Asymmetrical Ion-Channel Model Inferred from Two-Dimensional Crystallization of a Peptide Antibiotic

R. Ionov,* A. El-Abed,* A. Angelova,† M. Goldmann,‡ and P. Peretti*

*Groupe de Recherche en Physique et Biophysique, Université René Descartes, 75270 Paris Cedex 06, France; †College of Sciences Leonardo da Vinci, BG-1000 Sofia, Bulgaria; and ‡Laboratoire Physico-Chimie Curie, Section de recherche, Institut Curie, 75231 Paris Cedex 05, and Laboratoire d'Utilisation du Rayonnement Electromagnetique, Université Paris Sud, 91405 Orsay, France

ABSTRACT The structural organization of ion channels formed in lipid membranes by amphiphilic α-helical peptides is deduced by applying direct structural methods to different lipid/alamethicin systems. Alamethicin represents a hydrophobic α-helical peptide antibiotic forming voltage-gated ion channels in lipid membranes. Here the first direct evidence for the existence of large-scale two-dimensional crystalline domains of alamethicin helices, oriented parallel to the air/water interface, is presented using synchrotron x-ray diffraction, fluorescence microscopy, and surface pressure/area isotherms. Proofs are obtained that the antibiotic peptide injected into the aqueous phase under phospholipid monolayers penetrates these monolayers, phase separates, and forms domains within the lipid environment, keeping the same, parallel orientation of the α-helices with respect to the phospholipid/water interface. A new asymmetrical, "lipid-covered ring" model of the voltage-gated ion channel of alamethicin is inferred from the structural results presented, and the mechanism of ion-channel formation is discussed.

INTRODUCTION

Diverse natural α-helical peptides are found to be active elements of the immune defense systems of humans, mammals, insects, etc. (Bechinger, 1997; Biggin and Sansom, 1999). Produced as a result of protein biodegradation, they exhibit broad-spectrum antimicrobial activity and are promising substances for overcoming the growing problem of resistance to the classical antibiotics (Kingman, 1994). An essential advantage of the α-helical peptide antibiotics is that they do not require specific receptor sites for their activity, because the mode of their action involves interaction with cell membrane lipids, formation of ion channels, or total membrane disruption.

Ion channels are key systems for receptor function, nerve, and brain processes, maintaining the life of organisms through the exchange of signals and fluid between cells and their environment (Marsh, 1996; Ashley, 1995; Nicholls et al., 1992). Simulating the operation of classical diodes and transistors, as well as of quantum-well devices of modern physics and electronics, voltage-gated ion channels could be utilized as molecular counters (Bezrukov et al., 1994) and could be of importance in the fields of bioelectronics and biocomputing. However, the general structural model of the voltage-gated ion channels is still under debate (Sansom, 1993). Single ion-channel function resembles phenomena of quantum physics (Bezrukov et al., 1994; Sansom, 1993) and could be understood from structural information provided by peptide and protein crystallography. Two-dimensional crystallization at interfaces has the potential to overcome the difficulties of crystallizing protein and peptide species (Möhwald, 1993).

Amphiphilic α-helical peptides readily adsorb at phospholipid/water and air/water interfaces and can be studied by the Langmuir monolayer technique. Interest in the study of Langmuir and Langmuir-Blodgett films began in the fields of membrane biophysics (Leblanc and Salesse, 1994; Roberts, 1990; Wang et al., 1997, Borissevich et al., 1996; Sackmann, 1996), optics and microelectronics (Leblanc and Salesse, 1994; Roberts, 1990; Kuhn et al., 1993; Wang et al., 1997; Borissevich et al., 1996; Sackmann, 1996; Allen and Ashwell, 1992), with modeling of low-dimensional systems, as well as in studies of phase transitions and intermolecular interactions (Möhwald, 1993; Leblanc and Salesse, 1994; Roberts, 1990; Kuhn et al., 1993; Wang et al., 1997; Borissevich et al., 1996; Sackmann, 1996; Allen and Ashwell, 1992; Ionov and Angelova, 1995a,b; Angelova et al., 1994). Because the primary action of ion-channel-forming peptides is to interact with the phospholipid monolayer leaflets of membranes, Langmuir monolayers are suitable systems for the study of these interactions at phospholipid/water interfaces, to determine the structural organization of the peptide molecules at the interface and to model the initial steps of ion channel formation.

The voltage-gated ion channels formed in lipid membranes by the natural α-helical peptide antibiotic alamethicin show current-voltage asymmetry and discrete conductance multilevels in single-channel ion-current characteristics (Sansom, 1993, 1998; Gordon and Haydon, 1975; Hanke and Boheim, 1980; Wooley and Wallace, 1992; Vodyanoy et al., 1983; Hall et al., 1984; Bezrukov et al., 1998). The appearance of multilevels could be explained by the presence of a hydrophilic, circular pore of a discretely varying

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Address reprint requests to Dr. Radoslav Ionov, Groupe de Recherche en Physique et Biophysique, Université Rene Descartes (Paris V), 45 rue des Saints Pères, 75270 Paris Cedex 06, France. Tel.: 33-1-4286-2046; Fax: 33-1-4286-2085; E-mail: r.ionov@usa.net.

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radius due to the addition or removal of peptide monomers of a molecular diameter of ~1 nm (Sansom, 1993; Boheim, 1974; Opsahl and Webb, 1994). There are two structural possibilities for the formation of such pores by the α-helical peptide alamethicin. The first one involves the creation of a pore by aggregation of the α-helices into a bundle perpendicular to the phospholipid/water interface of the membrane (the "barrel-stave" (BS) model; Boheim, 1974; Fox and Richards, 1982). The helix axis is oriented parallel to the pore axis.

The second possibility, which we shall consider and which has not been explored so far, includes pore formation by aggregation of the hydrophilic C termini of alamethicin helices in the plane parallel to the phospholipid/water interface. Thus the α-helix axis within the plate-like alamethicin aggregate is oriented perpendicular to the pore axis (see Fig. 5 *A*) and hence parallel to the membrane/water interface. In the initial work of Fox and Richards (1982), the BS model was proposed as one possibility for the channel organization of alamethicin. However, this model does not explain some recently reported ion current measurements, current/voltage asymmetry, and structural data related to peptide insertion into phospholipid bilayers (Taylor and de Levie, 1991; Bechinger, 1997; Sansom, 1993). The orientation of the alamethicin helix in bilayer vesicles and multilamellar liposomes is under debate (Sansom, 1993; Wu et al., 1995), and the necessity for modification of the BS model has become obvious (Taylor and de Levie, 1991; Bechinger, 1997).

Using direct structural methods, we show that alamethicin forms stable two-dimensional crystals in which the α-helical axis is parallel with respect to the air/water and phospholipid/water interfaces under the physiological conditions corresponding to ion current experiments. On the basis of these structural findings, a novel, "lipid-covered ring" (LCR) ion channel model (presented in Fig. 5) is proposed.

MATERIALS AND METHODS

Alamethicin (Sigma; MW 1959.9) was spread at the air/water interface from a chloroform/methanol (20:1) solution or injected from ethanol solution into the aqueous subphase supporting insoluble phospholipid monolayers. The aqueous solution contained 0.1 M NaCl (pH 7.0), which was adjusted with 1×10^{-3} M phosphate buffer (Na_2HPO_4/NaH_2PO_4, 1:1 molar ratio, p.a. grade; Merck). Deionized pure water with a resistivity of 10^{18} Ω was used.

The lipids used (1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), 1,2-myristoyl-sn-glycero-3-phosphatidylethanolamine (DMPE), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylglycerol (DPPG), and octadecylamine (ODA)) were supplied from Avanti Polar Lipids (Alabaster, AL) or Sigma-Aldrich (France).

A Langmuir trough was used to vary the surface pressure of the lipid monolayers.

Fluorescence microscopy at the air/water interface (McConnell et al., 1984; Weis and McConnell, 1984) was used to study the two-dimensional domains of alamethicin. The fluorescent probe used was a 7-nitrobenz-2-oxa-1,3-diazol-4-yl-amino-phospholipid dye (NBD dye) labeled at the C12 position (N-3786; Molecular Probes). The dye/alamethicin and dye/lipid molar ratios were 1:800.

Synchrotron x-ray diffraction from Langmuir monolayers was performed at Laboratoire d'Utilisation du Rayonnement Electromagnetique (Orsay, France) (Fradin et al., 1998; Renault et al., 1998).

Strong electrical fields perpendicular to the lipid/water interface were applied by placing a planar electrode at a distance of ~1 mm above the Langmuir monolayer and a second electrode inside the aqueous subphase of 0.1 M NaCl. Electric potentials of up to 2.5 kV were applied.

The surface charge measurements were performed by the method described by El-Abed et al. (1995).

RESULTS AND DISCUSSION

Alamethicin is an antibiotic peptide made up of 19 amino acid residues and one amino alcohol (Fox and Richards, 1982) (see Fig. 1, *inset*, and Fig. 5 *E*), in the following sequence:

1 5 10
Ac-Aib-L-Pro-Aib-L-Ala-Aib-L-Ala-L-Gln-Aib-L-Val-Aib-

15 20
Gly-L-Leu-Aib-L-Pro-L-Val-Aib-Aib-L-Glu-L-Gln-L-Phol,

where Aib denotes α-aminoisobutyric acid, and Phol denotes phenylalaninol. The 3D molecular structure of alamethicin (Fox and Richards, 1982; Sansom, 1993; You et al., 1996) is α-helical, as shown in Fig. 1 (*inset*) and Fig. 5 *E*. The cross-sectional areas parallel and perpendicular to the helix axis are ~3.20 nm^2 and ~0.8 nm^2, respectively. The

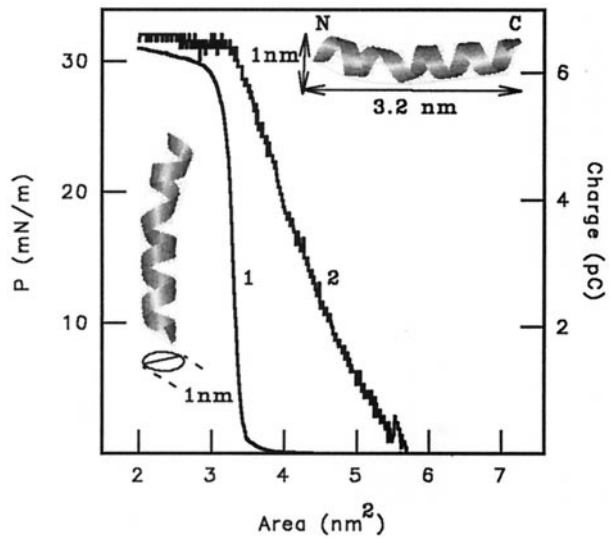

FIGURE 1 (*Curve 1*) Typical surface pressure/area isotherm of an alamethicin monolayer spread on a 0.1 M NaCl aqueous subphase of pH 7.0 at 22°C. (*Curve 2*) Surface charge/area dependence of an alamethicin monolayer on pure water a subphase at 22°C. The inset shows the x-ray-data-based (Fox and Richards; 1982) 3D molecular model of the alamethicin α-helix and the cross-sectional areas for parallel (3.2 nm^2) and perpendicular (0.8 nm^2) orientations of the peptide at the air/water interface. The C and N termini of alamethicin are indicated.

amino (N) terminus of the peptide is acetylated (Ac-Aib), and the C-terminal residue is alcohol (Phol). The ionizable group is Glu^{18}. Under physiological conditions (pH around 7.0–7.4), the peptide is in uncharged (Opsahl and Webb, 1994). The hydrophilic amino acid residues, which expose polar substituents to the aqueous environment, are Gln^{7}, Glu^{18}, Gln^{19}, and $Phol^{20}$. The Aib residues, which are largely involved in the alamethicin structure and contribute to its α-helicity, are hydrophobic in nature (White and Wimley, 1998). The peptide is amphiphilic because its polar hydrophilic groups are at the C terminus or lie along a narrow hydrophilic strip parallel to the helix axis (Fox and Richards, 1982; see the 3D peptide presentations in Fig. 5). The majority of the amino acid residues, including the N terminus, are hydrophobic in nature. Therefore, alamethicin has the potential to form monolayers at the air/water interface.

Peptide orientation parallel to the air/water interface evidenced by surface pressure/area monolayer isotherms

The Langmuir monolayer isotherm of the pure peptide alamethicin demonstrates that it forms stable monolayers at the air/water interface (Fig. 1, *curve 1*). The steep rise of the isotherm and the compressibility coefficient of 2.9 m/N (at $\pi = 20$ mN/m) indicate a solid-like structural organization of the alamethicin monolayer. An area per molecule of 3.2 nm^2 was determined at a surface pressure of 20 mN/m. This indicates that the peptide molecules are oriented with their α-helix axis parallel to the air/water interface. A much smaller molecular area of $\sim$0.8 nm^2 would be expected for a perpendicular orientation of the helix axis with respect to the interface (Fig. 1, *inset*). The "collapse" of the monolayer occurred at a reproducible π value of 29 mN/m. The plateau region of monolayer "collapse" continues up to zero molecular areas, thus demonstrating that reorientation does not take place at molecular areas of $\sim$0.8 nm^2.

It was also found that the peptide adopts a parallel orientation with respect to the air/water and phospholipid/water interfaces when it is cospread with diverse lipid species in mixed monolayers or is injected under phospholipid monolayers. Typical surface pressure/area isotherms of mixed monolayers of the peptide alamethicin with dioleoylphosphatidylethanolamine (DOPE) (a system in which high ion-current asymmetry has been established; Sansom, 1993; Vodyanoy et al. 1983; Hall et al., 1984) are presented in Fig. 2. The nearly linear variation of the mean molecular area with the molar fraction of alamethicin in the monolayers, X_a (see Fig. 2, *inset*), indicates that the planar orientation of the peptide does not change in the mixed lipid/peptide monolayers. The negligibly small deviations of the mean molecular areas from an "ideal" dependence on X_a (weighted-average values), together with the observation of two monolayer "collapse" pressures, the lower one being

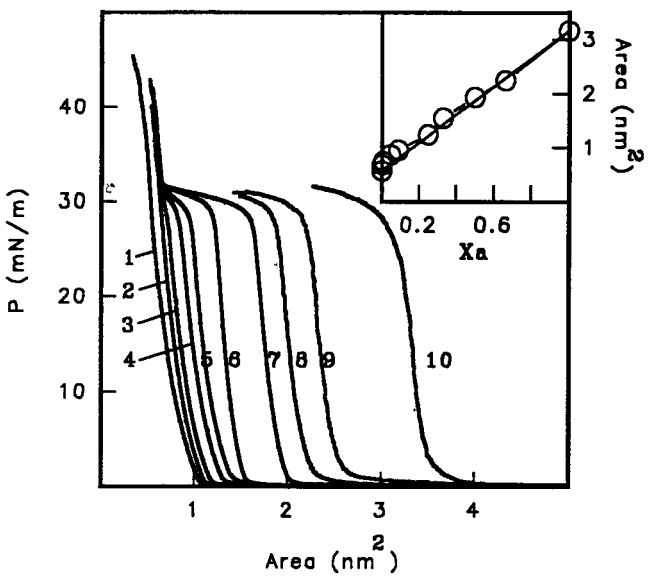

FIGURE 2 Surface pressure/area isotherms of mixed monolayers of alamethicin (*curve 10*) and 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) (*curve 1*) at 22°C on a 0.1 M NaCl aqueous subphase (pH 7.0) at different peptide/lipid molar ratios: 1:300 (*curve 2*), 1:100 (*curve 3*), 1:20 (*curve 4*), 1:10 (*curve 5*), 1:3 (*curve 6*), 1:2 (*curve 7*), 1:1 (*curve 8*), 2:1 (*curve 9*). The behavior of the isotherms is also representative for mixtures of alamethicin with 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), 1,2-myristoyl-sn-glycero-3-phosphatidylethanolamine (DMPE), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylglycerol (DPPG), and octadecylamine (ODA). The inset shows the variation of the mean molecular area with the molar fraction of alamethicin, X_a, in the binary peptide/lipid monolayers. The solid line shows the "ideal" mixing/demixing dependence.

independent of the monolayer composition at $X_a > 0.01$, demonstrate the two-dimensional immiscibility (Angelova et al., 1995, 1997; Leblanc and Salesse, 1994; Borissevich et al., 1996) of alamethicin and phospholipid molecules in the binary monolayers.

Solid-like two-dimensional peptide domains imaged by fluorescence microscopy at the air/water interface

Lipid/peptide immiscibility was confirmed by investigation of the domain organization of lipid/alamethicin monolayers by fluorescence microscopy. This method, first introduced by McDonnell (McConnell et al., 1984; Weis and McConnell, 1984), provides direct imaging of the nucleation and growth of dynamic domain structures (Möhwald, 1993; Vollhardt, 1993, 1996; Angelova et al., 1996) at fluid monolayer/water interfaces. If the peptide mixes homogeneously with phospholipid molecules, such as DOPE, which form fluid phase monolayers, a bright featureless spot should be observed in the fluorescent microscopy images. However, if alamethicin separates in the binary monolayers and forms

solid-like domains, then these domains should be visible as dark two-dimensional crystals, as shown in Fig. 3.

The fluorescent microscope measurements demonstrate the formation of large-scale (several μm in length) solid-like alamethicin aggregates at the air/water interface (Fig. 3 *A*). The peptide helices aggregate and crystallize at a surface pressure of 0.1 mN/m, and the lateral domain length varies from ~30 to 200 μm.

When mixed alamethicin/DOPE monolayers are compressed beyond the alamethicin "collapse" pressure, the fluorescent microscopy images reveal that the peptide crystals are squeezed from the interface because of their penetration beneath the phospholipid monolayers.

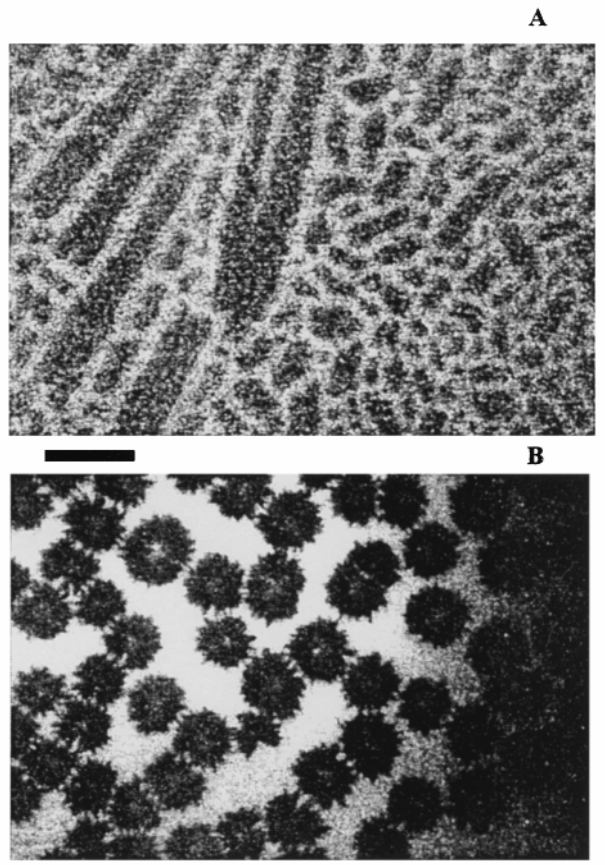

FIGURE 3 Fluorescence microscopy images. (*A*) Pure alamethicin monolayer at a surface pressure of 0.3 mN/m. The domain width is ~10 μm and the domain length varies from 30 to ~200 μm. The bar corresponds to 50 μm. (*B*) Alamethicin domains formed in DOPE monolayers upon alamethicin injection in the aqueous subphase followed by stepwise monolayer decompression from $\pi = 36$ to $\pi = 28$ mN/m with a step of 1mN/m. The equilibration time after every decompression step was 30 min. Alamethicin was injected from ethanol solution in the aqueous subphase to a total concentration of 1×10^{-7} M. The aqueous subphase was 0.1 M NaCl at pH 7.0, and the temperature was 22°C. The fluorescent NBD probe was soluble in fluid-phase monolayers such as DOPE. The dye/alamethicin and dye/lipid molar ratios were 1:800.

Crystalline structure of two-dimensional peptide aggregates deduced from synchrotron x-ray diffraction investigation at grazing angles

We studied the structural organization of the alamethicin helices in the crystalline aggregates at the air/water interface by means of a direct synchrotron x-ray diffraction method. Three x-ray peaks with wavevectors of $Q_{10} = 6.518$ nm^{-1}, $Q_{01} = 1.853$ nm^{-1}, and $Q_{11} = 6.655$ nm^{-1} were recorded at a surface pressure of 20 mN/m. They define an orthorhombic two-dimensional crystalline lattice with lattice parameters of $\mathbf{a} = 0.9635 \pm 0.0004$ nm, $\mathbf{b} = 3.389 \pm 0.009$ nm, and an angle between the vectors **a** and **b** of $\gamma = 93.87°$ (Fig. 4 *B, inset*). The area per molecule estimated from the x-ray spacing values corresponds to the molecular area for alamethicin obtained from the monolayer isotherm (Fig. 1, curve 1). The values of **b** and **a** predict an α-helix of length 3.389 nm and radius 0.4817 nm.

Fig. 4 *A* shows the surface pressure dependence of the alamethicin crystalline peak corresponding to a spacing of 0.9635 nm (lattice parameter **a**). With the increase of the surface pressure from 7 to 25 mN/m, the peak position varied from 0.9770 nm to 0.9419 nm. The value of γ remained within 0.25° of a right angle increasing with surface pressure. The peak intensity rose with surface pressure, while the peak width remained approximately constant and close to the experimental resolution of the system (0.07 nm^{-1}), determined by Soller slits. At a surface pressure of 25 mN/m, a correlation length, *R*, higher than 79 nm was estimated for the single Gaussian peak Q_{10}, using the Scherrer expression (Ionov and Angelova, 1994), $R = 0.88\lambda/[\Delta(2\theta)\cos\theta_n]$, where $\Delta(2\theta)$ is the half-width of the Bragg peak (in radians), θ_n is the angular position of the *n*th peak ($n = 1$), and λ is the x-ray wavelength, 0.1488 nm. The magnitude of *R* indicates that more than 79 α-helical molecules are associated in every two-dimensional peptide monocrystal. At lower surface pressures (<25 mN/m), the higher wavevector diffraction region was decomposed into two peaks with wavevectors of Q_{10} and Q_{11}. The second peak at Q_{11} was better expressed at low temperatures (Fig. 4 *B*). The intensity of the peak at Q_{01} (Fig. 4 *C*) was low, which made it difficult to study its surface pressure dependence. The x-ray spectra were reproducible, and every spectrum was recorded several times. The structural investigation revealed that the alamethicin helices adopt a planar orientation with respect to the fluid air/water interface and aggregate into two-dimensional crystals in pure alamethicin monolayers and in the presence of insoluble lipid molecules in mixed lipid/peptide monolayers.

Monolayer surface pressure/area measurements under an electric field reveal extremely high energy for peptide reorientation from a parallel to a perpendicular orientation with respect to the lipid/water interface

In the BS model, the "dipolar" mechanism of alamethicin insertion into lipid membranes has been based on the as

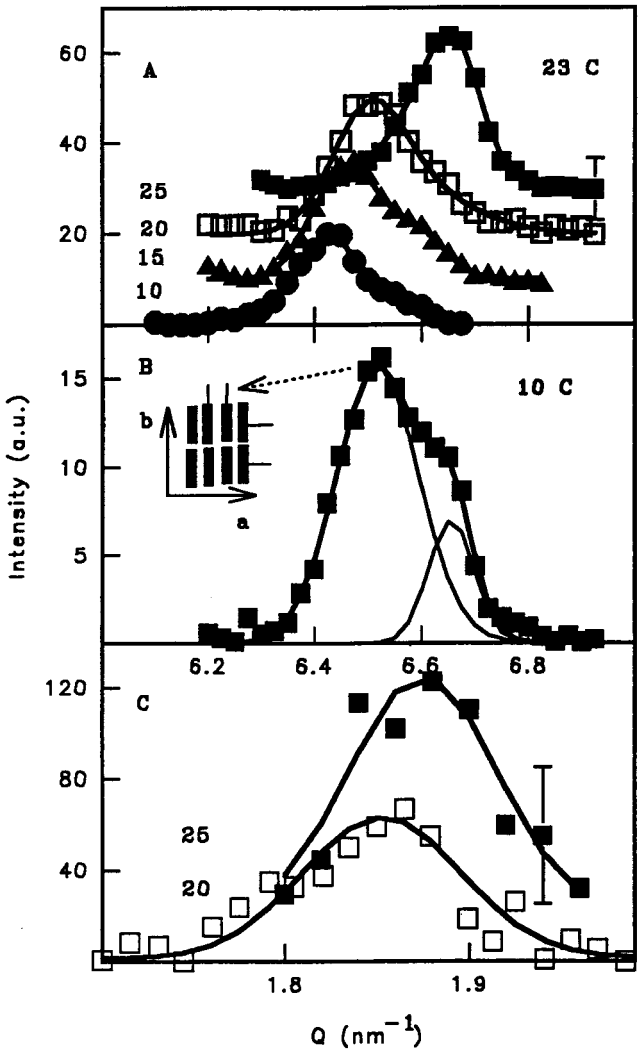

FIGURE 4 (*A*) Surface pressure dependence of the x-ray peak of the short in-plane spacing (*a*) of a pure alamethicin monolayer at 23°C. The numbers on the x-ray patterns indicate the surface pressure values in mN/m. The wavevector $Q = 2\pi/d$, where d is the Bragg spacing. The standard error of the x-ray experimental points determined by Poisson statistics is indicated in the figure by a vertical linear bar. (*B*) Short in-plane spacing peak at a surface pressure of 20 mN/m and a temperature of 10°C. The spectrum is decomposed into two Gaussian peaks (Q_{10} and Q_{11}). The inset shows the two-dimensional orthorhombic unit cell of the alamethicin crystals with short (*a*) and long (*b*) in-plane periods. (*C*) Surface pressure dependence of the long in-plane spacing (*b*) x-ray peak at 23°C. The experimental data were fitted using a Gaussian function. The detector noise and the baseline were subtracted for all spectra.

sumption that 1) the peptide dipole parallel to the helix axis (~60 D) will reorient perpendicular to the membrane interface, and 2) the helix will penetrate into the lipid bilayer upon the application of positive electric field (typically $\sim 10^7$ V/m for the ion current experiments) to the side of the membrane where the peptide is injected (*cis* side). If the polarity of the field is reversed, the helix should be pulled out of the bilayer. The energy of peptide reorientation from a planar to a perpendicular (BS model) configuration at the lipid/water interface has been supposed to be very low (Sansom, 1993).

One may expect to detect the α-helix reorientation in Langmuir monolayer experiments if the force applied for monolayer compression is higher than the electrical force necessary for molecular dipole reorientation in ion current measurements. The peptide reorientation upon monolayer compression at the lipid/water interface could be easily established, taking into account the essentially different areas of 3.2 nm^2 and 0.8 nm^2 corresponding to the parallel and perpendicular orientations of alamethicin helices, respectively. The results presented in Figs. 1 and 2 indicate that peptide reorientation does not take place at the interface because the work for monolayer compression to $\pi \approx 20$ mN/m of both pure alamethicin and mixed alamethicin/phospholipid monolayers (~5*kT*) is about an order of magnitude larger than the estimated work of the electrical field (the field at which the onset of the ion current occurs varies from 4 to 80 mV; Vodyanoy et al., 1983) necessary for alamethicin dipole reorientation. Mixing of alamethicin with ionic lipids, which creates charged interfaces and could induce dipole reorientation, yielded the same effect. These results show that the planar configuration of the amphiphilic peptide helices is very stable, and the perpendicular orientation at the interface requires extremely high energy.

To prove that the alamethicin helices adopt a stable planar configuration at the lipid/water interface, we placed a planar electrode ~1 mm above the monolayer and a second electrode inside the aqueous subphase of 0.1 M NaCl. The top electrode covered the entire monolayer surface when the film was compressed to a surface pressure of ~25 mN/m, while it covered about half of it when the monolayer was expanded to zero pressure. Electric potentials of up to 2.5 kV were applied while the peptide monolayers were compressed. These experiments were performed with positive and negative potentials applied to the top electrode for both pure alamethicin and mixed lipid/alamethicin monolayers. The obtained surface pressure/area isotherms were the same as those in Figs. 1 and 2, indicating that the total force of the monolayer compression and the external electric field is lower than that necessary to disturb the planar orientation of the α-helices. The same conclusion was drawn by verifying the variation of the surface pressure upon application of the electrical field on the multibilayer structure formed after the "collapse" of the mixed monolayers at a fixed total area of the Langmuir trough. The stable planar orientation of the peptide helices might be due to hydrogen bond formation between water molecules of the subphase and the polar amino acid residues (Gln^7, Glu^{18}, Glu^{19}, and Phol^{20}) of alamethicin aligned along the helix axis.

Surface charge measurements (Fig. 1, *curve 2*) indicated that the planar configuration of the alamethicin helices is preserved after the two-dimensional/three-dimensional

transformation of the monolayer (i.e., the monolayer "collapse"). The surface charge registered on the top planar electrode is proportional to the component of the helix dipole perpendicular to the monolayer interface (El-Abed et al., 1995). Hence the alamethicin reorientation should cause a dramatic change in the surface charge because of its strong dipole moment directed along the helix axis. The compression of the monolayer at a constant velocity led to a linear increase of the total surface charge and the establishment of a plateau region after the monolayer "collapse." This result, obtained with both pure and mixed monolayers, demonstrated an absence of molecular reorientation of the peptide helices at the interface and within the multibilayer structure formed after the "collapse" of the mixed alamethicin/lipid monolayers.

Therefore, the correct model of the peptide ion channel should take into account 1) the stability of the planar orientation of alamethicin; 2) its immiscibility with phospholipids at interfaces; and 3) the tendency of the peptide toward two-dimensional crystallization. The results of our interfacial and structural studies of alamethicin differ from earlier data obtained with alamethicin of undefined purity (Chapman et al., 1969). The MW of alamethicin reported in that work was about twice that of the purified compound used here.

An asymmetrical lipid-covered ring model of the ion channels formed by α-helical peptides

On the basis of the structural, morphological, thermodynamic, and electrical results, we propose a LCR model for the ion channel of alamethicin (Fig. 5). The model includes a plate-like peptide ring covered with a lipid monolayer, an arrangement that imparts structural asymmetry to the channel. In the model the peptide helices form two-dimensional aggregates, with a central hydrophilic ring-like cavity (Fig. 5 *A*, *top view of the ring*) formed by the polar hydrophilic C-terminal amino acid groups (including the ionizable Glu^{18}-COOH residue). The radius, *r*, of the pore formed by the aggregation of *n* helices could vary discretely (in a quantum-like way) by the addition or subtraction of helical monomers with a radius *R* (Fig. 5 *A*). The plane of the peptide aggregate is parallel to the phospholipid/water interface and is accommodated on the monolayer *cis* side of the lipid membrane (Fig. 5 *B*, *side view of the ring*). The polar surface of the peptide helix is oriented toward the water phase, while its hydrophobic part is toward the phospholipid hydrocarbon tails of the adjacent (*trans* side) monolayer of the membrane. The C- and N-terminal groups make energetically favorable contacts with the hydrophilic and the hydrophobic regions of the membrane, respectively.

The asymmetry of the ion channel structure implies an asymmetry of corresponding ion current characteristics. The partial negative charge of the cavity formed within the *cis* monolayer creates a potential well for cations and a poten-

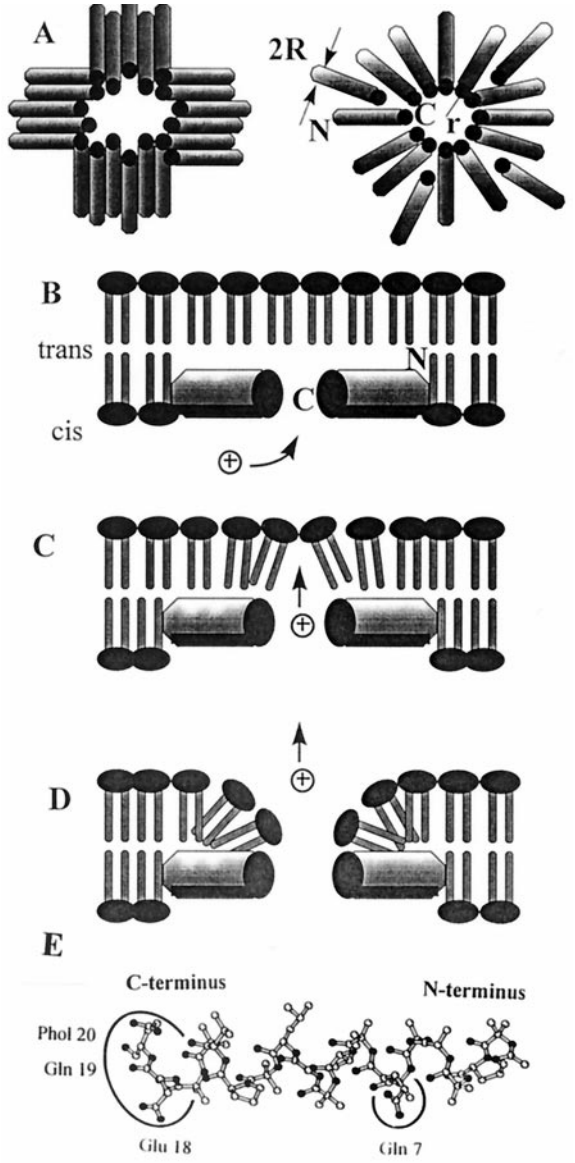

FIGURE 5 Lipid-covered ring (LCR) model of the alamethicin ion channel. (*A*) Top views of two types of possible ring-like organizations, possessing a central hydrophilic pore, formed by the aggregation of alamethicin helices oriented parallel to the phospholipid/water interface. *R* is the alamethicin helix radius, and *r* is the pore radius. The C and N termini of the peptide molecule are indicated by the letters C and N. (*B*) Side view of the channel. The peptide ring is embedded in the *cis* side phospholipid monolayer of the membrane (i.e., the side where alamethicin was injected into the aqueous phase). The channel is closed by the *trans* side phospholipid monolayer, which covers the alamethicin ring. The letters C and N indicate the C and N termini of the peptide. (*C*) Upon the application of a weak positive electrical field to the *cis* side monolayer, cations (denoted by +) enter the hydrophilic cavity of the ring and disturb the relatively fluid hydrophobic tails of the phospholipid molecules of the *trans* monolayer. (*D*) Strong positive electrical fields are able to open the channel because of the force applied by the cation to the *trans* monolayer. The darker areas on the peptide helix represent the hydrophilic regions of the molecule. (*E*) Ball-and-stick molecular model of alamethicin, based on x-ray diffraction data (Fox and Richards, 1982). The hydrophilic regions of the molecule are indicated.

tial barrier for anions. Therefore, the magnitudes of the electrical field needed for the cations and anions to wriggle within the cavity will be different. If one applies a positive electric field to the *cis* side of the membrane, cations will enter within the potential well of the cavity of the *cis* monolayer formed by the hydrophilic C termini of the alamethicin aggregate (Fig. 5 *B*). The applied potential will be distributed over the entire *trans* monolayer. The presence of hydrophilic cations within the alamethicin cavity will disturb the *trans* side phospholipid monolayer of the membrane and, in particular, its hydrophobic tail portion (Fig. 5 *C*). The degree of deformation of the *trans* monolayer will be dependent on the cation energy and the monolayer elastic properties. If the energy of the cation is high enough in the presence of a strong electrical field, the channel will open because of the force applied by the cation to the *trans* membrane monolayer, and the cation will pass through the membrane (Fig. 5 *D*). If the applied electric field is of the opposite sign (i.e., negative field applied to the *cis* side of the membrane), the energy of the anions should be sufficient to overcome both potential barriers, 1) of the cavity and 2) of the *trans*-side monolayer. Therefore, a higher negative voltage would be necessary to open the channel and to allow anions to pass through the membrane. The stronger the lateral interactions in the headgroup region of the lipid monolayer, the higher the ion energy needed to disturb the *trans*-side monolayer and to open the ion channel. Therefore, the highest ion-current asymmetry would be expected for lipid membranes constituted by PE derivatives, which exhibit a strong tendency for lateral hydrogen bond formation.

The LCR model proposed here retains the main advantages of the BS model in explaining 1) the quantum-like single-channel ion current dependence on the helix diameter (as discussed above) and 2) the increased stability of selected ion-current levels upon linkage of the C termini of the α-helices by flexible tethers (You et al., 1996; Matsubara et al., 1996) due to the same physical reasons that pertain to the BS model.

In addition, the helices in the LCR model are oriented parallel to the membrane/water interface, consistent with direct structural measurements, and the LCR model explains several other ion channel properties:

1. The structural asymmetry of the channel in the LCR model explains the ion current asymmetry (see the discussion above).

2. The LCR model explains (as discussed above) the highest ion-current asymmetry experimentally established with PE membranes (Sansom, 1993; Vodyanoy et al. 1983; Hall et al., 1984), while the other models do not account for this effect.

3. The proposed LCR model fits recent experimental data of Taylor and de Levie (1991), who found "reversed" alamethicin conductance states in lipid bilayers when positive conductance was present at the moment of fast voltage reversal and an increased probability of these states with decreasing temperature and increasing magnitude of negative voltage. The results of Taylor and de Levie (1991) were not explained by the BS model. According to our LCR model, if the voltage is rapidly reversed, the reversed current could flow during the relaxation time needed for the *cis* (or *trans*) side monolayer to close the channel, as a result of the presence of lateral membrane pressure (usually higher than 30 mN/m). The higher the magnitude of the negative voltage, the higher the "reversed" ion current and the probability of ion flow needed to hydrodynamically maintain the open channel. With decreasing temperature, the probability that the channel will stay open rises because of the increased time of relaxation of the *trans* side (or *cis* side) monolayer because of its decreased fluidity.

The LCR model could also fit the following recently reported findings:

4. Higher probability of finding the channel in its higher conductance states with rising membrane tension (Opsahl and Webb, 1994). Higher tension, t, corresponds to lower lateral lipid pressure, p ($\Delta t = -p$). As will be shown below, a decrease in lateral lipid monolayer pressure leads to incorporation of more alamethicin molecules within the monolayer, which increases the probability of reaching a higher channel radius (Fig. 5 *A*) and higher conductance states.

5. Dependences of the conductance states on the lipid monolayer curvature (Keller et al., 1993). States of higher conductance have been reported to be more probable in DOPE, a lipid that could form nonlamellar structures of high curvatures, than in DOPC, a lipid that forms bilayer structures of zero curvature. DOPE bilayers require higher alamethicin concentrations for channel formation. This experimental observation is related to the higher energy needed to break hydrogen bonds in the PE headgroup region of the DOPE monolayers. The inverted-cone shape of DOPE molecules (which is the reason for the high monolayer curvature of its assemblies; Israelachvili, 1991) allows a larger space to be opened in the headgroup region of the DOPE monolayers as compared to that of DOPC. This increases the probability that larger numbers of alamethicin molecules will penetrate the DOPE monolayers and form channels of higher radii.

On the mechanism of peptide penetration and formation of plate-like aggregates in phospholipid monolayers

The alamethicin concentrations typically used in single-channel ion current measurements are higher than 10^{-7} M. Our experiments on peptide adsorption at the air/water interface reveal that the surface pressure rises steeply from 1 mN/m to ~27 mN/m as the alamethicin concentration is increased from $\sim 3 \times 10^{-8}$ to 9×10^{-8} M. Therefore, beneath densely packed *cis* monolayers (of a lateral pressure

of ~30–50 mN/m in lipid bilayer membranes), a high surface concentration of alamethicin aggregates should be present. The alamethicin helices could penetrate the *cis* side monolayers of the membranes by two mechanisms: the collision of the alamethicin aggregates present beneath the *cis* monolayers or local fluctuations in the lateral pressure of the *cis* monolayers to values less than 30 mN/m. Such fluctuations are typical for fluid-phase lipid membranes such as DOPE and DOPC (Tristram-Nagle et al., 1998).

To prove that alamethicin aggregates in phospholipid monolayers with a decrease in the lateral surface pressure, the following experiment was performed. A phospholipid monolayer was compressed to ~36 mN/m in the Langmuir trough, and alamethicin was injected from ethanol solution under the monolayer to a total concentration of $\sim 1 \times 10^{-7}$ M in the aqueous subphase. The lipid monolayer was decompressed to rapidly decrease the surface pressure. Surface pressure reduction to below 31 mN/m, caused by monolayer expansion, was always followed by a pressure increase, indicating that alamethicin molecules begin to adsorb at the interface and to penetrate the phospholipid monolayer. The adsorption process at the interface was followed by fluorescent microscopy. Initially, the sizes of the nuclei of peptide aggregates were very small. Obviously, these nuclei were centers of two-dimensional crystallization (Vollhardt, 1996, 1993; Angelova et al., 1996) of the adsorbed alamethicin, because upon further decompression of the phospholipid monolayers the peptide aggregates grew and became increasingly visible in the microscopy images (Fig. 3 *B*). Hence the local reduction in the lateral membrane pressure favored the formation of plate-like peptide aggregates at the interface. The fluctuations of the surface pressure could contribute to either an increase or a decrease in the number of peptide helices associating in an aggregate and, correspondingly, to a change in the radius of the pore, which determines the size of the peptide channel.

Relevance of the proposed model with a parallel helix-axis orientation at the membrane interface to lipid bilayers

1. This study was initiated with the expectation of verifying the perpendicular orientation of alamethicin helices at the membrane/water interface suggested by the BS model. Direct structural measurements were made under physiological conditions for alamethicin ion channel operation at membrane interfaces. Direct structural evidence for a perpendicular orientation of the alamethicin helices at the lipid bilayer/water interface has not been published. The initial event in the formation of the ion channel is the interaction of the alamethicin molecules with a membrane monolayer. A key element in the BS model is the existence of a hydrophobic region of ~2.5 nm in the phospholipid bilayers, where the alamethicin barrel is formed either spontaneously or by electric field induction. The hydrophobic/hydrophilic balance determines the molecular orientation at the air/water interface and the formation of Langmuir monolayers. Alamethicin helices in pure peptide monolayers at the air/water interface or embedded in a hydrophobic lipid environment (~2–3 nm in thickness) in mixed monolayers at such an interface are subjected to the same physical conditions that they would be if they were immersed in the hydrophobic region of a lipid bilayer. Therefore, if the perpendicular alamethicin helix orientation is more probable for lipid bilayers, it should be characteristic also for Langmuir monolayers and for multibilayers formed after monolayer "collapse." This orientation should be formed spontaneously or induced either by monolayer compression or by electrostatic fields. However, our results clearly show a preference for parallel orientation of the alamethicin helix relative to the lipid/water interface.

2. The BS model involves an energetically unfavorable contact between the hydrophobic N terminus of the alamethicin helices and water molecules. This problem does not arise in our LCR model (see Fig. 5).

3. The structural effect of incorporation of alamethicin into DOPE bilayer dispersions prepared under the same physiological conditions as those for the operation of ion channels has recently been studied by us (Angelova et al., 1999), using time-resolved synchrotron x-ray diffraction. The induced transition of DOPE from an inverted hexagonal (H_{II}) phase into a cubic Q^{224} phase upon incorporation of alamethicin has been explained by the lateral expansion of the DOPE headgroup area and effective increase in the radius of DOPE monolayer curvature as a result of the incorporation of parallel oriented alamethicin helices in the headgroup region of the lipid/water interface. This explanation is consistent with monolayer studies and the ion channel model proposed here. A perpendicular incorporation of alamethicin helices into DOPE bilayers would not explain the phospholipid phase transition (Angelova et al., 1999). In hydrated DOPC/alamethicin mixtures, alamethicin also adopts an orientation parallel to the DOPC membrane bilayer/water interface. A detailed time-resolved x-ray diffraction study of DOPC/alamethicin systems has been presented elsewhere (Angelova et al., 1999).

4. There is increasing evidence in recent literature of a preference for a parallel orientation of the alamethicin helix with respect to the membrane interface. Fourier transform infrared (Greenhall et al., 1998), NMR, and Raman (Banerjee et al., 1985) measurements have demonstrated interactions of the alamethicin helices with the lipid headgroups and not with the acyl chains in the hydrophobic region of the bilayer membranes. Molecular areas of ~3.0–3.50 nm^2 have been estimated (Wu et all., 1995) from x-ray diffraction studies of supported multibilayers at low alamethicin concentrations. According to our study these areas correspond to parallel orientation of the alamethicin helices at the phospholipid bilayer/water interface.

The proposed LCR model avoids some of the inconsistencies of the BS model and satisfies the structural results and recently reported ion current data. The model accounts for various features of the ion current measurements and explains the functions of both voltage-gated and gradient-controlled ion channels formed by weakly hydrophobic peptides and proteins.

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