How Do Channel- and Pore-Forming Helical Peptides Interact with Lipid Membranes and How does this Account for their Antimicrobial Activity?

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Abstract: Animals and plants defend themselves against pathogenic micro-organisms by the rapid mobilization of polycationic helical amphipathic peptides. Interactions with membranes induce optimal orientation and mutual structural changes, allowing for example to form transbilayer ion channels or pores whose properties are compared in this review. Physicochemical studies of peptide-lipid interactions provide attractive approaches for drug design.

Key-words: channel-forming peptides, defense peptides, amphipathicity, lipid composition, cellular targets, planar lipid bilayers, conductances

INTRODUCTION

The emergence of microbial resistance to classical antibiotics is increasingly becoming a serious medical problem and is fostering the interest in the discovery of new antimicrobial peptides with a view of potentially treating infection. Unlike conventional antibiotics whose targets are specific proteins, these peptides which belong to the innate immunological system exert their activity directly on the cellular membrane lipids meeting the obvious requirement for rapid killing of invading pathogens through membrane permeabilization. They usually have a broad activity spectra and have the common property of being amphipathic, that is molecules with two faces, with one being hydrophilic or positively-charged, and the other neutral and hydrophobic.

The range of structures adopted by antimicrobial peptides is large : linear amphipathic and hydrophobic α−helices, cyclic peptides forming β−sheets, lipopeptides, etc... Given this diversity as well as the various cellular targets, it is safe to recognize at the outset that it is likely that several modes of action are involved. Perhaps the best known membrane permeabilization mechanism is through channel formation, as extensively studied with alamethicin (for review, see e.g. [14, 26]). With this peptide, channels are formed by transmembrane conducting aggregates or bundles of helical monomers with the hydrophilic helical sectors defining the ion pathway whereas hydrophobic residues contact the surrounding lipid acyl chains, hence the barrel-stave model [70]. However, this model is far from being exclusive, and less well defined permeabilizing structures made up of mixed lipid and peptide molecules, for which the term 'pores' should be preferred, may form with other peptides. In addition, even less specific mechanisms have been proposed, such as membrane destabilization or disruption by strong association of the lipid headgroups with peptides predominantly remaining flat on the bilayer (carpet-like model [71]).

To write another review on such a popular subject seems tentalizing after a considerable number of excellent previous reviews attempting to keep abreast of the burgeoning literature in the field (in chronological order): [70, 67, 4, 57, 35, 29, 36, 23]. Consulting the PubMed database alone for 'defense peptides' yields about 2200 articles published between 1965 and march 2001. Most of them concentrate in the last few years: 630 articles from 1998 to the year 2000 inclusive, i.e. an average of 20 per month. For the purpose of this mini-review, I shall focus on linear polycationic helical peptides, especially those that have been shown to form pores or channels as assayed by conductance studies in planar lipid bilayers. It will become apparent that the ability to form transbilayer ion channels or pores, and their 'resolution', is correlated to the relative importance of the helical hydrophilic and hydrophobic sectors.

SUMMARY OF EXPERIMENTAL METHODS: ANTIMICROBIALS ASSAYS VS. CONDUCTANCE MEASUREMENTS IN PLANAR LIPID BILAYERS.

Since it is impractical to perform electrophysiology directly on microbial and bacterial targets, it is important to realize that both types of activity of these antimicrobial peptides have to be investigated with widely different methods and conditions (essentially as regards lipid composition and concentrations), and thus some caution must be exerted before any inference can be made as to the molecular mechanisms involved in antimicrobial activity from conductance studies in planar lipid bilayers.

Briefly, in antimicrobial assays, minimal inhibitory concentrations (MIC) are determined for instance on 96-well microtitration plates as the lowest peptide concentration at

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which there is no change in optical density or by following the color change of phenol red resulting from acidification of the culture medium during growth. Minimal lethal concentrations (MLC) are measured by counting colonies after the incubation of cells with peptides and plating on solid culture medium. Light or electron microscopy can also be performed to follow bacteria morphologies and membrane potential monitored by fluorescent potential-sensitive dyes, as 3,3'-dipropyl-2,2'-thiadicarbocyanine iodide (diS-C3-5). These methods are detailed in e.g. [7].

Membrane permeabilization can be assessed through the leakage of fluorescent probes entrapped into liposomes but channel and pore formation is best studied through electrical measurements (conductance) on planar lipid bilayers exposed to the same peptides. Ideally, macroscopic (resulting from the recruitment of hundreds or thousands channels) and single-channel conductances are recorded to yield such parameters as voltage-dependence, concentration-dependence, oligomerization state of the conducting aggregates, channel duration and ion selectivity. These conductance methods and parameters will be briefly recalled and illustrated below while discussing alamethicin properties.

THE DIVERSITY OF α−HELICAL ANTIMICROBIAL PEPTIDES AND COMPARED EXTENT OF THE POLAR ANGLE

Antimicrobial helical peptides have been isolated from fungi, bee venom, frog skin, insect hemolyph and a host of other tissues. In Fig. **1**, Edmunson helical wheels (projections of residues on a plane) of some of the best-known polycationic helical peptides are compared. These were chosen amongst those for which pore-formation is best documented and to illustrate the effects of an increasing hydrophilic sector, or polar angle. Alamethicin, although not polycationic, has been included as a reference (archetype) for voltage-dependent channel formation. The essential properties of these peptides will now be reviewed and compared, especially as regards their channel- or pore-forming properties, before general conclusions can be drawn on common features but also on more specific mechanisms underlying antimicrobial activities of this class of amphipathic peptides.

ALAMETHICIN

Alamethicin, a 20 residue-long peptide produced by the fungus *Trichoderma viride*, is certainly one of the best studied models for peptide-membrane interactions [15], especially as regards its channel-forming properties [26]. Its sequence may look peculiar when compared to the polycationic peptides discussed below, since it contains eight Aibs (α−amino isobutyric acid, a non-coded residue) and a C-terminal phenylalaninol, but studies on synthetic analogues showed that these residues were not essential in channel formation [63]. Since some consensus has been attained with the 'barrel-stave' model for alamethicin mode of action, it is convenient to briefly survey alamethicin properties before reviewing data available with the polycationic antimicrobial for which, it must be stressed at the outset, the modes of action are still a matter of debate.

Crystallographic and Nuclear Magnetic Resonance studies have unambiguously shown that the N-terminal half of alamethicin assumes an α−helical structure, followed by residues 10 and 11 that cannot form intramolecular H-bonds due to the kink introduced by Pro14, a 3_{10} helical turn and finally a more variable secondary structure for the C-terminus [30, 31]. In the helical wheel representation where all residues of the helix are projected on a plane, only two polar residues (Gln7 and Gln18) define a rather modest hydrophilic sector (see Fig. **1**).

The orientation of alamethicin helices depends both on the physical state of the bilayer and the peptide concentration. Oriented circular dichroism experiments have shown that in hydrated lipid membranes in the fluid state and for high molar peptide/lipid (P/L) ratios, the peptide is oriented along the bilayer normal [45]. However, in normal conditions (intermediate range of P/L), the peptide at rest without any applied voltage does not fully span the bilayer. The C-terminal residues would anchor the peptide at the bilayer interface whereas the kink (at an average angle of 30°) would allow the N-terminus to significantly dip into the hydrocarbon core. In my view, the most convincing experiment for this location involves photo-crosslinking between an alamethicin residue near the N-terminus and the lipid aliphatic chain [52]. As confirmed by Electron Spin Resonance spectroscopy, the N-terminus would be located about midway between the two bilayer leaflets [2] and hence would be able to sense the voltage changes across the membrane dielectric.

Once incorporated into planar lipid bilayers under applied voltage, alamethicin displays unique conductance properties characterized by a high voltage-dependence of macroscopic current-voltage (I-V) curves and multi-state single-channel behavior. Since the methods used for conductance measurements are identical for the peptides discussed here, they shall now be briefly recalled. In the macroscopic conductance configuration, relatively large area (e.g. diameter: 200 μm) are preferred for planar lipid bilayers which can be 'painted' or 'folded' from lipid monolayers (see e.g. [40]) over a hole in the septum between the two hemi-chambers containing the electrolyte solution (e.g. 1 M KCl both sides). The *cis*-side is defined as the side of peptide addition and the application of a positive voltage to this side results in a positive current if cations flow from the *cis* to the *trans*-sides. Fig. **2A** shows macroscopic Current-Voltage curves developed by neutral lipid bilayers exposed to two alamethicin concentrations and submitted to a slow volage ramp. These curves reflect the steep exponential recruitment of many channels (several thousands) only for positive voltages, if the peptide was added to the *cis*-side. The voltage-dependence is quite high, with an e-fold change in conductance (V_e, see Fig. **2A**) of only 5 mV. The threshold for the development of this exponential branch is shifted to the left (reduced voltage) as alamethicin concentration is increased. An e-fold change in concentration will result in a shift (V_a) of about 50 mV. <N>, the apparent and mean number of alamethicin monomers that form the transmembrane conducting structures can be estimated [38] from concentration- and voltage-dependences, as $\langle N \rangle = V_a / V_e \approx 10$.

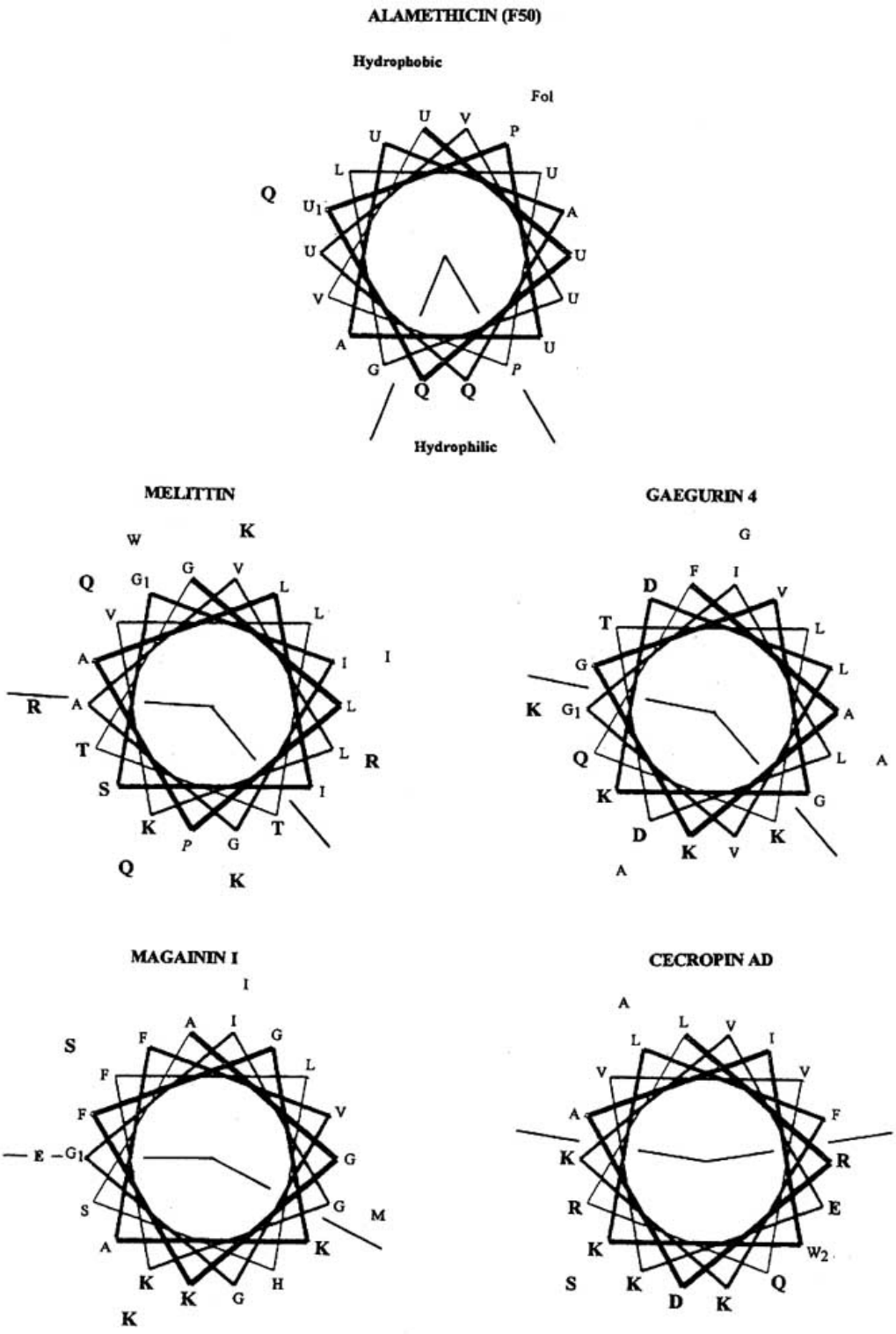

Fig (1). Helical wheel representation of some polycationic antimicrobial peptides compared to alamethicin (at the top). Polar and charged residues are shown in bold and hydrophilic sectors delineated by solid lines are facing downwards. Note the increasing polar angle in this series.

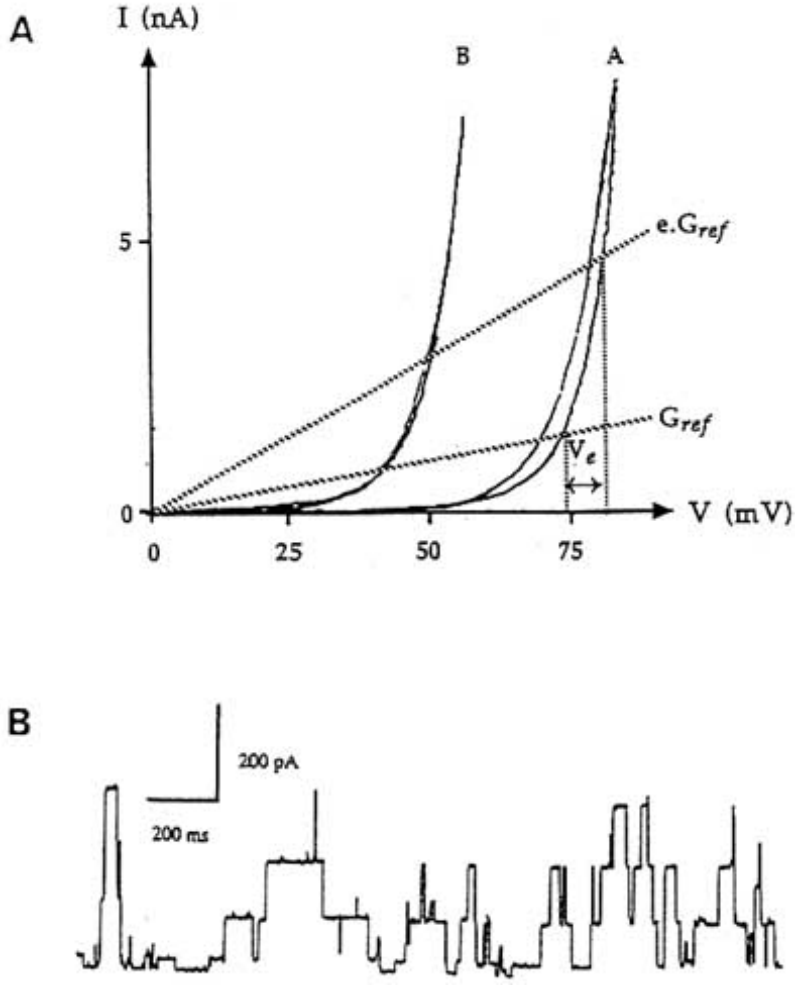

Fig (2). Channel activity induced by alamethicin. A) Macroscopic current-voltage curves for an alamethicin analogue at two concentrations in the *cis*-side: $5x10^{-8}$ M (curve A) and 10^{-7} M (curve B). The electrolyte solution was 1 M KCl (buffered to pH 7.4) both sides of a neutral planar lipid bilayer, submitted to a voltage ramp. G_{ref} is an arbitrary reference conductance and V_e is the voltage-dependence parameter. B) Typical single-channel condutance fluctuations stressing the non-integral mutistates (openings are upward fluctuations). The peptide concentration was $4x10^{-9}$ M and the applied steady-state voltage was 80 mV. (from [8, 42], with permission from Elsevier).

Single-channel conductance experiments are best studied at reduced alamethicin concentration and with small bilayers formed at the tip of patch micropipettes [40]. Unitary conductance fluctuations are characterized by at least five or six open substates spanning a broad conductance range (20 pS to several nS). The conductances of these substates are not equal, and thus do not result from parallel identical channels, but obey a geometrical progression. This pattern is still the best evidence for the 'barrel-stave' model of channel formation, each substate representing a bundle of α-helices of different size. In other words, single-channel transitions result from the uptake and release of individual monomers within an helical transmembrane bundle whose central pore is lined by the hydrophilic surfaces of the helices arranged in a crown-like fashion.

Although alamethicin discovery stemmed from early observation of antimicrobial activity, studies devoted to this topic are scanty, by contrast to those dealing with conductances. Recently, using the mycoplasma *Spiroplasma melliferum* as a cellular target, minimal inhibitory concentrations (MICs) were compared for a series of synthetic alamethicin analogues [8]. There was a good correlation between channel-forming properties and MICs: those analogues yielding larger channels through a higher number of monomers <N> in the 'barrel-stave' were the most efficient in the antibacterial assay.

MELITTIN

Melittin is a 26 residue-long peptide isolated from the venom of the honey bee *Apis mellifera* and is well known for its membrane-disruptive properties [22]. Despite being devoid of Aib, melittin is often considered as structurally- and functionally-related to alamethicin. However, melittin

bears six positive charges, most of them clustering near the C-terminus, and the hydrophilic or polar face is much wider than with alamethicin (Fig. **1**). Its scope of activity is somewhat also wider than alamethicin since, in addition to increased membrane permeability for ions, lysis at higher concentration, a further effect of melittin is to induce aggregation of some membrane proteins, as demosntrated for band 3 in erythrocytes [17] and for bacteriorhodopsin in reconstituted systems [43].

Both ^{1}H-NMR of melittin in solution in methanol [3] and the crystal structure [75] agree to depict an overall structure: two α−helical segments (between residues 1 and 10, and 13-26) with a kink of about 30° around positions 11 and 12 due the proline 14, as in alamethicin. However, by contrast to the latter, the C-terminal part of the peptide is more ordered and there is no evidence in melittin for 3_{10} helical turns. This overall structure remains similar for membrane-bound melittin [6] except that the last 4-5 C-terminal residues tend to uncoil [79].

As high peptide/lipid (P/L) ratios induce non-bilayer lipid structures [27], we shall limit the discussion to low P/L which is also more relevant to the conditions used for conductance studies in planar lipid bilayers. A surface location had been suggested by an early fluorescence and Raman spectroscopy study which showed that melittin-bilayer interactions were more sensitive to the nature of lipid headgroup than to the nature of the acyl chain [51]. As with alamethicin, the orientation of melittin is dependent upon the physical state of the bilayer: in fully hydrated bilayers, increasing the temperature above the lipid phase transition change melittin orientation from parallel to perpendicular, relative to the bilayer plane [79]. The degree of melittin binding to liposomes was found sensitive to the bilayer curvature, the partition coefficient for SUV (small unilamellar vesicles) being 20 times that for binding to approximately planar bilayer domains (freeze-thaw liposomes) [6]. Whereas earlier studies provided evidence for tetrameric aggregates within the bilayer [80], time-resolved fluorescence energy transfer measurements using melittin as donor and a modified melittin as acceptor led to the conclusion that melittin in fluid membranes was essentially monomeric [49]. Only P/L ratios above 1/200 and high ionic strength favors some aggregation.

Macroscopic current-voltage curve induced by melittin in planar lipid bilayers look similar to those of alamethicin but with a reduced voltage-dependence. Indeeed, V_e, the voltage shift for an e-fold conductance increase is 22 mV for the unmodified natural melittin (as compared to 5-6 mV for alamethicin). Although bursts of single-channel activity broadly similar to those of alamethicin could be recorded, events are much more rapid and hardly resolved. Only by raising the electrolyte concentration up to 5 M NaCl was it possible to record discrete multi-level channel openings [39].

Quite interestingly, acetylation of melittin which neutralizes all charged groups except the two arginines near the C-terminus and which would thus significantly reduce the hydrophilic helical sector enhances voltage-dependence (V_e = 11 mV, i.e. still twice alamethicin value) and increases the apparent number of monomers forming the pores from four as initially found for natural melittin [76] to eight (i.e. equivalent to alamethicin). Single-channel durations are also increased suggesting that the absence of charges stabilizes the pore [74]. The high content of positive charges in natural melittin accounts for the anion selectivity of the pores, over cations. The picture that emerges for melittin pores is thus still compatible with the barrel-stave model as initially propounded for alamethicin, but with a more transient lifetime. Besides, the lack of trypsin access to melittin N-terminus when negative voltage is applied to the inside of liposomes binding external melittin adds further evidence for transmembrane crossing during pore formation [64]. Finally, as pore formation is coupled to peptide translocation (and lipid flip-flop), mixed pores may be more common than initially thought and since the pore sizes depend on the peptide density on the bilayer, these sizes tend to decrease upon peptide translocation. Melittin molecules are internalized into lipid vesicles upon the desintegration of many short-lived pores. [60].

GAEGURIN

Amongst the α−helical polycationic peptides selected for the purpose of this mini-review, gaegurins stand apart simply because hardly ten papers are devoted to them (since 1994) as compared to more than 400 for alamethicin, 1400 for melittin, 250 for magainins and nearly 300 for cecropins. Nevertheless, it is worthwhile to compare gaegurins properties to both melittin since gaegurin 4 presents a similar polar angle but lacks a central proline and to magainins whose polar angle is wider. Among six peptides isolated from the skin of the korean frog *Rana rugosa* [65], gaegurin 4 is 37 residue-long and being the most abundant, it is assumed to play a role in the innate defense system. The solution structure of gaegurin 4 studied by circular dichroism and NMR spectroscopy in membrane-mimicking systems revealed two amphipathic α−helical segments connected by a flexible hinge (between residues 11 and 15 with the K**G**V**G**K motif). The C-terminal disulfide bridge between Cys31 and Cys37 does not have a significant influence on conformation and antimicrobial activity since they are both maintained in the reduced form of the peptide [66].

Indeed, gaegurin 4 displays a broad range of activity against procaryotic cells, especially against Gram-positive and Gram-negative bacteria and fungi, with little hemolysis of human red blood cells at usual concentrations. Pore formation was recently studied in neutral and acidic planar lipid bilayers [50]. In those bilayers that showed resolved discrete channel-like activity, multilevels spanning nearly two orders of magnitude of single-channel conductances were ususally observed. Low peptide concentrations, in the nM range i.e. three orders of magnitude lower than the minimal inhibitory concentrations vs. bacteria, favored only one conductance level. The latter is significantly higher in acidic than in neutral bilayers : 120 vs. 65 pS in symmetrical 100 mM KCl. These pores appear most often in the open state, with quite fast closing events that are favored at negative voltage, even though the peptide was added to the *cis*-side. However, there is no evidence for a significant voltage-

dependence since macroscopic current-voltage curves appear ohmic and fail to show any exponential or supra-linear branch.

To summarize, although these conductance experiments in planar lipid bilayers show that gaegurin 4 forms pores, the properties of the latter appear quite different from alamethicin and melittin. In particular, current fluctuations are faster and the loss of high voltage-sensitivity is likely to be due to the lack of a central proline. Geagurin 4 pores are thus prone to induce a background leak. The higher conductances obtained in phosphatidylserine (PS)-containing membranes may explain the cytoxicity of this class of peptides, along with magainins and cecropins (see below) against cancer cells, since the latter selectively express high PS levels [78].

MAGAININS

Discovered in 1987, magainins are certainly the best-studied α−helical polycationic antimicrobial peptides. Two 23 residue-long analogs, magainins 1 and 2, conferring resistance to infection and allowing wounds to quickly heal, were isolated from the frog *Xenopus leavis* skin [87]. They show a broad spectrum of antimicrobial activity, both again Gram-negative and -positive bacteria, fungi and protozoa. Much higher concentrations are needed to lyse vertebrate cells, such as erythrocytes, but magainins exhibit antitumor activity against some transformed cell lines [18]. The basis of this selectivity has been investigated on model membranes. Indeed, magainins have a high apparent affinity toward negatively-charged membranes, hence bacterial membranes and not for eukaryotic ones (also containing cholesterol which decreases the binding), but only through electrostatic interactions and not through specific binding to anionic lipids [84].

Evidence for an α−helical conformation of membrane-bound magainin is provided by circular dichroism and NMR studies. Helicity raises significantly when magainin 2 interacts with acidic lipid vesicles [83] and two-dimensional ^{1}H NMR spectroscopy not only confirms that magainin is completely α−helical except for a few terminal residues in various model membrane environments [34, 56], but also suggests the structure to be slightly curved with the bend centered at residues Phe 12 and Gly 11. Furthermore, solid-state NMR experiments on oriented samples of phospholipid bilayers associated with magainin show that the helix axis lies in the plane of the bilayer [5]. Magainin analogues bearing tryptophans at different positions in the sequence were used to determine the orientation relative to the membrane. It was confirmed that the peptides predominantly under the dimeric form were lying flat on the bilayer surface but vesicular leakage of entrapped calcein was associated with significant burying of the Trps (blue-shift) suggesting a transmembrane orientation [58]. The initial rate of magainin-induced leakage being dependent on the fourth power of the peptide concentration, tetramers are most likely forming the pores, in broad agreement with the conductance studies in planar lipid bilayers discussed below.

In the planar bilayer assay, relatively large magainin concentrations and high applied voltage are needed to disclose a weakly voltage-dependent (see Fig. **3A**) and anion-selective conductance in neutral bilayers [24, 25]. However, single-channel occurences are rare and short-lived and, contrasting with the multistate alamethicin behavior, only one level was observed within a single experiment but that level could differ in separate experiments as shown in Fig. **3B**. Conductances as high as 2 nS can be encountered. In negatively-charged bilayers, a very similar analogue (magainin 2) induces another type of electrical activity with single-channel currents best observed in the early stage of peptide addition and a cationic selectivity [19]. Thus, magainins promote two kinds of pore structures according to which lipid bilayer composition is used. In any case, the inference that antimicrobial activity is probably reflecting the pore-forming properties of magainin is strengthened by the direct patch-clamp recording of a channel-like activity in mouse transformed cell lines that was concentration-dependent [37]. Another recent study adds further indirect support for magainin pores: the perturbing effect of magainins on lipid chain order, measured with electron paramagnetic resonance, was compared to synthetic peptides and no correlation was found with their relative ability to permeabilize acidic lipid vesicles [11].

Not only magainins rapidly dissipate membrane electrochemical potential but they promote lipid transfer between the two leaflets of the bilayer, cancelling membrane asymmetry and this is coupled to peptide translocation along the walls of the pore. Indeed and contrasting with the barrel-stave ('all-peptide' pore) model for alamethicin that may still apply for magainin in neutral bilayers, magainins pores are mostly lipid+peptide mixed pores [57] and these are electrostatically stabilized by negatively-charged lipids [19]. This arrangement is independently confirmed in neutron in-plane scattering. This method detects structures in the membrane whose neutron scattering-length densities are higher or lower than that of a pure bilayer and the use of D_2O filling the water-filled pores improved the resolution. At low magainin concentration, its adsorption both expands the membrane laterally and decreases the bilayer thickness. Above a concentration threshold, the very high adsorption energy drives the lipid+peptide system to lower energy configurations and pores are observed [54]. The diameter of the water-filled cavity is estimated to 35 Å, i.e. twice than with alamethicin [41]. In fact, the data do not fit a barrel-stave arrangement, since it would require about 11 magainin monomers instead of the experimentally observed 4-7 [82]. Instead, a 'toroidal' model is proposed in which the lipid headgroups bend back to intercalate between the transmembrane peptide helices [54].

Among the factors that may modulate activity and specificity, the hydrophobic moment was found more important than hydrophobicity. No strong correlation exists between the latter and antimicrobial effects as shown with model peptides, but on the other hand hemolytic activity is increased by the replacement of charged residues by hydrophobic ones in the hydrophilic regions [10]. In a first systematic study on magainin analogues, the polar angle (subtendended by the hydrophilic sector of the helix) was varied between 80° and 180°, the other structural parameters such as hydrophobicity, the hydrophobic moment and the number of charged residues being maintained roughly

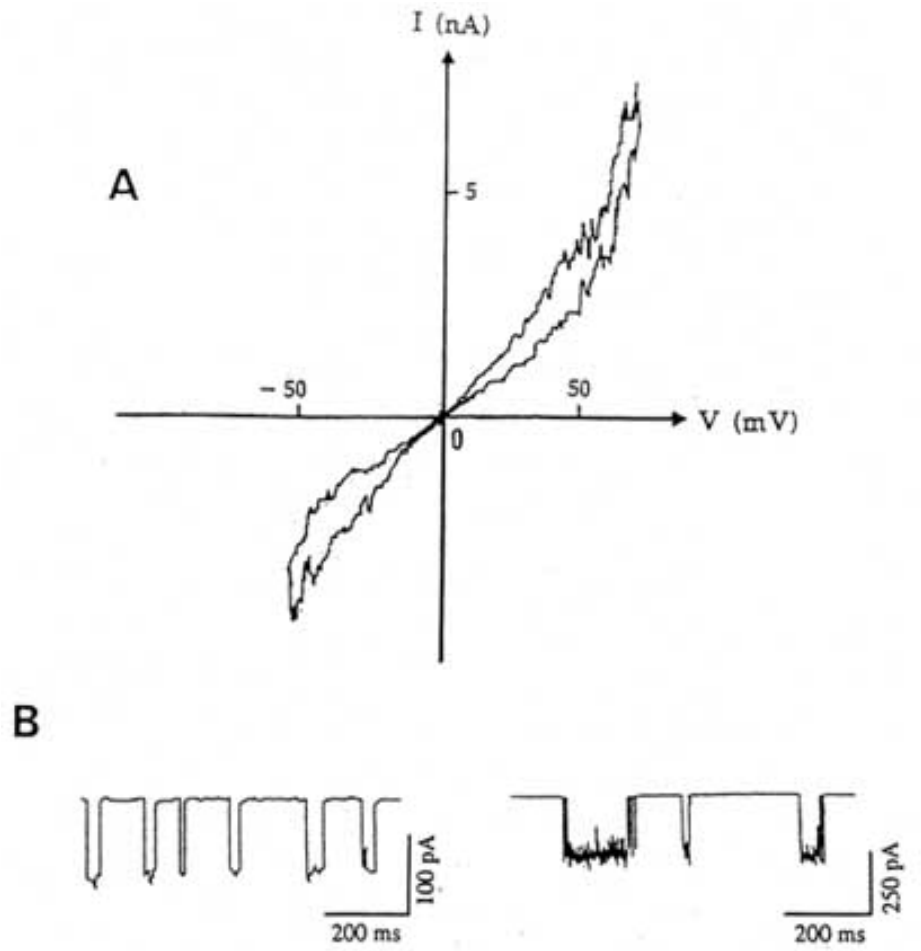

Fig (3). Pore activity induced by magainin 1 (to be compared to Fig. **2**).

A) Macroscopic current-voltage curve for $3x10^{-6}$ M magainin both sides of a neutral planar lipid bilayer bathed by buffered 1 M KCl. Note the weak voltage-dependence. **B)** Two instances of single channel-like bursting activity. Concentrations and voltages were $5x10^{-7}$ M and –120 mV on the left, $2.5x10^{-6}$ M and –220 mV on the right. Conductances were about 750 and 1150 pS, respectively. (modified from [24]).

constant [20]. Although both overall antibacterial and hemolytic activities were enhanced with large positively-charged sector, this was attributed to greater lipid affinity and the permeabilizing effect of membrane-bound peptides were in fact greater with analogues with smaller hydrophilic sector that reduced electrostatic repulsion between transmembrane helices. This trend has been recently confirmed with model peptides composed almost exclusively of alanine and lysine. Despite a lower affinity toward acidic lipid vesicles, the analogue with a polar angle of 100° exhibited higher membrane permeabilization activity (higher rate of pore formation), a greater flip-flop rate, as well as enhanced antimicrobial activity than an analogue with a 180° polar angle. Peptides of this former class form pores composed of fewer lipid molecules than peptides with a wide polar angle [77]. In another more recent systematic study, the number of positive charges was varied between three and seven whereas appropriate hydrophobic properties were conserved. The optimum peptide charge for antimicrobial activity and selectivity (i.e. reduced hemolysis) turned out to be five and interestingly, reinforcement of electrostatic interactions with highly negatively charged membranes led to reduced activities [21].

CECROPINS

Polycationic defense peptides are also part of insects immune response. The *cecropia* moth and other silkworms react to bacterial infection by synthesizing peptides [12], the best characterized of which being cecropins A, B and D containing 37, 35 and 36 residues, respectively. The sequence is characterized by the concentration of charged residues in the N-terminal part of cecropins, in contrast to magainins and melittin where they are more evenly distributed. A mammalian counterpart (cecropin P1) was later isolated from porcine small intestine [53]. The antibacterial spectrum of cecropins is large including both Gram-positive and Gram-negative bacteria, but eukarotic cells are resistant [13].

The structure of cecropins was first determined by Circular Dichroism and NMR in water/organic solvents mixtures [32, 73] as a long α−helix in CecP1 whilst perturbation brought about by the Gly-Pro hinge motif at positions 23-24 in some of the analogues (e.g. CecA) defines two amphipathic helices. The basic residues concentrate in the C-terminal helix with a polar angle that is somewhat wider than in magainins (see Fig. **1**). This structure is essentially confirmed with membrane-bound cecropins (to lipid multibilayers) by ATR-FTIR (Attenuated Total Reflection-Fourier Transform Infra Red) spectroscopy. This technique also allowed to evaluate CecP1 orientation wich was found preferentially parallel to the membrane [33]. Nevertheless, with the more flexible CecB interacting with phospholipid bilayers, spin-label Electron Spin Resonance studies support a significant embedment of the more hydrophobic C-terminal helix [47]. Finally, under applied potential, simulation (molecular dynamics) studies failed to detect any transmembrane location [33] (but this was performed with CecP1, i.e. with no hinge), in contrast with alamethicin for instance [9].

Nevertheless, a conductance study in planar lipid bilayers do show some pore-activity. As for magainins, relatively high peptide concentrations, in the 10^{-7} - 10^{-6} M range, were required to induce macroscopic current-voltage curves which were symmetrical even though the peptide was added to only one side of the bilayer [16]. There was virtually no voltage-dependence, the curves being slightly superlinear in negatively charged bilayers, without any steep exponential branch, whereas in positively charged ones, the current saturates with voltage. The later behavior is also observed in cholesterol-containing, negatively-charged bilayers. Overall, cholesterol (up to 20% , wt/wt) reduce the effectiveness of the peptide about 60-fold, thus explaining the lack of effect on mammalian cells. The sign of the reversal potential under an applied salt gradient indicated an anion selectivity [16]. Rather badly resolved single-channel events were recorded, most often on top of a significant background or leak conductance. Unit conductances ranged for natural cecropins (A, B and D) from 7 to 160 pS in 100 mM NaCl, but can reach 2 nS with the hybrid molecule cecropin AD which contains residues 1-11 of cecropin A and residues 12-37 of cecropin D. This enhanced conductance correlates with increased antibacterial activity [16].

In a three-dimensional molecular model of cecropin channels [28], the peptides are first assumed to interact through their highly conserved N-termini and form anti-parallel dimers. The charged N-terminal helices would remain flat at the bilayer interface whereas, due to the bend allowed by the Gly23-Pro24 motif, the more hydrophobic C-terminal helices span the bilayer. Pores of type I, accounting for the smaller conductances, would be formed by six adjacent C-terminal helices with the narrowest part of the pore delineated by Gln31 and Gln34. Due to the initial dimeric arrangement, an hexagonal lattice is formed with a rather high density of pores and surface helices, thus realizing a compromise between barrel-stave and carpet-like models (see below, Discussion). In pores of type II that may be induced by the lateral bilayer expansion caused by type I pores, C- and N-terminal helices swap their relative positions in a concerted movement, keeping the dimer arrangement that prevent the charged residues to encounter the bilayer hydrophobic core. Nevertheless, in doing so, the N-terminal helices are likely to drag negatively-charged lipid headgroups resulting in much larger conductances associated with lethality [28]. An essentially similar model of mixed lipid-peptide pores has also been proposed for magainin [19].

DISCUSSION

The experimental requirements to be met in planar bilayer conductance assays in order to conclude that a peptide is a channel former are quite stringent, as recently recalled [43]. In particular, since channel formation is a stochastic process, a sufficiently large number of events as well as the current-voltage curves must be recorded. It may added that, whenever possible, macroscopic conductance should also be studied and there must be consistency between these two levels of investigation. This is clearly the case for alamethicin since there is an excellent agreement between <N> , the mean number of monomers forming the barrel stave as derived from macroscopic conductance analysis and the most probable single-channel substate. In addition, a simple geometric model allows to calculate the diameter and hence the conductances of pores made of N monomers (helix end diameter ~ 10 Å) arranged in a crown array [70]. The quite good agreement between the single-channel conductances of the different substates and this model recently refined by taking into account the actual structure of the permeation pathway validates the dynamic 'barrel-stave' model.

It must be acknowledged that evidence for channels or pores is less compelling with the highly charged amphipathic helical peptides than with e.g alamethicin whose high voltage-sensitivity and multistate behavior are unique functional signatures. For peptides as magainins, some authors claim a 'channel-like' activity not directly involving a transmembrane orientation of peptides : "the unpacking of lipids will form a pore of unpredictable size" [43]. Since many spectroscopic studies, albeit witout any applied transmembrane voltage, detect the association of magainins and cecropins with the lipid headgroups and suggest that these peptides would remain parallel to the bilayer surface, a 'carpet-like' mechanism was proposed [71]. In the latter, the binding of amphipathic α-helical monomers through their basic residues interacting with negatively charged lipid headgroups would be followed by a rotation of the peptide such that hydrophobic residues face the hydrophobic core of the bilayer. Finally, cell lysis would reflect the peptide-induced lipid packing disruption. As a complement to this carpet-like model, the additional concept of 'molecular electroporation' assumes that lipidic pores could be created if a reagent that bind to a lipid bilayer carries a sufficient charge density to provide an electrostatic potential at least 0.2 V across the bilayer [62] and leading to some dielectric breakdown. NK-lysin, for which the bilayer assay was inconclusive [68], is given as an example supporting this notion. Even with alamethicin, the barrel-stave model which appears to be the object of a consensus has been recently questioned ! A radically different mode of action, termed 'asymmetrical lipid ring' states from data

obtained from synchrotron radiation X-ray diffraction that alamethicin would remain flat, not only at rest but also under the influence of the electric field [48]. 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. Even with the highly charged melittin and magainins, pores are detected through oriented circular dichroism and neutron scattering only when the helices are oriented perpendicularly to the plane of the bilayers. However, intead of the barrel-stave model which would strictly apply to alamethicin, 'toroidal pores' are assumed i.e. the water core or lumen is lined by both the peptides and the lipid headgroups [86].

Despite the obvious deterioration of channel properties and the less well defined supramolecular assemblies underlying pore activity by the peptides with larger polar angles, the body of evidence summarized in this review still supports the conclusion that channel or pore formation, causing the dissipation of membrane potential and subsequent cell lysis, is the main or primary physiological effect of these peptides. Even with cecropins, the most effective pore-forming analogue (cecropin AD) was also the most potent as regards antibacterial activity against several test organisms [16]. Although the alternative mechanisms discussed above may occur with the highly charged peptides, they are not exclusive of pore formation (see e.g. [55]) since, similarly to alamethicin and gramicidin, transmembrane orientation of magainin and pores were detected in oriented circular dichroism studies and confirmed by neutron in-plane scattering [54, 86]. The orientational transition (from flat to transverse) occurs above a critical peptide / lipid (P/L) molar ratio of around 1/30. The effect of voltage would be to reduce the free energy differences between the two orientations for much lower P/L and, only for a minute fraction of the total surface-bound peptide, pore fluctuations can be detected in conductance experiments.

A fundamental difference in the mechanism of pore formation by alamethicin and the highly positively-charged peptides discussed here is that the intermediate state of monomer insertion and lateral diffusion [42] prior to channel or pore build-up which is implicit for alamethicin cannot occur with the latter peptides, because of the very high energy cost for the bilayer crossing of charged residues in the monomer form. Rather, a two-state model assuming direct transitions between monomers lying flat on the bilayer and transmembrane oligomeric pores is more plausible [46]. Thus, instead of regular fluctuations of the pore size through uptake and release of inserted monomers laterally diffusing in the bilayer as with alamethicin, pores of different fixed sizes may be stochastically recruited with the positively-charged peptides. Nevertheless, there is no evidence that these pores result from already pre-formed aggregates at the interface, prior voltage application. Within the series of peptides discussed here, note that as the polar angle becomes larger, the apparent number of monomers per conducting aggregate tends to decrease, e.g. 8-10 for alamethicin vs. around 4 for magainin. However, pores formed by the latter are larger since lipid headgroups participate to the pore lining. The enhanced coupled lipid and peptide flip-flop or redistribution likely explains that the expression of channel fluctuations is much more transient with the highly charged peptides, hence the restricted channel statistics.

We have seen above that within a series of alamethicin synthetic analogues, antimicrobial activity was correlated to the oligomeric pore size as defined in conductance experiments. Can such a correlation be further extended when polycationic peptides are considered as well ? In one of a rare study endeavouring such a comparison, minimal inhibitory concentrations exhibited by various antimicrobial peptides on a series of six species of mollicutes (bacteria without any cell wall) were similar for alamethicin and melittin (bacteriocidal) and much lower than for magainin 2 and cecropins (bacteriostatic) [7]. Thus, poor channel-formers were also less efficient in antibacterial assays, at least with this class of micro-organisms. Pending more systematic comparisons and further basic biophysical studies, it would seem that the loss of voltage-dependence is detrimental to antimicrobial activity. Presumably the strong electrostatic interaction between surface negative charges of bilayers and peptide positive charges impedes efficient voltage-driven membrane insertion of the peptides. With alamethicin-like peptides, low concentrations would readily permeabilize polarized membranes whereas the ohmic conductance or leakiness induced by highly charged peptides might partly compensate their lower voltage-dependence with a more sustained action but at the price of much higher concentrations.

Synergy between couple of peptides have been demonstrated, especially between magainin 2 and PGLa, which is another peptide secreted by the skin granular glands of the african frog *Xenopus laevis*. PGLa presents the same number of lysines than magainin 2 but with a different distribution along the helix such that the polar angle is significantly smaller. Interestingly, PGLa was found to form pores more effectively than magainin 2 and when both were used as a 1:1 mixture, the antibacterial activity (vs. E. coli) was increased almost 10-fold [61]. The exact mechanism underlying this synergy has not been elucidated yet, nor conductance studies been performed with the complex, but it is tempting to suggest that somehow the smaller polar angle of PGLa reduces the geometrical (and energy) strain imposed by the very large magainin hydrophilic sectors to the transmembrane assembly. The search for increased activity and selectivity has also motivated the construction of hybrid peptides. In an effort to combine the strong lytic activity of melittin with the prokaryotic membrane specificity of cecropins, hybrids between portions of these two parent peptides were synthesized. Out of the 30 hybrids assayed, the most interesting analogues were shorter than cecropins and linked the first eight residues of cecropin N-terminal to the eighteen first residues of melittin as the C-terminal. The hybrid molecule produced a potent antibiotic activity without hemolytic activity [81]. This hybrid was devoid of any proline but the GIG motif at the junction between the two moeities provided the flexible hinge necessary for activity. The deletion of this motif indeed rendered the peptide inactive, as well as cancelling an excessive β−turn structure provided by a GPG hinge [72].

In conclusion, physicochemical research on peptide-membrane interactions has continued to progress steadily

and it has emerged that peptide structure, orientation, and activity on membranes reflect the balance of a number of weak forces. Simple helical peptides have a structure flexible enough to be influenced by subtle changes in membrane composition and their orientation relative to the bilayer are quite sensitive to peptide-lipid ratio. Channel formation was convincinly demonstrated with the first classical antimicrobial peptides (alamethicin and gramicidin) by electrical measurements on planar lipid bilayers and at least within a series of voltage-dependent channel-forming-peptides, this behavior was found to underlie antimicrobial activity. However, it must be realized that even with alamethicin under experimental conditions that are compatible with conductance, the vast majority of peptide molecules remains oriented parallel to the bilayer plane. Only a minute fraction is actually engaged in the transmembrane conducting helical bundles under applied voltage [1]. Such a quasi-consensus has not been reached yet with the polycationic amphipathic peptides discovered and studied more recently. Although alternative modes of action stressing the disruption of membrane lipid packing have been proposed, the balance of evidence from both spectroscopic and electrical data seems to favor pore formation as a unifying principle for antibacterial activity. In particular and in our view, the apparent heterogenous size of unitary conductances observed in a series of planar bilayer conductance experiments with peptides presenting a large polar angle does not preclude a pore mode of action, but simply a somewhat stochastic recruitment of peptide molecules in transmembrane aggregates eventually dragging lipids as well. On the other hand, voltage-induced lipid bilayer disruptions would lead to unresolved and erratic fluctuations or/and irreversible dielectric breakdown. Obviously, more basic research is required to definitely settle this question, especially in designing conductance experiments in conditions closer to antimicrobial assays, e.g. as regards membrane composition. Whatever their exact mode of action, antimicrobial helical peptides hold great potential against conventional antibiotics-resistant bacteria and accordingly, they are increasingly being clinically tested (see e.g. [36, 23].

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