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REVIEW

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Helical kink and channel behaviour: a comparative study with the peptaibols alamethicin, trichotoxin and antiamoebin

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Abstract Kinks or bends introduced in peptides and proteins by ''helical distorter'' residues such as proline, other imino acids and glycine, especially when these are in close proximity in the sequence, are increasingly recognized as playing an essential role in the gating of channel-forming peptides as well as of physiological ion channels. Peptaibols are useful simple models for the much more complex biological ion channels, especially voltage-gated ones. In this short review, we compare the monomeric structures of three selected peptaibols (alamethicin, trichotoxin and antiamoebin) that widely differ with regards their near-central kink angles and dipolar moment orientations. These structural features are then shown to be correlated to the different patterns of channel activity, both at the macroscopic and single-channel levels of investigation.

Keywords Barrel stave · Channel-forming-peptides · Conductances · Planar lipid bilayers · Secondary structure

Introduction

Peptaibols are fungal peptides rich in the non-coded α-aminoisobutyric acid (Aib); they are a large family, consisting of more than 300 members whose sequence are collected in the Peptaibol database (http://www.cryst.bbk.ac.uk/peptaibol) (Whitmore et al. 2003). Peptaibols are potent antimicrobial agents, many of which appear to form pores or channels in biomembranes. The latter activity has been characterized by electrical conductance methods when these peptaibols interact with planar lipid bilayers. As mentioned by Whitmore and Wallace (2004) in this issue, both their distribution (the number of peptaibols) and their activity potency is strongly biased towards the longest ones (20 residues), especially alamethicin (for review, see e.g. Woolley and Wallace 1992; Duclohier and Wroblewski 2001). There is presently a consensus for the mode of action of the long peptaibols whose helical length matches the standard bilayer thickness with the ''barrel-stave'' model. As the length decreases, especially below 18 residues, the situation is much less clear and several mechanisms have been proposed, e.g. bilayer thinning and ''en masse'' destabilization, on-line dimers, carrier-like modes of transport.

In this review are summarized the structural and functional data previously obtained with three different peptaibols of decreasing length: alamethicin (20 residues), trichotoxin_A50E (18 residues) and antiamoebin (16 residues), whose crystal structures are known (Fox and Richards 1982; Snook et al. 1998; Chugh et al. 2002). Apart from different lengths and from the amino acid sequences shown in Fig. 1, it is apparent that these three selected peptaibols present similar, but subtly different, patterns of so-called key residues (in bold): helix-distorting ones (underlined: Pro, other imino groups and Gly) and H-bond donors (Gln near the end of the first N-terminal third). The likely importance of these residues, at least for alamethicin, has been addressed in a number of studies (e.g. Duclohier et al. 1992; Molle et al. 1996; Kaduk et al. 1997; Asami et al. 2002). There is growing evidence for the importance of dynamic helix bending in the gating of physiological channels (Kuo et al. 2003; Miyazawa et al. 2003), although voltage gating may operate by additional mechanisms (Jiang et al. 2003), as first suggested by peptaibol studies (see e.g. Sansom and Weinstein 2000). Hence, in this review we focus on the influence of helix-distorting residues and their distribution in monomer structures of the

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Alamethicin_F50 AcU**P**UAUA**Q**UVU**G**LU**P**VUU**QQ**Fol

Trichotoxin_A50E AcU**G**ULU-**Q**UUUAAU**P**LUU-**Q**Vol

Antiamoebin AcFUUUJ**G**LUU**OQ**J**O**U**P**Fol

Fig. 1 Alignment of the amino acid sequences (one-letter codes) of the peptaibols discussed in this review. The N-terminal residue is acetylated (Ac) whereas the C-terminal residue is an amino alcohol. Glutamines, which can be involved in intermolecular H-bonding, are in *bold*. The potential helix breakers, Gly, Hyp, and Pro, are *bold and underlined*. U is Aib (α-aminoisobutyric acid), O is Hyp (hydroxyproline) and J is Iva (isovaline)

above-mentioned three peptaibols, and their correlation with conductance properties.

Before doing so, it might be useful to rapidly recall the essential behaviour of these selected peptaibols. Alamethicin, a 20-residue-long peptaibol, in planar lipid bilayers develops a conductance whose voltage dependence is as high (Gordon and Haydon 1975) as the sodium system of nerve fibres (Hodgkin et al. 1952). In addition, in the presence of protamines and under stringent conditions, a behaviour even more typical of excitable membranes, namely negative resistance and action potentials, has been demonstrated (Mueller and Rudin 1968) and recently further characterized (Duclohier and Spach 2001). There is a consensus about the alamethicin structure (for review, see Cafiso 1994) and the dynamic "barrel-stave" model (Boheim 1974) to account for the voltage dependence and the multi-states of unitary conductance, reflecting the sequential uptake and release of amphipathic monomers into conducting aggregates. Trichotoxin_A50E, an 18-residue peptaibol, induces macroscopic current–voltage curves with a moderate voltage sensitivity and the concentration dependence suggests the channel may be hexameric. Trichotoxin exhibits a much higher cation/anion selectivity than alamethicin and it produces one main conductance level, as opposed to the multiple open levels of alamethicin. As for antiamoebin, a 16-residue-long and significantly bent peptaibol, a carrier-like mechanism was first put forward to account for its mode of lytic action (Snook et al. 1998). Experimental evidence was subsequently provided for channel activity in planar lipid bilayers under certain circumstances (Duclohier et al. 1998). Macroscopic current–voltage curves are mainly ohmic, i.e. the rate of pore formation is voltage independent, and single-channel events have a large amplitude on top of a background conductance, which may account for the previously proposed carrier-like activity.

Conductance methods in planar lipid bilayers: a reminder

The activity induced by peptaibols was assayed in planar lipid bilayers both in the macroscopic and in the single-channel conductance configurations, according to methods previously described (see e.g. Duclohier et al. 1992). Briefly, in the macroscopic conductance configuration, the activity of hundreds or thousands of channels are recorded with current–voltage (*I–V*) curves displayed by virtually solvent-free bilayers (Montal and Mueller 1972) doped with the pore-forming peptides and submitted to slow-voltage linear ramps. Typically, the duration of each voltage excursion, e.g. between +150 and −150 mV, was of the order of 1 minute. Voltage is delivered via an Ag/AgCl electrode in the *cis* side (the side of peptide addition and the positive side for electrical conventions). Currents are measured via a second electrode in the *trans* side connected to the amplifier and current–voltage converter. For recording single-channel activity, bilayers are made at the tip of patch pipettes (Hanke et al. 1984) and a standard patch-clamp apparatus is used. In all cases, the lipids used to form bilayers were a neutral mixture from Avanti polar lipids (Alabaster, Ala., USA): 1-palmitoyl-oleoyl-phosphatidylcholine (POPC)/dioleoyl-phosphatidylethanolamine (DOPE), molar ratio 7/3.

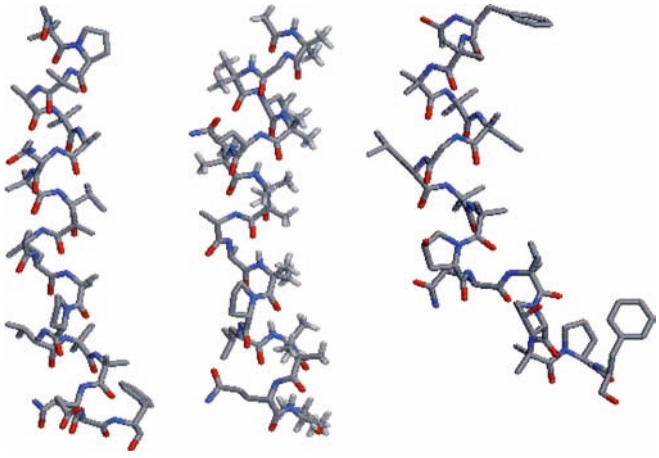

Fig. 2 Comparison of alamethicin (*left*), trichotoxin (*centre*) and antiamoebin (*right*) monomer structures, emphasizing the differences in how straight or curved their helices are, and the kink angles. Crystallographic coordinates were taken from the Protein Data Bank with the following codes: 1AMT for alamethicin, 1M24 for trichotoxin_A50E and IJOH for antiamoebin. The figures were made using RASMOL software in stick mode (Sayle and Milner-White 1995). The N-termini are facing upwards. Figure courtesy of B.A. Wallace

Comparison of monomeric structures

The crystallographic structures of the monomers are shown in Fig. 2. Apart from a slightly different length, the most striking feature is the curvature of the peptide molecule. Whereas in trichotoxin it is limited to 8–10° (Chugh et al. 2002), there is a significant kink angle betwen C- and N-terminal moieties in alamethicin of 20–35° (Fox and Richards, 1982; Breed et al. 1995), which is much more pronounced in antiamoebin at 58° (Wallace et al. 1999). As a result, the dipole moment of antiamoebin is at a more acute angle relative to the main

Table 1 Correlation of structural and main functional parameters for alamethicin, trichotoxin and antiamoebin. $< N >$ is the mean apparent number of monomers per transmembrane conducting barrel stave

Peptaibol	Kink angle (°)	Dipole moment (D)	Voltage dependence V_e (mV) and $< N >$	Single-channel pattern
Alamethicin	20–35	54.1	5 mV, 8–10	Multi-states
Trichotoxin	8–10	37	18 mV, 6	One open state
Antiamoebin	58	26.8	$\sim\infty$, 4	One open state

helical axis than in the other two instances. The values of the calculated dipole moments (Chugh et al. 2002) are listed in Table 1. At least for alamethicin, they are in reasonable agreement with the broad range (40–75 D, depending on the polarity of the solvents) experimentally determined for alamethicin (Yantorno et al. 1982).

Comparison of functional properties in planar lipid bilayers

Macroscopic conductance: voltage dependence and apparent oligomerization

A typical macroscopic current–voltage curve induced by alamethicin interacting with neutral planar lipid bilayers is shown as curve 1 in Fig. 3. Under a voltage threshold which is concentration dependent, the transmembrane current is negligible. Above that threshold, there is an exponential branch whose steepness is characterized by V_e, the voltage increment resulting in an e-fold (×2.8) conductance change. V_e for alamethicin is only about 5 mV. The characteristic voltage (V_c) at which the exponential branch crosses a reference conductance (i.e. an arbitrary conductance well above any bare bilayer leakiness, dashed line) shifts towards a lower voltage when the alamethicin concentration is increased. V_a is the V_c shift when that concentration is increased by e (2.8). The apparent number of monomers per transmembrane conducting bundle or "barrel stave" can then be simply estimated as $N = V_a/V_e$ (demonstrated by Hall et al. 1984). For alamethicin in standard planar bilayers (i.e. whose thickness matches the helical length), $N = 8$–10. The fact that octamers are the most probable oligomerization state is reflected in the single-channel pattern (see below).

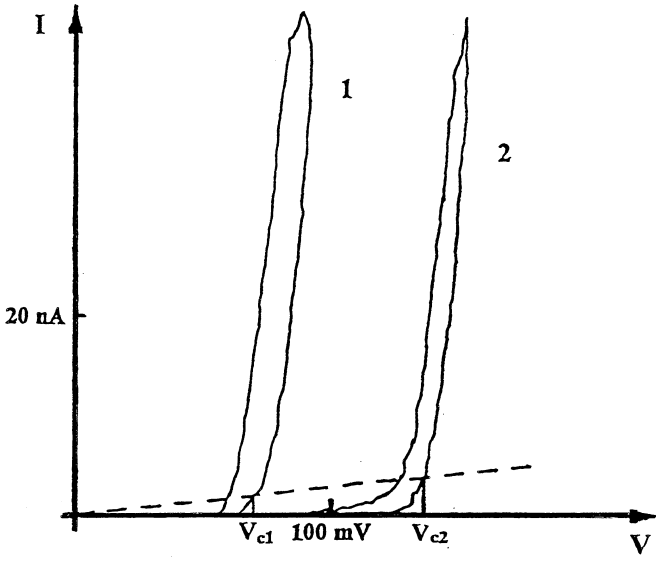

Fig. 3 Macroscopic current–voltage (I–V) curves induced by alamethicin (*curve 1*) and its P14A-substituted analogue (*curve 2*) under the same conditions: a concentration of 8×10^{-8} M on the *cis* side of a POPC/DOPE (7/3) bilayer bathed both sides by 1 M KCl, 10 mM Hepes (pH 7.4). The *dashed line* represents a reference conductance of 25 nS defining the characteristic voltages V_c as intercepts with the early rising phase of the exponential branches. Figure from Kaduk et al. (1997)

We reasoned that if the Pro-induced helical kink was important for alamethicin insertion, then its substitution by alanine, for instance, could result in a significantly reduced voltage dependence. As shown by curve 2 in Fig. 3, this proved not to be the case (Duclohier et al. 1992). Apart from a very significant shift towards higher voltage at a similar concentration (the voltage required to open a similar number of channels is roughly doubled), the voltage dependence per se or the steepness of the exponential branch is unchanged. Note also that the current hysteresis between the raising and falling limbs of the response is narrower with the Pro14A substitution, an indication of faster kinetics as confirmed by the single-channel analysis (see below and Fig. 4).

Gly is another potential "helix distorter", often found for instance in extra-membraneous loops, especially when in close proximity to Pro (within one helical turn, 3–4 residues apart). Out of more than 300 peptaibol sequences examined, there is a very significant number (130) of examples of the Gly-X-X-Pro motif, and none with contiguous Pro and Gly or with only one intervening residue (Wallace, personal communication). Thus, another alamethicin analogue where both Pro14 and Gly11 (Fig. 1) were substituted with alanines was synthesized and assayed. Apart from still faster kinetics than with P14A substitution alone, the high voltage dependence of the macroscopic conductance was preserved, albeit at the price of higher peptide concentration. This was unexpected, especially when confronted with electron paramagnetic resonance spectroscopy, which showed that only the double mutation (P14A + G11A) resulted in a significant straightening of the helix (Jacob et al. 1999).

With trichotoxin_A50E, the steepness of the exponential branch is quite modest, $V_e \approx 18$ mV on average, and V_a, the concentration-dependent parameter (see above), is $\sim$115 mV. Thus, here the apparent and mean number of helical monomers per conducting bundle is

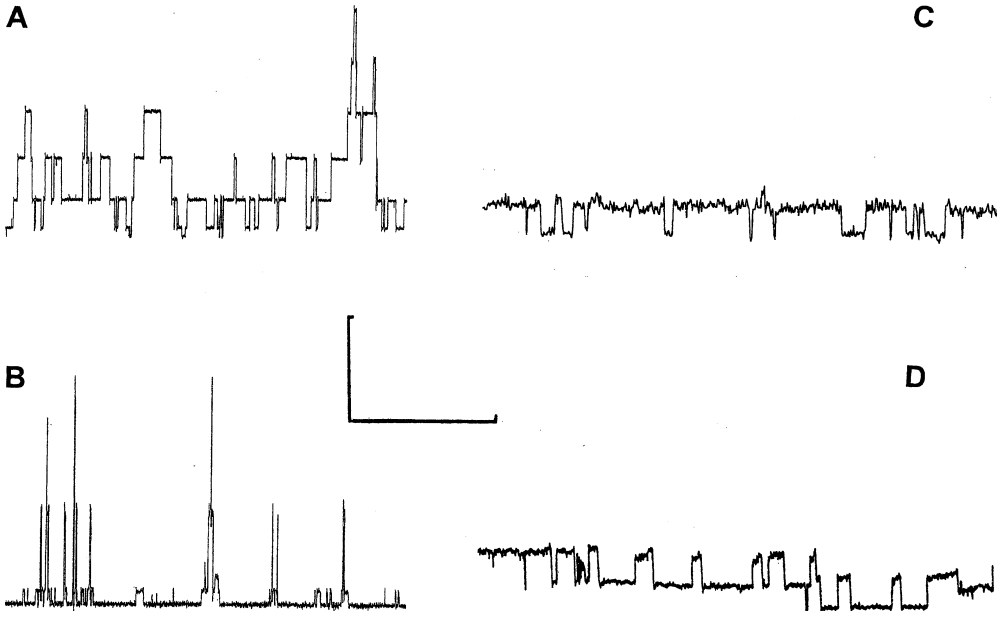

Fig. 4 Compared single-channel traces for alamethicin (**A**), its Pro14-Ala substituted analogue (**B**), trichotoxin_A50E (**C**) and antiamoebin (**D**) under the same experimental conditions (POPC/DOPE, 7/3, bilayers; 1 M KCl) except for peptide concentrations and applied voltages (*cis* side): 5×10^{-9} M and 120 mV for **A**, 3×10^{-8} M and 130 mV for **B**, 2.5×10^{-7} M and 180 mV for **C** and 10^{-7} M and 90 mV for **D**. In all cases, openings are upward deflections. *Horizontal time scale bar*: 1 s for all. *Vertical transmembrane current scale bar*: 400 pA for traces **A** and **C**, 200 pA for **B** and 40 pA for **D**. Adapted from Kaduk et al. (1997) (**A** and **B**), Duclohier et al. (unpublished data, **B**) and Duclohier at al. (1998) (**D**)

$N = V_a/V_e = 6$ (rounded), i.e. significantly lower than for alamethicin (Duclohier et al., manuscript in preparation). The recent finding that a molecular model made of a parallel hexameric bundle of trichotoxin yields single-channel conductance quite compatible with experimental measurements adds confidence to the oligomerization state derived from macroscopic conductance analysis (Duclohier et al., in preparation).

Finally, as for antiamoebin, there remains hardly any typical voltage-dependent behaviour, a leaky-like or ohmic component being predominant in macroscopic current–voltage curves. Only high concentrations and high applied voltages disclose an exponential branch superimposed on that background conductance whose concentration dependence argues for tetramers as the transmembrane conducting units (Duclohier at al. 1998).

Single-channel conductance: kinetics, multi versus single open states

Typical patterns of single-channel activity displayed by the selected peptaibols are shown in Fig. 4. Alamethicin single-channel activity is characterized by continuous fluctuations between closed and well-resolved open multi-states (Fig. 4A). The increments between these latter follow a geometrical progression, reflecting a sequential increase and decrease of the pore diameter through uptake and release of monomers in the barrel stave. The pore lumen is lined by hydrophilic residues Q6, Q18–19 together with G11 and P14 carbonyls of adjoining transmembrane helices. The single-channel conductance reached by the upper open states is rather high (up to 5–10 nS in 1 M KCl), but the lowest open state has a conductance (20 pS) much more in line with physiological ion channels (Hanke et al. 1980). This first open state most likely results from a tetramer, as suggested by studies on template-assembled tetramers (Duclohier et al. 2003) and dimers (Okazaki et al. 2003). The Pro14Ala substitution has a drastic effect on channel kinetics and level distribution (Fig. 4B). Although the voltage dependence of the macroscopic conductance was unchanged (Fig. 3), the single-channel fluctuations are much more rapid and lower-sized transmembrane conducting aggregates are favoured. This is even more pronounced when both Pro14 and Gly11 are substituted by Ala: the kinetics are still faster, hardly resolved even though the macroscopic conductance voltage dependence is virtually unchanged (Jacob et al. 1999).

The two other peptaibols, trichotoxin and antiamoebin, display strikingly different single-channel activity patterns (Fig. 4C and D, respectively). Trichotoxin_A50E fails to show open multi-states but only one rather noisy level (on top of a significant leak) is disclosed (Fig. 4C). Since trichorzins PA, another group of 18-residue long peptaibols, do still show an alamethicin-like multi-state pattern of single-channel activity, albeit much faster (Duval et al. 1998), the trichotoxin peculiar behaviour is not mainly due to the shorter helix. The most notable difference in the respective sequences is the lack of any near-central Gly in trichotoxin. Furthermore, the conductance of that trichotoxin single level is considerably smaller than the most probable alamethicin one and, together with the macroscopic conductance analysis, suggests a hexamer as the conducting unit, as opposed to 8- to 10-mers for alamethicin. Finally, with antiamoebin (Fig. 4D), the single-channel conductance is even smaller than with trichotoxin_A50E (90 pS vs. 700 pS, under the same experimental conditions) and argues, with the concentration dependence of the macroscopic conductance, for tetramers, but significantly different (O'Reilly and Wallace 2003) from the regular alamethicin ones.

Discussion: correlating functional and structural features

From the present comparative survey of the main structural and functional properties of three selected peptaibols, it is apparent that the conductance properties are strongly correlated with helical bends and resulting helical dipoles (Table 1). However, contrasting with what was initially assumed, it is far from clear that the kink angle actually changes during the first gating step, i.e. transmembrane insertion (Tieleman et al. 2001). Two main classes of models have been proposed for the way by which the alamethicin-like voltage-gated channels operate. These are the conformational change models and the helix dipole models. Hall et al. (1984) proposed a conformational transition between a bent structure and a linear one as the basic gating event. This model is related to the one proposed by Fox and Richards (1982), although the open and closed states are not the same as above. Models stressing the role of the helix dipole moment include those where the helix flips across the membrane and those where the helices in the channel move with respect to each other (see e.g. Menestrina et al. 1986). These proposals remain speculative since the structure of alamethicin in the lipid bilayer is not known in detail, although simulation (molecular models/dynamics) studies presently give a fairly detailed and plausible view of the channel in its lipid and aqueous environment (Tieleman et al. 2002). At rest, without any applied voltage, the C-termini of alamethicin and trichotoxin are assumed to lie flat at the bilayer interface, whereas their N-termini (after the near-central Pro) would bury to a different extent in the lipid hydrocarbon core. The higher kink angle for alamethicin would allow a greater embedment in the lipid matrix (at an average depth of 10 Å) and thus this end of the dipole would experience a greater voltage drop than for trichotoxin, hence the higher voltage dependence. As for antimoebin, the situation is likely to be quite different. Its highly curved shape would favour significant embedment in the bilayer, accounting for the observed background ohmic conductance.

The first hint at the Pro-induced kink in the helix as a prospective and general mechanism for physiological ion channel gating dates from the early 1980s, stemming from alamethicin studies, both structural and functional. It now appears that helical kinks also play a significant role in much more complex systems where extracellular signals are transduced across membranes via conformational changes in the transmembrane domains (TMs) of ion channels and G-protein-coupled receptors (GPCRs). Experimental and simulation studies indicate that such conformational switches in transmembrane α-helices can be generated by proline-containing motifs that form molecular hinges (Cordes et al. 2002). For instance, mutational studies in gap junctions have unambiguously shown a role for a proline in the second transmembrane segment in the mechanism of voltage-dependent gating of connexin32: the channel open conformation corresponds to a more bent TM2 helix, and the closed conformation corresponds to a less bent helix (Ri et al. 1999). However, hinges in transmembrane helices are not confined to proline-containing sequence motifs, as evidenced by the glycine-promoted hinge in the M2 helix of the nicotinic acetylcholine receptor, which lines the pore and plays a key role in the gating of this channel (Law et al. 2003). As a matter of fact, in the above-mentioned study (Ri et al. 1999), out of a series of other mutations, the Pro $\rightarrow$ Gly mutation was the least perturbing one. Finally in a broader context, in chicken muscle adenylate kinase, mutating Pro17 (to Gly or Val) not only leads to lower secondary structural content, but also to poorer affinity to substrates and reduced catalytic efficiency (Sheng et al. 2001), and in the recently solved crystal structure of the lactose permease of *Escherichia coli* (reviewed by Abramson et al. 2003), conformational flexibility of the hydrophilic cavity is conferred by Pro- and Gly-induced kinks and bends.

In conclusion, the significance of proline and other helix distorters is certainly one of the topics in which studies of simple model peptides, whether available natural ones or designed synthetic ones, have been of great value in the understanding of more complex systems.

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