Artificial Membrane Excitability Revisited and Implications for the Gating of Voltage-Dependent Ion Channels

H Duclohier[1] and G Spach[2]

1 *Interactions Cellulaires et Moleculaires, UMR 6026 CNRS Universite de Rennes I, Rennes Cedex, France*

2 *UMR 6522 CNRS 'Polymeres, Biopolymeres, Membranes', Universite de Rouen, 76821 Mont Saint Aignan, France*

Abstract. Excitability phenomena in planar lipid bilayers doped with alamethicin and protamines have been first described by Mueller and Rudin (Nature 217, 713–719, 1968) These properties are reinvestigated here with virtually solvent free bilayers made of synthetic phospholipids doped with alamethicin charged component (Glu18) and protamine or other synthetic basic polypeptides After retrieving the narrow set of experimental requisites allowing negative resistance and action potentials to develop, the potencies of different basic polypeptides were compared Poly arginines were found to be by far the most efficient We also describe a transient increase of current amplitude upon addition of calcium that may reflect a lateral phase separation and conversely a gradual decrease of negative resistance due to tetrodotoxin, a potent sodium channel blocker Functional modulations are correlated with conformational changes assayed in circular dichroism alamethicin ellipticity in small unilamellar vesicles is markedly reduced upon protamine addi tion, only if the ionic strength is in the same low range that is compatible with regenerative conductance properties These results are discussed in the framework of current models of ion channels gating

Key words: Voltage-gated ion channels — Channel-forming peptides — Basic polypeptides — Negative resistance — Action potentials — Secondary structures

Introduction

Alamethicin is a 20 residue-long peptaibol, i e a peptide rich in Aib (α aminoisobutyric acid), developing in planar lipid bilayers a cationic conductance as highly voltage-dependent (Eisenberg et al 1973, Boheim 1974, Gordon and Haydon 1975) as sodium channels of nerve fibres (Hodgkin and Huxley 1952, Hille 1984) In the presence of protamines and under stringent conditions, a behaviour more typical of

Correspondence to Dr Herve Duclohier, Interactions Cellulaires et Moleculaires UMR 6026 CNRS Universite de Rennes I, Batiment 14, Campus de Beaulieu, 35042 Rennes Cedex, France E mail Herve.Duclohier@univ-rennes1.fr

excitable membranes, namely negative resistance and action potentials, was demonstrated (Mueller and Rudin 1968). In spite of the abundant literature dealing with alamethicin, excitability phenomena induced by this amphipathic peptide in conjunction with basic polypeptides in planar lipid bilayers have apparently not been subjected to further experimental investigations since this initial demonstration, apart from another early work (Boheim 1972). However, the molecular interpretation partly based on a carrier mechanism was biased by the ring structure that was assumed for alamethicin.

There is presently a consensus about alamethicin structure (for review, see e.g. Cafiso 1994) and also, to a large extent, to the dynamic "barrel-stave" model (Sansom 1991) initially propounded after the first multi-states of single-channel conductances were recorded (Boheim 1974). Given these progresses and more generally the better comprehension of lipid-peptide interactions both at the interface and within the membrane (see e.g., Cafiso 1999), we set out to reinvestigate the alamethicin-basic polypeptides system. We took advantage of "virtually solvent-free" bilayers (Montal and Mueller 1972) formed with better defined lipids, more accurate as artificial membranes than the old decane-oxidized cholesterol painted bilayers and used a purified alamethicin fraction.

After the redefinition of the proper experimental conditions necessary to retrieve those macroscopic excitability phenomena, we concentrated upon the dynamic behaviour of the system. Then we compared the efficiencies of poly-lysines and poly-arginines to natural protamines for their ability to promote negative resistance when used in conjunction with alamethicin, before illustrating the effects of calcium ions and tetrodotoxin (a well known sodium channel blocker). The modifications brought about by basic polypeptides to alamethicin single-channel conductance properties are also described. Finally, the transition from the normal monotonic exponential voltage-dependence to the regenerative or excitable properties is shown to be correlated with changes in the alamethicin secondary structure, namely a decreased α-helical content.

Materials and Methods

Materials

Natural alamethicin was a generous gift from Upjohn Co. (Kalamazoo, MI, USA). The main fraction (Rf 30 or "fraction 4") which is negatively-charged near its C-terminus (Glu18) was purified on a C18 semi-preparative HPLC column (Waters Instruments Inc., Rochester, MN, USA), the mobile phase being ethanol : H_2O (65 : 35) and absorption detected at 210 nm. Protamine-sulfate and other basic polypeptides (poly-lysine, 52 kDa and poly-arginine, 40 kDa) were from Sigma (St Louis, MO, USA). The lipids used to form planar bilayers, 1-2-palmitoyloleoyl-phosphatidylcholine, 1-2-palmitoyloleoyl-phosphatidylserine, dioleoylphosphatidyl-ethanolamine (abbreviated later as POPC, POPS and DOPE, respectively) were supplied by Avanti Polar Lipids (Alabaster, ALA, USA) whereas egg phosphatidyl-

choline used for small unilamellar vesicles (SUVs) was from Sigma. Hexane and hexadecane (spectroscopic grade) were purchased from Fluka.

Macroscopic and single-channel conductances

In the macroscopic conductance configuration, virtually solvent-free bilayers (Montal and Mueller 1972) of relatively large area were used. Briefly, the lipid mixture whose composition is indicated in figure legends was spread as a 10 mg/ml solution in hexane on top of electrolyte solutions (NaCl or KCl, whose ionic strength was in the range 1–50 mmol/l as stated in figure legends, always buffered with 10 mmol/l HEPES, pH 7.4). After solvent evaporation, bilayers were formed by the folding procedure, i.e. lowering and then raising the buffer level on one side over a 150–200 μm hole in a 25 μm thick PTFE septum sandwiched between two glass hemi-chambers. The usual sign conventions apply: positive voltage delivered *via* an Ag/AgCl electrode in the *cis*-compartment, to which peptides are added, results in positive current (cations flowing from *cis* to *trans*). The latter was measured with a second Ag/AgCl electrode in the *trans*-side and fed to a Keithley amplifier (model 427, Cleveland, OH, USA). Either the voltage-clamp or the current-clamp modes of recording electrical events were used. To record macroscopic current-voltage (I-V) curves, we employed triangular waveforms of voltage command whose speed was low enough (5 mV/s) to ensure that currents reach steady-state values (as a rule 3–5 runs are superimposed), as independently tested with square voltage pulses of increasing amplitudes. To record single-channel current fluctuations, bilayers were formed at the tip of patch pipettes by the tip-dip method (Hanke et al. 1984), the electrolyte solution being 1mol/l KCl, 10 mmol/l HEPES, pH 7.4. After checking bare bilayer stability and electrical silence, alamethicin was added to the *cis*-side from a stock solution in methanol, the final concentration of which never exceeding 1% (v/v) in the bilayer chamber. Control experiments showed no apparent influence of this maximum methanol concentration upon the electrical parameters (capacitance and conductance) of the bilayer.

Circular dichroism

Circular dichroism (CD) spectra were recorded with a Mark V Jobin-Yvon dichrograph from peptides (alamethicin alone or with protamine) interacting with egg lecithin small unilamellar vesicles in suspension (final lipid concentration: 1 mmol/l) in a 10 mmol/l Na-Phosphate Buffer (NaH_2PO_4 + Na_2HPO_4) supplemented with either 15 or 150 mmol/l NaCl, pH 7.4. The path length was 0.01 cm and the response time constant was set at 5 s. Several (5–10) runs were averaged in the 190–270 nm range at room temperature. Blank spectra with lipid vesicles were substracted to yield alamethicin spectra. The mean residue ellipticity was estimated from the amplitude of the 190–192 and 222–224 nm peaks relating to published standards (Chang et al. 1978). The effect of protamine addition (in large excess as compared to alamethicin) on alamethicin ellipticity was inferred from spectra substraction:

CD [alamethicin + protamine with vesicles] – CD [protamine alone with vesicles].

Results

Induction of a negative resistance branch in Current-Voltage curves and dynamic behaviour

Planar lipid bilayers doped with relatively large aqueous concentrations (of the order of 10^{-7} 10^{-8} mol/l) of alamethicin and submitted to slow voltage-ramps (5 mV/s) develop a characteristic pattern of current-voltage (I-V) curves a highly voltage-dependent exponential branch above a threshold which is concentration dependent (see curve 1 in Fig 1) Asymmetrical I-V curves reflect the one-sided addition of alamethicin and the polarity of the peptide dipole a formal negative charge at the N-terminus would reverse the asymmetry (Hall et al 1984) The hys teresis between raising and falling limbs of the voltage ramp reflects the average duration of single channels, slower channels being associated to larger hystereses In such macroscopic conductance experiments, thousands of conducting transmembrane aggregates can be recruited with voltage

Although this classical behaviour (Eisenberg et al 1973, Gordon and Haydon 1975) can be observed under a variety of experimental conditions (especially as regards to lipids, ionic strength, etc), only a narrow set of stringent conditions would allow the transition shown in Fig 1 (from curve 1 to curve 2) upon addition of protamines there is a drastic reduction of threshold down to 0 mV, an increased hysteresis and moreover the development in the lower quadrant of a so called "neg ative resistance" branch The term is in fact borrowed from solid-state physics and electrophysiology (where it qualifies membrane excitability) and accounts for the current increase even as the driving force is decreased In our hands, we found that the following criteria (reflected in the legend of Fig 1) must be satisfied to induce negative resistance

incorporation of negatively-charged lipids in the membrane-forming solution,
low ionic strength for electrolyte solutions on both sides of the bilayer,
alamethicin aqueous concentration in the *cis* side sufficiently large to develop the exponential voltage-dependent branch at low threshold (around 50 mV),

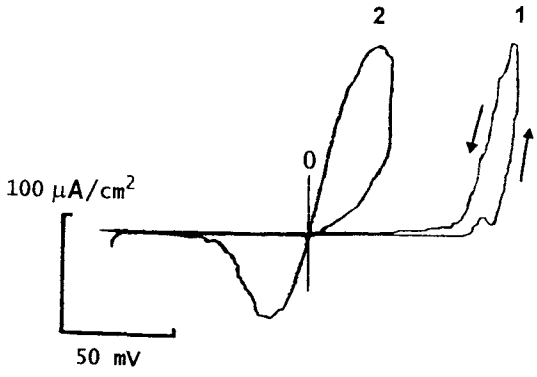

Figure 1 Macroscopic Current Voltage (I V) curves in a negative ly charged (POPS DOPE, 1 1) planar lipid bilayer bathed both sides by 25 mmol/l KCl, 10 mmol/l HEPES, pH 7 4 and with 10 ' g/ml alamethicin (Rf 30) before (curve 1) and after addition of 5×10^{-5} g/ml protamine sulfate (curve 2) to the same compartment (*cis*-side) Voltage ramp sweep 5 mV/s and room temperature

– above all, an adequate protamine/alamethicin bulk concentration ratio (for instance, a mass ratio of 50 had been used in Fig. 1).

The maximum development of the negative resistance was only attained after the doped bilayer (alamethicin + protamine) had been submitted to a succession of voltage ramps. A steady-state response was reached after 6–7 runs in the particular example of Fig. 1, where the durations of the raising and falling limbs of the voltage ramps were equal (20 s). The highly dynamic behaviour of the system is further illustrated in Fig. 2A in which the steepness of the voltage excursion was varied for both branches; as a rule, slowing down the rising phase and speeding up the falling phase of the voltage ramp favours larger negative resistance amplitude.

Two metastable conductance states

Fig. 2B shows that the 'Alamethicin + Protamine system' submitted to slow voltage-ramps evolves between two limiting states of high and low macroscopic conductances (branches I-I′ and II, respectively). If the voltage excursion is stopped at any given point on the developing exponential branch (D, for instance, on branch II of Fig. 2B), and the voltage clamped at the actual value, the membrane current increases towards the corresponding point (E) on the upper conductance curve with kinetics that are function of the applied voltage (not shown). This current readjustment suggests that the apparent hysteresis in macroscopic I-V curves would in fact be due to a time- and voltage-dependent transition of the transmembrane conducting aggregates between the two limiting states of low and high conductances. The reversibility of the different branches was also tested: only C–D, A–B and E–B′ were fully reversible with the sign of voltage excursion and would represent two metastable conductance states.

Compared efficiencies of different basic polypeptides, modulation by calcium and sensitivity to the sodium channel blocker Tetrodotoxin

The efficiencies of poly-arginine and poly-lysine of the same molecular weight range (40 and 52 kDa, respectively) to develop negative resistance branches in macroscopic I-V curves were compared to protamine-sulfate, the natural "Alm-modifier" used by Mueller and Rudin (1968). Poly-arginine was by far the most potent, both in terms of aqueous concentration and negative resistance amplitude (Fig. 3). Despite a higher degree of polymerization, poly-lysine was found less efficient than natural protamines, albeit for a similar concentration range. The characteristic pattern of all Dose-Response curves is an optimal concentration above which response amplitudes fall.

The addition of 0.1 mmol/l $CaCl_2$ only to the *cis*- or positive side (as for the peptides) led to a significant and transient increase of the negative resistance amplitude (Fig. 4 A). After the peak occuring about 12 min after $CaCl_2$ addition, the steady-state plateau was twice higher, on average, than the initial amplitude. This most likely reflects lateral phase separation of the negatively-charged lipids resulting in local peptide concentration changes (see e.g., Mittler-Neher and Knoll

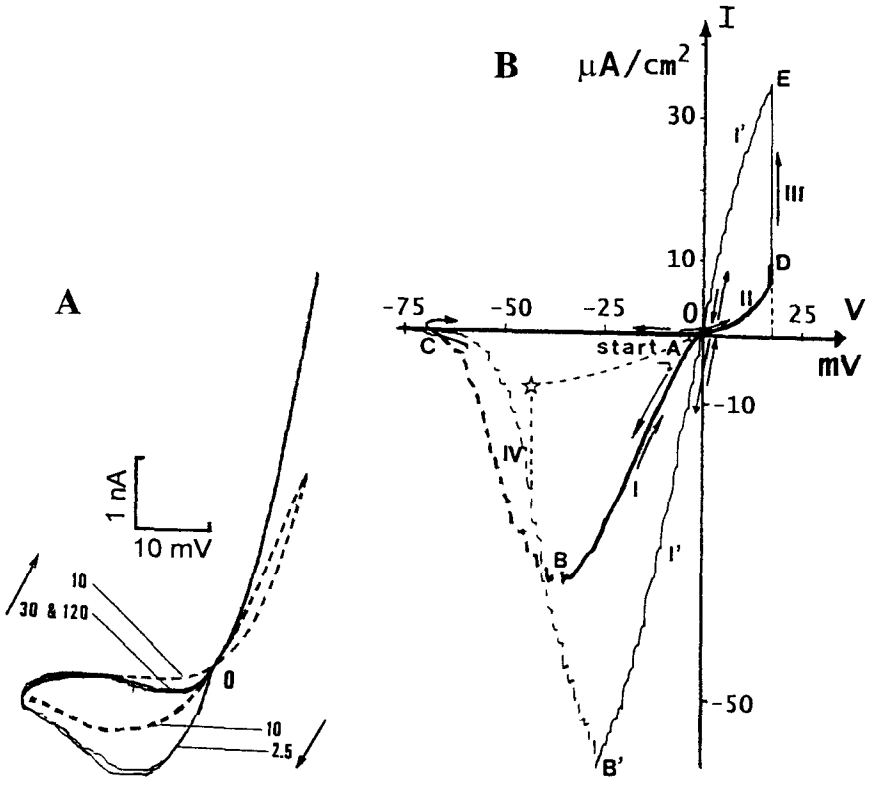

Figure 2. A. Dynamic behaviour illustrated by modulation of the negative resistance amplitude by different speeds of voltage excursion during the ascending and descending limbs of the ramp (whose directions and durations in seconds are indicated next to the curves) The I-V curve shown as a broken line was recorded with equal durations (10 s) for the rising and falling limbs of the voltage ramp During the course of the same experiment, both decreasing the falling limb duration (to 2 5 s) and increasing the rising limb duration (to 30, then 120 s) greatly enhanced transmembrane currents (continuous line) **B.** Two metastable states high and low conductances (branches I-I′ and II of I-V curve, respectively) Same conditions as above (A) and as in Fig 1, in particular 25 mmol/l KCl, 10 mmol/l HEPES, pH 7 4 on both sides of the bilayer At point D, the voltage excursion was stopped and the system evolved spontaneously from the low to the high conductance states Reversible branches are shown as continuous lines and irreversible portions as broken lines The response to the first voltage cycle is represented as a thick line whereas the thin line corresponds to the second cycle

1993) Conversely, the addition of tetrodotoxin, a classic sodium channel blocker, at a physiologically-relevant concentration (in the micromolar range) gradually decreased negative resistances, to about one third of the initial value in the example shown in Fig. 4B.

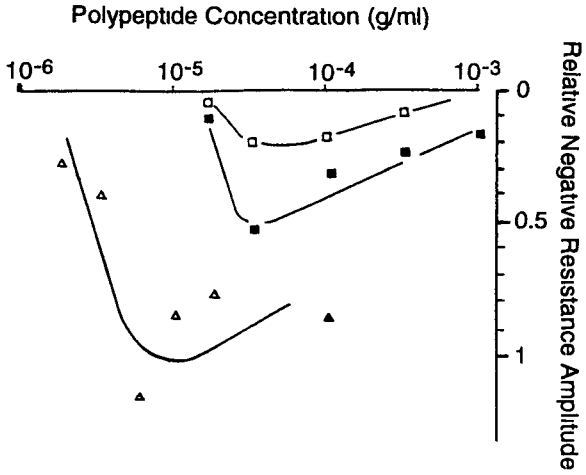

Figure 3. Relative amplitude of the negative resistance branch of I-V curves as a function of bath (*cis*-side) concentrations of basic polypeptides, empty squares poly-lysine (52 kDa), filled squares protamine sulfate, triangles poly-arginine (40 kDa) Same buffered electrolyte solution as above (25 mmol/l KCl, 10 mmol/l HEPES, pH 7 4)

Action potentials and modifications of the single-channel conductance pattern

A very asymmetric ionic gradient (KCl 50 mmol l^{-1}/1 mmol l^{-1}, *cis/trans*) im posed across the doped bilayer (Alm + Poly-Arg) incorporating negatively charged lipids, induced a large negative resting potential difference, thus indicating a cationic selectivity in the resting state Stimulation by a supra-threshold current clamp depolarization elicited trains of action potentials However, as shown in Fig 5, the latter often evolved with dampening oscillations towards the upper voltage level The electrical spike first excursion is from a resting membrane potential of −90 mV to around +80 mV, i e quite close to theoretical reversal potentials for K^+ and Cl^- (−100 and +100 mV, respectively, taking into account the applied salt gradient)

With an alamethicin/protamine mass ratio as low as 10^{-3}, the modifications brought upon the single-channel activity are quite significant as shown in Fig 6 Compared to the normal pattern, protamine induces a shortening of mean open durations, flickering of the open substates and a downward shift of the most probable single-channel substate (from substate 3 to substate 2) Here, the ionic strength is raised to 1 mol/l KCl (symmetrical) for a better resolution of the multi-state pattern, but since decreasing the salt concentration should also decrease the electrostatic screening effect on the charged binding site (Glu18), the block probability of protamine upon alamethicin single-channel events is expected to be much enhanced in the 'macroscopic conductance' configuration (15 to 50 mmol/l NaCl or KCl), as experimentally confirmed (Rink et al 1994)

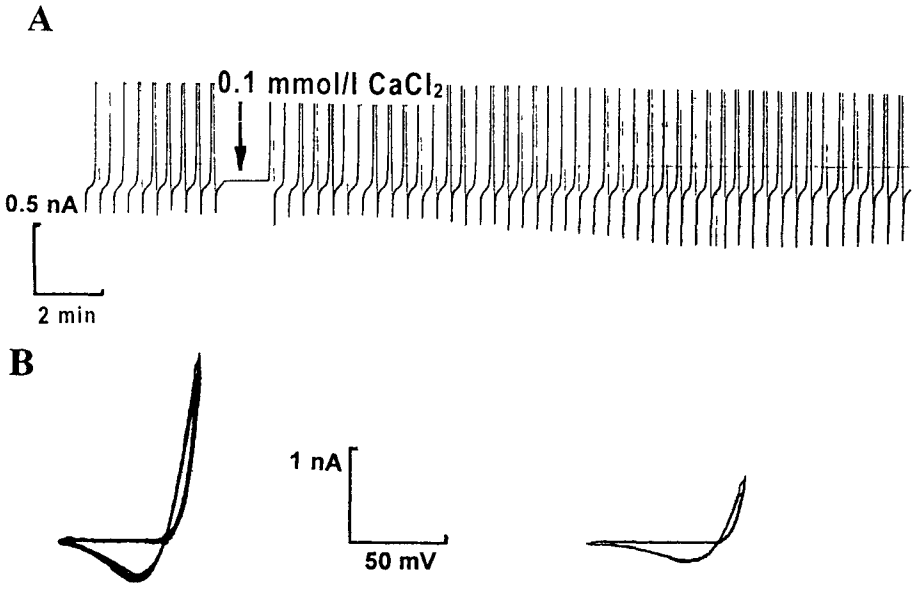

Figure 4. A. Addition of $CaCl_2$ to the *cis*-side transiently increases the current during the negative branch. Chart recording shows current responses to successive and identical I-V curves on a contracted time scale (3 runs per minute) Same buffered electrolyte solution as above (25 mmol/l KCl, 10 mmol/l HEPES, pH 7.4). **B.** Addition of 3 μmol/l TTX to the *cis*-side decreases transmembrane current. The voltage excursion in I-V runs is from -50 to $+25$ mV in 20 s and back in 2 s. Electrolyte on both sides: 15 mmol/l NaCl, 10 mmol/l HEPES, pH 7.4. Record on the left shows the initial response with superimposed sweeps (every 2 mn) during a lapse of time equivalent to the one needed for the TTX assay Record on the right shows the remaining response (two superimposed sweeps) 10 min after tetrodotoxin addition.

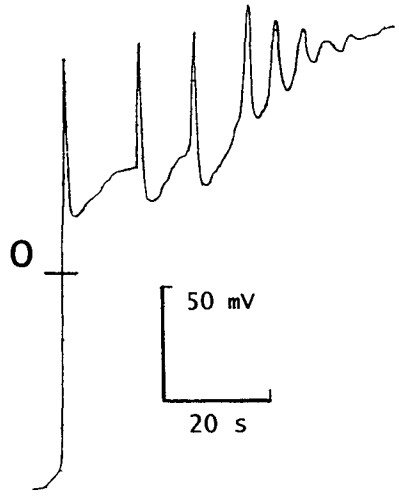

Figure 5. Action potentials and oscillations in space-clamp conditions. The lipid bilayer was made by apposition of two POPC : DOPE POPS (3.5/4/2 5) monolayers and bathed by 50 mmol·l^{-1}/1 mmol·l^{-1} KCl (*cis/trans*) buffered (10 mmol/l HEPES, pH 7.4) solutions The *cis*-side also contained 2×10^{-7} g/ml alamethicin Rf 30 and $2\,5\times10^{-6}$ g/ml poly-arginine (40 kDa).

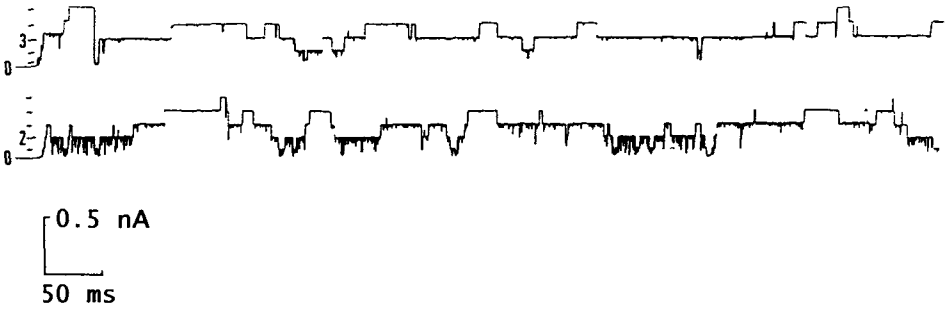

Figure 6. Compared single-channel traces displayed at 130 mV by alamethicin (Rf 30, 10^{-7} g/ml) alone (upper trace) and in the presence of protamine sulfate (10^{-4} g/ml) also in the *cis*-side (lower trace) 1 mol/l KCl, 10 mmol/l HEPES, pH 7 4 on both sides of a lipid bilayer formed at the tip of a patch pipette from a POPC DOPE POPS Chol (2 5/3/2/2 5) monolayer

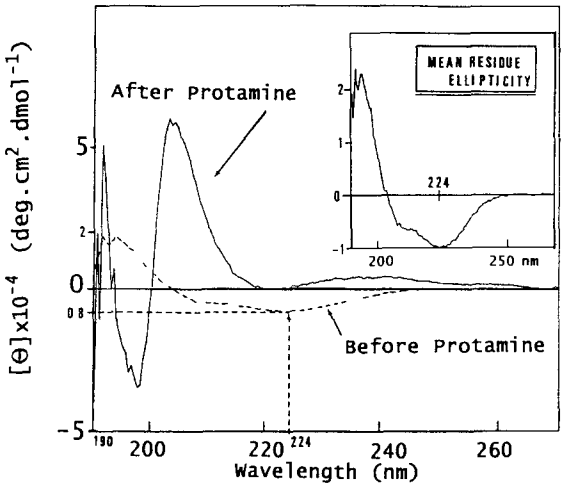

Figure 7. Changes in circular dichroism spectra of alamethicin (0 1 mg/ml) interacting with egg PC SUVs induced by protamine (1 mg/ml) in the low ionic strength medium (10 mmol/l phosphate buffer + 15 mmol/l NaCl) The inset shows the normal alamethicin spectrum on a reduced ellipticity scale [Θ], the mean residue ellipticity

Correlations with conformational changes

The effects of protamine upon the secondary structure of alamethicin interacting with lipid vesicles was investigated in circular dichroism (CD) spectroscopy. Since protamine is in large excess and its random coil conformation (checked in independent experiments) is unlikely to be much affected by the alamethicin-lipid system, we considered it safe to infer its effects from the subtraction of spectra

CD [alamethicin + protamine in vesicles] − CD [protamine alone with vesicles].

Fig 7 compares CD spectra of alamethicin before and after addition of protamine in a low ionic strength buffer (15 mmol/l NaCl, 10 mmol/l Na-phosphate buffer, pH 7 4) It is clear that most of the initial alamethicin helicity is lost upon the interaction with the highly-positively charged polypeptide The resulting spectrum is indicative of an unstructured coil chain In a higher ionic strength medium (150 mmol/l NaCl) in which no excitability phenomena could be observed, this transition is much less sharp and there is a remaining significant helicity

Discussion

Although alamethicin stands as a paradigm for the 'barrel-stave' mode of action of amphipathic peptides, excitability phenomena promoted by interaction with polyamines or basic polypeptides curiously seem to have been neglected since the pioneering studies (Mueller and Rudin 1968, Boheim 1972) Experimental difficulties in reproducing and extending the initial results may partly explain this apparent lack of interest We felt that a reinvestigation of the alamethicin-protamine system was justified by the fact that earlier interpretations were partly based on a cyclic structure for alamethicin complexing cations and on a conductance component mediated by protamines on their own, both assumptions that later proved to be wrong (Martin and Williams 1976, Rink et al 1994) Besides, polyamines exert various effects on membranes especially through interactions with channels and receptors (for review, see e g Williams 1997) For instance, the rectification and negative resistance of macroscopic I-V curves of Kir potassium channels reflect a voltage-dependent block by intracellular spermine (Lopatin et al 1994)

With peptaibol analogues of alamethicin lacking Pro2 (trichorzianins), we also recorded negative resistances, albeit with smaller amplitude A still more modest amplitude for the negative resistance branch of I-V curves was also observed with the neutral trichorzianin (Gln18) suggesting that the formal negative charge near the C-terminus (Glu18 in alamethicin Rf 30), although a favourable factor, is not a prerequisite for the kind of interaction with basic polypeptides that is needed to develop excitability Negative resistance was recently more consistently and easily retrieved with an alamethicin dimer at a lower concentration of one order of magnitude (Duclohier et al 1999)

In summary, the main effects of protamine and other basic polypeptides on the macroscopic alamethicin I-V curves are twofold 1 drastic reduction of the voltage threshold for the development of the exponential branch and, 2 induction of a negative resistance branch in the negative quadrant of I-V curves The first effect can be accounted for by an enhanced surface density of alamethicin due to decreased electrostatic repulsions with the negatively-charged bilayers when the polycationic protamine or $(Arg^+/K^+)_n$ are bound to the latter In addition, this complexation of the negative surface charges (only on one side) is likely to induce some phase separation in the mixed charged zwitterionic bilayers, although this could be limited by the flexible structure of these polycations (see Ikeda et al 1990) More conspicuously, the latter are likely to increase the transmembrane voltage drop

and consequently enhance alamethicin conductance Indeed, the current densities attained in this study at the peak of the negative resistance branches are much higher than those previously reported when using neutral PC- or PE-decane painted bilayers (Boheim 1972, Eisenberg et al 1973) Likewise, action potentials amplitude can reach 170 mV and thus are quite 'robust' when compared with those delivered by natural excitable membranes

However, the simple explanation which stresses overruling influence of basic polypeptides on the interfacial concentration of alamethicin and on the voltage drop is unlikely to account for all aspects of artificial excitability reported here We propose that as positive voltage continues to increase (or after a time-dependent process upon constant voltage, see arrow on branch DE in Fig 2B), basic polypeptides are repelled into alamethicin transmembrane aggregates The inner hydrophobic barrel of the latter would then be lined with positive charges, accounting for the reversal of ion selectivity from cation in normal alamethicin channels to anion selectivity in modified or mixed alamethicin-basic polypeptides channels It should be stressed here that basic polypeptides *per se* cannot induce significant conductances as confirmed in another study (Rink et al 1994) In agreement with a partial block of the pore, as shown in Fig 6, that is consistent with previous findings of the above-mentioned study, the modified channels no longer close at 0 mV, but at a negative voltage after the alamethicin-polyamines electrostatic and dipolar interactions had been released (at points B and B′ on Fig 2B, for instance) After this transition which seemed to be dependent on the maximum current attained at the end of the positive voltage excursion and on the speed of the falling branch, channels turn cation-selective again but rapidly close because of the inherent asymmetrical voltage gating of alamethicin (if confined to one side of the bilayer) Once negative resistance is established, the system becomes unstable in current-clamp configuration and conditions for excitability are met an applied inward current will make the system oscillate between the two branches and fire repetitive action potentials By analogy with Teorell's oscillator, the U or N-shaped I-V curve can be seen as a cut across a folded surface where the alamethicin/basic polypeptide concentration ratio would be the third variable (z-axis) along with current and voltage (y and x axes)

These effects of protamine can tentatively be put into perspective with the voltage-gating of more complex and physiological channels which is controlled by S4 transmembrane segments (Sigworth 1993) These voltage sensors are very highly conserved throughout the super-family of such channels and present arginines every third position (Noda et al 1984) Protamine sequences do share this feature, albeit to a limited extent For instance, bull protamine has the following aminoacid sequence (Mazrimas et al 1986)

ARYRCCLTHSGSRCRRRRRRRCRRRRRRRFGRRRRRRRVCCRYTVIRCTRQ

Besides transiently modifying ion selectivity, protamine could thus act as a voltage-sensor and assist alamethicin gating, especially in reducing the voltage threshold From a structural point of view, the interaction of protamine with

alamethicin bundles may impose an overall stretched conformation coupled with high voltage-sensitivity and anion selectivity Our circular dichroism studies indeed show, at rest and in low ionic strength, a stretched out conformation for alamethicin Since the negatively charged phospholipid headgroups are less screened at low ionic strength, alamethicin Rf 30, which bears a formal negative charge, would be more easily drawn out of the membrane interior by protamines, than at high ionic strength, and more likely to adopt a non-helical conformation This holds only for the resting membrane potential situation Positive voltages can counter this effect, by pushing the N-ends (positive part of the peptide dipole) of alamethicin together with the highly-positively charged protamines into the membrane

The molecular interpretation proposed above is in line with a thermodynamic approach "reinterpreting the sodium channel gating in terms of a phase transition between a transmembrane S4 α-helix and a channel-helix", the latter being in a coil like conformation during activation (Benndorf 1989) Helix-coil transitions for the S4 segments in voltage-activated ion channels have also been proposed on the basis of the ferroelectric nature of these proteins (Leuchtag 1994), a property also assumed in a previous discussion of negative resistance in terms of ion hydration energies (Tredgold 1973) The 'gating alternative' (as recalled by Sigworth 1993) *vs* the outward motion and solvent exposure of unperturbed S4 helices (Yang et al 1997) has been recently given some credence in a circular dichroism study of a solvent-dependent conformational flexibility of isolated voltage-sensors (Helluin et al 1998)

Finally, the mechanism proposed here remains compatible with the phenomenology put forward by analogy with 'active' electronic devices in which it was proposed that an applied voltage would change the free energy difference between two metastable channel configurations of different conductivities or selectivities (Mueller and Rudin 1963) Excitability-bistability phenomena and electrical oscillations have been the subject of many theoretical as well as experimental investigations in a variety of systems (for review, see Larter 1990) Only a single instance shall be mentioned here in the context of the present study a lipid-impregnated cellulose ester membrane filter was shown to reproducibly induce voltage oscillations under constant current when alamethicin and protamine were present in the same hemi-chamber whose salt concentration was higher than the other These oscillations interpreted as arising from a cationic/anionic selectivity switching were controlled by bacteriorhodopsin, a light activated proton pump (Ikematsu et al 1995) Natural excitable membranes can display these same phenomena when studied under similar conditions as artificial model systems For instance, the squid giant axon internally and externally perfused with a few millimolar $CoCl_2$ show periodic voltage changes when submitted to a sustained inward current (Terakawa 1981) However, theoretical studies suggesting that temporal oscillations might be coupled to spatial patterns in fluid mosaic membranes (Fromherz 1988) have not yet been subjected to experimental tests

In conclusion, in this study proper experimental conditions, especially negatively-charged phospholipids and low ionic strentgh electrolyte solutions were speci-

fied in order to record excitability phenomena with alamethicin and basic polypeptides interacting with planar lipid bilayers Poly-arginines were found to be the most efficient polycations to develop this behaviour. Calcium ions and tetrodotoxin modulated negative resistance in opposite ways, through lateral phase separation and pores blockade, respectively. The macroscopic behaviour was correlated to a blocking effect of polycations on upper single-channel conductance levels of alamethicin and to a reversed ion selectivity. Finally, these electrophysiological modulations are ascribed to some polycation-induced alamethicin uncoiling that is put into perspective with the voltage-gating of physiological ion channels

Acknowledgements. I am indebted to Prof G Boheim (Rhur Universitat, Bochum, Germany) for introducing me to some of the bilayer techniques (the experiment illustrated in Fig 6 was performed with him in his laboratory), and for useful discussions and suggestions I also thank Dr G Spach and J Y Dugast (UMR 6522 CNRS, Université de Rouen, France) Supported by GDR CNRS 1153 and 790

References

Benndorf K (1989) A reinterpretation of Na channel gating and permeation in terms of a phase transition between a transmembrane S4 α-helix and a channel-helix Eur Biophys J **17**, 257—271

Boheim G (1972) Erregbarkeit Schwarzer Lipid-Membranen Ein beitrag zur klarung der erregungsvorgange in dem durch alamethicin und protamin modifizierten membranmodellsystem Ph D thesis, Universitat Aachen (Germany)

Boheim G (1974) Statistical analysis of alamethicin channels in black lipid membranes J Membr Biol **19**, 277—303

Cafiso D S (1994) Alamethicin a peptide model for voltage gating and protein-membrane interactions Annu Rev Biophys Biomol Struc **23**, 141—165

Cafiso D S (1999) Interaction of natural and model peptides with membranes Curr Top Membr **48**, 197—228

Chang C T , Wu C S , Yang, J T (1978) Circular dichroic analysis of protein conformation inclusion of β-turns Anal Biochem **91**, 13—31

Duclohier H , Kociolek K , Stasiak M , Leplawy M T , Marshall G R (1999) C-terminally shortened alamethicin on templates influence of the linkers on conductances Biochim Biophys Acta **1420**, 14—22

Eisenberg M , Hall J E , Mead C A (1973) The nature of the voltage-dependent conductance induced by alamethicin in black lipid membranes J Membrane Biol **14**, 143—176

Fromherz P (1988) Spatio-temporal patterns in the fluid-mosaic model of membranes Biochim Biophys Acta **944**, 108—111

Gordon L G , Haydon D A (1975) Potential-dependent conductances in lipid membranes containing alamethicin Phil Trans R Soc London, Ser B**270**, 433—447

Hall J E , Vodyanoy I , Balasubramanian T M , Marshall G R (1984) Alamethicin, a rich model for channel behavior Biophys J **45**, 233—247

Hanke W , Methfessel C , Wilmsen H U , Boheim G (1984) Ion channel reconstitution into planar lipid bilayers on glass pipettes Bioelectrochem Bioenerg **12**, 329—339

Helluin O , Bendahhou S , Duclohier H (1998) Voltage-sensitivity and conformational change of isolated S4L45 fragments from sodium channels are tuned to proline Eur Biophys J **27**, 595—604

Hille B (1984) Ionic Channels of Excitable Membranes Sinauer Associates Inc , Sunderland, Mass

Hodgkin A L , Huxley A F (1952) A quantitative description of membrane current and its application to conduction and excitation in nerve J Physiol (London) **117**, 500—544

Ikeda T , Yamaguchi H , Tazuke S (1990) Phase separation in phospholipid bilayers induced by biologically active polycations Biochim Biophys Acta **1026**, 105—112

Ikematsu M , Iseki M , Sugiyama Y , Mizukami A (1995) Oscillatory phenomena in model membranes electrical oscillations in lipid-impreganted membrane filter induced by alamethicin and controlled by bacteriorhodopsin BioSystems **35**, 123—128

Larter R (1990) Oscillations and spatial nonuniformities in membranes Chem Rev **90**, 355—381

Leuchtag H R (1994) Long-range interactions, voltage sensitivity, and ion conduction in S4 segments of excitable channels Biophys J **66**, 217—224

Lopatin A N , Makhina E N , Nichols C G (1994) Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification Nature **372**, 366—369

Martin D R , Williams R J (1976) Chemical nature and sequence of alamethicin Biochem J **153**, 181—190

Mazrimas J A , Corzett M , Campos C , Balhorn R (1986) A corrected primary sequence for bull protamine Biochim Biophys Acta **872**, 11—15

Mittler-Neher S , Knoll W (1993) Ca(2+)-induced lateral phase separation in black lipid membranes and its coupling to the ion translocation by gramicidin Biochim Biophys Acta **1152**, 259—269

Montal M , Mueller P (1972) Formation of bimolecular membranes from lipid monolayers and a study of their properties Proc Natl Acad Sci U S A **69**, 3561—3566

Mueller P , Rudin D O (1963) Induced excitability in reconstituted cell membrane structure J Theor Biol **4**, 268—280

Mueller P , Rudin D O (1968) Action potentials induced in bimolecular lipid membranes Nature **217**, 713—719

Noda M , Shimizu S , Tanabe T , Takai T , Kayano T , Ikeda T , Takahashi H , Nakayama H , Kanaoka Y , Minamino N , Kangawa K , Matsuo H , Raftery M A , Hirose T , Inayama S , Hayashida H , Mayata T , Numa S (1984) Primary structure of *Electrophorus electricus* sodium channel deduced from cDNA sequence Nature **312**, 121—127

Rink T , Bartel H , Jung G , Bannwarth W , Boheim G (1994) Effects of polycations on ion channels by neutral and negatively-charged alamethicins Eur Biophys J **23**, 155—165

Sansom M S (1991) The biophysics of peptide models of ion channels Prog Biophys Mol Biol **55**, 139—236

Sigworth F J (1993) Voltage gating of ion channels Q Rev Biophys **27**, 1—40

Terakawa S (1981) Periodic responses in squid axon membrane exposed intracellularly and extracellularly to solutions containing a single species of salt J Membr Biol **63**, 51—59

Tredgold R H (1973) A possible mechanism for the negative resistance characteristic of axon membranes Nature New Biol **242**, 209—210

Williams K (1997) Interactions of polyamines with ion channels Biochem J **325**, 289—297

Yang N , George A L Jr , Horn R (1997) Probing the outer vestibule of a sodium channel voltage sensor Biophys J **73**, 2260—2268

Final version accepted October 13, 2001