_{\nu-1,\,\nu}$ (s^{-1})	$k_{\nu,\,\nu-1}$ (s^{-1})	τ_ν (ms)	τ_ν (ms)
1	0.68					73	66
2	20.0	29.4	10.6	5.7	0.15	236	127
3	55.4	2.76	6.7	4.1	1.45	431	185
4	22.7	0.410	7.6	0.83	2.0	356	150
5	1.23	0.054		0.41	7.1	154	94

symmetric with respect to positive and negative voltages because trichotoxin A-40 was present in identical concentrations on both sides of the membrane. With a voltage sweep-rate of 10 s/cycle, i.e., 24 mV $\cdot$ s^{-1}, a slight hysteresis was seen, which disappeared at a reduced sweep-rate of 2.4 mV $\cdot$ s^{-1}.

A half-logarithmic plot of the conductance $\lambda = I/V$ versus voltage for the curve of Fig. 2a, obtained with a voltage sweep-rate of 2.4 mV $\cdot$ s^{-1}, is shown in Fig. 2b (open circles). The voltage-dependent part of the conductance $(\lambda - \lambda_0)$ (where λ_0 is the zero-voltage conductance), which fits into a straight line approximation (Fig. 2b), may be characterized by the parameters V_c and α_λ [13]. V_c is defined by the voltage at which $(\lambda - \lambda_0)$ reaches 100 μS $\cdot$ cm^{-2} for 1 M KCl and a given antibiotic concentration. α_λ is obtained from the slope of the straight line in Fig. 2b by the equation

$$\lambda \propto \exp\{\alpha_\lambda \cdot u\} \qquad (1)$$

where $u = FV/RT$ is the reduced voltage. The evaluation yields for positive voltages $V_c^+ = 47.7$ mV, $\alpha_\lambda^+ = 3.5$ and for negative voltages $V_c^- = 46.2$, $\alpha_\lambda^- = 3.3$. The experimental reproducibility is given by $V_c \pm 5$ mV and $\alpha_\lambda \pm 0.5$. Comparison with data of alamethicin R_F 30 [13] shows that the same V_c value is obtained at a quarter of the concentration of alamethicin (1 M KCl, 25°C) and that α_λ is about twice as large with alamethicin R_F 30 as with trichotoxin-A 40.

As with suzukacillin [18], a zero voltage conductance λ_0 appears in the presence of trichotoxin A-40, but it is even faster, even if the system is maintained at zero voltage. It will be shown that the increase in λ_0 is very much accelerated by a transient application of a voltage across the membrane.

Voltage-jump current-relaxation experiments

The trichotoxin A-40-induced conductance depends on pretreatment of the membrane, as observed with alamethicin [13]. However, λ_0 increases much faster with trichotoxin A-40, especially if a high voltage is applied. Therefore, the experiments have been carried out under conditions of high λ_0.

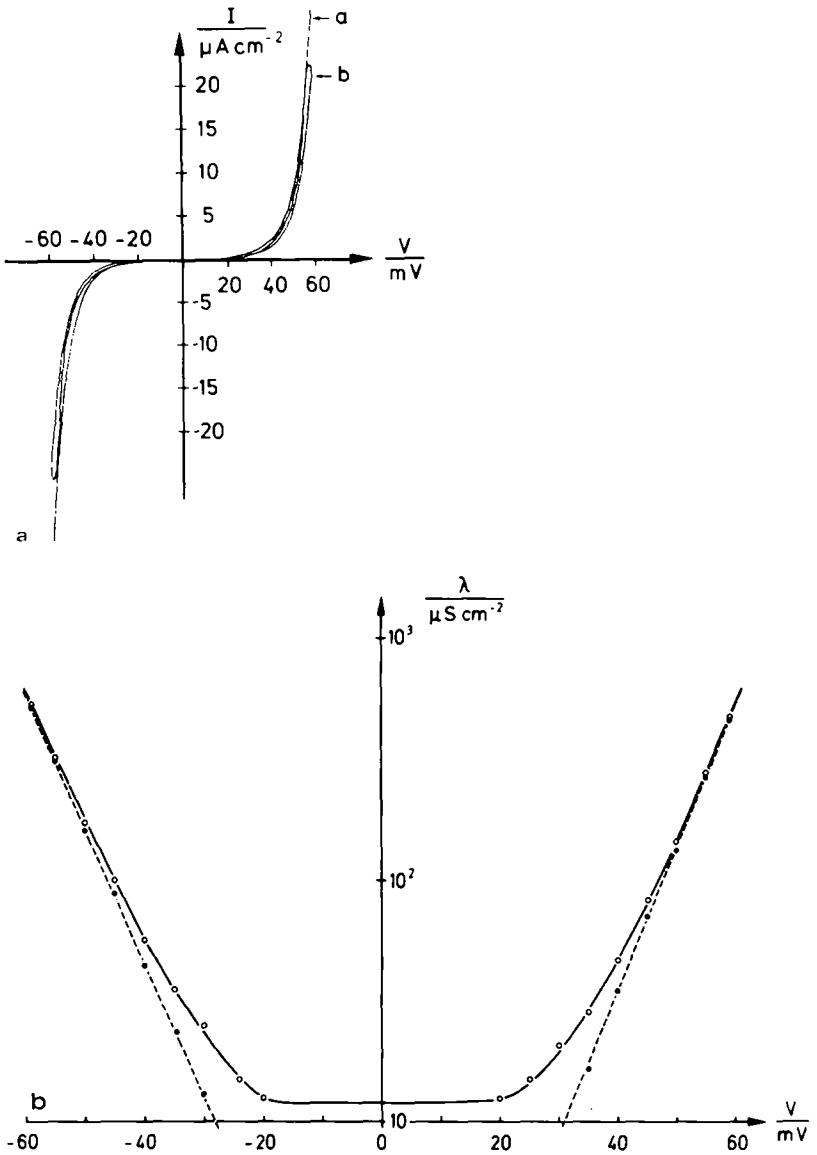
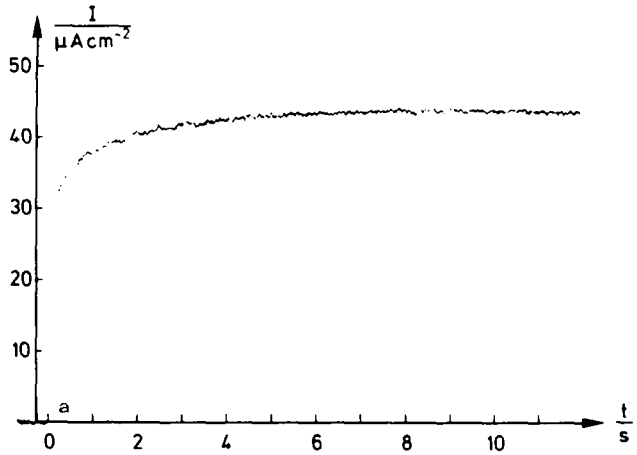


Fig. 2. (a) Current-voltage characteristic of a trichotoxin A-40-modified phosphatidylcholine membrane. The curves were recorded with a voltage sweep rate of (a) 100 s/cycle and (b) 10 s/cycle. Membrane solution, 1% di-(18 : 1)-phosphatidylcholine in decane; salt solution, 1 M KCl, unbuffered; antibiotic concentration, 1.0 $\mu g \cdot cm^{-3}$ trichotoxin A-40; temperature, 25°C. (b) Half-logarithmic plot of the conductance $\lambda = I/V$ of curve (a) versus voltage (open circles). The filled circles are obtained by subtracting the zero voltage conductance $\lambda_0 = 12\ \mu S \cdot cm^{-2}$ from λ. For details and evaluation see text.

A typical time course of a current-relaxation after a voltage-jump is shown in Fig. 3. In contrast to the results obtained with alamethicin [4,10,13] and suzukacillin [18], the current-relaxation curve in the presence of trichotoxin A-40 cannot be described at a low time resolution (in the s range) by a single first-order relaxation process (Fig. 3a). The half-logarithmic plot of the relaxation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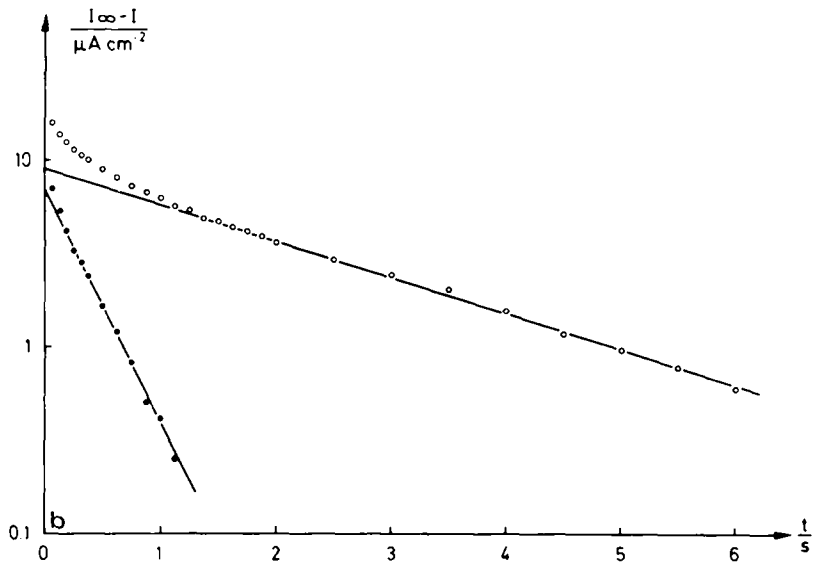
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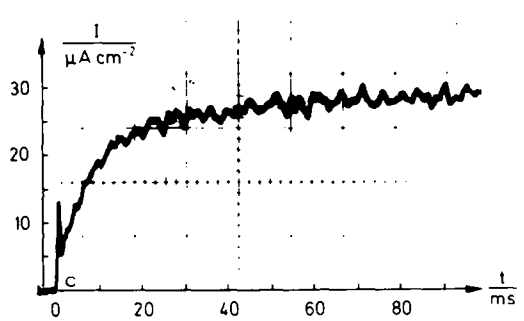
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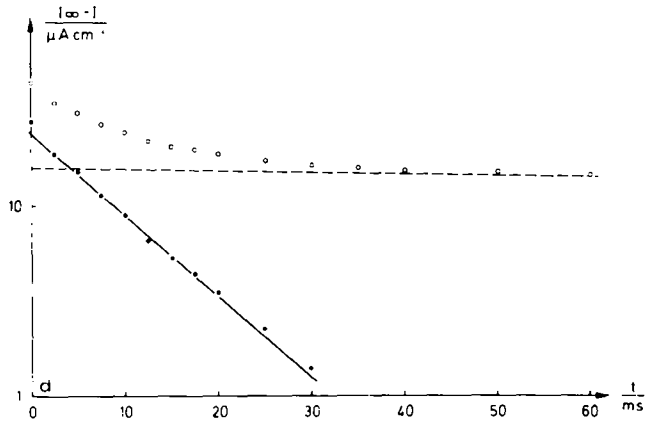

Fig. 3. Current relaxation after a voltage jump from 0 mV to 63 mV. Membrane solution, 1% di-(18 : 1)-phosphatidylcholine in decane; salt solution, 1 M KCl, unbuffered; antibiotic concentration, 1.0 $\mu g \cdot cm^{-3}$ trichotoxin A-40; temperature, 25°C. (a) At a low time-resolution in the s-range the current-relaxation curve does not exhibit a simple first-order relaxation process. (b) A half-logarithmic plot of the relaxation amplitude $I_\infty - I$ versus voltage shows that the curve may be fitted by two relaxation processes of first order. (c) At a time resolution in the 10 ms-range a further relaxation process of large amplitude is observed. (d) A half-logarithmic plot of the relaxation amplitude $I_\infty - I$ versus voltage demonstrates that this fast process may be fitted by a first-order differential equation within the limits of experimental error.

amplitude $I_\infty - I$ versus V shows that at least two first-order relaxation processes exist (Fig. 3b). At an increased time resolution in the 10 ms range a third relaxation is seen (Fig. 3c). This relaxation may be fitted by a single differential equation of first order within the limits of experimental accuracy (Fig. 3d). The fast relaxation process occurs in the same time range as the fast processes observed with alamethicin [13] and suzukacillin [18]. In addition to the appearance of a third relaxation process another feature of the trichotoxin A-40 system becomes apparent. The amplitude of the fast process is larger than those of the slow processes. With alamethicin the amplitude of the single slow relaxation process is considerably larger than that of the fast process [13]. This different behaviour seems to reflect a difference in the pore nucleation properties of these antibiotics as discussed later. The response of the trichotoxin A-40 system to an abrupt change in the voltage polarity was tested. Fig. 4 demonstrates that the conductance which is developed by application of a positive voltage disappeared in a very fast reaction after polarity change. A conductance of comparable amplitude and with the same fast and slow relaxations was observed at the corresponding negative voltage. An abrupt change back to the initial positive voltage gave the same result. This is typical of the alamethicin system [22,23] and of its analogues. The pores exhibit an intrinsic asymmetry which leads to different responses to positive or negative voltages. On the other hand, the voltage dependence of gramicidin A pore-formation is nearly quadratic [24].

(i) Addition of trichotoxin A-40 to the aqueous salt solution. A series of voltage-jump current-relaxation experiments was carried out with trichotoxin A-40 added to the aqueous salt solution. In order to achieve a satisfactory reproducibility of the experimental results during subsequent recordings for a given membrane, the system was maintained at zero voltage for 1 h, and then

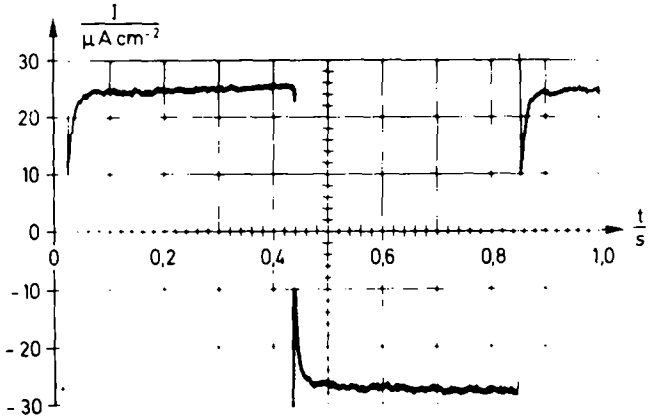

Fig. 4. Response of the trichotoxin A-40 system to an abrupt sign reversal of the applied membrane voltage. The conducting pores exhibit an intrinsic asymmetry. A conductance which appears at the positive voltage of +50 mV vanishes in a very fast reaction after voltage-sign reversal. The development of the conductance at −50 mV procedes just in the same way as at +50 mV. The final conductances at the positive and negative voltage are comparable, since trichotoxin A-40 was present in identical concentrations on both sides of the membrane. Membrane solution, 1% di-(18 : 1)-phosphatidylcholine in decane; salt solution, 1 M KCl, unbuffered; antibiotic concentration, 1.0 $\mu g \cdot cm^{-3}$ trichotoxin A-40; temperature, 25°C.

pretreated by a few voltage pulses from 0 mV to the highest measuring voltage used (63 mV) until a constant value in relaxation time and amplitude was reached. The final voltage was reduced in steps of 6 or 7 mV down to 7 mV. At each voltage 3 relaxation curves were recorded and the whole series was repeated twice. The following quantities have been determined from the analysis of current-relaxation experiments: the relaxation time τ_v, τ_s, τ_f of the very slow, slow and fast relaxation processes, respectively, the final steady-state conductance, λ_∞, and the conductance values λ_{v0}, λ_{s0}, λ_{f0} obtained by extrapolation of the very slow, slow and fast relaxation processes to $t = 0$, respectively. λ_{f0} is identical to the initial conductance $\lambda(t = 0)$. The results are presented in Fig. 5 together with the standard deviation of the experimental data. It can be seen from Fig. 5a that the initial (zero voltage) conductance, λ_0, remains constant, whereas λ_∞ increases steeply with the voltage. The voltage dependence of the quantities determined experimentally is expressed by α_i-parameters in analogy to Eqn. 1 [13]. The value of $\alpha_{(\lambda_\infty - \lambda_0)}$ which applies to the voltage-dependent part $(\lambda_\infty - \lambda_0)$ of the steady-state conductance is listed among other data in Table III.

A comparison of the results obtained with the different antibiotic analogues alamethicin R_F 30, suzukacillin A and trichotoxin A-40 is given in Table III. Suzukacillin A is the most active analogue followed by alamethicin R_F 30 and trichotoxin A-40. The smallest amount of antibiotic needed to induce a conductance of 100 $\mu S \cdot cm^{-2}$ at a reference voltage of $V_c \approx 50$ mV, under otherwise identical conditions was obtained with suzukacillin A. However, it was with alamethicin R_F 30 that the voltage dependence of the steady-state conductance was the strongest and with trichotoxin A-40 the weakest.

In Fig. 5b the amplitudes $\lambda_{v\infty}$, $\lambda_{s\infty}$, $\lambda_{f\infty}$ of the very slow, slow and fast relaxation processes, respectively, are drawn. The amplitudes are given by $\lambda_{v\infty} = \lambda_\infty - \lambda_{v0}$, $\lambda_{s\infty} = \lambda_{v0} - \lambda_{s0}$ and $\lambda_{f\infty} = \lambda_{s0} - \lambda_0$. It is observed that the amplitude

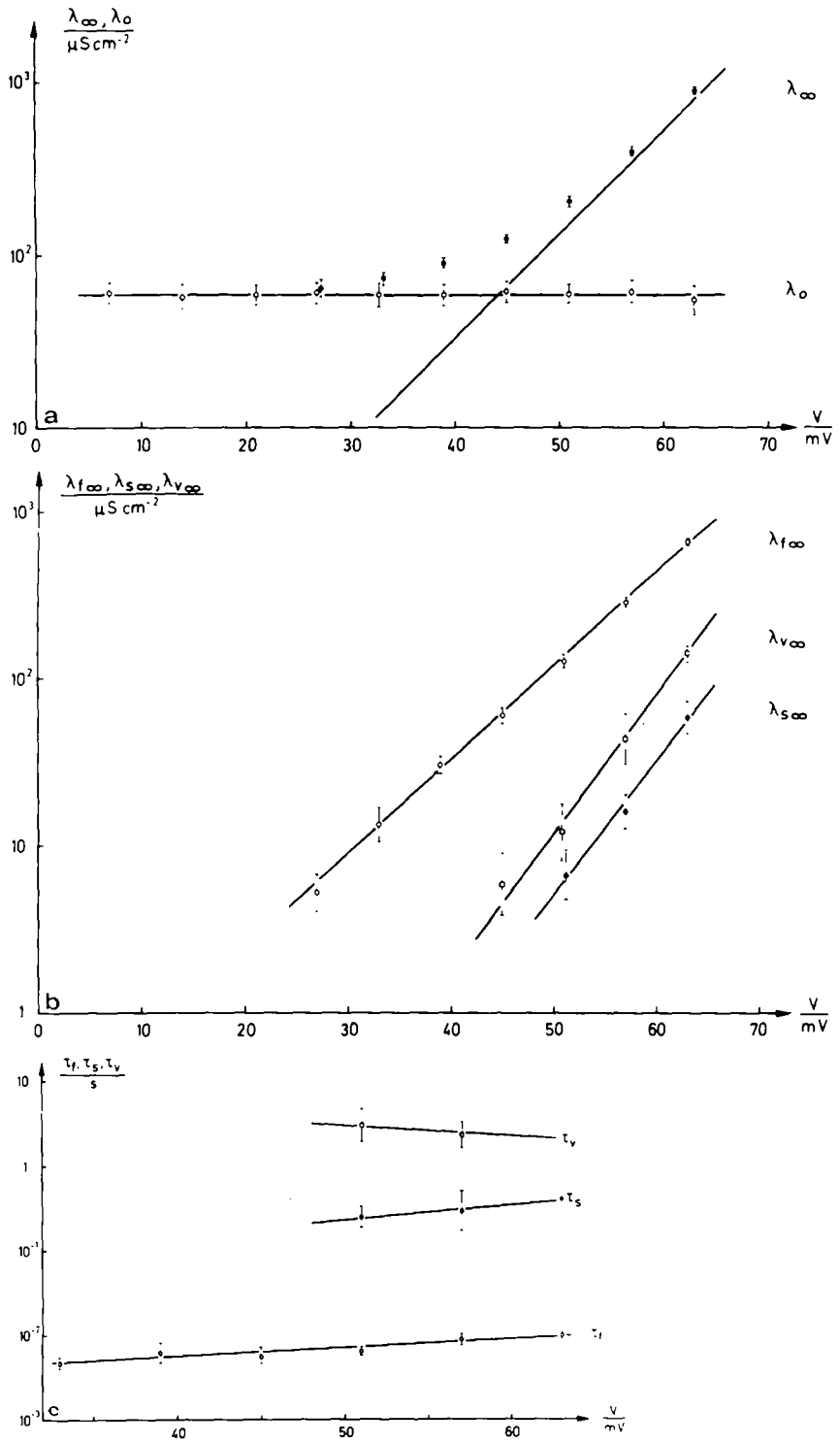

Fig. 5. Voltage dependence of (a) the final conductance, λ_∞, and the initial conductance λ_0; (b) the conductances $\lambda_{f\infty}$, $\lambda_{s\infty}$, $\lambda_{v\infty}$ which were obtained from the fast (○), slow (●) and very slow (□) relaxation processes, respectively; (c) the corresponding relaxation times τ_f, τ_s, τ_v. Trichotoxin A-40 was present in both aqueous compartments. Voltage jumps started from 0 mV. For details see text. Membrane solution, 1% di-(18 : 1)-phosphatidylcholine in decane; salt solution, 1 M KCl, unbuffered; antibiotic concentration, 1.0 $\mu g \cdot cm^{-3}$ trichotoxin A-40; temperature, 25°C.

TABLE III

COMPARISON OF THE RESULTS OF VOLTAGE-JUMP CURRENT-RELAXATION EXPERIMENTS IN THE PRESENCE OF ALAMETHICIN R_F 30 [13], SUZUKACILLIN A [18] AND TRICHOTOXIN A-40

See Figs. 5, and 6, and for definition of the experimental parameters see text. Membrane solution, 1% di-(18 : 1)-phosphatidylcholine in decane; salt solution, 1 M KCl, unbuffered; temperature, 25°C.

	Alamethicin R_F 30		Suzukacillin A	Trichotoxin A-40
Antibiotic concn. ($\mu g \cdot cm^{-3}$)	0.25	0.25	0.15	1.0
λ_0 ($\mu S \cdot cm^{-2}$)	1	110	5	60
V_c (100 $\mu S \cdot cm^{-2}$) (mV)	47		50	48
V_c (1 $mS \cdot cm^{-2}$) (mV)		40		
$\alpha_{(\lambda_\infty - \lambda_0)}$	6.0	5.0	4.4	3.6
$\frac{\lambda_{v\infty}(V_c)}{\lambda_\infty(V_c) - \lambda_0}$	—	—	—	0.08
$\alpha_{\lambda_{v\infty}}$	—	—	—	4.9
$\frac{\lambda_{s\infty}(V_c)}{\lambda_\infty(V_c) - \lambda_0}$	0.98	0.9	0.94	0.03
$\alpha_{\lambda_{s\infty}}$	6.3	5.3	4.7	4.7
$\frac{\lambda_{f\infty}(V_c)}{\lambda_\infty(V_c) - \lambda_0}$	0.02	0.1	0.06	0.89
$\alpha_{\lambda_{f\infty}}$				3.3
$\frac{\lambda_{s0}(V_c)}{\lambda_\infty(V_c)}$	0.03	0.2	0.11	0.91
$\alpha_{\lambda_{s0}}$	0.96	0.95	0.7	
$\tau_v(V_c)$ (s)	—	—	—	3
α_{τ_v}	—	—	—	—0.70
$\tau_s(V_c)$ (ms)	190	75	170	220
α_{τ_s}	2.6	2.4 (3.1)	2.1	1.05
$\tau_f(V_c)$ (ms)		3.1	4.6	7.1
α_{τ_f}		0.18		0.58

of the fast process is larger than $\lambda_{v\infty}$ and $\lambda_{s\infty}$ by about one order of magnitude. That is different with alamethicin R_F 30 and suzukacillin A. Besides the fact that only one slow relaxation is found with these compounds, the amplitude of the fast process is considerably smaller than that of the slow process. Table III demonstrates this difference by means of the ratios $\lambda_{v\infty}(V_c)/(\lambda_\infty(V_c) - \lambda_0)$, $\lambda_{s\infty}(V_c)/(\lambda_\infty(V_c) - \lambda_0)$ and $\lambda_{f\infty}(V_c)/(\lambda_\infty(V_c) - \lambda_0)$ estimated at the characteristic voltage, V_c. With trichotoxin A-40 all relaxation amplitudes exhibit a considerable voltage-dependence including $\lambda_{f\infty}$. In the case of the multi-pore analysis of alamethicin R_F 30 [13] and suzukacillin A [18], the quantity $\lambda_{s0} = \lambda_{f\infty} + \lambda_0$ has been evaluated. The λ_{s0} obtained was shown to possess only a weak voltage dependence. In the presence of trichotoxin A-40, λ_{s0} turns out to be strongly non-linear and curved to the λ_{s0}-axis in a λ_{s0} versus V plot.

The relaxation times τ_v, τ_s and τ_f are plotted in Fig. 5c. A comparison of the

time-ranges for the different antibiotic analogues indicates that the fast relaxation times observed with alamethicin R_F 30, suzukacillin A and trichotoxin A-40 correspond to each other. The same correspondence seems to hold for the slow relaxation times in the case of alamethicin R_F 30 and suzukacillin A and for the slow (medium) relaxation time observed with trichotoxin A-40. The additional very slow relaxation process seems to be specific to the trichotoxin A-40 system. Further evidence for this assignment of the relaxation times may be found in the voltage dependence of the τ_i. Contrary to α_{τ_f}, which is small and positive, and to α_{τ_s} which is larger and also positive in all cases, a negative value for α_{τ_v} is obtained, i.e. α_{τ_v} is reduced by a voltage increase. This behavi-

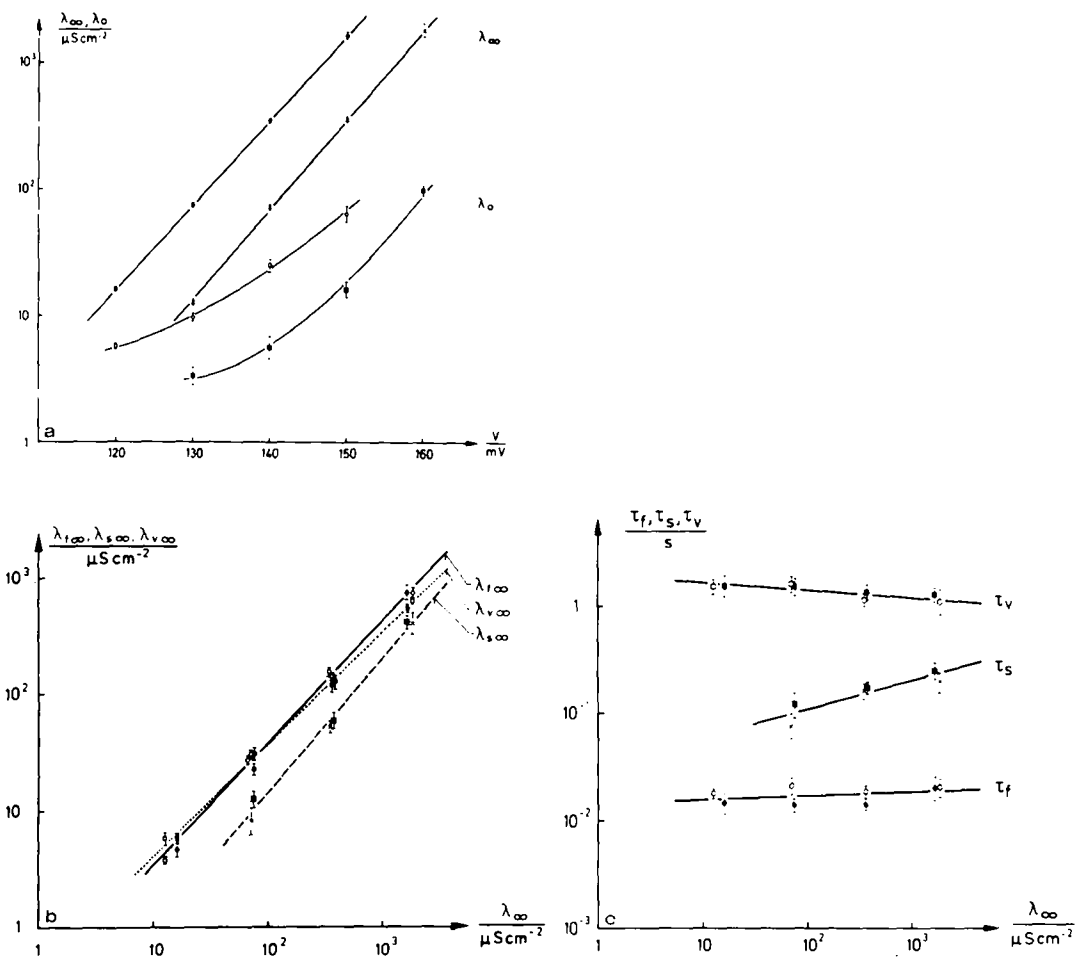

Fig. 6. Voltage of λ_∞ dependence of (a) the final conductance, λ_∞, and the initial conductance λ_0; (b) the conductances $\lambda_{f\infty}$, $\lambda_{s\infty}$, $\lambda_{v\infty}$ which were obtained from the fast (○, ●) slow (X, ⊕) and very slow (□, ■) relaxation processes, respectively; (c) the corresponding relaxation times τ_f, τ_s, τ_v. The two sets of points have been obtained with two different membranes. Trichotoxin A-40 was present in the membrane-forming solution at a ratio of approx. 250 phosphatidylcholine molecules to 1 trichotoxin A-40 molecule. Voltage jumps started from 0 mV. For details see text. Membrane solution, 1% di-(18 : 1)-phosphatidylcholine + 10^{-2}% trichotoxin A-40 in decane; salt solution, 1 M KCl, unbuffered; temperature, 25°C.

our is not observed for the alamethicin R_F 30 and suzukacillin A systems.

(ii) Addition of trichotoxin A-40 to the membrane-forming solution. In contrast to alamethicin R_F 30 and suzukacillin A, trichotoxin A-40 added from an ethanolic stock solution to the membrane-forming solution (phosphatidylcholine in decane) does not precipitate. Series of voltage-jump current-relaxation experiments were carried out at a molar ratio of about 250 : 1 phosphatidylcholine to trichotoxin A-40 (Fig. 6). At lower ratios the membranes became quite unstable. Fig. 6a indicates the characteristic voltage, $V_c \geqslant 130$ mV under these conditions. Approximately the same V_c value is obtained with a phosphatidylcholine membrane if 0.35 $\mu g \cdot cm^{-3}$ trichotoxin A-40 is added to the 1 M KCl solution. If the current-voltage curves were recorded with pause periods of 10 min in which the system was maintained at zero voltage, an increase in V_c due to the loss of trichotoxin A-40 into the aqueous phase could be observed. During 30 min V_c increased by about 10—15 mV.

Since the membrane broke easily at high voltages and high induced conductances, the measurements were started at the lowest voltage used during the experiments. The pulse amplitude was then increased stepwise by 10 mV. At each voltage three subsequent measurements were carried out. The two sets of results in Fig. 6 have been obtained with two different membranes. These two series were chosen from the great number of measurements made in order to show the range of experimental reproducibility and to demonstrate that the experimental variations are reduced if the system is analyzed as a function of the steady-state conductance, λ_∞. The measurements were started about 10 min after the membrane became black. Fig. 6a shows that immediately after membrane formation there is nearly no zero voltage conductance, λ_0. As the time proceeds, and especially after induction of large steady-state conductances, λ_0 increases up to 100 $\mu S \cdot cm^{-2}$. This means that no preformed pore structures of trichotoxin A-40 exist in the lipid-forming solution and that the pores are created only after the bimolecular lipid matrix was established.

The difference of 10 mV between identical λ_∞ values of the two sets of measurements in Fig. 6 indicates a difference of about 20% in the interfacial antibiotic concentration, as will be outlined later. The α_{λ_∞} values of the two sets are comparable; (●), $\alpha_{\lambda\infty} = 4.0$; (×), $\alpha_{\lambda_\infty} = 4.2$. As a consequence of the equivalence between voltage and trichotoxin A-40 concentration which was also found for alamethicin R_F 30 [13,14], it follows that the properties of the system can be characterized uniquely with respect to the steady-state conductance, λ_∞. The same λ_∞ value can be obtained for a given voltage-antibiotic concentration pair and for any decreased antibiotic concentration with a correspondingly increased voltage and vice versa.

Fig. 6b demonstrates that the relaxation amplitudes, $\lambda_{v\infty}$, $\lambda_{s\infty}$, $\lambda_{f\infty}$ of these different experimental sets agree within the limits of experimental error when plotted versus λ_∞. The amplitudes $\lambda_{v\infty}$ and $\lambda_{s\infty}$ are of a magnitude comparable to $\lambda_{f\infty}$. For the slopes of the straight lines in the double logarithmic λ_i versus λ_∞ plots one obtains $\alpha^*_{\lambda_{v\infty}} = 0.97$, $\alpha^*_{\lambda_{s\infty}} = 1.16$ and $\alpha^*_{\lambda_{f\infty}} = 1.05$. The values of voltage dependence indicating parameters obtained from the halflogarithmic λ_i versus V plots are $\alpha_{\lambda_{v\infty}} = 4.0$, $\alpha_{\lambda_{s\infty}} = 4.8$ and $\alpha_{\lambda_{f\infty}} = 4.3$.

Fig. 6c shows that the relaxation times τ_v, τ_s and τ_f, of the two sets are also of comparable values if plotted versus λ_∞. From the slope in a log τ_i/log λ_∞ plot

one obtains $\alpha^*_{\tau_v} = -0.072$, $\alpha^*_{\tau_s} = 0.269$ and $\alpha^*_{\tau_f} = 0.037$. With respect to a log τ_i/V plot this corresponds to $\alpha_{\tau_v} = -0.29$, $\alpha_{\tau_s} = 1.10$ and $\alpha_{\tau_f} = 0.15$.

Comparison of the data of Fig. 6 with those of Table III indicates that the properties of a trichotoxin A-40-modified system remain essentially unchanged whether the antibiotic is added to the aqueous salt solution or to the membrane forming solution.

Comparison of various alamethicin analogues

The activity of several alamethicin analogues with respect to the property of voltage-dependent pore formation in lipid bilayers was investigated. The analysis was carried out using as criteria the parameters V_c and α_λ which are obtained from the current-voltage characteristic of membranes modified by these antibiotics (see Fig. 2). The system was maintained at zero voltage and after the membrane became black the compartments were stirred for 10 min. After an additional 20 min the current-voltage curves were recorded by applying a triangle voltage pulse of 100 s per cycle. With this voltage sweep rate no hysteresis was seen in the case of the KCl salt. If the current-voltage characteristic was recorded after 60 min, a mean decrease in V_c of about 5 mV compared with the V_c value measured after 30 min was observed, whereas α_λ remained approximately unchanged. V_c was reproducible within $V_c \pm 6$ mV and α_λ within $\alpha_\lambda \pm 0.5$. At high voltages $V_c > 120$ mV the standard deviation amounted to ±10 mV. For a specific antibiotic, V_c was determined as a function of the antibiotic and salt concentration. In the case of 1 M KCl the voltage V_c at which $\lambda = 100\ \mu S \cdot cm^{-2}$ was chosen as references voltage.

Since the mean pore conductance, $\bar{\Lambda}$, of alamethicin [4,14] is a function of the ionic concentration, but to a first approximation not of the antibiotic concentration, the reference conductance λ at 0.1 M KCl was changed to $\lambda = 15.9\ \mu S \cdot cm^{-2}$ according to

$$\lambda = \bar{\Lambda} \cdot N_p \tag{2}$$

(N_p, total number of pores per cm^2 of membrane) with

$$\lambda\,(0.1\text{ M KCl}) = \frac{\bar{\Lambda}(0.1\text{ M KCl})}{\bar{\Lambda}(1\text{ M KCl})} \cdot \lambda(1\text{ M KCl}). \tag{3}$$

The systems are compared under identical N_p-concentration conditions. We mention that the ratio $\bar{\Lambda}(1\text{ M KCl})/\bar{\Lambda}(0.1\text{ M KCl}) = 6.3$ [14] is only approximately equal to the corresponding ratio of the bulk solution conductivities $\kappa(1\text{ M KCl})/\kappa(0.1\text{ M KCl}) = 8.68$ at 25°C.

Following the mathematical treatment of Boheim and Kolb [13] λ is described in terms of

$$\lambda \propto \exp\{\alpha_\lambda \cdot u\} \cdot (C_{AL})^{\delta_\lambda} \cdot (C_{salt})^{\epsilon_\lambda}. \tag{4}$$

The voltage dependence characterizing parameter α_λ is the same as in Eqn. 1. The parameter δ_λ which indicates the power dependence of λ on the alamethicin concentration, C_{AL} is given by [13]:

$$\delta_\lambda = \alpha_\lambda \frac{u_2 - u_1}{\ln\{(C_{AL})_1/(C_{AL})_2\}} \tag{5}$$

u_1, $(C_{AL})_1$ and u_2, $(C_{AL})_2$ are two different sets of the variables at which one obtains the same λ value. In analogy one obtains for the parameter ϵ_λ indicating the dependence on the salt concentration, C_{salt} [13]

$$\epsilon_\lambda = \alpha_\lambda \frac{u_2 - u_1}{\ln\{(C_{salt})_1/(C_{salt})_2\}} \tag{6}$$

The following antibiotics have been tested (Table IV); (1) Alamethicin R_F 30 with one phenylalaninol and a C-terminal COOH grroup.

(2) Alamethicin R_F 50 with one phenylalaninol and a C-terminal $CONH_2$ group. Table III gives additional evidence that alamethicin F 50, which was isolated and purified by Irmscher and Jung [20], is identical with alamethicin R_F 50.

(3) Suzukacillin A with one phenylalaninol, a C-terminal $CONH_2$ group and a larger N-terminal α-helix than alamethicin.

(4) Suzukacillin A'20 with no phenylalaninol group and an α-helix shorter than that of suzukacillin A but comparable to that of alamethicin.

(5) Suzukacillin B which seems to differ from suzukacillin A in that the 3 valines are replaced by leucine, alanine and α-aminoisobutyric acid.

(6) Trichotoxin A-40 with no phenylalaninol and a C-terminal COOH group.

(7) Trichotoxin A-40-OCH_3 with an esterified carboxyl group.

(8) Trichotoxin A-40-$(Ala)_2$-OCH_3, where the dipeptide residue -$(Ala)_2$-OCH_3 was linked to the free carboxyl group.

Two additional analogues, i.e., trichotoxin A-50 [19] and another phenylalaninol-free hydrolysis product of suzukacillin A called suzukacillin A'50 [20] have also been tested and found to be active, but have not been included in Table IV. According to Table IV the following activity sequences of the antibiotic analogues are found in 1 M KCl: suzukacillin A $>$ alamethicin F 50 $>$ alamethicin R_F 30 $>$ suzukacillin A'20 $>$ suzukacillin B $>$ trichotoxin A-40-OCH_3 $\approx$ trichotoxin A-40 $>$ trichotoxin A-40-$(Ala)_2$-OCH_3. In 0.1 M KCl: alamethicin F 50 $>$ suzukacillin A $>$ alamethicin R_F 30 $>$ suzukacillin A'20 $>$ suzukacillin B $>$ trichotoxin A-40-OCH_3 $>$ trichotoxin A-40 $>$ trichotoxin A-40-$(Ala)_2$-OCH_3. The most active antibiotic is characterized by the lowest V_c value at the same concentration and otherwise identical conditions. The data indicate that the neutral form of a specific antibiotic is more active than the possibly negatively charged analogue. The lack of the phenylalaninol group in a natural compound or the removal of this group by hydrolysis does not cause loss of the pore-formation property. A high power dependence of λ on the antibiotic concentration is found with all analogues. The power dependence of λ on the salt concentration, which is the square to cubic root of the antibiotic concentration dependence, is also observed with all analogues.

Salt specificity

The effect of different salts on the activity of three different alamethicin analogues was investigated in order to determine whether the voltage dependence of antibiotic pore formation is the result of a permanent dipole moment of the single molecule, or if it is caused by specific ion (cation)-antibiotic interactions. The direct measurement of the dipole moment of alamethicin of 67 Debye by Yantorno, Takashima and Mueller [25] has recently provided some

TABLE IV

CHARACTERISTIC VOLTAGE V_c AT WHICH $\lambda = 100\ \mu S \cdot cm^{-2}$ (1 M KCl) OR $\lambda = 15.9\ \mu S \cdot cm^{-2}$ (0.1 M KCl) AND EXPONENTIAL FACTORS α_λ, δ_λ AND ϵ_λ OF THE CURRENT-VOLTAGE CURVES INDUCED BY VARIOUS ALAMETHICIN ANALOGUES IN LIPID BILAYER MEMBRANES AT 1 M KCl AND 0.1 M KCl SALT SOLUTIONS, RESPECTIVELY

For definition of the parameters and experimental details see text. Membrane solution, 1% di-(18 : 1)-phosphatidylcholine in decane; temperature, 25°C.

Antibiotic	Antibiotic concn. ($\mu g \cdot cm^{-3}$)	Salt concn. (M)	V_c (mV)	α_λ	δ_λ	ϵ_λ
Alamethicin R_F 30	0.50	0.1	49	5.9	14.4	
	0.25	0.1	91	6.3		4.7
	0.25	1.0	47	6.4	10.5	
	0.10	1.0	86	6.1		
Alamethicin R_F 50	0.25	1.0	32	4.6	8.3	
	0.10	1.0	76	4.8		
Alamethicin F-50	0.50	0.1	31	5.1	11.5	
	0.25	0.1	72	4.9		3.3
	0.25	1.0	31	5.2	8.7	
	0.10	1.0	71	5.0		
Suzukacillin A-40	0.50	0.1	37	4.4	8.6	
	0.25	0.1	71	4.6		3.7
	0.25	1.0	23	4.4	9.4	
	0.10	1.0	75	4.1		
Suzukacillin A'20	0.50	0.1	63	4.1	9.9	3.0
	0.25	0.1	106	4.2		3.5
	0.50	1.0	21	4.3	8.1	
	0.25	1.0	56	3.9		
Suzukacillin B	0.50	0.1	94	4.2	9.0	2.8
	0.25	0.1	133	4.1		2.7
	0.50	1.0	54	3.9	8.8	
	0.25	1.0	93	4.0		
Trichotoxin A-40	2.0	0.1	41	3.2	12.1	
	1.0	0.1	101	4.0		3.2
	1.0	1.0	51	3.5	12.3	
	0.5	1.0	107	4.3		
Trichotoxin A-40—OCH_3	2.0	0.1	36	3.0	10.6	
	1.0	0.1	93	3.6		2.4
	1.0	1.0	52	3.1	11.0	
	0.5	1.0	108	3.9		
Trichotoxin A-40—$(Ala)_2$—)CH_3	2.0	0.1	78	3.3	12.6	
	1.0	0.1	137	4.2		3.6
	1.0	1.0	85	3.9	16.4	
	0.5	1.0	160	4.0		

support in favour of the first alternative. If this is true, then the salt effects should be of second order (e.g., like the salt specificity of the salting-out effect).

Salt specificity is measured by the method of current-voltage characteristics as described in the preceding section. Fig. 7 shows a set of current-voltage characteristics obtained with a trichotoxin A-40-modified membrane in the presence of 1 M NH_4Cl, 1 M $NH_2(CH_3)_2Cl$ and 1 M $N(CH_3)_4Cl$. With an increase in the number of substitutions, hydrogen-methyl, in the ammonium ion an increase in V_c is observed. The corresponding data and those of other salts are listed in Table V. The reproducibility of the results ranges within $V_c \pm 6$ mV

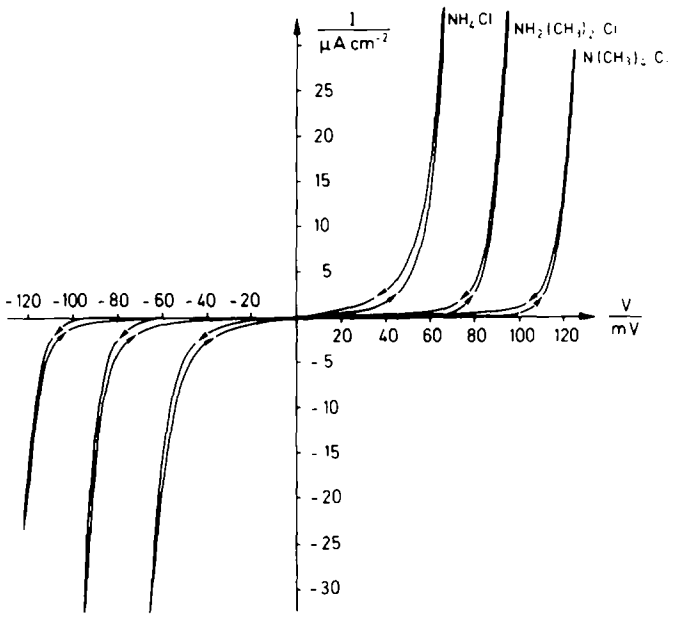

Fig. 7. Set of current-voltage characteristics of a trichotoxin A-40-modified lipid membrane in the presence of 1 M NH_4Cl, 1 M $NH_2(CH_3)_2Cl$ and 1 M $N(CH_3)_4Cl$, respectively. The triangle pulse lasted 100 s per cycle. Membrane solution, 1% di-(18 : 1)-phosphatidylcholine in decane; antibiotic concentration, 1 $\mu g \cdot cm^{-3}$ trichotoxin A-40; temperature, 25°C.

and $\alpha_\lambda \pm 0.5$. The deviations were larger in case of 1 M $LaCl_3$ and 1 M KCl + 100 μM $UO_2(CH_3COO)_2$. With several salts a hysteresis behaviour was observed at a pulse period of 100 s per cycle. Then the mean value between the upper and lower trace at positive voltages was evaluated.

Since the mean pore conductances, $\overline{\Lambda}$, were only measured for a small number of salt species, the reference conductance $\lambda(V_c)$ was varied with the change in the bulk solution conductivity, κ, instead of the change in $\overline{\Lambda}$ as expressed in Eqn. 3. Nevertheless, the data of Table V give a satisfactory picture of the salt specificity of the parameters V_c and α_λ. Table V demonstrates that the discrimination abilities of alamethicin R_F 30, suzukacillin A and trichotoxin A-40 to different alkali cations are poor. Even in the case of suzukacillin a difference between the V_c values for Rb^+ and Li^+ of about 50 mV would correspond only to a 2.5-fold change in the antibiotic concentration in the presence of 1 M KCl (Table IV). A comparison between carrier antibiotics like valinomycin, nonactin, etc., which exhibit a specific carrier-cation binding [26], and alamethicin-type antibiotics shows that the intercationic selectivity of the latter is quite small. According to Table Vb, in the case of KCl/LiCl mixtures the pore-formation properties seem not to be a linear combination of those found with KCl and LiCl alone. A change in the diameter of the cation (Table Vc) by a factor of 2 by means of the methyl-substituted ammonium ions caused a change in V_c of approx. 60 mV with trichotoxin A-40, which again corresponds to a difference of a factor of 2 in the antibiotic concentration at 1 M KCl. In addition, Table Va indicates that the variation of the type of anions results in V_c changes

TABLE V

CHARACTERISTIC VOLTAGE (V_c) AT WHICH $\lambda = 100 \cdot \kappa$ (1 M salt i)/κ (1 M KCl) $\mu S \cdot cm^{-2}$ AND EXPONENTIAL FACTOR (α_λ) OF THE CURRENT-VOLTAGE CURVES INDUCED BY ALAMETHICIN R_F 30, SUZUKACILLIN A AND TRICHOTOXIN A-40 FOR THE 1 M ALKALI CHLORIDE AND VARIOUS OTHER SALT SOLUTIONS

Pulse period was 100 s per cycle. For definition of the parameters and experimental details see text. Membrane solution, 1% di-(18 : 1)-phosphatidylcholine in decane; temperature, 25°C.

(a) Alamethicin R_F 30 (0.25 $\mu g \cdot cm^{-3}$)

Salt solution	V_c (mV)	α_λ	κ (mS · cm^{-2})	$\lambda(V_c)$ (μS · cm^{-2})
1 M LiCl	71	6.8	71.2	63.6
1 M NaCl	48	6.8	83.8	74.8
1 M KCl	46	6.7	112	100
1 M RbCl	64	7.2	115	103
1 M CsCl	69	7.4	115	103
1 M NH_4Cl	53	6.4	112	100
1 M $N(CH_3)_4Cl$	88	6.7	69.6	62.1
1 M Tris · HCl	79	6.5	45.1	40.3
1 M Glucosamin · HCl	47	5.5	44.5	39.7
1 M $LaCl_3$	114	2.3	125	112
1 M KCl + 100 μM $UO_2(CH_3COO)_2$	99	3.3	112	100
1 N $MgCl_2$	68	5.4	72.9	65.1
1 N K_2SO_4	48	7.1	82.3	73.1
1 N Na_2SO_4	51	7.2	59.4	53.0
1 M $NaNO_3$	64	6.6	76.3	68.1
1 M $NaCH_3COO$	29	7.0	49.3	44.0
1 M $Tl CH_3COO$	93	6.3		≈40

(b) Suzukacillin A (0.10 $\mu g \cdot cm^{-3}$)

1 M LiCl	112	3.0	71.2	63.6
1 M NaCl	82	3.9	83.8	74.8
1 M KCl	75	4.1	112	100
1 M RbCl	60	3.5	115	103
1 M CsCl	71	3.9	115	103
1 M KCl	75	4.1	112	100
0.75 M KCl + 0.25 M LiCl	81	4.3	101	90.2
0.5 M KCl + 0.5 M LiCl	89	3.6	90.2	80.5
0.25 M KCl + 0.75 M LiCl	118	3.2	80.1	71.5
1 M LiCl	112	3.0	71.2	63.4
1 M Tris · HCl	100	4.2	45.1	40.3
1 M Tris · HCl *	38	4.0	45.1	40.3
1 M $CaCl_2$ *	37	3.3	128	115

(c) Trichotoxin A-40 (1.0 $\mu g \cdot cm^{-3}$)

1 M LiCl	77	3.1	71.2	63.6
1 M NaCl	53	3.4	83.8	74.8
1 M KCl	51	3.5	112	100
1 M RbCl	55	3.6	115	103
1 M CsCl	53	3.1	115	103
1 M NH_4Cl	51	2.9	112	100
1 M NH_3CH_3Cl	64	3.8	96.6	86.3
1 M $NH_2(CH_3)_2Cl$	85	4.0	83.7	74.7
1 M $NH(CH_3)_3Cl$	101	4.1	75.3	67.2
1 M $N(CH_3)_4Cl$	115	3.8	69.6	62.1

* 0.25 $\mu g \cdot cm^{-3}$ suzukacillin A.

of a range similar to that obtained with different kinds of cations. The introduction of divalent cations (Mg^{2+}, Ca^{2+}) or a divalent anion (SO_4^{2-}) does not significantly change V_c or α_λ. This means that the pore-formation properties are not influenced by the valency of the cation. If ions which are known to interact with the lipid matrix were introduced (La^{3+}, UO_2^{2+} see Table Va) a pronounced change in V_c and α_λ could then be observed. Thus, the ion-lipid matrix interactions lead to more significant alterations of the antibiotic activity at lipid membranes than the ion-antibiotic interactions. Since we did not observe a pronounced ionic specificity in the action of the antibiotics, the dipole moment concept of the alamethicin molecule seems to be more likely. In contrast to the experiments with gramicidin A [27] in the presence of Tl^+ (Table Va) no divergent behaviour of the alamethicin pore could be observed.

Discussion

The single-pore experiments have shown that trichotoxin A-40 forms pore structures similar to those created by alamethicin R_F 30, alamethicin R_F 50 [6] and suzukacillin A [18]. The occurrence of fast spikes is also observed with alamethicin R_F 30 [14] and suzukacillin A [18] but not as frequently as with trichotoxin A-40. The nature of these spikes is not yet completely understood, although the analysis of Kolb and Boheim [14] seems to indicate that they may be caused by short-lived pores which do not achieve a steady-state in pore state fluctuations (formation of aggregates of under-critical size).

The multi-pore experiments show that trichotoxin A has a weaker voltage dependence than alamethicin R_F 30 and suzukacillin A. According to the theoretical analysis of Boheim and Kolb [13], such a result indicates that the mean state of a fluctuating trichotoxin A-40 pore is lower than that of an alamethicin R_F 30 pore under otherwise identical conditions. Additional evidence for this interpretation is given by the data presented in the section describing single-pore experiments.

In the prescence of trichotoxin A-40 a third, very slow relaxation process occurs which is not found in the alamethicin R_F 30 and suzukacillin system. The equivalences between the two other relaxation processes and single-pore phenomena were established by comparing the time scales (same order of magnitude) and degree of voltage dependence. The fast relaxation time, τ_f, which was assigned to the mean life time, τ_p, of the most probable pore-state was twice as large with trichotoxin A-40 as with alamethicin R_F 30 (Table III). Approximately the same ratio was found by direct measurement of τ_p (Table II). The slow relaxation time, τ_s, was assigned to the mean life-time of a fluctuating pore. With each antibiotic this time constant showed the strongest voltage dependence (α_{τ_s} positive). In the case of alamethicin R_F 30 and suzukacillin A the largest relaxation amplitude was observed with the pore formation/decay (slow) process in contrast to the situation with trichotoxin A-40. There the relaxation amplitude of the pore-growth (fast) process was the dominant one. This seems to reflect a difference in the pore nucleation and pore-growth properties of these antibiotics. In the case of alamethicin R_F 30 and suzukacillin A the weak voltage dependence of λ_{so} is consistent with the interpretation that the mean conductance, $\overline{\Lambda}$, of a pore increases with voltage as a consequence of

a shift in pore state to higher levels. With trichotoxin A-40 the voltage dependence of $\lambda_{so} = \lambda_{f\infty} + \lambda_0$ is much stronger. In view of the arguments presented above a possible explanation would be a voltage-dependent shift of the aggregates of under-critical size to fluctuating pores. This shift would be accompanied with a strong conductance increase of a single pore.

The very slow relaxation process of the trichotoxin A-40 system which occurs in the range of seconds is different from the other very slow process which was observed in the range of minutes to hours with alamethicin R_F 30 [13] and suzukacillin A [18]. The latter process, which is seen also with trichotoxin A-40 (e.g., the slow appearance of the zero voltage conductance) was attributed to a slow change of the equilibrium distribution between different conformations of α-helical content. In polar solvents an α-helix content of about 20% was found, which changed to approx. 40% in more apolar solvents [17,28,29].

The relaxation process which takes place in the s-range at least 1 h after the membrane became black might be the result of reactions at the membrane interphase. Possible explanations would be a voltage-induced formation of additional preaggregates or a redistribution of monomers between existing preaggregates. Similar redistribution processes were discussed for the saturation range of alamethicin R_F 30 pore formation [13] and for the suzukacillin A inactivation process [18]. However, a conclusion must be reached by additional experiments.

The comparison of the different alamethicin analogues shows that suzuka-sequently, a highly hydrophobic character does not favour the pore formation of alamethicin-type antibiotics. Substitution of the possibly negatively-charged solution, this antibiotic seems to be more hydrophobic than the others. Consequently, a highly hydrophobic character does not favour the pore formation of alamethicin-type antibiotics. The substitution of the negatively-charged $-COO^-$ group by the neutral $-CONH_2$ or $-COOCH_3$ groups does not change the pore-formation properties significantly. Substitution by the Ala-Ala-OCH_3 dipeptide, however, reduces the antibiotic activity. Eisenberg, Kleinberg and Shaper [16] have reported that an alamethicin analogue synthesized by condensation with dansyl-cadaverine forms pores which have only about 50% of the conductance of the natural compound.

The hydrolysis product suzukacillin A'20 lacks the N-terminal proline and the phenylalaninol group. It is still pore forming and its activity is insignificantly reduced compared to the natural suzukacillin A. Trichotoxin A-40, which consists of only one proline and from which phenylalaninol is missing by nature, is also active. This means that none of the two groups are essential parts of the ion-conducting pore.

From the investigation of the salt specificity one may conclude that the nature of the salt is not important for antibiotic pore formation. It is influenced primarily by the activity of the aqueous solution and by other second-order concentration effects, possibly the salting-out effect [8]. This is consistent with the observation that the permanent dipole moment of alamethicin seems to be quite large [25]. It ranges from approx. 40 Debye in a 10% ethanol in dioxane solvent to approx. 80 Debye in a 40% ethanol in dioxane solvent. Significant changes in the pore-formation properties are observed only with

ions which are known to interact with the lipid matrix (Table Va). Since a strong dependence is reported [6,12] of alamethicin pore formation on the kind of lipid, this result is understandable.

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