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Electric field dependence of alamethicin channels

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Circular dichroism (CD) of alamethicin embedded in vesicular membranes from outside, and its change, upon imposing Donnan potentials across the membrane, was measured. The changes in CD suggested a decrease in a helicity and increase in β structure with the membrane potential positive inside and vice versa when thepotential was positive on the outer side of the vesicles from where the alamethicin was inserted into the membrane. The Donnan potential was created by entrapping the polyacrylate (PA^-) in the vesicles and changing the salt concentration outside or by adding different concentrations of PA^- or polyethyleneimide (PEI^+) at the outside of vesicles with $2 \cdot 10^{-5}$ M salt inside. The effect of the potential on the CD spectra and thus the alamethicin conformation is independent on the type of the polyelectrolyte employed for the Donnan potential generation.

Introduction

Alamethicin, a 20 amino acid linear polypeptide from the fungi *Trichoderma viride* [1] is known as a voltage gated channel former in lipid membranes [2–7].

It was shown [3] that the channel conductance is increasing exponentially with the applied voltage, and the pore, once opened, may have several conductance states, each having voltage dependent conductivity. The alamethicin conformation was studied by NMR [13–15], infrared spectroscopy [6,12] and X-ray diffraction [8]. Most of the results show a very high degree of helicity. Circular dichroism results [16–19] also show that the alamethicin conformation strongly depends on its environments.

Alamethicin penetration into the lipid bilayer remains a disputed problem. Fluorescence studies [11] showed that alamethicin analogs penetrate lipid bilayers with the long axis perpendicular to the surface, but aggregation models are controversial. Some authors [7,18] support the idea that alamethicin is adsorbed to the bilayer as monomers, and the external electric field contributes to monomer association, to channel formation by insertion of the so formed oligomers into the lipid membrane, and eventually to the channel opening. Alternatively, it is claimed that the oligomer aggregation and their insertion into the lipid layer are field independent and the field only induces pore opening [8]. Neither conductance studies on alamethicin analogs [9], nor theoretical considerations [10] had brought further clarifications of the problem.

In our present paper we present evidence that the electric field affects the conformation of alamethicin interacting with vesicular lipid membranes. The results can be interpreted as an effect of the electric field on the insertion of alamethicin into the membrane and on the conformation of the inserted polypeptides

Materials and Methods

Alamethicin from *Trichoderma viride* was purchased from Sigma and used without further purification.

Small sonicated unilamellar vesicles were prepared from egg phosphatidylcholine (Lipid Products, Surrey); in a solution contained $2 \cdot 10^{-5}$ M NaCl and 0.1 M glucose by sonication in a bath sonicator (Lab. Suppl. Co. Hicksville, NY) until a nearly clear dispersion was obtained. After vesicle preparation, small aliquots of alamethicin in methanol (10 μl) were added to about 1.8 ml of vesicle icontaining solution. The final lipid concentration was 1 mg/ml and the final polypeptide concentration was 150 μg/ml. The polypeptide to lipid ratio was about 1 : 20.

After 5 min, during which all the alamethicin entered the lipid bilayer, different amounts of 0.1 N (normality equals molarity in monomeric units, since the degree of polymerization $\approx$ 100000, the PA^- molarity is negligibly small) sodium polyacrylate (PA^-) solution were added. Free alamethicin binds cations and tends to interact with PA^- to form gels. Lack of gelation with

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added PA^- indicates that most of the alamethicin is fully incorporated into the membranes. The final polyacrylate concentrations were between 0 and 0.1 N. The concentrations of Na^+ and glucose added up to 0.1 M to maintain isotonicity. Due to the permeability of alamethicin channels to small ions, different polyacrylate concentrations resulted in different Donnan potentials across the membrane.

In order to reverse the field direction, we prepared big phosphatidylcholine (PC) vesicles by injection of PC solution in pentane [20] into a solution containing 0.1 M sodium polyacrylate. After vesicles formation, the polyacrylate from outside the vesicles was removed by treatment with excess (about 100 equivalents) anion exchange resin (Bio-Rad, Richmond, CA) for 4 h. The collected residue suspension was then centrifuged twice at $2500 \times g$ to remove the remaining resin. The final polyacrylate concentration outside the vesicles was measured by precipitation with $CaCl_2$ and by measuring the light scattering at 350 nm of the resuspended precipitate. For calibration we used precipitates obtained from polyacrylate solutions at different concentrations.

$CaCl_2$ induced also a partial aggregation of the vesicles due to its interaction with lipids which produced a background light scattering. To subtract this background we subtracted the scattering from vesicles in glucose solution without polyacrylate and with Ca^{2+} added. After the ion exchange resin treatment, the vesicles were dialyzed for 12 hours against solutions containing $2 \cdot 10^{-5}$ M NaCl and 10^{-1} M glucose and 0.03 M NaCl and 0.01 M glucose. The alamethicin was added 10 min before any measurement. After the ion exchange treatment, only 0.5 mg/ml phospholipid remained in the sample as determined by phosphate measurements. This was taken into account when adding alamethicin, in order to obtain a 1:20 polypeptide to lipid ratio.

As an alternative method to obtain a negative inside Donnan potential across the vesicular membrane, we prepared vesicles as in the first case (i.e., in glucose solution), and added polyethyleneimide (PEI^+) instead of sodium polyacrylate after incubation with alamethicin. The polyethyleneimide concentrations as well as the polypeptide to lipid ratio were about the same as polyacrylate. The use of polyethyleneimide for establishing Donnan potential enabled us to measure also the electric field intensity by using the potential sensitive fluorescent dye $DisC_2$ [24], which could not be done with polyacrylate which binds the dye and induces its quenching. The dye dissolved in methanol was added to lipid vesicles samples, having 0.2 mg/ml lipid. The final dye concentration was 10^{-8} M. Its fluorescence in the presence of lipids was about half of its fluorescence in their absence. After addition of 0.1 M polyethyleneimide, we added alamethicin and measured the fluorescence decay with a Perkin-Elmer luminescence spectrophotometer. The same experiment was repeated with several alamethicin concentrations between 1 to 10 μg/ml.

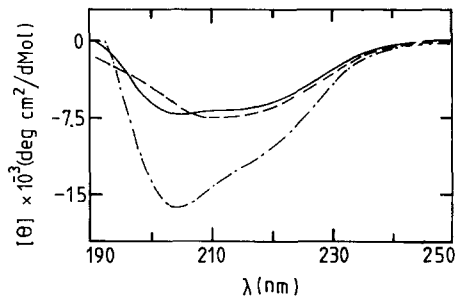

Fig. 1. Far UV CD spectra of alamethicin solutions. ———, 20 mg/ml methanol solution diluted 200-fold in water; - - - - - -, same, in the presence of 0.1 M sodium polyacrylate; ·–·–·, 10^{-5} M alamethicin in methanol.

The effect of the different electric potentials on protein conformation was estimated by far ultraviolet circular dichroism (CD).

The CD spectra were obtained with a JASCO 500 spectropolarimeter, using a 0.2 mm pathlength cylindrical cuvette, and performing 16 scans (between 190 and 260 nm and 20 nm/min scan speed) per sample. The samples containing 0 and 0.1 N polyacrylate were sonicated for 10 min and then remeasured.

Every sample prepared for CD measurement had its blank, i.e., an identical sample but without alamethicin. Each blank was subtracted from its corresponding sample, so we were able to separate the induced field-modification of the CD spectrum of alamethicin from the polymer-induced effects.

As further control, CD spectra of alamethicin in water and methanol without lipid and the effect of polyacrylate on these CD spectra were also measured (Fig. 1).

The Donnan potential, $\Delta\psi$, was calculated from the Donnan equilibrium fulfilling the requirement for electroneutrality and that the anion and cation distribution between the two sides of the membrane is determined by the potential

$$\Delta\psi = \frac{RT}{F} \ln \frac{a^+_{out}}{a^+_{in}} = \frac{RT}{F} \ln \frac{a^-_{in}}{a^-_{out}} \tag{1}$$

where a is the activity of ionic species involved.

When we add different quantities of 0.1 N $Na_\nu PA^{\nu-}$ to the vesicular suspension prepared in salt concentration C (here $C = 2 \cdot 10^{-5}$ M) and 0.1 M glucose to maintain equal osmotic pressure inside-outside, requirements of equilibration ($[Na^+]_{out}[Cl^-]_{out} = [Na^-]_{in}[Cl^-]_{in}$ at electroneutrality demands a flux of neutral NaCl into the vesicles. The increase in osmotic pressure due to the increase in NaCl concentration in the vesicles is negligible and the vesicular volume is considered to

remain constant. The Donnan potential at equilibrium becomes then:

$$\Delta\psi = \frac{RT}{F}\ln\frac{f[\mathrm{PA}]+C-\Delta C}{C+(v_o/v_i)\cdot\Delta C} = \frac{RT}{F}\ln\frac{C+(v_o/v_i)\cdot\Delta C}{C-\Delta C} \qquad (2)$$

where $f = 0.2$ is the PA^- counterions osmotic or activity coefficient in the absence of salt, taken to be the effective counterion contribution to the activity [21,22], [PA^-], the polymer concentration in monomeric units. ΔC is the decrease in salt concentration in the outer solution due to equilibration after addition of the polyacrylate, it is calculated by equating the logarithmic terms. v_o/v_i is the outer to inner volume ratio (200) as determined from the measured internal volume and the lipid concentration. $\Delta\psi$ was similarly calculated for PEI^+ outside and PA^- inside the vesicles.

In the case when PA^- was inside the vesicles and the salt concentration in the outer phase, C_o was obtained by equilibrium dialysis, the Donnan potential is given by

$$\Delta\psi = \frac{RT}{F}\ln\frac{f[\mathrm{PA}]+\Delta C}{C} = \frac{RT}{F}\ln\frac{C}{\Delta C} \qquad (3)$$

The equation of the membrane potential to the Donnan potential is an idealization assuming that no segment of the polysalts penetrates the membrane.

Results

Fig. 1 shows the free alamethicin CD spectra in methanol and in water as well as the influence of sodium polyacrylate on the alamethicin spectrum in water. The direct contact between alamethicin clusters and sodium polyacrylate induces crosslinking and gel formation, which we had to resuspend by strong agitation. The resulting CD spectrum is affected by absorption flattening and scattering [23]. The major influence of the polyacrylate on alamethicin solution appears to be a lowering of ellipticity in the range of 190–205 nm and its small enhancement above 210 nm. We considered subsequently only data obtained in the range of 200–240 nm for secondary structure estimations, since the noise level below 200 nm did not permit adequate accuracy.

The suppressed ellipticity around 200 nm resulting from clustering of alamethicin oligomers around PA^- is absent when alamethicin is embedded in lipid membranes. The same solid line spectrum obtained in the absence of PA^- presented in Fig. 2a is obtained within experimental error when alamethicin is added to PC vesicles prepared by sonication in 0.1 M PA^- with the PA^- in the outer phase exchanged by Cl^- and thus obtaining 0.1 M NaCl outside (potential < 3 mV). The spectrum remains the same after adding 0.1 M PA^- to the outer phase.

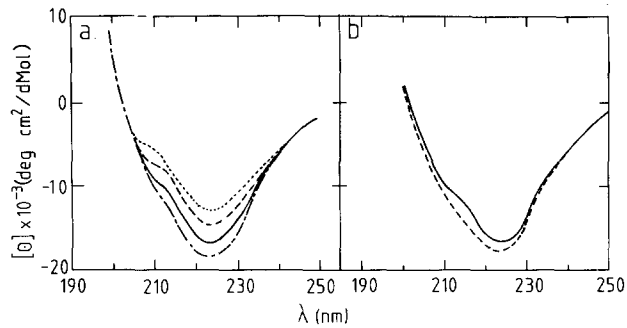

Fig. 2. (a) CD spectra of alamethicin embedded in PC vesicles prepared in $2\cdot10^{-5}$ M NaCl (alamethicin concentration 150 μg/ml, lipid concentration 1 mg/ml). (polypeptide/lipid molar ratio 1 : 20) in the presence of added or entrapped PA^-. ———, no PA^- (identical spectra were obtained with 0.1 M PA^- inside the vesicles and 0.1 M NaCl outside (Donnan potential ≈ 2 mV); - - - - -, with 0.03 M PA^- outside the vesicle; ·····, with 0.1 M PA^- outside the vesicles; ·–·–·, with 0.1 PA^- inside the vesicles. PA^- outside exchanged for Cl^- and dialyzed against $2\cdot10^{-5}$ M NaCl and 0.02 M glucose. (b) CD spectra of polyethylene imide-affected alamethicin vesicles. ———, 150 μg/ml alamethicin embedded in PC vesicles prepared in $2\cdot10^{-5}$ M NaCl (lipid concentration 1 μg/ml); - - - - -, same vesicles with 0.1 M polyethyleneimide added to the outer phase.

Fig. 2a shows the CD spectra of alamethicin for different electric potentials either positive inside the vesicles (polyacrylate added to vesicles with low salt content and with alamethicin embedded in the membrane) or positive outside (PA^- in the vesicles). When the polyacrylate concentration outside increases, we may notice the decrease in the absolute value of the CD signal at 220 nm and the appearance of a shoulder at shorter wavelength. When the potential is positive out the outside of the vesicle surface, the effect is reversed, i.e., the CD band at around 220 nm is increased. The effect does not depend on the way the field is created, namely by PA^- in the vesicles or polyethyleneimide (PEI^+) in the outer phase, as shown in Fig. 2b.

Fig. 2b shows the PEI^+ effect on the CD spectra increasing the helicity at 222 nm with its concentration. Cascio and Wallace [18] showed that the CD spectra depend on the lipid to alamethicin ratio. The effect of potential on the CD spectrum remained practially the same when the lipid/alamethicin molar ratio was changed within an order of magnitude. Fig. 3 shows the electric field effect on the alamethicin CD spectrum when the polypeptide/lipid ratio is 1 : 100 (lipid concentration is increased 5-fold). One may observe that the effects are similar to those shown in Fig. 2a. The similarity of the results in Figs. 2a, 2b and 3 indicates that the conformation of alamethicin is a function of membrane potential and its direction only, irrespective of the experimental condition under which the potential has been generated. The dashed CD curves show the effect of the sonication on the samples. Sonication cancels the field by mixing outside and inside contents of the vesicles. We notice that after sonication the

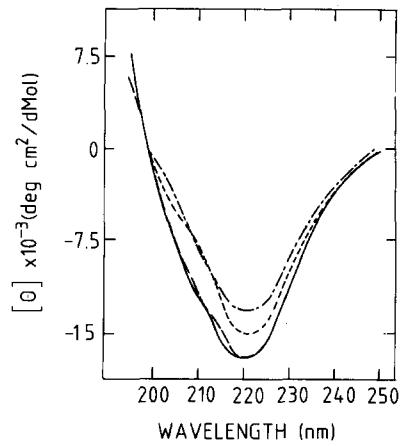

Fig. 3. CD spectra of alamethicin (150 μg/ml) in PC vesicles $2 \cdot 10^{-5}$ M NaCl inside (5 mg/ml) in the absence (———) and in the presence (·–·–·) of 0.1 N PA^- added outside the vesicles. The polypeptide/lipid ratio is 1:100. The short dashed line (- - - - - -) represents the CD spectra of the sample with the added PA^- after 15 min sonication. The long dashed line (— — —) after 15 min sonication of the sample without PA^-.

ellipticity of the field affected alamethicin increased, although the unperturbed value by the electric field was not restored. It seems that PA^- is protecting the alamethicin containing vesicles and in the bath sonicator their rupture could not be completed. In Fig. 4 the change in ellipticity, $\Delta\theta$, is presented under different experimental conditions as a function of the calculated transmembrane potential. The experimental points obtained under the different conditions are a continuous curve. Table I shows the secondary structure calculated form the ellipticity at different potential differences across the membrane. The evaluation of the alamethicin conformation was done similarly to that of bacteriorhodopsin under the influence of electric field [24], namely, by fitting the basis spectra for α helix, random coil and β forms presented either by Chen et al. [25] or by Chang et al. 26]. There is a trend toward β structure as the positive inside potential increases and toward α

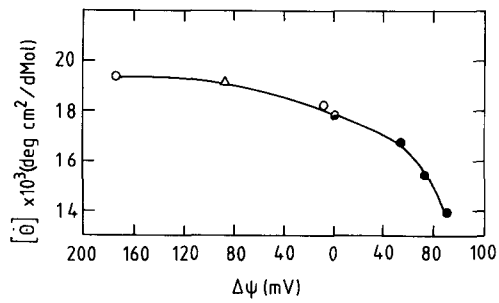

Fig. 4. The molar ellipticity of alamethicin as function of Donnan potential calculated from Eqn. 2. The experimental points were obtained by PA^- addition outside the vesicles (●), by preparing the vesicles with 0.1 M PA^- and then dialyzing against NaCl (○) or by adding PEI^+ outside the vesicles (△). See text for details about generating the electric field.

TABLE I

Alamethicin conformation at different membrane potentials

The polarity corresponds to the inner phase of the vesicles.

$\Delta\psi$ (mV)	$[\delta]_{223}$ (deg·cm²·dmol⁻¹)	α-helix (%)	β-helix (%)	β-turn	Unordered (random)
89	12900	48	40	3	9
72	14400	63	16	5	16
55	15700	68	13	4	15
0	16800	74	8	5	12
−89	18100	79	10	6	5
−173	18400	81	8	5	6

helicity at opposite polarity. Fig. 5 shows the fluorescence quenching of the fluorescent dye $DisC_2$ [5], when a Donnan potential is applied. The procedure is described in the experimental section. Addition of alamethicin in the absence of a polyelectrolyte does not have any effect on the fluorescence. Addition of PEI^+ to the outer phase induces Donnan potentials positive outside across the membrane. The potential increases the concentration of the dye on the inner negative side of the membrane, which causes additional adsorption of the dye and further quenching. The so induced quenching is a function of the membrane potential which can be determined by calibration with the K^+ gradient and valinomycin. The Donnan potential in these experiments was induced by PEI^+ as PA^- binds the cationic dye and causes fluorescence quenching even in the absence of vesicles. The halftime of quenching (i.e., the time required for the polypeptide to allow the Donnan equilibrium plus the time required for the quenching) is of the order of 1 min, the same order of magnitude as in the case of insulin-adipocyte interaction [27]. It may be noticed that the final fluorescence value is independent of the alamethicin concentration (about 2 μg/ml), but the kinetics of the fluorescence decay is faster when more alamethicin is present.

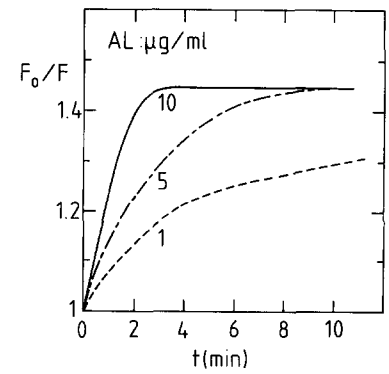

Fig. 5. Time dependence of fluorescence quenching of the potential sensitive dye $DisC_2(5)$ in presence of 0.1 mg/ml DC vesicles in $2 \cdot 10^{-5}$ M NaCl upon addition of 0.1 M PEI^+ and different amounts of alamethicin to the outer phase. Excitation wavelength was 620 nm and emission wavelength 670 nm.

Discussion

No diffusion potential can be maintained across a membrane which is unselectively leaky to small ions. If the membrane is not permeable to polyions, Donnan potennetials and stable electric fields across the membrane can be established (Fig. 5). The potential inferred from the fluorescence quenching of the potential sensitive cationic dye $DisC_2$ is only about two thirds of that calculated by Eqn. 2. There may be several reasons for this discrepancy: one is the possible adsorption of PEI^+ on the membrane surface with subsequent effect on the dye adsorption. On the other hand the calculated potential may be overestimated if the osmotic coefficient is lower than 0.2. However, at this point we merely wanted to show that Donnan potential provides a means to generate membrane potentials when the membrane is unselectively permeable to small ions. Donnan potential may, therefore, also play an important role in contributing to the potential of cell membranes and to the cross membrane electric fields. According to our results, this field generates a conformational change in alamethicin molecules. The conformational change increases with the field strength and its direction depends on the field polarity. To illustrate this, 223 nm ellipticities are represented in Fig. 4 as function of the Donnan potential calculated from Eqn. 2. When the vesicle inside is positive, the field induces a transition from helix to more extended (β) structures (Table I). If negative potential is applied at the vesicle interior, one observes an increase in the helicity. Conductivity data [4] show that either positive or negative potentials induce conductance of the alamethicin channel, but, as shown in black lipid membrane experiments [3], if the alamethicin is present at only one side of the membrane, only potentials positive at the alamethicin-containing side induce pore opening. In the present experiments α helicity increases at positive potential on the outer side of the vesicles from where alamethicin penetrates the membrane.

Our experiments show that when the electric field across the vesicular bilayer membrane is negative outside, there is a decrease in α helicity and increase in β structure. Electric field in the opposite direction enhances α helicity. This kind of conformational change can be brought about by a direct effect on the alamethicin molecules inserted in the membrane in a preferential direction, but it can also affect the insertion of alamethicin either as monomers or as oligomeric aggregates with subsequent effect on the conformation. This is in keeping with a fixed direction of the inserted alamethicin molecules. If the field would determine the direction of the inserted dipoles its effect on the conformation should be the same irrespective of tis direction. The applied field across the membrane can modulate the channel conductance by direct effect on the insertion and on the conformation of the inserted components. Comparing the present data with the conductivity data when alamethicin has access only to one side of the membrane one can conclude that cross membrane field, positive on the alamethicin side enhances its insertion and α helicity. Opposite polarization favors β structure and may inhibit insertion.

Acknowledgements

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