J. Membrane Biol. 184, 1–12 (2001)
DOI: 10.1007/s00232-001-0077-2

The Journal of
Membrane
Biology

Topical Review

Voltage-Dependent Pore Formation and Antimicrobial Activity by Alamethicin and Analogues

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Received: 30 June 2001

Introduction

Despite the long-standing interest for membrane-active peptides, alamethicin remains the best studied simple model of pores formed by the intramembrane aggregation of amphipathic α-helices. The demonstration of artificial membrane excitability developed by planar lipid bilayers doped with alamethicin [70] indeed struck the minds of many a biophysicist, including electrophysiologists, at a time when the protein nature of ion channels was still a matter of conjecture. Since 1967, the number of articles devoted to alamethicin doubles every decade to presently attain 500 and thus this peptaibol, i.e., a peptide rich in α-aminoisobutyric acid (Aib) or α-methylalanine (α-MeA), opened a classical chapter in biophysics with offshoots such as lipid-peptide interactions and the host of studies presently devoted to antimicrobial peptides (for reviews, *see* e.g., [32, 43]).

Discovered in cultures of the fungus *Trichoderma viride* [64], alamethicin was assumed to be a cyclic peptide when the 'barrel-stave' model was put forward [6, 12]. An NMR spectroscopy study establishing the correct and linear sequence (*see* the Table) in 1976 [63] was quickly followed by the chemical synthesis of a very similar peptide, with which the essential functional properties were retrieved [36]. After the pioneering studies mentioned above [70], alamethicin single-channel events were recorded with multiple open states [38] and their detailed kinetic analysis led to the proposition of the 'barrel-stave' model [12]. In the early eighties, the high-resolution (down to 1.5 Å) crystallographic structure of alamethicin [33] was a landmark, from which new structural studies sprang also allowing the formulation of hypotheses on the voltage-dependent membrane insertion and the architecture of the oligomeric channel.

The purpose of this review is to provide a comprehensive account of the presently available functional and structural data on alamethicin and peptaibols, emphasizing the most recent advances and trends. We shall also concentrate upon comparative macroscopic and single-channel conductances in planar lipid bilayers and antimicrobial activities of alamethicin, related peptaibols and synthetic analogues designed to test specific residues. Within this class of membrane-active peptides, it is shown that antimicrobial activity obeys the same rules as defined by conductance assays. Several reviews published in the last decade and dealing with this field under different aspects ought to be mentioned [98, 76, 18, 7, 74].

Macroscopic Conductance Data Allow an Estimation of the Pore Sizes that is Compatible with Single-Channel Studies

A functional signature of alamethicin is the very high voltage-dependence of the macroscopic conductance, i.e., the conductance developed by hundreds or thousands of channels, in planar lipid bilayers. After peptide addition to the *cis*-side of the bilayer chamber and par-

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Key words: Ion channels — Amphipathic α-helix — Peptaibols — Planar lipid bilayers — Conductances — Antibacterial activity

titioning to the bilayer, which is rapid (the bilayer/water partition coefficient being about 10^5 at an alamethicin concentration of 3×10^{-8} M, corresponding to a lipid/peptide ratio of 80 [35]), the bilayer submitted to slow voltage triangular waveforms delivers highly asymmetric *I-V* curves. Conductance is negligible in the negative voltage direction and only develops in the positive quadrant along a steep exponential branch once a voltage threshold, which is concentration-dependent, has been reached. The voltage-dependence of this branch is quantified by V_e, the voltage shift resulting in an *e*-fold increase in conductance (*e*: natural logarithm base). V_e for alamethicin being only 4–5 mV, the conductance induced by this simple peptide in planar lipid bilayers is as efficient as regards voltage-dependence as, e.g., the sodium channel in excitable membranes. Asymmetrical *I-V* curves reflect the confinement of the peptide on only one side of the bilayer and as time elapses after many voltage sweeps, alamethicin migrates from *cis*- to *trans*-sides, favored by the building up of conducting bundles which also induce lipid 'flip-flop' [40]. *I-V* curves subsequently tend to become symmetrical.

Macroscopic conductances were studied as a function of ionic strength, various salts and especially the aqueous concentration of alamethicin. The threshold for the development of the exponential branch decreases according to a logarithmic mode as the ionic strength increases [31], and alamethicin pores are weakly cation-specific, mostly excluding divalent cations, but there is no real ion selectivity within monovalent ions, except for the lowest single-channel conductance level (*see* below). However, the most extensively studied topic is the alamethicin concentration-dependence. With V_a being the shift of the voltage threshold resulting from an *e*-fold change in aqueous alamethicin concentration, $\langle N \rangle$, the apparent and mean number of monomers involved per channel [42] can be simply calculated as the ratio of V_a and V_e, the voltage-dependent parameter defined above:

$$\langle N \rangle = V_a/V_e.$$

There is an agreement between this analysis and $\langle N \rangle$ estimated from the most probable single-channel substate, if four helices are assumed to form the smaller conducting aggregate. Indeed, alamethicin unitary conductance fluctuations are characterized by at least five or six multistates whose conductances obey a geometrical progression, each transition resulting from the uptake or release of individual monomers within the conducting bundle. The probability distribution is centered on substates 3–5 and the quasi-ohmic conductance of each substate presents a rather low voltage-dependence of on- and off-rate constants [31, 12]. The smallest conductance substate (20 pS in 1M KCl), presumably resulting from a tetramer, is impermeable both to Ca^{2+} and Cl^- [44] and the sequence of normalized unitary conductances (1:4:20:45:75:110) remains roughly identical within alamethicin natural and synthetic analogues.

Peptide Length/Bilayer Thickness Mismatch and Cholesterol Influence Pore Sizes and Channel Stability

Cholesterol is a well known biomembrane vicosity 'buffer', and increasing its mole fraction very significantly lengthens the mean open-channel lifetimes, although higher voltages are required to form a given number of alamethicin channels. An analysis of on- and off-rates of channel formation showed that the main influence of cholesterol addition is not simply through viscosity but through an increased membrane dipolar potential and/or a larger alamethicin adsorption on membranes [58]. The dependence of the number of monomers forming the pores upon the bilayer thickness was studied in bilayers made up of monoglycerides with chain length varying between 14 and 22 carbon atoms (bilayer core thickness from 19.5 to 29 Å). All macroscopic conductance parameters are modified with the thickness increase such that $\langle N \rangle$, corrected for a constant gating charge per monomer of 0.5 electronic charge, varies between 4 and 11 from the thinnest to the thickest bilayers, respectively [42], in agreement with a theoretical study, suggesting that a good matching between peptide helical length and bilayer thickness favors larger oligomers [86]. This is also confirmed with phospholipids by neutron-scattering experiments: the mean number of alamethicin monomers in conducting aggregates is lower in dilauroyl-PC ($N = 8$) than in diphytanoyl-PC ($N = 11$) [45].

A series of natural analogues of alamethicin (i.e., peptaibols) 14 to 20 residues long (*see* the Table and also www.cryst.bbk.ac.uk/peptaibol [96]), offered the opportunity to complement these studies by assaying the influence of helical length and sequence on channel size and duration, with the host bilayer thickness remaining constant in most of these experiments. In agreement with a previous study on a synthetic analogue of the Alm-dUL series [27], longibrachin (20 residues) single-channel conductance fluctuations are faster than those displayed by alamethicin [21], suggesting that Pro2 favors the transient anchoring of the N-terminus on the other side of the bilayer. In trichorzianins (19 residues), not only Pro2 is missing but the near-central glycine is replaced by a serine. To resolve the very fast single-channel events induced by these analogues, it was necessary to significantly reduce the bilayer thickness through the use of monopalmitolein. As with alamethicin, the charged analogue induced more asymmetrical macroscopic *I-V* curves than the neutral one [65]. With bilayers made of neutral and electrically-negative phospholipids containing cholesterol and out of the four trichorzianin analogues assayed (Table), the most efficient was the one

presenting both Glu18 and Trpol. The spectacular lenghtening of channel duration induced by the latter aminoalcohol reflects both a larger dipolar moment and the steric hindrance of the aromatic side chain [26].

As for antiamoebin (16 residues), its functional properties in planar lipid bilayers are radically different from the above-mentioned peptaibols and do not obey the dynamic 'barrel-stave' model. In neutral phospholipids and relatively high peptide concentrations, a transition between an ohmic conductance that reflects a carrier mechanism and a voltage-dependent component was observed [29] making this peptaibol another rare case for which lipid-modulated types of membrane ion transport can be demonstrated. Finally, the sequence of harzianins HC (14 residues) is made up of repeated (Aib-Pro-X-Y) motifs and they are structured in a subtype of 3_{10} helix giving them a sufficient length to span the bilayer despite the small number of residues. Macroscopic *I-V* curves are symmetrical with a high voltage-dependence and the size of the conducting bundles ($\langle N \rangle = 8$) is similar to alamethicin. However, the typical pattern of unit conductance substates in geometrical progression is not observed any longer but instead only one level, indicating fixed-size pores [62].

In summary, a gradual channel destabilization occurs in bilayers of similar thickness as the overall length of the investigated peptaibols decreases, illustrating the importance of the peptide-length/bilayer-thickness mismatch notion.

Phosphatidylethanolamine Favors Channel Build-up Through an Increased Surface Tension and Bilayer Thinning

In contrast to bilayers containing, e.g., valinomycin functioning according the 'carrier' mode, those incorporating pore formers do not show any abrupt conductance transition near T_t, the lipid phase transition temperature [13]. The effects recorded, as well as those resulting from the inclusion of a small percentage of phosphatidic acid [49], are indicative of a phase separation between pure lipid domains and mixed lipid + peptide domains. Hydrostatic pressure variations acting mainly on the lipid matrix exert effects on alamethicin conductances: a ten-fold increase of tension applied through suction to a bilayer formed at the tip of a patch pipette shifts the open probability towards high-conductance substates and decreases channel lifetimes [72]. The analysis of the data is in quite good agreement with the 'barrel-stave' model since the pore diameter expansion is compatible with the uptake of additional peptide monomers whose cross section area is 80 $Å^2$ [75].

Some phospholipids with small headgroups, such as dioleoylphosphatidylethanolamine (DOPE), are prone to form inverted hexagonal instead of lamellar phases and favor spontaneous curvature or surface tension if the lamellar organization is imposed as, e.g., in planar lipid bilayers. This generally affects peptide-lipid interaction (for review, *see* [19]). In the case of alamethicin, these phospholipids shift the single-channel probability distribution towards higher conductance substates [56] and this is strongly correlated with spontaneous curvature or surface tension. This set of functional studies can be put into perspective with two more recent spectroscopic investigations. In phosphatidylethanolamine and phosphatidylcholine binary lipid bilayers, increasing the mole fraction of the former impedes alamethicin binding to the lipids, in agreement with the larger peptide concentrations that are needed to develop similar conductances. This larger free energy of binding is linearly correlated with the spontaneous curvature or surface tension of the PE-rich bilayers concomitant with a local bilayer thinning that could be induced by the peptide [60]. This latter effect is indeed observed through X-ray diffraction by diphytanoylphosphatidylcholine multilayers: in the range of peptide/lipid 1:150 to 1:50, membrane thinning is linear and can reach as much as 2 Å. For each adsorbed alamethicin molecule, the lateral expansion of the lipid headgroup region corresponds to the size of the peptide lying flat at the interface and extends up to 100 Å around each peptide [100]. However, above a critical peptide/lipid molar ratio (around 1/40 with diphytanoylphosphatidylcholine), alamethicin inserts spontaneously in the bilayer whose thickness increases [45]. The thinning effect of alamethicin lipids is confirmed in another recent investigation using time-resolved X-ray diffraction, which also shows the coexistence of lipid domains with associated and non-associated helical peptide [1]. In DOPE dispersions, the inverted hexagonal (H_{II}) phase for high alamethicin concentrations (10^{-4} M) is maintained, and above a critical peptide concentration two new cubic structures appear and their pattern of expression depends both on concentration and temperature.

In asymmetric bilayers made of a phosphatidylethanolamine (PE) and phosphatidylserine (PS) leaflets, calcium ions initially confined on the PE side can cross the bilayer through alamethicin channels and then, accumulating at the other interface (PS-side) reduce the 'interfacial potential' component of the voltage drop and thus contribute to close alamethicin channels [41]. This is also observed with long-chain quaternary ammonium ions and with local anesthetics, which are thus able to cross alamethicin pores [24]. Finally, replacing NaCl or KCl by $LaCl_3$ reduces mean open lifetimes by about one order of magnitude and the most probable single-channel conductances fall back to lower substate values [37]. This likely reflects a faster lateral diffusion of alamethicin monomers following lipid cluster formation due to the complexation of lipid headgroups with trivalents cations.

Alamethicin Forms α-Helices with a Central Kink and a More Flexible C-Terminus

Based on conformations adopted by alamethicin fragments (residues 1–6, 7–13, 11–16) and on previous studies on the stereochemistry of Aib-rich peptides, a body of spectroscopic studies first suggested for the 1–17 fragment a 3_{10}-helix, resulting in a more extended structure than the classical α-helix. As for the C-terminal polar tripeptide, this was found much more flexible [71].

Alamethicin crystals diffracting at high resolution (1.5 Å) were obtained by Fox and Richards (1982) after dilution in acetonitrile of an alamethicin (negatively-charged form)/methanol solution at 100 mg/ml [33]. Three independent monomeric forms are identified, but their structures are quite close, with a predominant α-helix from the N-terminus up to the kink introduced by proline 14. Due to this kink, whose angle varies between 35° and 39°, carbonyl oxygens from Aib10 and Gly11 cannot form intramolecular H-bonds, but rather interact with water. The analysis of the intramolecular H-bonding network shows a $4 \rightarrow 1$ H-bond between positions 12 and 15 and this 3_{10} conformation prevails above Pro14 in a variable extent [33]. The interest of the crystallographic study also lies in the clear definition of hydrophilic and hydrophobic sectors, in the modeling of conducting oligomers and in the formulation of hypotheses on the role of specific residues (*see* below). Gln7 and Glu/Gln18 sidechains together with backbone carbonyls near the center of the molecule, especially Gly11 [94], define a narrow strip parallel to the main axis and on the convex side of the bent helix. Gln19 sidechain does not take part in this hydrophilic sector but is tilted away on the helix side. The alamethicin molecule is thus amphipathic (albeit to a limited extent) from two points of view: polar groups are preferably segregated in the C-terminal part and together with Gln 7, Gly 11 and possibly Pro14, they delineate a narrow longitudinal hydrophilic sector.

To some extent, alamethicin structure depends upon the physical state of the lipid bilayer to which it is bound or in which it is embedded: it is helical from residue 1 to residue 12 above the lipid transition temperature (T_t) whereas below T_t the helix extends up to position 16 [93]. In hydrated lipid membranes in fluid state and for high molar peptide/lipid ratios favoring peptide aggregation, helical segments are orthogonal to the bilayer plane. Bringing back the temperature below T_t reorients these segments parallel to the interface. Although helicities as derived from circular dichroism in the same study seem under-estimated as compared to values issued by Raman spectroscopy, in dimyristoylphosphatidylcholine the alamethicin helicity content is quite comparable to values issued from the crystal [20]. Alamethicin in turn exerts reciprocal influence on the lipid matrix: phosphorus and deuterium NMR showed that alamethicin incorporation into lecithin multibilayers particularly influences lipid headgroups instead of the aliphatic core, even for high peptide/lipid molar ratio [4].

Pro-induced Kink has Found an Optimal Position in Alamethicin and Inter-helical H-Bonds Stabilize the Pore Assembly

Since Pro is often found at very conserved positions within some transmembrane segments of numerous transport membrane proteins, an important structural and functional role is attributed to kinks, e.g., to favor helix aggregation [97]. Pro *cis/trans* isomerization may play a role in regulatory processes [52] and is implied in opening-closing mechanisms of ion channels or receptors [15]. Indeed, one of the main issues at stake in NMR studies focusing on alamethicin conformational dynamics in various solvents and lipids had important implications for the voltage-dependent activation of alamethicin channels and possibly of physiological channels: could the kink introduced by Pro play the role of a hinge between the C- and N-terminal parts? A global reorientation of alamethicin or an alignment of both parts under the electric field could favor transmembrane insertion, the first step in channel-building. In SDS micelles and with the help of simulated annealing with angular and distance NMR constraints, several straight as well as curved forms had been unambiguously demonstrated. Conversion between these forms reflects a degree of rotational freedom between positions 10 and 12, due to the lack of H-bonds with Pro14 [34].

The assumed involvement of Pro in voltage-dependent gating was tested with synthetic alamethicin analogues since natural peptaibols most often differ by more than one residue and since the solid-phase synthesis of peptaibols was mastered only recently. An Aib-devoid template, in which all eight Aibs were replaced by leucines (Alm-dUL, see the Table 1), was first synthesized and characterized. All the essential conductance properties that are typical of alamethicin were conserved, except the mean duration of all sublevels, which was reduced by an order of magnitude [66]. A conformational study performed on this analogue showed an increased α-helicity compared to alamethicin (whose Aibs favor some 3_{10} helical conformation) and thus a slight shortening of the molecule [14]. For a constant thickness of the host bilayer, this could explain the relative channel destabilization, but one has also to take into account the larger steric hindrance of Leu sidechains, especially at position 3, which may hinder a good matching of monomers within the aggregate [16]. Further residue deletions or additions in the C-terminal part, together with the replacement of the amino-alcohol by an amide, were without significant functional influence, thus confirming the weak specific role of this region [67]. The conservation of essential functional and structural properties of

the parent molecule after all its eight Aibs out of 20 residues have been replaced by Leu whilst preserving Gln7 and Pro14, then allowed the substitutions affecting the latter residues to be assayed.

The role of Pro14 was first tested by alanine substitution on Alm-dUL. Although the apparent mean number of monomers per channel is reduced and unitary conductance fluctuations become faster, the high voltage-sensitivity typical for alamethicin is strictly conserved [27]. This contrasts not only with results from a similar study on melittin [23], but also with the modulation of the voltage-dependence of sodium channel voltage-sensors (S4 segments) according to the presence and position of prolines [47]. As already suggested [61], proline influence on peptide dynamics and functional properties is thus strongly dependent upon the neighbouring peptide sequence. This is confirmed with analogues even closer to alamethicin since Aib residues were kept in the sequence. When 'Pro-scanning' is performed, displacing its position between 11 and 17 [55], the most significant effect remains on channel kinetics, i.e., much faster events for substitutions on either side of the natural position. It is clear that proline occupies an optimal position (14) in alamethicin and most of the long-sequence peptaibols, favoring long channel duration and large size with relatively weak peptide concentrations. These conclusions hold in a subsequent investigation of the effects of the same 'Pro-scanned' alamethicin analogues on cytotoxicity, incidentally demonstrating that the primary mode of action of alamethicin-like peptides on these functions is based on a 'channel-type' membrane permeabilization [22].

This work on the role of proline was further extended with another analogue, in which not only Pro14 was substituted with alanine, but also Gly11, another residue often found in bends and linkers. Parallel structural studies measuring interactions between a nitroxide, a spin labeling group, at the C-terminus and the Cα hydrogens at different positions along the backbone allowed an estimation of distances and thus of flexibility [53]. Compared to alamethicin, the substituted P14A analogue presents similar C-terminus-Cα distances and, accordingly, this substitution does not affect helix overall structure. On the other hand, the additional substitution G11A does significantly increase the average C- to N-termini distances. However, if unitary conductance fluctuations are even faster than with P14A alone, voltage-dependence is still unchanged. These residues, although favoring channel stability, do not appear essential in voltage sensitivity as one might have expected. Thus, the assumed realignment of alamethicin N- and C-termini under the influence of an electric field may well not be an absolute requirement for insertion within the bilayer, as is also suggested by very recent molecular dynamics simulations [91].

The other key residue is Gln7, which, through its sidechain bearing an hydroxyl group, can help stabilizing transmembrane bundles of parallel helices, thanks to the formation of a crown-like network of intermolecular H-bonds. Upon the replacement of this residue in Alm-dUL by Asn (shorter sidechain but still allowing intermolecular H-bonds) and Ser (much shorter sidechain), opening probability is reduced especially in the case of the second substitution [68]. This agrees with a molecular modeling/dynamics study that clearly shows a gradual reduction of H-bond number as the sidechain at position 7 is made shorter. The effect is most pronounced with Ser, with which H-bonds are indirect, i.e., mediated by a water molecule between two contiguous Ser sidechains [16]. A previous study employing ^{1}H-NMR on fragments of suzukacillin, an alamethicin natural analogue (*see* the Table 1), had already suggested the possible role of Gln in stabilizing aggregates of channel-forming peptides [50].

Alamethicin Inserts via its N-terminus that Responds to Voltage and Laterally Aggregates to Form Funnel-shaped Pores of Varying Sizes

Since conductance parameters are not significantly modified by the deletion of the last three C-terminal residues, it is indeed the N-terminus that reacts to voltage changes: a positive voltage repels the positive end of the peptide dipole into the bilayer core and finally allows the crossing. The 'barrel-stave' model further assumes their lateral aggregation to form transmembrane helical bundles with continuous uptake and release of individual monomers. Remarkably, this general scheme has survived, even if a quite recent study proposes a radically different model termed 'asymmetrical lipid ring' [51]. From data obtained from synchroton radiation X-ray diffraction, this latter model states that alamethicin would remain flat not only at rest but also under the influence of the electric field. The latter would only act on the lipid polar headgroups of the other leaflet (opposite to the side of alamethicin addition), swinging them towards peptide C-termini, thus completing the hydrophilic pore. However, this study was performed on Langmuir lipid monolayers, where the control of applied voltage seems rather difficult. Besides, another recent study adds further experimental evidence for the effective transmembrane crossing of alamethicin: annexin on the *trans*-side prevents the voltage-induced release in the bulk of alamethicin initially added on the *cis*-side [85]. In the 'barrel-stave' model, the parallel orientation of helices within alamethicin bundles is henceforth the object of a consensus. It is, however, possible that a few helices might remain antiparallel, as postulated to explain 'persistent' or long-lived channels induced by the sudden reversal of the normally activating voltage with the neutral form of alamethicin and at low temperature [89].

Table 1. Sequences of selected peptaibols discussed in this review

Peptide	Sequence																				
						5						10					15				
Alamethicin (F30 I)	*Ac*	**U**	**P**	**U**	**A**	**U**	**A**	**Q**	**U**	**V**	**U**	**G**	**L**	**U**	**P**	**V**	**U**	**U**	E	**Q**	**Fl**
Alamethicin (F50)	*Ac*	**U**	**P**	**U**	**A**	**U**	**A**	**Q**	**U**	**V**	**U**	**G**	**L**	**U**	**P**	**V**	**U**	**U**	Q	**Q**	**Fl**
Alm-dUL	*Ac*	L	P	L	**A**	L	**A**	Q	L	**V**	L	G	**L**	L	P	**V**	L	L	E	**Q**	Fa
Alm-dUL-F20W	*Ac*	L	P	L	**A**	L	**A**	Q	L	**V**	L	G	**L**	L	P	**V**	L	L	E	**Q**	Wa
Alm-dUL-P2A	*Ac*	L	A	L	**A**	L	**A**	**Q**	L	**V**	L	G	**L**	L	P	**V**	L	L	E	**Q**	Fl
Alm-dUL-P14A	*Ac*	L	P	L	**A**	L	**A**	Q	L	**V**	L	G	**L**	L	A	**V**	L	L	E	**Q**	Fl
Alm-G11A+PI4A	*Ac*	U	P	U	**A**	U	**A**	Q	U	**V**	U	A	**L**	U	A	**V**	U	U	E	**Q**	Fl
Alm-dUL-Q7N	*Ac*	L	P	L	**A**	L	**A**	N	L	**V**	L	G	**L**	L	P	**V**	L	L	E	**Q**	Fl
Alm-dUL-Q7A	*Ac*	L	P	L	**A**	L	**A**	A	L	**V**	L	G	**L**	L	P	**V**	L	L	E	**Q**	Fl
Suzukacillin	*Ac*	U	A	U	A	U	A	Q	U	U	U	G	L	U	P	V	U	U	Q	Q	Fl
Longibrachin A I	*Ac*	**U**	**A**	**U**	**A**	**U**	**A**	**Q**	**U**	**V**	**U**	**G**	**L**	**U**	**P**	**V**	**U**	**U**	Q	**Q**	**Fl**
Longibrachin B II	*Ac*	**U**	**A**	**U**	**A**	**U**	**A**	**Q**	**U**	**V**	**U**	**G**	**L**	**U**	**P**	**V**	**U**	**U**	E	**Q**	**Fl**
Trichorzianin TA IIIc	*Ac*	**U**	**A**	**A**	**U**	U	**Q**	**U**	**U**	**U**	**S**	**L**	**U**	**P**	**V**	**U**	**I**	**Q**	Q	Wl	
Trichorzianin TB IIIc	*Ac*	**U**	**A**	**A**	**U**	U	**Q**	**U**	**U**	**U**	**S**	**L**	**U**	**P**	**V**	**U**	**I**	**Q**	E	Wl	
Trichorzianin TA VII	*Ac*	**U**	**A**	**A**	**U**	J	**Q**	**U**	**U**	**U**	**S**	**L**	**U**	**P**	**V**	**U**	**I**	**Q**	Q	Fl	
Trichorzianin TB VII	*Ac*	**U**	**A**	**A**	**U**	J	**Q**	**U**	**U**	**U**	**S**	**L**	**U**	**P**	**V**	**U**	**I**	**Q**	E	Fl	
Trichorzin PA V	*Ac*	**U**	**S**	**A**	J	**J**	**Q**	U	**V**	**U**	**G**	**L**	**U**	**P**	**L**	**U**	**U**	**Q**	Wl		
Trichorzin PA IX	*Ac*	**U**	**S**	**A**	J	**J**	**Q**	U	**V**	**U**	**G**	**L**	**U**	**P**	**L**	**U**	**U**	**Q**	Fl		
Zervamicin ZII B	*Ac*	W	I	Q	J	I	T	U	L	U	O	Q	U	O	U	P	Fl				
Antiamoebin	*Ac*	F	U	U	U	J	G	L	U	U	O	Q	J	O	U	P	Fl				
Harzianin HC III	*Ac*	**U**	N	**L**	**U**	**P**	S	V	**U**	**P**	J	**L**	**U**	**P**	**Ll**						
Harzianin HC VIII	*Ac*	**U**	N	**L**	**U**	**P**	A	V	**U**	**P**	J	**L**	**U**	**P**	**Ll**						
Harzianin HC IX	*Ac*	**U**	N	**L**	**U**	**P**	A	I	**U**	**P**	J	**L**	**U**	**P**	**Ll**						
Harzianin HC X	*Ac*	**U**	Q	**L**	**U**	**P**	A	V	**U**	**P**	J	**L**	**U**	**P**	**Ll**						

The names of nonstandard residues, and their three- and one-letter codes are: α-aminoisobutyric acid, Aib, U; isovaline, Iva, J; hydroxyproline, Hyp, O. C-terminal amino alcohols abbreviations are: Fl for phenyl-alaninol, Wl for tryptophanol, Ll for leucinol. The C-termini of the first two synthetic alamethicin analogues are Phe-NH_2 (Fa) and Trp-NH_2 (Wa)

A more limited reorientation as a voltage-driven transmembrane insertion from a quasi-flat position at the interface at rest remains the most probable mechanism. However, instead of viewing alamethicin monomers as straight helical rods on the interface, partly buried within lipid headgroups, as in the earlier 'barrel-stave'-model versions, we favor a significant embedment of the N-terminal part (up to the kink due to Pro14) in the bilayer hydrophobic core without crossing it and making a kink up to ~ 30° with the C-terminal part remaining flat at the interface. Although this angle fluctuates in the dynamic fluid bilayer, the N-terminal end of the peptide dipole (ie., the positive end) would be at an average depth of 10 Å within the bilayer and thus able to sense a voltage drop about one third of that applied across the whole bilayer. In other words, in the initial step of alamethicin insertion, transmembrane electric field changes would actually be effective on only one third of the alamethicin dipolar moment (estimated to 75 Debye units in octanol [80, 102], dielectric constant $e \sim 3.4$). Besides, covalent photo cross-linking between carbene groups on lipid aliphatic chains and alamethicin N-terminus confirms this partial inclusion of the peptide molecule within the bilayer, in the absence of voltage [59]. On the other hand, the question of whether alamethicin aggregates at the bilayer interface, prior to any application of voltage, does not seem to be settled. Membrane-capacitance changes recorded at voltages below the threshold for conductance development [92], together with the slopes in absorption isotherms followed by circular dichroism [81,87], were interpreted as reflecting interactions between monomers and argued that the main and limiting step in channel formation would be a voltage-dependent partitioning of alamethicin between the aqueous and the lipid phases. However, concentrations used in both approaches are in widely different ranges and it appears unlikely that voltage-dependent transmembrane insertion might occur without a partial embedding of alamethicin molecules in the bilayer, since potential difference can only be efficient across the membrane dielectric. Moreover, EPR studies also disagree with a simple voltage-dependent partioning from the bulk. Ion fluxes induced by alamethicin in lecithin vesicles incorporating a protonophore and submitted to a pH gradient were followed through ΔpH-sensitive spin labels [3]. Owing to the large surface/volume ratio in this system, nearly all alamethicin molecules were bound to lipids. Ion fluxes induced by alamethicin were super-linear, i.e., voltage-dependent. In addition, also at odds with the conclusions of the above-mentioned CD studies, the cooperativity observed in EPR experiments [2, 3] cannot reflect peptide aggregation at the interface. This is so because in all examined

conditions and especially for high peptide/lipid molar ratios, alamethicin remained monomeric and the fraction actually involved in pore formation under applied voltage represented only a quite small percentage of the total [5]. This is in agreement with the lateral diffusion coefficients of a fluorescent alamethicin analogue in planar lipid bilayers, monitored at rest and under applied voltage [46].

Finally, probable conformational changes of alamethicin between monomers at rest and those involved in the conducting bundles remain the main open question. The possibility of a field-induced transition from β-strand or -coil for residues near the C-terminus in the 'off' state to an all α-form in the 'on' state has been considered (e.g., [42]). This idea began to receive experimental validation: alamethicin ellipticity was recorded on vesicles submitted to a Donnan potential established by different concentrations of polyelectrolytes or salts and monitored by a potential-sensitive fluorescent dye [17]. Positive potentials applied to the external side (alamethicin-addition side) does slightly increase helicity: from 75% to 80% at 0 and 100 mV, respectively. The β-strand content is hardly affected, whereas random conformation is roughly halved. This increased helicity, however modest, does certainly favor stable insertion. Nevertheless, there was some influence of the polyelectrolyte (polyacrylate) used for the Donnan potential on alamethicin ellipticity and in this experimental system, the transmembrane potential difference is likely to rapidly dissipate as soon as pores are formed. We believe that only the coupling of simultaneous spectroscopy with electrical measurements on planar lipid bilayers could afford more definitive answers as regards these activation mechanisms [25,46]. In the transmembrane conducting aggregates, hydrophobic residues of each alamethicin helix are interacting with the lipid aliphatic chains, whereas the juxtaposed hydrophilic sectors define the aqueous pore. A simple geometric model allows calculation of the diameter and hence the conductances of pores made of N monomers (helix end diameter ~10 Å) arranged in a crown array [44, 76]. The quite good agreement between the single-channel conductances of the different substrates and this model, recently refined by taking into account the actual structure of the permeation pathway [84], validates the dynamic 'barrel-stave' model. Fig. 1 summarizes the molecular steps of alamethicin channel formation.

An essential finding of simulation (molecular modeling and dynamics) studies was that alamethicin bundles are more stable when monomers are tilted 20° away from each other and when they interact through their N-terminals, leaving the C-terminals to form a funnel-shaped mouth [77]. At the end of simulations of bundles ($4 < N < 8$), the helices' N-termini are found separated by 9.5 Å, and lateral sidechains of Gln7 delineate the narrowest portion of the lumen to which also Gly11 carbonyl oxygens are exposed. Lateral sidechains of Gln 19 are deflected and C-terminal amino-alcohols form an aromatic belt at the interfacial region, contributing to anchor the bundle to the lipid polar headgroups. These simulation methods also allow a comparison of alamethicin analogues otherwise studied for their conductances in order to predict the effects of some residue substitutions. For instance, the synthetic analogue Alm-dUL, in which all eight Aibs of native alamethicin are replaced by Leu residues, yields bundles whose helices tend to remain more parallel between them than in the parent molecule. This Aib→Leu substitution also reduces pore hydration, especially at positions 3 and 10 [16]. As discussed above, these findings are correlated to shorter mean channel lifetimes. Water molecule inclusion in these calculations not only shows favorable interaction with Gln7 polar side chains translating into significantly reduced translational and rotational diffusions of H_2O, but also an antiparallel alignment of water and peptide dipoles [90].

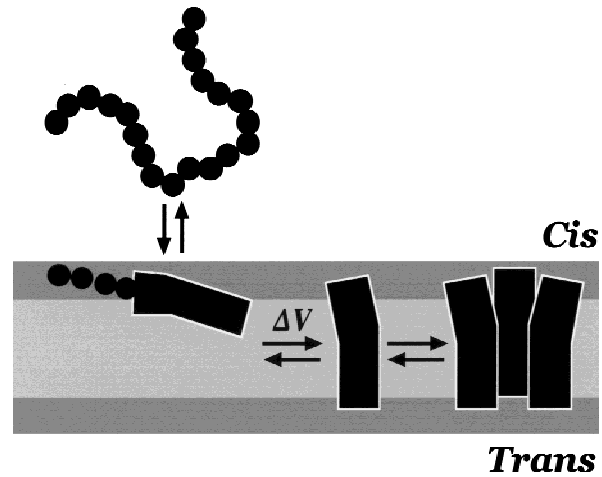

Fig. 1. Schematic diagram summarizing the essential steps of voltage-driven alamethicin channel formation. The lipid bilayer is represented as a slab with its hydrophobic core (light-shaded) ~30 Å thick and the twin polar headgroups layers. The overall length of the helical and transmembrane form of alamethicin matches the hydrophobic core thickness. Alamethicin is shown disordered in the aqueous phase, *cis*-side, in equilibrium with the membrane-bound form (on the left). In this state, alamethicin becomes largely helical with its N-terminal part slightly buried in the hydrophobic core and making an angle averaging ~30° with the bilayer plane. The first C-terminal residues (up to four or five) are still in a nonhelical conformation, within the polar headgroups region. An applied positive field (*cis*-side) pushes the N-terminus (positive end of peptide dipole) through the bilayer and allows a full crossing for a minute proportion of the membrane-bound alamethicin molecules. The kink angle would remain relatively constant. This transmembrane state of alamethicin monomers is highly unstable (hydrophilic residues being buried) but through lateral diffusion and collision with other transmembrane monomers, conducting bundles (barrel-staves) rapidly form. The size of the latter is fluctuating ($3–4 < N < 12$) through uptake and release of monomers. Half an hexamer is shown on the right.

Alamethicin bundles are presently studied by these simulation methods in interaction with explicit lipid bilayers in an aqueous ionic environment [78]. In this situation, the hexameric bundle is more stable when Glu18 sidechains are protonated, and the pore constriction at the level of Gln7 has a diameter of 2.5 Å. A partial dehydration of K^+ is thus possible in this region, although one must allow for some sidechain flexibility.

Antimicrobial Activities Are Correlated to Pore Sizes

In contrast with the extensive range of biophysical studies related to alamethicin pore formation and the involved molecular mechanisms, fewer studies have been devoted to its antimicrobial activity, despite alamethicin's discovery being due to the observation of its antibiotic activity [64]. In one of the early studies, a large activity spectrum had been found against bacteria from the intestinal tract of ruminants [54]. The peptide is active against fungi and Gram-positive bacteria but seems uneffective against the Gram-negative ones. This is probably due to the fact that the lipopolysaccharides (LPS) of the outer membrane of Gram-negative bacteria form a strong diffusion barrier against hydrophobic molecules such as peptaibols. Alamethicin and trichorzins PA are furthermore bactericidal for species of the class *Mollicutes*, including several pathogens for man and farm animals (mycoplasmas), and for insects and plants (spiroplasmas) [8, 9]. Minimal inhibitory concentrations ranged from 1 to 12.5 μM whereas in the same conditions, cationic animal defense peptides such as magainin 2 and cecropins proved quite uneffective. Using *Acholeplasma laidlawii* and the bee pathogen *Spiroplasma melliferum* as targets, it was shown that, similar to melittin and gramicidin S, the antibacterial activities of peptaibols were due to membrane permeabilization. The potentiometric and DNA-staining fluorescent probes (3,3′-dipropyl-2,2′-thiadicarbocyanine iodide and DAPI, respectively) revealed that the depolarization of the membrane triggered an irreversible cell lysis with the loss of cell DNA [8–10].

There is a consensus that membrane permeabilization and its subsequent depolarization are the primary if not the only mechanism of the antibiotic activity of membrane-seeking peptides. However, since cell membranes are much more complex than the liposomes and the planar lipid bilayers used as model systems in a large number of biophysical studies, one may wonder whether the permeabilization mechanisms deduced from these studies are also valid for biomembranes. In order to clarify the mechanisms of alamethicin antibiotic activity, antibacterial activities (again using *S. melliferum* as a target cell) were compared with conductance properties on planar bilayers for alamethicin and the series of synthetic derivatives described above (of the Alm-dUL series). These experiments showed a good correlation between antibiotic activity and pore size as estimated by $\langle N \rangle$, the apparent mean number of monomers involved in conducting bundles and derived from macroscopic conductances [10]. Specifically, the larger the pore size the lower the minimal inhibitory concentration (MIC), i.e., the higher the antibiotic activity. In particular, at both ends of the spectrum, MICs were 12.5 and 100 μM for Alm-dUL ($\langle N \rangle = 10$) and for the P14A analogue ($\langle N \rangle = 6$), respectively. Furthermore, as in conductance experiments, peptide length influences the antibacterial activity of peptaibols: trichorzins PA, which are two residues shorter than alamethicin, are 2 to 4 times less active than the latter on the same bacteria [9]. Altogether, these observations are quite consistent with the bactericidal activity of alamethicin being based upon the barrel-stave mechanism. However, since peptide concentrations differ by two orders of magnitude in the two kinds of experiments, one has to assume that there is a substantial alamethicin reserve at the membrane interface, not expressed as pores in standard bilayer conductance experiments, but which can be recruited for antibacterial activity according to the same barrel-stave mechanism. This conclusion cannot be extended for other membrane-active peptides, e.g., cationic amphipathic peptides, whose antibacterial activities can reflect either lipid-matrix structural defects or large and ill-defined peptide aggregates ('carpet' or 'raft' mechanisms). This is notably the case of defense peptides such as magainins and cecropins endowed with much larger hydrophilic sectors probably hampering the formation of stable transmembrane helix bundles [43, 83].

Alamethicin Channel Properties Can Be Modulated through Chemical Design

The last few years witnessed the development of new experimental approaches, taking advantage of improved chemical methods of synthesis and modification in order to control the number of alamethicin monomers in conducting bundles on the one hand, and to design alamethicin derivatives with new and possibly more specific properties on the other hand. It should also be mentioned that alamethicin continues to serve as a 'molecular tool' in various areas of membrane research. One of the latest examples is a study devoted to a mitochondrial permeability pore (MTP) that could be involved in apoptosis and where the Ca^{2+}-induced swelling was compared to the one triggered by alamethicin [57].

If the 'barrel-stave' model is valid, i.e., if the multiple single-channel conductance levels do really reflect uptake and release of individual monomers within transmembrane conducting bundles, then it should be feasible to stabilize given conductance substates by controlling

their oligomerization states. The main alternative mechanisms assume sequential conformational changes and gating in a fixed-size bundle or an ensemble of parallel pores of different but fixed sizes. The chemical construction of molecules with well defined sizes and spatial arrangement by covalent coupling of peptides on templates has first been applied to peptide motifs mimicking physiological channels or receptors, eg., transmembrane segments presumed to form the pore of voltage-gated calcium channels [39], and then to melittin [73]. As for alamethicin, progress in this area was slower and limited to small-sized oligomers. Since transmembrane insertion mainly occurs via the N-terminus and since the C-terminus exerts a much smaller influence on conductance properties, chemical reactive groups of templates were coupled to the latter. Templates have to remain flexible enough and present a geometry that does not perturb the normal parallel laying of helical motifs. For instance, bis (*N*-3-aminopropyl)-1,7-heptanediamide (BAPHDA), was used to covalently couple at both its extremities an alamethicin molecule on its C-terminal amino-alcohol [103]. Single-channel conductance traces showed that some alamethicin-like substates were stabilized, especially the one corresponding to an hexamer, i.e., in the present case, three dimers forming the pore. However, there was no evidence that high voltage-dependence was conserved since no macroscopic conductance experiments were reported for this analogue. When peptide motifs are coupled to contiguous Lys residues, both macroscopic and single-channel conductances become atypical (especially with a very dampened voltage-dependence), most probably reflecting helix misalignment. A single alanine introduction between the two lysines is sufficient for normal behavior retrieval [30]. Not only does the voltage-dependence remain high (V_e = 6 mV), but also a negative resistance region in *I-V* curves, a signature for excitable systems, develops under much more favorable conditions than with monomeric alamethicin.

A few alamethicin derivatives have been recently designed to react to specific chemicals. The linkage of a pyromelittate group to the alamethicin C-terminus through three additional negative charges, increasing electrostatic repulsion between monomers, impedes the building-up of conducting bundles unless ionic strength is raised, especially through Ca^{2+} addition [99]. Conductances of alamethicin derivatives bearing ferrocenoyl groups (Fc-Alm) at the C-terminus are sensitive to redox properties of the medium [79]. In the same framework, two (or more) short peptaibols were linked through functional groups whose conformation was modulated by the coordination of a metal ion. Two or three trichogin motifs 10–11 residues long (ie., too short on their own to span a lipid bilayer) have been linked to either side of tris (2-aminoethyl) amine via 4-carboxy-1-methylbenzene used as a spacer [82]. The binding of Zn(II) to these functional groups leads to a 'basket-like' structure with the peptide chains pointing in the same direction, and thus the complex is inactive. Zn(II) complexation with EDTA releases this conformational constraint and, by allowing a much more extended structure now able to cross the bilayer, induces leakage of liposome-entrapped carboxyfluorescein. Finally, alamethicin ionic specificity can be manipulated by changing the C-terminal electrical charge. Alamethicin on its own forms moderately cation-specific pores (mostly excluding Ca^{2+}), but the Gln18 $\rightarrow$ Lys substitution in a covalent dimer reverses the permeability ratio P_K/P_{Cl} [88]. Once the selectivity has been further improved, this kind of compounds might prove helpful as a molecular tool for studying, for instance, anion channel-related diseases.

Summary and Open Questions

Alamethicin remains, after thirty years of investigation, a fruitful model for ion channels. The range of experimental as well as theoretical studies devoted to this molecule is impressive and alamethicin continues to serve as a standard for the setting up and testing of new approaches (recently: calibration of pores and 'molecular Coulter counter' [11]). Newly discovered natural or designed molecules, candidates as pore-formers and/or antimicrobial agents, can hardly escape being compared to alamethicin. However, alamethicin and defense peptides such as magainins and cecropins most likely exert their effects in quite different ways [48], as would also be suggested (albeit indirectly) by the lack of success in co-crystallizing alamethicin with lipids, in contrast to what has been recently achieved with magainin and protegrin [101].

The 'barrel-stave' model paved the way for a better understanding of the much more complex physiological channel proteins. A natural offshoot is thus the 'peptide strategy', consisting of conformational and functional studies of synthetic or designed peptides mimicking transmembrane segments of natural ion channels or membrane receptor proteins (*see,* e.g., [28, 69]). The main outcome of comparative studies of natural and synthetic analogues is the robustness of the voltage-dependence property, except for antiamoebin. Finally, recent comparative conductance and antibacterial assays on a series of alamethicin analogues show that both sets of parameters are correlated: the larger the pores, the more efficient the peptide.

No doubt that along with gramicidin, for instance [94], alamethicin will continue to serve as a relevant ion channel model, especially as regards voltage-sensitivity. Since alamethicin monomers are predominantly lying flat at the membrane interface, the voltage-dependent transmembrane insertion and lateral mobility within the

lipid matrix are crucial in the building-up of pores (*see* Fig. 1). Further experiments need to be devised to elucidate the pore build-up steps that are consecutive to transmembrane insertion. Particularly, helix interactions within the conducting bundle could possibly be tracked by monitoring the self-quenching of labeled monomers, energy transfer (FRET) and fluorescence correlation spectroscopy. A newly available technique, SRCD (Synchrotron Radiation Circular Dichroism) [95], when combined with electrophysiological conditioning and recording on planar bilayers, could prove useful for measuring conformational changes during channel formation and voltage gating. Finally, the increasing need for new classes of antibiotics due to the continuous emergence of (multi)resistant pathogens has strongly stimulated, during the past decade, research on the antimicrobial properties of membrane-active peptides. Generally speaking, three main objectives remain, namely, to elucidate the mechanism of interaction with target membranes, to explain the factors determining the activity spectrum, and to enlarge the repertoire of available peptides. The ultimate goal is to use the acquired knowledge for designing and producing by genetical and/or chemical engineering new pharmacophores that might ultimately be used as drugs to combat infectious diseases. The complexity of microbial membranes compared to simple, artificial models should be taken into account, as well as diverse resistance factors, if one is to expect rapid progress in the field. In this context, it is symptomatic that the biomedical potential of peptaibols, including alamethicin, is still far from having been fully explored and evaluated.

This work was supported by GDR CNRS 790 'Protéines et peptides membranotropes'. We thank L. Béven, B. Bodo, D.S. Cafiso, J.-Y. Dugast, S. Ladha, G. Molle, S. Rebuffat, M.S.P. Sansom, G. Spach and B.A. Wallace for their friendly collaboration.

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