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Effects of polycations on ion channels formed by neutral and negatively charged alamethicins

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Abstract. The effects of the peptide polycations salmon protamine ($M_r = 4332$, $z = +21$) and poly-L-lysine ($M_r \cong 100{,}00$, $z \cong +775$) on ion channels formed by synthetic alamethicin Alm-F30 (one negative charge), natural Alm-F50 (neutral) and phosphorylated Alm-F50 (two negative charges) reconstituted in planar lipid bilayers have been studied at the single channel level. It was observed that both polycations in micromolar concentrations transiently block ion permeation through the channels formed by each alamethicin analogue, although in case of the neutral Alm-F50 to a significantly lesser extent. Poly-L-lysine showed to be more effective than protamine in blocking these channels. If either polycation is present in the cis-compartment, blockade occurs only at cis positive membrane voltages. At constant polycation concentration, dwell times in the blocked state increase when salt concentration is lowered, and decrease at acidic pH with an apparent pK of 4.8. Mean lifetime of blockade events shortens when membrane voltage is increased, which suggests that both polycations may permeate through the oligomeric alamethicin channels if conductance levels are > 2. We suggest that blockade is caused by electrostatic binding of a single polycation molecule to the C-terminal channel mouth; in case of Alm-F30, Glu18 has to be considered as the putative binding site. Our results provide further evidence for the barrel-stave model and a parallel orientation of dipole monomers in the channel aggregate, the C-termini facing the membrane side with the more positive membrane potential.

Key words: Alamethicin – Ion channel blockade – Planar lipid bilayers – Polycations

1. Introduction

Alamethicin, a linear polypeptide isolated from the mold fungus *Trichoderma viride*, is known to increase the conductivity of lipid bilayer membranes in a strongly voltage-dependent manner (Mueller and Rudin 1968; Eisenberg et al. 1973; Roy 1975; Boheim and Kolb 1978; Kolb and Boheim 1978). The peptide consists of nineteen amino acids and one amino alcohol, phenylalaninol, at the C-terminus. Other uncommon features are the acetylated N-terminus and the high content of the unusual amino acid 2-methylalanine (α-aminoisobutyric acid, aib). The latter amino acid stabilizes the mainly helical secondary structure, as shown by x-ray diffraction (Fox and Richards 1982), circular dichroism (Jung et al. 1975), ^{13}C-NMR (Schmitt and Jung 1985 b) and 2D-NMR measurements (Esposito et al. 1987). Owing to its extraribosomal synthesis, there is a considerable microheterogeneity among naturally synthesized alamethicins. An extract usually yields two major components, Alm-F30 and Alm-F50. The sequences of these analogues (see Table 1) differ in only one residue: Glutamate in position 18 of Alm-F30 is replaced by glutamine in Alm-F50. Hence, the C-terminus of Alm-F30 carries one negative charge whereas Alm-F50 is neutral. In order to be able to work with uniform channel formers we used highly pure synthetic Alm-F30 (Schmitt and Jung 1985 a) and HPLC purified natural Alm-F50 (Irmscher and Jung 1977). In addition to these analogues, we also examined the synthetic alamethicin F50-phosphate (Alm-F50-P). The primary structure of this peptide is identical with natural Alm-F50, except for the C-terminal phenylalaninol which is esterified to phosphoric acid. Hence, the peptide's C-terminus carries two negative elementary charges at neutral pH.

Electrophysiological experiments show that the conductivity of an alamethicin-doped bilayer depends exponentially on membrane voltage, if it exceeds a so-called characteristic voltage. The characteristic voltage itself is a function of the 4th to 6th power of salt concentration as well as the 6th to 9th power of alamethicin concentration. The latter fact indicates that the conducting unit of alamethicin consists of several monomers. As is well known, this macroscopic conductivity results from the voltage-dependent formation of ion channels by the peptide (Gordon and Haydon 1972; Eisenberg et al. 1973; Boheim 1974). The alamethicin pore fluctuates between

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up to twelve discrete conductance states and the conductance ratio of two neighbouring levels approaches one at high conductance states. The conductance state most probably adopted by the pore is the higher, the higher the membrane voltage and the lower the salt concentration. A straightforward explanation for this peculiar fluctuation pattern is given by the "barrel-stave-model" (Baumann and Mueller 1974; Boheim 1974). According to this model, a central permeation pathway is surrounded by helical monomers building up the channel. Fluctuations between various conductance states can then easily be explained by uptake and release of monomers into and from the pore aggregate, thus changing pore diameter and conductance. There is some experimental evidence that the C-termini of the monomers in the aggregate are all oriented to the membrane side with the more positive electrostatic potential (Boheim et al. 1983), which might be due to the rather high dipole moment of the α-helical monomer (Hol et al. 1978; Schwarz and Savko 1982). The dipole moment vector of each monomer in the aggregate is then aligned in parallel with the electric field vector across the membrane. These considerations gave rise to various models to account for the voltage-sensitivity of pore formation (for review see Woolley and Wallace 1992).

Effects of the basic peptide protamine on the alamethicin multi-channel system have already been published (Mueller and Rudin 1968). These authors reported a significant decrease in the characteristic voltage of the alamethicin-induced macroscopic conductivity in planar bilayers. With suitable concentrations of the polycation, the characteristic voltage of the system became negative, resulting in a range of negative slope conductivity in the current/voltage relationship. In addition, Mueller and Rudin demonstrated that under certain conditions oscillations and action potentials could be displayed by the alamethicin/protamine system. In the present work, we studied the effects of the peptide polycations protamine ($z = +21$, $M_r = 4332$) and poly-L-lysine ($z \cong +775$, $M_r = 100{,}000$) on Alm-F30, Alm-F50 and Alm-F50-P at the single channel level at low temperature (4 °C).

2. Materials and methods

a. Synthesis of alamethicin F30 (Alm-F30)

Highly pure and crystalline Alm-F30 was obtained by segment condensation of purified and characterized intermediates as described previously (Schmitt and Jung 1985a). The product was uniform in various HPLC and TLC systems and free of racemic amino acids. Intermediates of the synthesis and the final product were characterized by FAB-MS, circular dichroism and ^{13}C-NMR (Schmitt and Jung 1985b). Very recently we confirmed the high purity of our synthetic Alm-F30 by electrospray-MS (Metzger and Jung, unpublished).

b. Isolation and purification of alamethicin F50 (Alm-F50)

The precultivation and fermentation of the Alm-producing strain *Trichoderma viride* NRRL 3199 were done essentially as described (Irmscher and Jung 1977). However, optimizations of the fermentation and isolation procedures resulted in 100–150% improvements in the yield of isolated Alm-F50 (Franz and Jung, unpublished). Briefly, the isolation of the peptide antibiotic from the filtered culture medium was optimized by adsorption of the resin XAD-8. After washing with water Alm was eluted with methanol/water mixtures with increasing methanol content. In order to remove polyols (added against foam formation in the fermenter) the combined Alm containing fractions were evaporated to dryness, dissolved in chloroform/methanol (3:1, v/v) and Alm was precipitated by addition of diethylether. The precipitate was dissolved in chloroform/methanol (1:1, v/v) and chromatographed on Sephadex LH 20 using the same solvent mixture as eluent (column 50 × 2 cm; 3 ml/min). Alm containing fractions were pooled and evaporated to yield crude Alm (450 mg). The preparation consited of Alm-F50 as the main component according to analytical and preparative HPLC on Nucleosil C18 (7 µm) using methanol/water (85:15, v/v) as eluent. Finally, preparative HPLC yielded highly pure Alm-F50 according to amino acid analysis, TLC and FAB-MS.

c. Synthesis of alamethicin F50-phosphate (Alm-F50-P)

The purified component Alm-F50 isolated from the culture of *Trichoderma viride* NRRL 3199 was phosphorylated at the C-terminal hydroxyl group of phenylalaninol as follows (Bannwarth and Trzeciak 1987). A solution of Alm-F50 (12.7 µmol), tetrazole (100 µmol) and dibenzyloxidiisopropylaminophosphin (100 µmol) in acetonitrile (20 ml) was stirred under water-free conditions and in argon atmosphere for 1 h. After adding the same reagents Alm-F50 was quantitatively esterified after 1 h according to thin layer chromatography. For oxidation to the trialkylphosphate m-chloroperbenzoic acid (211 µmol) was added. After 90 min the solution was diluted with dichlormethane (100 ml), washed with saturated sodium bicarbonate solution, dried over sodium sulfate and evaporated *in vacuo* to yield an oily residue. After adding pentane (50 ml) the product (50 mg) precipitated and was chromatographed on silica gel (6 g; 0.063–0.04 mm) by eluting with chloroform containing 10%, 17% and 25% methanol followed by chloroform/methanol (3:1, v/v) containing 1%, 2% and 3% water. Fractions no. 17–28 (10 ml) were evaporated and diethylether was added to precipitate alamethicin F50-dibenzylphosphate (Alm-F50-PO(OBzl)$_2$). Yield: 21 mg (74%). Calcd. for $C_{106}H_{164}N_{23}O_{27}P$ 2223.6, found (FAB-MS in thioglycerol): 2246.4 ± 0.6 $[M+Na]^+$ main peak, 2224.8 ± 1.0 $[M+H]^+$, and 2156.4 $[M-C_6H_5CH_2]^+$.

Alm-F50-PO(OBzl)$_2$ (19 mg, 8.54 µmol) in ethanol (50 ml) was hydrogenated in the presence of 5% Pd/C (20 mg) for 24 h. After addition of water (25 ml), filtration (0.45 µm filter from Millipore), and evaporation *in vacuo* the crude product (13 mg) was chromatographed on silica gel (1.5 g) using chloroform/methanol (5:4, v/v) with increasing water content (1 to 10%). The product was converted to its sodium salt Alm-F50-PO $(O^-Na^+)_2$ (5.2 mg) using a Dowex cation exchanger. Additional

product (7.0 mg) could be recovered by working up fractions from the first chromatographic step. Total yield was 12.2 mg (68.4%). High-performance thin-layer chromatography on silica gel in chloroform/methanol/water (65:25:4, v/v/v) using the spray reagents molybdate for phosphate groups and chlorine/tolidine for peptide bonds revealed a uniform product. Calcd. for $C_{92}H_{150}N_{23}O_{27}PNa$ 2066.4, found by FAB-MS in thioglycerol: 2066.6 ± 0.7 (single mass peak). The amino acid analyses from hydrolyzates (5.6 N HCl, 24 h) of Alm-F50 (and Alm-F50-P) were identical: Glx 3.10 (2.71), Pro 1.90 (2.11), Gly 0.98 (0.95), Ala 1.97 (2.09), Val 2.05 (2.06), Leu 1.00 (1.00), NH_3 3.23 (3.33). ^{31}P-NMR showed a single resonance peak as expected for alkylmonophosphates.

d. Experiments in lipid bilayers

Planar lipid bilayers were prepared from solutions of either 1,2 POPC or 80% 1,2 POPC/20% 1,2 DOPE in *n*-decane (1.2% w/v). Lipids were purchased from Avanti Polar Lipids, Alabaster, Alabama, USA, and used without further purification. Salmon protamine sulfate and poly-L-lysine hydrobromide ($M_r \cong 100{,}000$ D) were obtained from Sigma, St. Louis, USA, and used as delivered. Table 1 shows the primary structures of the alamethicin analogues and salmon protamine.

All experiments were carried out in KCl solutions at 4 °C. The sign of membrane voltage refers to the cis-compartment with respect to the grounded trans-compartment. Unless otherwise noted, peptides had always been added to cis.

We used cuvettes made from PMMA (Perspex), the hole on which the bilayer was painted being 0.1 mm in diameter. This setup resulted in a total of membrane- and stray capacitances of about 25–45 pF. Current measurements were carried out with a homemade high-bandwidth current-to-voltage converter providing a maximum bandwith of 16 kHz with the given membrane capacitances and a transimpedance of 1 mV/pA. The amplifier's output was passed through a three-pole Bessel low-pass filter at a corner frequency of 10 kHz (measurements in 1.0 M and 0.5 M KCl) or 3 kHz (measurements in 0.2 M KCl). Recordings were stored on a DAT recorder (DTR 1200, Biologic).

For data evaluation on an IBM-compatible personal computer, the current tracks were digitized at a rate of 250 kSamples/s and 8 bit resolution. We used self-written programs to evaluate block probabilities and mean lifetimes of the blocked state.

Table 1. *Upper part:* Primary structures of the alamethicin analogues used in this work. I: Alm-F30; II: Alm-F50; III: Alm-F50-P. The symbol α denotes α-aminoisobutyric acid, Pheol phenylalaninol. Note that Alm-F30 carries one negative charge at Glu18, which is exchanged with Gln in Alm-F50. *Lower part:* Primary structure of salmon protamine. Positively charged residues are framed; the peptide carries 21 elementary charges (from: Ando et al. 1973)

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        1     5          10        15        20
I   Ac α P α A α A Q α V α G L α P V α α E Q  Pheol
II  Ac α P α A α A Q α V α G L α P V α α Q Q  Pheol
II  Ac α P α A α A Q α V α G L α P V α α Q Q  Pheol-PO3 2-

1          5          10        15          20
P [R R R R] S S S [R] P V [R R R R R] P [R] V S

21        25          30  32
[R R R R R] G G [R R R R]
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3. Theory

We used a simple mass action model to describe the dependency of block probability on polycation concentration and conductance level of the channel. Our basic assumption is that a blockade event is caused by electrostatic binding of a polycation molecule to an alamethicin channel aggregate, whereby mean lifetimes of the channel states are significantly longer than blockade times. It is further assumed that

i. Each channel aggregate may only bind one polycation molecule at a time, i.e. 1:1 stoichiometry.
ii. A given conductance level is confined to two possible states, either "open" or "blocked".
iii. The reaction of complex formation between channels and polycations is assumed to equilibrate.

Under these assumptions, we can develop the following reaction scheme:

$$\mathrm{Alm} + \mathrm{PC} \underset{k_{\mathrm{open}}}{\overset{k_{\mathrm{block}}}{\rightleftharpoons}} \mathrm{Alm{-}PC}$$

(Alm ≡ alamethicin-channel; PC ≡ polycation; Alm–PC ≡ channel-polycation-complex; k_{block} and k_{open} are the rate constants for complex formation and dissociation).

As we are observing a sequence of elementary events in time rather than an ensemble of reacting molecules, it seems reasonable to introduce the probabilities p_{block} and p_{open} of the two allowed states instead of the concentrations of channels and complexes:

$$p_{\mathrm{block}} = t_{\mathrm{block}}/(t_{\mathrm{block}} + t_{\mathrm{open}}) \text{ and } p_{\mathrm{open}} = t_{\mathrm{open}}/(t_{\mathrm{block}} + t_{\mathrm{open}}), \tag{1}$$

where t_{block} and t_{open} denote dwell times for the channel in the corresponding states. It follows that

$$p_{\mathrm{block}} + p_{\mathrm{open}} = 1. \tag{2}$$

We therefore obtain the kinetic equation

$$dp_{\mathrm{block}}/dt = k_{\mathrm{block}}\, c_{\mathrm{PC}}(1 - p_{\mathrm{block}}) - k_{\mathrm{open}}\, p_{\mathrm{block}} \tag{3}$$

where c_{PC} denotes the concentration of the polycation. Assuming equilibrium and solving for p_{block}, Eq. (3) yields

$$p_{\mathrm{block}} = K\, c_{\mathrm{PC}}/(1 + K\, c_{\mathrm{PC}}) \quad \text{with} \quad K = k_{\mathrm{block}}/k_{\mathrm{open}}. \tag{4}$$

K is the association equilibrium constant for polycation binding to alamethicin channels. Its value is determined by the work to be done for the binding reaction. Here we have to consider not only a chemical, but also an electrostatic work term:

$$K = \exp\{-(\Delta G^0 + W_{\mathrm{el}})/RT\} \tag{5}$$

ΔG^0 is the standard Gibbs free energy change due to the binding reaction, R the gas constant and T the absolute temperature. W_{el} denotes the electrostatic work done during the reaction. If we assume the blockade events to be statistically independent from each other, we can evaluate the rate constant k_{open} from the lifetime distribution of the blockade events. If $N(t)$ is the number of complex events, we have

$$dN/dt = -k_{open} \cdot N. \tag{7}$$

With the starting condition $N(t=0) = N_0$, the solution of (7) is

$$N(t) = N_0 \exp\{-t/\tau\} \quad \text{with} \quad \tau = 1/k_{open}. \tag{8}$$

The parameter τ is the mean lifetime of the blocked state. According to the "rate theory" (Eyring et al. 1949), the value of a reaction's rate constant depends on the Gibbs free energy of the transition state. The potential energy of the transition state can be split into a chemical and an electrostatic part, and we obtain the following expression for k_{open}:

$$k_{open} = (kT/h) \exp\{-(\Delta G^{0*} + W_{el}^*)/RT\} \tag{9}$$

k is Boltzmann's constant and h Planck's constant. The energy parameters have in general the same meaning as in (5), but refer to the transition state.

In this work, the rate constant k_{open} was obtained by fitting Eq. (8) to the lifetime distribution histograms of the blockade events. Owing to the limited recording bandwidth, we had to correct the observed block probability values for events which were too short to be evaluated and were therefore missed. The correction factor was calculated as follows:

$$\begin{aligned} p_{block,real} &= p_{block,observed} \cdot (t_{block,real}/t_{block,observed}) \\ &= p_{block,observed} \cdot \exp\{1/\pi \cdot \tau \cdot f_G\} \end{aligned} \tag{10}$$

with f_G as the cutoff frequency of the recording setup. $1/(\pi f_G)$ is then simply the 10–90% rise time of our setup, since events shorter than this time are neglected by the evaluation program.

To describe electrostatic effects at charged lipid membranes adjacent to electrolyte solutions, the theory of Gouy and Chapman is commonly used (Aveyard and Haydon 1973; McLaughlin 1977). The application of this theory to our problem, i.e. calculating the electrostatic potential at the channel mouth in order to quantify the electrostatic work terms in Eqs. (5) and (9), is problematic owing to the discreteness of charges of both the channel and the polycation. A reasonable treatment of the problem would require detailed knowledge of the charge distribution on both reacting species and has not been considered in this work.

4. Results

4.1. Alamethicin F30 and protamine

At ionic strengths between 0.2 and 1.0 M, micromolar concentrations of protamine block ion permeation through alamethicin F30 channels. With the polycation present in the cis-compartment, blockade occurs only if membrane voltage is positive. Figure 1 shows a current trace obtained with 30 nM Alm-F30 in both compartments and 30 μM protamine cis in 0.5 M KCl.

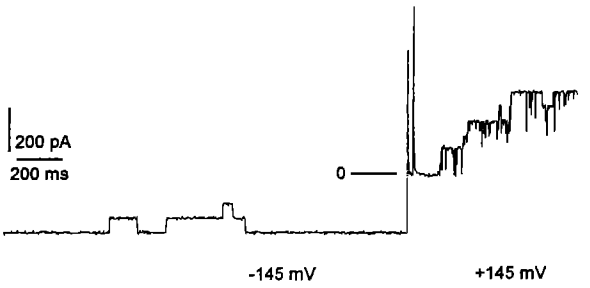

Fig. 1. If protamine is present in the cis-compartment, blockade only occurs at positive membrane voltages. The figure shows a current recording in 0.5 M KCl, 30 μM protamine cis. At negative voltages, the channel is not blocked; as the sign of membrane voltage changes, a new channel forms which is transiently blocked by the polycation. Note that channel blockade is already established when the alamethicin channel forms. Temperature: 4 °C, 30 nM Alm-F30 in both compartments. The horizontal bar indicates zero current level

This effect can be easily explained if we assume that the C-terminal mouth of the alamethicin channel aggregate faces that membrane side with the more positive potential. At positive voltages, the polycation may bind to Glu18; at negative voltages, the N-terminal mouth points to the compartment with the polycation, however since there is no anionic binding site, blockade does not occur.

Block probability increases as the salt concentration decreases, a behaviour which is predicted by the Gouy-Chapman-Theory. At high salt concentrations, mobile counterions exert a strong screening effect on the charged binding site, thus reducing the electrostatic potential in its vicinity. This, in turn, reduces the probability that a polycation molecule approaches the binding site. Figure 2 shows current fluctuation traces of Alm-F30 in 1.0, 0.5 and 0.2 M KCl, and Fig. 3 shows a plot of the block probability versus protamine concentration for the fourth conductance state at three ionic strengths.

The observed increase of the association constant with decreasing ionic strength is due to significantly prolonged mean lifetimes of the blocked state, i.e. k_{open} decreases.

At 0.2 M ionic strength, the association constant K depends on the conductance level of the channel. Under this condition, values of this parameter are the higher, the lower the conductance level. The mean lifetime τ of the blocked state is inversely related to the conductance level at all ionic strengths tested. Table 2 shows numeric values of the association constants K and the mean lifetimes τ of the blocked states.

In 0.1 M KCl, protamine concentrations higher than 10 μM changed the fluctuation pattern from the well-known alamethicin-type to bursts of very short-lived, spike-like events. We observed this effect, which may be considered as a destabilization of the channel aggregate, in 0.05 M KCl at submicromolar protamine concentrations. The term destabilisation is used for the fact that the interaction of protamine with alamethicin monomers prevents its assembly to aggregates.

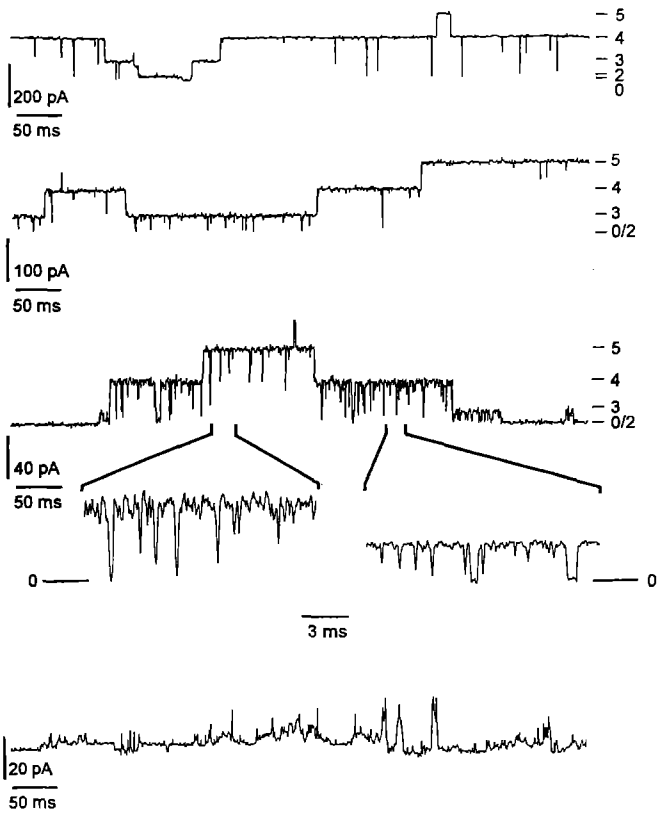

Fig. 2. Current fluctuations of alamethicin F30, 2.3 μM protamine cis, membrane voltage +135 mV, 4°C. Ionic strength (from top to bottom) 1.0 M, 0.5 M, 0.2 M and 0.05 M. Alamethicin concentrations were 15 nM, 30 nM, 50 nM and 100 nM, respectively. Block probability increases as the ionic strength decreases. In 0.05 M KCl, however, protamine at the given concentration destabilizes the pore aggregate

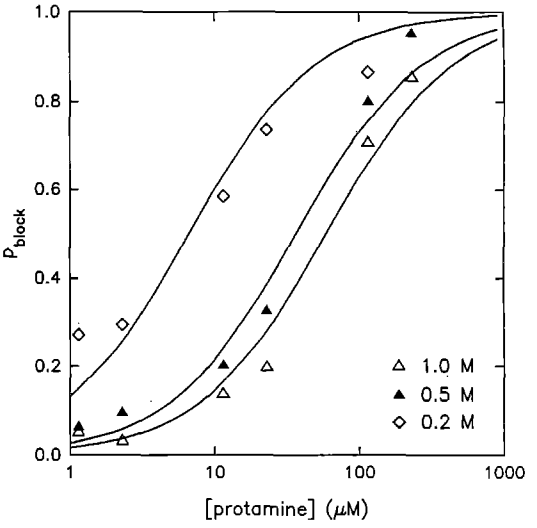

Fig. 3. Block probability vs. protamine concentration for the fourth conductance state of Alm-F30 at various KCl concentrations. Experimental data were fitted according to Eq. (4). The association constant K is obtained from the fit. Values are listed in Table 2.

Table 2. Association constants K and mean lifetimes τ of the blocked state for alamethicin F30 and protamine

Ionic strength	3rd level		4th level		5th level	
	K	τ	K	τ	K	τ
(M)	(l/M)	(μs)	(l/M)	(μs)	(l/M)	(μs)
1.0	$1.5 \cdot 10^4$	88	$1.7 \cdot 10^4$	61	$1.4 \cdot 10^4$	36
0.5	$2.2 \cdot 10^4$	129	$2.7 \cdot 10^4$	71	$2.3 \cdot 10^4$	41
0.2	$2.9 \cdot 10^5$	673	$1.5 \cdot 10^5$	309	$6.2 \cdot 10^4$	178

If the glutamic acid in position (18) is the binding site for protamine, lowering the pH of the electrolyte solution should have an effect on permeation blockade owing to protonation of this residue. For this reason, we measured the block probability as a function of pH. Figure 4 shows the results.

As expected, block probability decreased with decreasing pH. The experimental data for the third and the fourth conductance level have been fitted by titration curves of the form

$$p_{block} = (p_{block,neutral} - p_{block,acid})/(1 + 10^{pK-pH}) + p_{block,acid}$$

where $p_{block,neutral}$ denotes the block probability at neutral pH and $p_{block,acid}$ its limit at acidic pH. The fits yield an apparent pK of 4.8 for both states. Evaluation of the fifth state was not possible, because at pH < 5.5 τ values were shorter than the time resolution of our recording setup. The same problem occurred for levels 3 and 4 at pH < 4.0, so the limit of block probability at very low pH can only be estimated. By comparison with Alm-F50 (see below) we assumed that this limit is different from zero. To fit our data, we took an estimate of a limit 10% below the value at pH 4.0.

We also examined the effect of the transmembrane electric field by making experiments at constant protamine and salt concentrations but different membrane

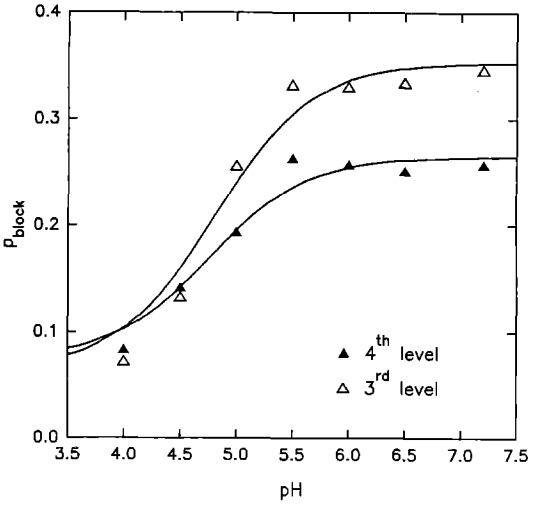

Fig. 4. Block probability vs. pH for the third and fourth conductance level of Alm-F30. Measurements were made in 0.5 M KCl, 30 μM protamine added to cis. By fitting the data with titration curves, apparent pK values of 4.8 were obtained for both levels

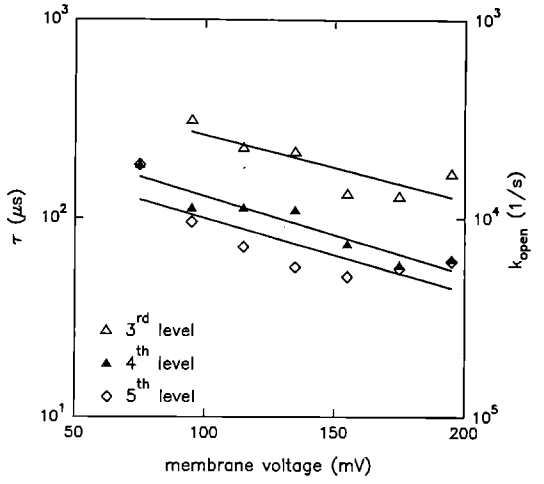

Fig. 5. Mean lifetime τ of the blocked state (*left ordinate*) and rate constant k_{open} (*right ordinate*) vs. membrane voltage for the third, fourth and fifth conductance level of Alm-F30. The voltage dependence is nearly the same for the three conductance states, as the semi-logarithmic plot yields straight lines with almost identical slopes

voltages. It was found that the mean lifetime τ of the blocked state decreases exponentially with increasing membrane voltage, as shown in Fig. 5.

The voltage dependence of blockade is nearly the same for the third, fourth and fifth conductance state of Alm-F30, as the slopes of the straight lines in the semilogarithmic plot of τ vs. membrane voltage (Fig. 5) are almost identical for the three conductance states. The mean lifetime of the blocked state decreases e-fold when membrane voltage is increased by about 120 mV, which corresponds to 0.2 formal reaction charges for the dissociation reaction. This voltage dependence suggests a permeation of protamine through the channel conductance states, the bound polycation may be eluted through the channel lumen.

It should be noted that aside from inducing permeation blockade, protamine has two other effects on Alm-F30 channels. First, the lifetimes of both the channel aggregate and the conductance states are significantly shortened in the presence of the polycation. Second, lower conductance states are stabilized when protamine is present. This might be due to screening of the negative charge of Glu18 by the polycation, as will be discussed later.

4.2. Alamethicin F30 and poly-L-lysine

The peptide polycation poly-L-lysine ($M_r \cong 100{,}000$, $z \cong +775$) also blocks ion permeation through Alm-F30 ion channels. If the polycation is added to the cis-side, blockade is only observed at positive membrane voltages. As in case of protamine, we conclude that poly-L-lysine only binds to the C-terminal mouth of the channel.

In contrast to protamine, poly-L-lysine does not completely block the current through alamethicin F30 channels. Even in the blocked state, the current decreases only to about 20% of the open channel current. This value is independent of the conductance level. Figure 6 shows

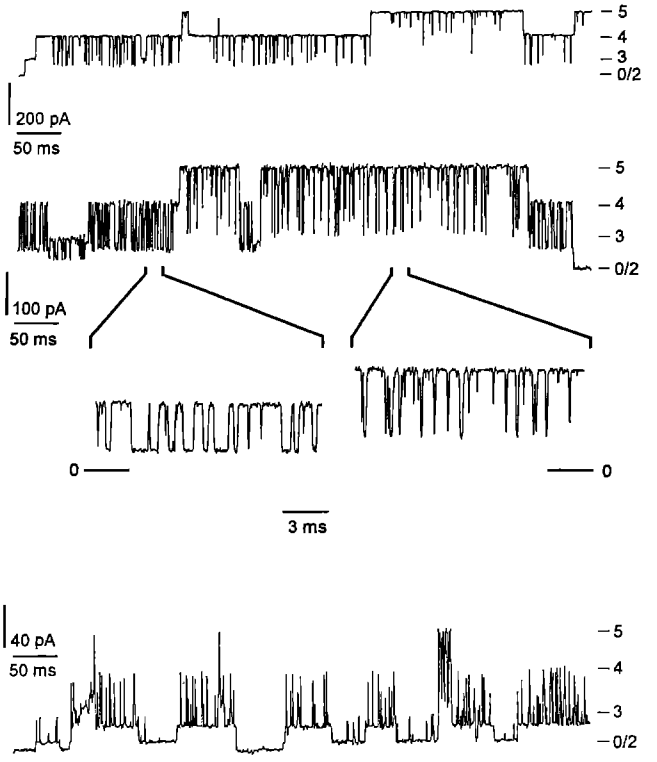

Fig. 6. Single channel current fluctuations of Alm-F30 in presence of 5 μM poly-L-lysine cis at +140 mV membrane voltage. Temperature: 4°C. *Upper trace:* 1.0 M KCl; *middle trace:* 0.5 M KCl; *lower trace:* 0.2 M KCl. The applied alamethicin concentrations were 15 nM, 30 nM and 50 nM, respectively

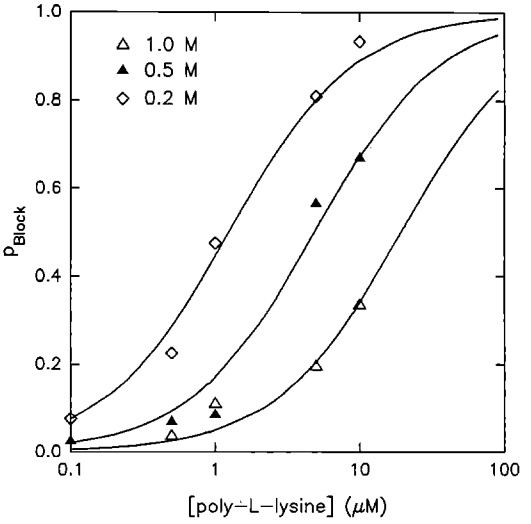

Fig. 7. Block probability versus poly-L-lysine concentration for the fourth conductance state of alamethicin F30 at different ionic strengths. For a given concentration of the polycation, block probability increases as ionic strength decreases. The experimental data were fitted with Eq. (4)

current fluctuations of Alm-F30 in the presence of 5 μM poly-L-lysine cis at different ionic strengths.

Our mass action model gives a good description of the dependence of block probability on polycation concentration. Figure 7 shows a plot of block probability versus

poly-L-lysine concentration for the fourth conductance state of Alm-F30 at different ionic strengths.

The association equilibrium constant K and the mean lifetime τ of the blocked state increase with decreasing ionic strength, which indicates electrostatic binding. Compared with protamine-induced blockade, both K and τ are significantly larger with poly-L-lysine. Data for the third conductance state could not be evaluated as τ became so long for this level that the blocked state could hardly be distinguished from the channel's closed state. Table 3 gives the values for K and τ.

At ionic strengths at or below 0.5 M, K depends on the channel's conductance level. Under this condition, the value of K is significantly larger for the fourth level compared to the fifth. The mean lifetime of the blocked state is longer for the fourth conductance state at all ionic strengths tested. At low salt concentrations, e.g. 0.1 M and 0.05 M KCl, a destabilization of the channel aggregate in the presence of micromolar poly-L-lysine concentrations was observed.

We also examined the membrane voltage dependence of the poly-L-lysine induced blockade. As with protamine, τ decreases exponentially with increasing membrane voltage and an elution of the bound poly-L-lysine molecule into the channel lumen has to be postulated. Figure 8 shows the results of experiments performed at constant ionic strength (0.5 M) and constant poly-L-lysine concentration (5 μM) but different membrane voltages.

The voltage dependence of τ is nearly the same for the fourth and the fifth conductance state. An e-fold increase of τ is achieved by an increase of membrane voltage of about 23 mV, the voltage dependence thus being about fivefold stronger compared to protamine. The dissociation of the bound polycation therefore involves about one formal reaction charge. We conclude that poly-L-lysine permeates conductance levels 4 and 5 far better than protamine.

The shortening of both channel- and conductance state lifetimes and the stabilization of low conductance states found with protamine were also observed in presence of poly-L-lysine. This similarity suggests the same mechanism of action for both polycations. This will be discussed in more detail later.

4.3. *Alamethicin F50 and protamine/poly-L-lysine*

The sequence analogue Alm-F50 lacks the C-terminal free negative charge because of the substitution of glutamate in position (18) with glutamine. In spite of this, it is also blocked by both polycations, but to a significantly lesser extent than its charged analogue Alm-F30. Protamine concentrations of about 0.1 mM or poly-L-lysine concentrations of about 10 μM are required to induce a perceptible frequency of blockade events. Unfortunately, the lifetimes of these blockade events are too short to be evaluated even at lower ionic strength. Thus, our results remain qualitative.

If the polycation is added to the cis-compartment, blockade events are only observed if the membrane voltage is positive. Obviously, both polycations only interact with the C-terminal mouth of the Alm-F50 channel. As noted before, the sequence of Alm-F50 does not contain an anionic residue which could act as polycation binding site. The helical structure of the alamethicin monomer, however, gives rise to a negative electrostatic potential at the C-terminal channel mouth. We shall consider this in the discussion.

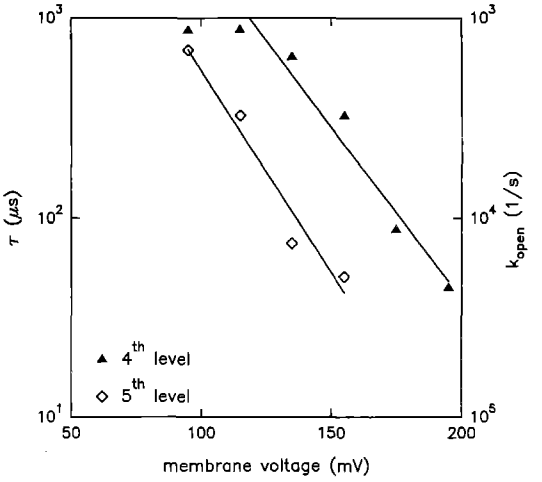

Fig. 8. Mean lifetime τ of the blocked state (*left ordinate*) and rate constant k_{open} (*right ordinate*) versus membrane voltage for the fourth and the fifth conductance state of Alm-F30. τ decreases exponentially with increasing membrane voltage, indicating a permeation of poly-L-lysine through the two conductance states tested

Table 3. Association constants K and mean lifetimes τ of the blocked state for alamethicin F30 and poly-L-lysine

Ionic strength	4th level		5th level	
	K	τ	K	τ
(M)	(l/M)	(μs)	(l/M)	(μs)
1.0	$5.2 \cdot 10^4$	162	$5.7 \cdot 10^4$	57
0.5	$2.1 \cdot 10^5$	606	$9.0 \cdot 10^4$	103
0.2	$8.1 \cdot 10^5$	1050	$2.4 \cdot 10^5$	195

The extent of blockade is only weakly affected by the salt concentration. Effects of the polycations on channel and conductance level lifetimes or conductance level distribution were not observed. Figures 9 and 10 show single channel current fluctuations of Alm-F50 in the presence of either 115 μM protamine or 10 μM poly-L-lysine at different ionic strengths.

4.4. *Alamethicin-F50-phosphate and protamine/poly-L-lysine*

Alamethicin-F50-phosphate shows single channel current fluctuations of the well-known alamethicin type, consisting of bursts with transitions between several non-integral conductance levels. Although its sequence is the same as that of Alm-F50, apart from two negative charges at the very end of the C-terminus, its electrophysiological appearance is quite different. The lifetimes of both the

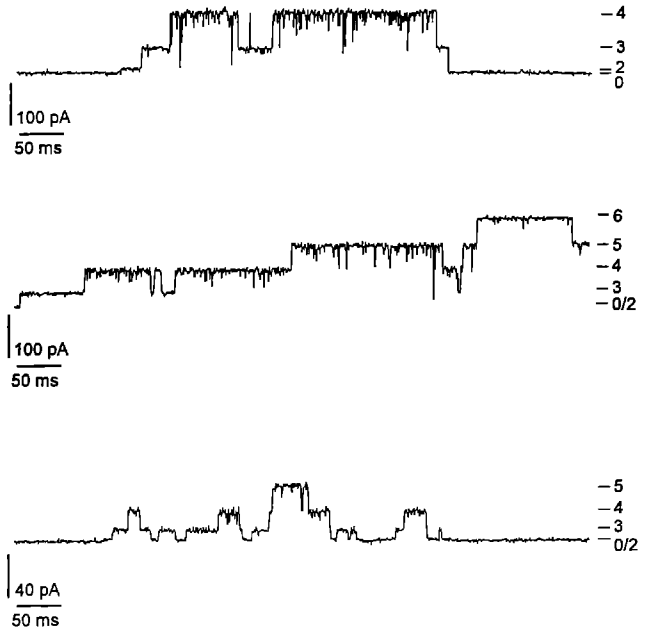

Fig. 9. Single channel current fluctuations of alamethicin F50 in presence of 115 μM protamine cis at a membrane voltage of +140 mV. Temperature: 4°C. *Upper trace:* 1.0 M KCl; *middle trace:* 0.5 M KCl; *lower trace:* 0.2 M KCl. Alamethicin concentrations were 5 nM, 7.5 nM and 13 nM, respectively. Owing to the limited recording bandwidth (3 kHz) in 0.2 M KCl, most of the very short-lived blockade events are not visible in this trace

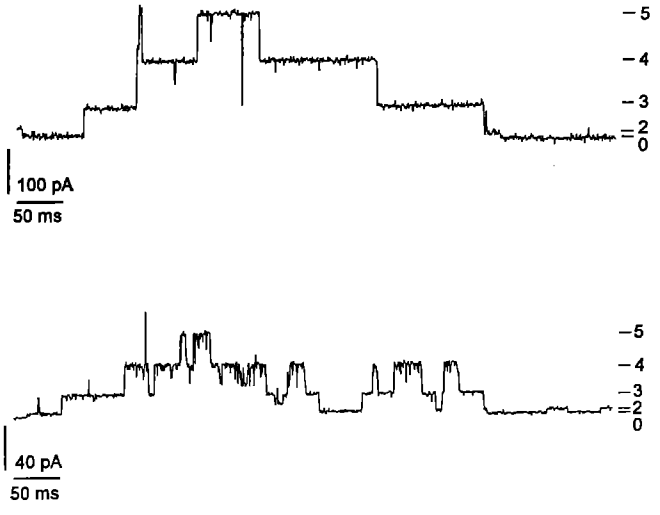

Fig. 10. Single channel current fluctuations of alamethicin F50 with 10 μM poly-L-lysine cis, applied membrane voltage +140 mV. *Upper trace:* 1.0 M KCl; *lower trace:* 0.2 M KCl. Alamethicin concentrations were 5 nM and 13 nM, respectively. Temperature: 4°C

pore aggregate and the conductance levels are significantly prolonged compared with Alm-F50 and even Alm-F30. The probability distribution of the conductance levels shows a shift towards higher levels and resembles Alm-F30. We conclude that the phosphate group esterified to the C-terminal phenylalaninol exerts a considerable stabilization on the pore aggregate.

Ion channels formed by alamethicin-phosphate are blocked by protamine as well as by poly-L-lysine. When

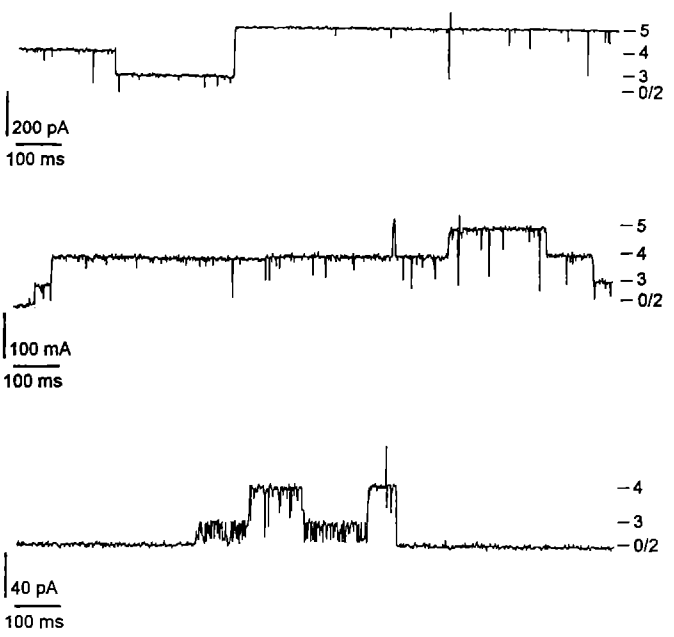

Fig. 11. Single channel current fluctuations of alamethicin-F50-phosphate in presence of 2.3 μM protamine cis at a membrane voltage of +145 mV and different ionic strengths. Temperature: 4°C. *Upper trace:* 1.0 M KCl; *middle trace:* 0.5 M KCl; *lower trace:* 0.2 M KCl. Concentrations of alamethicin F50-phosphate were 100 nM, 160 nM and 380 nM, respectively. Please note the high alamethicin concentrations required to obtain single channel current fluctuations at this membrane voltage

Table 4. Association constants K and mean lifetimes τ of the blocked state for alamethicin F50-phosphate and protamine

Ionic strength	3rd level		4th level		5th level	
	K	τ	K	τ	K	τ
(M)	(l/M)	(μs)	(l/M)	(μs)	(l/M)	(μs)
1.0	$1.2 \cdot 10^4$	112	$1.0 \cdot 10^4$	90	$8.7 \cdot 10^3$	63
0.5	$2.0 \cdot 10^4$	226	$2.1 \cdot 10^4$	143	$2.3 \cdot 10^4$	72
0.2	$3.4 \cdot 10^5$	790	$3.1 \cdot 10^5$	227	$1.0 \cdot 10^5$	221

polycations are present in the cis-compartment, blockade is only observed at positive membrane voltages. As in the case of Alm-F30 and Alm-F50, this may be explained by polycations binding exclusively to the C-terminal mouth of the channel aggregate.

Figure 11 shows single channel current fluctuations of Alm-F50-P in the presence of 2.3 μM protamine cis. One can see that the appearance of current fluctuations and blockade events closely resembles that of Alm-F30.

The association constant K for protamine binding to Alm-F50-P and the mean lifetime τ of the blocked state are functions of both ionic strength and the conductance level of the channel aggregate. As in the case of Alm-F30, both parameters increase with decreasing ionic strength. Table 4 shows the values determined for τ and K.

At 1.0 M salt concentration, the association constant K for protamine binding to Alm-F50-P is lower compared with Alm-F30. A significantly stronger binding of protamine by Alm-F50-P, as would be expected from its

higher C-terminal charge, is only found at 0.2 M ionic strength. We shall come back to this in the discussion. The mean lifetime τ of the blocked state, however, is longer compared to that with Alm-F30 under the same conditions.

The 775-residue poly-L-lysine blocks alamethicin-F50-phosphate channels as well as those formed by Alm-F30. As for the latter, blockade occurs only at cis positive membrane voltages if the polycation is added to the cis side. Blockade of these two alamethicin analogues by poly-L-lysine looks very similar, as may be seen in Fig. 12, which shows current fluctuations of Alm-F50-P channels in the presence of 5 μM poly-L-lysine cis at different salt concentrations. Values of the parameters K and τ are listed in Table 5.

For a given concentration of the polycation, K and τ increase when the ionic strength decreases. From the higher C-terminal charge of Alm-F50-P, one might expect significantly higher values of both parameters compared to Alm-F30. In the case of the association constant K, this expectation is met at ionic strengths below 1.0 M.

Table 5. Association constants K and mean lifetimes τ of the blocked state for alamethicin F50-phosphate and poly-L-lysine

Ionic strength	4^{th} level		5^{th} level	
	K	τ	K	τ
(M)	(l/M)	(μs)	(l/M)	(μs)
1.0	$6.7 \cdot 10^4$	210	$4.9 \cdot 10^4$	73
0.5	$2.5 \cdot 10^5$	572	$2.2 \cdot 10^5$	223
0.2	$2.3 \cdot 10^6$	1180	$9.0 \cdot 10^5$	856

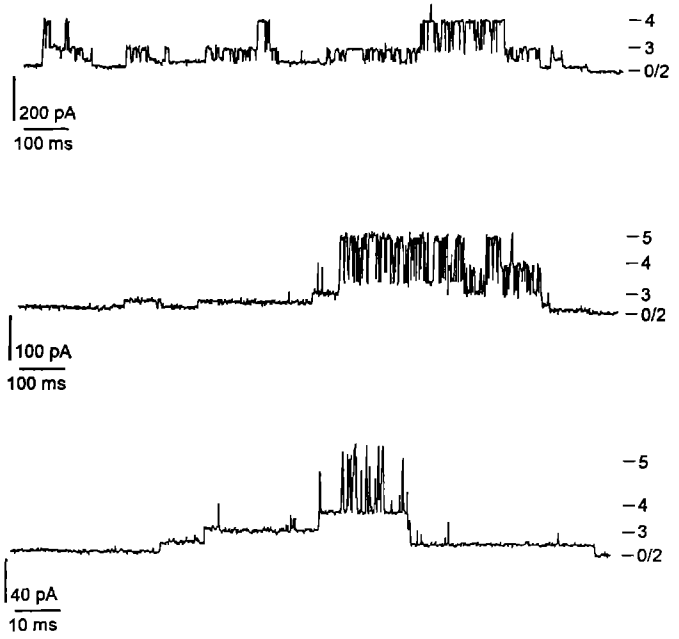

Fig. 12. Single channel current fluctuations of alamethicin F50-phosphate at different salt concentrations and 5 μM poly-L-lysine cis. Recordings were made at +145 mV membrane voltage and 4°C. *Upper trace:* 1.0 M KCl; *middle trace:* 0.5 M KCl; *lower trace:* 0.2 M KCl. Alamethicin-F50-phosphate concentrations are the same as in Fig. 11

In addition, the values of K as well as of τ are larger for the fourth compared to the fifth conductance state at these salt concentrations. Poly-L-lysine proves also in the case of Alm-F50-P to be more effective in blocking than protamine.

Besides blocking ion permeation through Alm-F50-P channels, both polycations stabilize lower conductance states of the peptide, i.e. the probability distribution of the observed conductance states is shifted towards lower conductance states. In addition, the lifetimes of both the pore aggregate and the conductance levels are drastically shortened in the presence of the polycations. If we recall that same observation for Alm-F30 ion channels, we see again that shielding of the C-terminal charge by polycations appears to significantly affect the stability of the pore aggregate as well as of certain conductance levels.

5. Discussion

In this work, we have shown that the peptide polycations protamine ($z = +21$, $M_r = 4332$) and poly-L-lysine ($M_r \cong 100{,}000$, $z \cong +775$) in micromolar concentrations block ion permeation through ion channels formed by alamethicin F30, alamethicin F50 and alamethicin-F50-phosphate. In order to give a molecular interpretation of this effect, we refer to the "barrel stave" model as proposed by Baumann and Mueller (1974) and Boheim (1974). We further consider the α-helical alamethicin monomer as an electrical dipole with half a negative elementary charge at the C-terminus and half a positive elementary charge at the N-terminus (Hol et al. 1978; Boheim et al. 1983; Schwarz and Savko 1982). These helical monomers are supposed to surround the central permeation pathway, their dipole moment vectors aligned in parallel to each other and the transmembrane electric field vector. Thus, the C-terminal mouth of the channel aggregate is always oriented towards the membrane side with the more positive electric potential. Fluctuations between different conductance states are explained as uptake and release of monomers into and from the aggregate. Experimental evidence for this model has been published by Hanke and Boheim (1980) and Boheim et al. (1983).

If the polycations are added to the cis-compartment, blockade events are only observed at positive membrane voltages. According to the model outlined above, an explanation for this phenomenon is that polycations bind exclusively to the C-terminal mouth of the channel aggregate. If we assume electrostatic binding, which suggests itself by the strong dependence of blockade on the salt concentration, the only anionic binding site in the primary structure of Alm-F30 is glutamate in position (18). At negative membrane voltages, the positively charged N-terminal part of the dipoles in the aggregate points to the cis-side, and binding does not occur.

If glutamate in position (18) is the polycation binding site, protonation of this residue should decrease the negative electrostatic potential at the C-terminal mouth and hence interfere with permeation blockade. Block probability in fact decreases with decreasing pH, the relation between these parameters is described by a simple titra-

tion curve. Data fitting yields an apparent pK of 4.8 for the third and the fourth conductance state, which is about the pK for a glutamate residue in a protein. The unknown limit of block probability at acidic pH restricts the accuracy of the measured pK to ± 0.15 pK-units. Gisin et al. (1977) published a pK-value of alamethicin F30-monomers in ethanol/water (3:7 v/v) of 5.2. However, one should be careful in comparing these values because the pK-value measured in this work is in fact the pK-value of the entire channel aggregate in the lipid environment, which might be somewhat different from the pK of a monomer in ethanol/water.

Further evidence for Glu18 as the binding site for polycations in Alm-F30 comes from comparison with Alm-F50, in which this residue is exchanged with glutamine. As was expected, blockade of this analogue starts at polycation concentrations one to two orders of magnitude higher than those required for Alm-F30. One may wonder why Alm-F50 is blocked by polycations at all because its primary structure does not contain any anionic residue. A possible origin for a negative electrostatic potential is the C-terminal dipole charge of the helix, which equals about half an elementary charge (Hol et al. 1978; Schwarz and Savko 1982). Phenylalaninol and the γ-carbonyl oxygen of glutamine in position (18) may very likely contribute to this. Considerations of the same kind also explain why the protonated Alm-F30 is still blocked by polycations.

Adding charge to the C-terminus of Alm-F50 by esterifying phenylalaninol(20) to phosphoric acid in Alm-F50-P greatly enhances blockade by polycations. This can be taken as an additional proof for polycations binding to the C-terminal mouth of the pore aggregate. From the larger C-terminal charge of Alm-F50-P compared to Alm-F30, one would expect significantly higher values of the association constant K and the mean lifetime τ of the blocked state for the former. However, this behaviour is only found at low salt concentrations. In 1.0 M KCl, the effect is just opposite to that expected. To understand this we have to consider the spatial charge distribution at the C-terminal pore mouth. Owing to electrostatic repulsion, the phosphate groups may be arranged with a maximum distance from each other. Assuming circular geometry for the pore mouth, this results in a considerable distance of the charged phosphate groups from the channel entrance. Hence, the electrostatic potential at the channel entrance will be significantly smaller than that immediately adjacent to the phosphate groups. This effect should be less pronounced at low ionic strength, as can be estimated from the increasing Debye length.

The "barrel-stave" model can account for the dependence of the association constant K and the mean lifetime τ of blockade on the conductance state of Alm-F30, which becomes significant especially at lower ionic strengths. If we assume that the channel aggregate consists of n monomers, each carrying one elementary charge e_0, the total C-terminal charge will be

$$z = n \cdot e_0.$$

If each of the n monomers is of diameter a, the lumen area of the "barrel stave" pore aggregate is (Hanke and Boheim 1980)

$$A = n^2 a^2 / 4\pi.$$

We obtain for the charge density $\sigma = z/A$

$$\sigma = 4\pi \cdot e_0 / n a^2.$$

The charge density is inversely related to the number of monomers in the aggregate and therefore decreases with increasing conductance level. As the electrostatic potential is proportional to the surface charge density, the potential-dependent parameters τ and K decrease as well.

The mean lifetime τ of the blocked state becomes significantly shorter at more positive membrane voltages. This indicates that both polycations permeate through channel structures which correspond to the third to fifth conductance states of Alm-F30. The voltage dependence of τ, as described by the voltage change necessary to achieve an e-fold change of this parameter, is about five-fold larger for poly-L-lysine compared to protamine. This points to a better mobility of the former in the channel lumen. We shall compare the diameter of the polycations with the diameter of the third state's channel lumen, which is estimated as 10.5 Å by Hanke and Boheim (1980) and 12.6 Å by Bezrukov and Vodyanoy (1993). There is evidence that the secondary structure of both polycations in solution is roughly globular at neutral pH (Walton and Blackwell 1973; Gatewood et al. 1990). Diameters of about 30 Å for salmon protamine and 80 Å for the 100 kD-poly-L-lysine are likely. It follows that both polycations have to undergo conformational transitions, e.g. becoming stretched out to a linear chain during permeation. The high electric field strength inside the channel, which is sensed by charged residues, makes this conceivable. At this point, one should remember that for a given polycation the voltage dependence of τ is almost the same for each of the tested conductance states. Hence, the channel diameter does not markedly influence polycation permeation, and we would rather suggest that the work of unfolding polycations limits their permeability through the channel lumen.

The better mobility of poly-L-lysine inside the pore is then due to a less rigid structure, which facilitates the transition from a "random coil" structure in the solution to a stretched-out conformation in the channel lumen. This flexible structure may also enable poly-L-lysine to approach the charged binding site more closely than protamine, which would be a straightforward explanation for its greater effectiveness in blocking Alm-F30 channels. The three proline residues in the primary structure of protamine may hinder the adoption of a stretched conformation.

The permeation of polycations has consequences for the validity of the mass action model, as it disturbs equilibrium between bound polycations and those free in solution. However, a steady-state is approximately given if the rate of permeation through the channel is small compared to the rate of polycation binding. If we consider the "worst case", i.e. bound polycations are solely eluted through the channel, we have the condition $k_{\text{block}} \gg k_{\text{open}}$ or, with Eq. (4), $K \cdot k_{\text{open}} \gg k_{\text{open}}$ for the validity of the model. With K being of the order of $10^4 - 10^5\ \text{M}^{-1}$, this condition is fulfilled to a good approximation.

In contrast to protamine, poly-L-lysine does not completely block ion permeation through the channel. For all the three conductance levels evaluated, current decreases during blockade to about 20% of the open channel current. As the current through the three conductance levels is decreased by a constant percentage instead of a constant value, one can not argue that the polycation molecule is simply too small to completely block the channel. A more reasonable explanation involves the Debye-Hückel theory, which predicts that the neighbourhood of the poly-L-lysine molecule will be depleted of potassium ions. Thus, the current through the channel in the presence of a bound polycation molecule is mainly anionic, carried by mobile counterions. Indeed, there is experimental evidence for anion selectivity of the alamethicin multi-pore system in the presence of polycations (Mueller and Rudin 1968). The mobility of these counter-anions is, however, markedly reduced compared to free solution, which results in a reduced channel conductance for all levels. In addition, alamethicin channels are slightly cation selective.

Aside from permeation blockade, poly-L-lysine and protamine exert some other effects on Alm-F30 channels. The lifetime of both the conductance states and the pore decrease significantly in the presence of polycations, and low conductance states are stabilized. In general, the single channel current fluctuations of Alm-F30 and Alm-F50-P in the presence of polycations resemble those of uncharged peptaibols like Alm-F50, paracelsin A or hypelcin A_1. A crucial role for the C-terminal structure and charge of peptaibols in stabilizing channel states has been shown earlier (Boheim et al. 1987). The significantly prolonged pore and state lifetimes of Alm-F50-P compared to Alm-F50 and the enhanced stability of high conductance states in the former peptide contribute to this assumption. It seems likely that a reduction or screening of C-terminal charge stabilizes low conductance levels as the weaker electrostatic repulsion between the monomers makes smaller aggregates energetically more favourable. Polycations might also interfere with the C-terminal intermolecular system of hydrogen bridges, which would explain shorter lifetimes of both the channel and the channel states. Aside from direct action on the channel aggregate, one could also imagine an interaction of polycations with membrane lipids. However, there is no experimental evidence for this hypothesis. On the contrary, we did not observe any leakage currents through the bilayer after the addition of polycations in the absence of alamethicin. Leakage currents are often associated with lipid/protein interactions and may be indicative for this phenomenon. Thus, we prefer a model of direct interaction between channel and polycation.

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