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Channel Properties of Template Assembled Alamethicin Tetramers‡

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Abstract: The multiple conductance levels displayed by the antibiotic alamethicin in planar lipid bilayers is explained by a dynamic 'barrel-stave' model, the conducting pore resulting from the aggregation of up to ten helical amphipathic helical monomers. However, the precise assignment of an oligomerization state to a particular single-channel conductance substate is far from being experimentally clear. In addition, it could be useful to tailor a given channel geometry to selectively allow the permeation of solutes with different molecular sizes, whilst retaining a high voltage-dependence. To control the aggregation state of the channel, the TASP (template assembled synthetic proteins) strategy was applied to synthesize structurally defined oligomers, i.e. dimer, trimer, tetramer. The modulation of conductance properties of three alamethicin tetramers with the length and flexibility of the linkers of the 'open' or linear template is described. It is shown that the introduction of an alanine between the contiguous lysines to which are tethered *C*-terminally modified alamethicin helical monomers stabilizes the open channel states, whereas the alanine substitution by Pro-Gly, a reverse beta-turn promoting motif, increases voltage-dependence and leads to single-channel conductance values more in line with the expected ones from a tetrameric bundle.

Keywords: template assembled synthetic proteins; peptaibols; channel forming peptides; conductances; voltage-dependence; planar lipid bilayers

INTRODUCTION

Alamethicin, a 20-residue long helical and amphipathic peptaibol, is the most intensively studied model peptide for voltage-gated ion channels. The amino acid sequence of its two major forms is:

2 7 11 14 18 20
Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-*X*-Gln-Phol

where *X* denotes Glu in the charged component (Rf30) and Gln in the neutral component (Rf50). Aib and Phol are α-aminoisobutyric acid, a non-coded residue, and phenylalaninol, respectively. The essential structural and functional features of alamethicin and analogues, including their correlation to antimicrobial activity have been

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periodically reviewed [1–4]. In planar lipid bilayers, alamethicin behaviour can be explained by the formation of transmembrane aggregates according to the 'barrel-stave' model [5]. The uptake and release of alamethicin helical monomers from channel aggregates, between 3–4 and up to 10–12 [6,7], account for multi-state conductance levels. One of the main goals of ongoing research in this field is to assign a particular conductance state to a given geometry of the conducting aggregate in accord with the high voltage-dependence of pore formation.

Structurally defined peptide oligomers can be prepared along the TASP (template assembled synthetic protein) strategy developed by the Mutter group [8,9]. This approach which covalently links monomeric motifs to various chemical scaffolds or templates has been applied to channel-forming peptides, including models of voltage-dependent calcium channels [10], of the acetylcholine receptor [11], of S4 sodium voltage sensors [12] and melittin [13]. The liposomal membrane permeability induced by assembled peptaibols was recently shown to be modulated by zinc(II) coordination to a tripodal metal ion ligand used as a template [14]. In the alamethicin case, structurally defined constructs derived from the monomer and its shortened sequences have been reported [15–18], resulting in some channel conductance substate stabilization. However, the voltage-dependence was most often significantly reduced.

Our previous study on defined alamethicin oligomers, i.e. dimer, trimer and tetramer, stressed the minimal requirements to be met for sufficient template flexibility with concomitant high voltage-dependence [19]. When alamethicin motifs are coupled to a template built of contiguous lysine residues, both macroscopic and single channel conductance become atypical. Most probably the polylysine template is not flexible enough to allow optimal parallel alignment of helical alamethicin monomers. A single alanine insertion between two lysines is sufficient for normal behaviour retrieval of the dimer [19]. In the same study, it was demonstrated that replacement of the last three alamethicin residues Glu-Gln-Phol by a simplified sequence Ala-Aib-Ala is of no consequence as far as macroscopic conductance is concerned, in agreement with previous studies on synthetic analogues [20]. Thus the *C*-terminally modified alamethicin Alm(1–17)-Ala-Aib-Ala is an advantageous monomer for the synthesis of template linked oligomers. Furthermore, the lack of bifunctional amino acids and the amino alcohol facilitates both the preparation of the monomer and template assembled oligomers.

A comparative conductance analysis was performed in planar lipid bilayers of three tetramers prepared from modified alamethicin and different linkers yielding increased template flexibility and spacing between the monomeric motifs (Figure 1). In the first tetramer (labelled TETRA-K4), the monomers were tethered to four contiguous lysines, whereas in the second more flexible one (labelled TETRA-A3), lysine residues were separated by alanine residues. The third tetramer (labelled TETRA-A2-PG) has been assembled on a template containing a central Pro-Gly sequence to facilitate

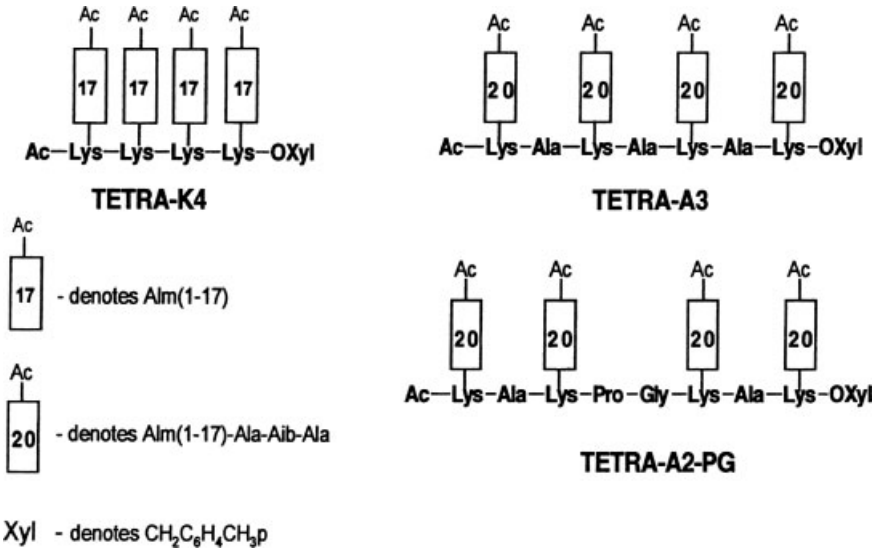

Figure 1 Structures of the tetramers investigated.

a reverse turn structure and to thus promote the aggregation of tethered helical peptides.

MATERIALS AND METHODS

Chemical Synthesis

The synthesis of Alm(1–17)-Ala-Aib-Ala, the building block of the template assembled bundles is a challenging task due to the low reactivity of sterically hindered Aib residues, the presence of acid labile Aib-Pro bonds and the increased racemization risk of proteinaceous amino acids in the sequence. As the research programme required access to a large amount of this peptide, it was decided to optimize classical solution synthesis according to the fragment condensation protocol. To preserve chiral integrity, the acyl donor segments had achiral or racemization resistant residues (Aib, Gly, Pro) on their *C*-termini. In the final condensation step, the acyl donor and acceptor segments Ac-(1–11)-OH and (12–20)-OBzl having *C*-terminal Gly and *N*-terminal Leu, respectively, were coupled on an unprecedented gram scale affording chromatographically pure Alm(1–17)-Ala-Aib-Ala-OBzl in a yield of 91%. Gas chromatography analysis on the column with a chiral stationary phase L-Chirasil-Val revealed satisfactory enantiomeric purity of the product.

The key building block Boc-Lys(20)-OXyl was used for stepwise synthesis of covalently bound dimer and trimer with the simultaneous formation of the template as a mouth of the channel. This strategy is conducive to controlling aggregate size and monitoring the purity of intermediate products. A further advantage is the possibility of obtaining dimer and trimer in one synthetic run. Step-by-step synthesis was no longer successful in the case of TETRA-A3. This oligomer was prepared by peptide bond formation between four molecules of Alm(1–17)-Ala-Aib-Ala and four lysine ε-amino groups in the template Ac-Lys-Ala-Lys-Ala-Lys-Ala-Lys-OXyl synthetized separately. Tetramer TETRA-A2-PG with the template containing the P-G sequence was assembled by the condensation of fragments Boc-Lys(20)-Ala- Lys(20)-Pro + Gly- Lys(20)-Ala- Lys(20)-OXyl. Details of the synthetic procedures for preparation of Alm(1–17)-Ala-Aib-Ala and tetramers together with the oligomers identification by mass spectrometry and characterization of their secondary structure by circular dichroism spectroscopy will be given elsewhere [21], as this present paper focuses on their comparative pore-forming properties in planar lipid bilayers.

Electrical Measurements on Planar Lipid Bilayers

The activity induced by the template assembled peptaibols was assayed in planar lipid bilayers (PLBs). Briefly, in the macroscopic conductance configuration, the activity of hundreds or thousands of channels was recorded with current–voltage (I–V) curves displayed by virtually solvent-free bilayers doped with the peptides and submitted to slow voltage ramps (1 cycle per min between e.g. $\pm$150 mV). The bilayer was made by 'folding' two lipid monolayers [22] (after solvent (hexane) evaporation) over a 150–200 μm hole in a PTFE film sandwiched between two half glass cells. The hole had been previously pretreated with a few μl of 4% hexadecane in hexane. The electrolyte on both sides was 1 M KCl, 10 mM Hepes (pH 7.4). Voltage was delivered via an Ag/AgCl electrode in the *cis*-side (the side of peptide addition and the positive-side for electrical conventions). Currents were measured via a second Ag/AgCl electrode in the *trans*-side connected to the amplifier and current–voltage converter. For recording single-channel activity at a steady-state applied voltage, the peptide concentration was reduced. Traces were analysed with the SES software (J. Dempster, Strathclyde University, Scotland). The lipid used to form bilayers was a neutral mixture from Avanti Polar Lipids (Alabaster, Alabama): 1-palmitoyloleoylphosphatidylcholine (POPC)/dioleoylphosphatidyl-ethanolamine (DOPE), molar ratio 7/3.

RESULTS

Conductance Properties of the Constrained Tetramer, TETRA-K4

These were investigated in our previous report, along with dimer and trimer where *C*-terminally modified alamethicin were coupled to open templates with contiguous lysines [19]. As shown in Figure 2, both the macroscopic and single-channel conductance behaviour of TETRA-K4 were untypical: the rising phase of the macroscopic currents in response to slow voltage ramps were greatly slowed down, whereas the falling phase was slightly steeper, in contrast to the response obtained with the standard monomeric alamethicin. The single-channel-like events were hardly resolved and the single 'open'

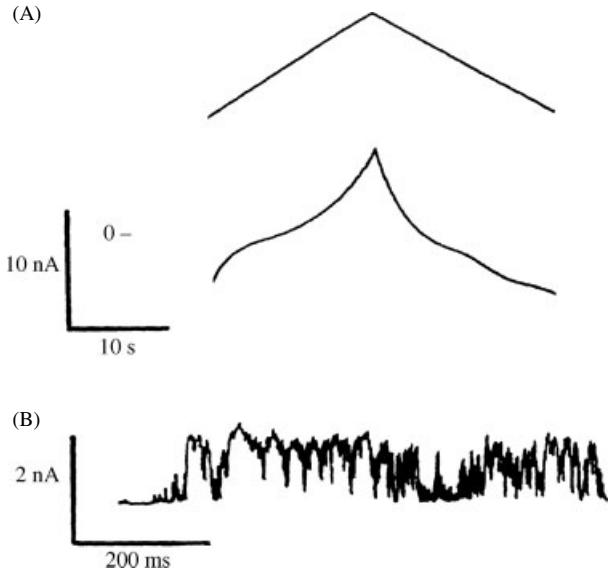

Figure 2 (A) Macroscopic current responses to ±100 mV voltage ramps induced by 2 nM of TETRA-K4 (*cis*-side). The electrolyte was 0.5 M KCl on both sides of a POPC/DOPE (7/3) bilayer. Room temperature. (B) Single-channel-like events at an applied steady-state voltage of 50 mV. (Modified from Duclohier *et al.*, 1999).

level averaged a very high conductance of ~40 nS, (i.e. about ten times the average single-channel level of monomeric alamethicin. Clearly, the constraints imposed a rather short and rigid template that did not allow a normal channel functioning of the assembly [19].

Macroscopic Conductance of TETRA-A3

Figure 3 shows the macroscopic current–voltage (I–V) curves induced by TETRA-A3 for two aqueous concentrations. In response to a slow voltage ramp (ensuring steady-state conductance), a transmembrane current developed above a concentration-dependent voltage threshold, but the exponential branch was less steep than with e.g. alamethicin [23]. Applying the classical analysis [24] that gives the apparent and mean number of monomers *per* transmembrane conducting bundle $\langle N \rangle = Va/Ve$, Va and Ve being the concentration- and voltage-dependent parameters or indices (voltage shifts yielding e-fold changes for thresholds): $Va \sim 85$ mV, $Ve \sim 30$ mV, then $\langle N \rangle \sim 3$ (rounded). The corresponding figures for native and monomeric alamethicin were 50–60 mV, 6 mV and $\langle N \rangle = 10$, respectively [23–25].

Single-channel Conductance of TETRA-A3

To record single-channel activity, either the applied voltage or the tetramer aqueous concentration was reduced. Two representative patterns of activity, at different concentrations and voltages are shown in Figure 4A and B. The lowest tetramer concentration as was used in macroscopic conductance experiments (2.5 nM, see above) and applying a transmembrane steady-state voltage of 40 mV, i.e. near the threshold at the foot of the exponential branch (see Figure 3), induced trains of regular current fluctuations. The continuous (40 s) single-channel recording of Figure 4A disclosed two open levels of equal conductance (500 pS) as analysed by the amplitude histogram (lower part of Figure 4A).

Further reducing the TETRA-A3 concentration in the bath (*cis*-side) but stepping the bilayer to increased voltage still induced a quasi multi-state behaviour (Figure 4B). However, the enlargement disclosed only two superimposed main open levels with conductances of 250 and 500 pS. These events

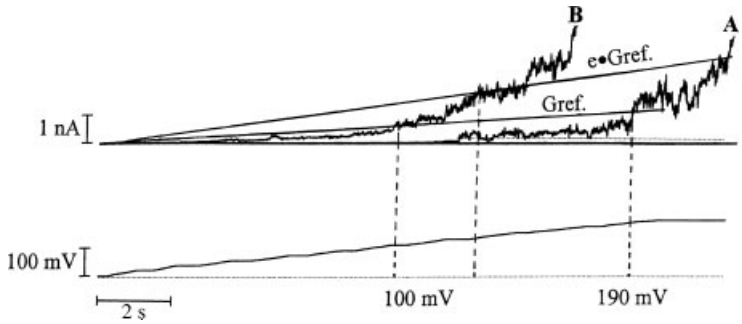

Figure 3 Macroscopic current–voltage (I–V) curves induced by TETRA-A3 at two aqueous (*cis*-side) concentrations: 2.5 nM (curve A) and 7.5 nM (curve B). The applied voltage ramp (lasting about 1 min) is shown below. An arbitrary linear conductance (Gref) to define voltage thresholds associated with the two current responses is shown as well as $e^{\bullet}$ Gref (to define Ve, the voltage-dependent index). 1 M KCl on both sides of a POPC/DOPE (7/3) bilayer.

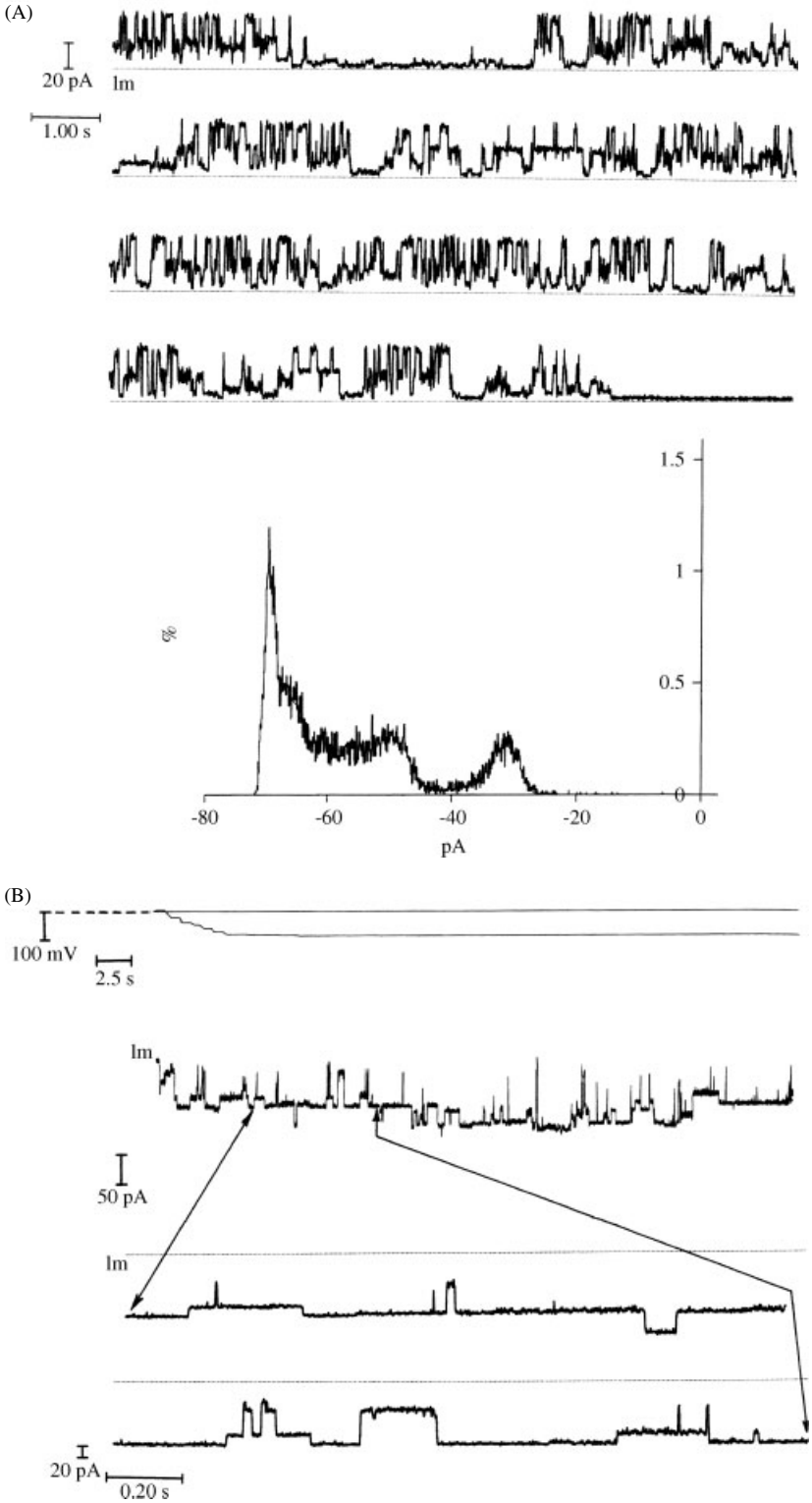

Figure 4 Single-channel activity induced by TETRA-A3. (A) A continuous recording of 40 s under an applied steady-state voltage of +40 mV in the presence of 2.5 nM (*cis*-side) tetramer. The associated amplitude histogram discloses two main open levels of equal conductance (~500 pS). (B) Another example at +80 mV and with a reduced concentration: 0.5 nM. The enlargement (bottom two traces) discloses two levels, at 250 and 500 pS.

were apparently independent, i.e. the larger channel did not result from the recruitment of two smaller ones. The mean open lifetime was in the 0.1–1 s range, i.e. at least 10 times slower than with the monomeric neutral alamethicin in the same conditions (lipids, temperature, ionic strength . . .) [26].

Conductance Properties of the Most Flexible Tetramer, TETRA-A2-PG

With the introduction of the Pro-Gly motif replacing Ala in the central linker, the voltage-dependence of macroscopic current–voltage (I–V) curves somewhat increased, from 30 mV (for TETRA-A3) to ~20 mV (for TETRA-A2-PG) (Figure 5A). With the latter, the apparent number of monomers per conducting bundle now reached the nominal value aimed for the designed construct, i.e. $\langle N \rangle \sim 4$ (rounded), *V*a remaining roughly unchanged. Accordingly, and although the single-channel-like events do not look as well defined as above, the unit conductance was reduced to 60 pS (Figure 5B).

DISCUSSION

Two previous studies reported the preparation and conductance properties of template-assembled alamethicin. In the first study, the first 18 residues of alamethicin were *C*-terminally attached to cyclic pseudopeptides [17]. The tetramer had a single-channel conductance of about 0.2 nS but there were no macroscopic conductance data, hence no voltage-dependence, reported. In the second and much more recent study, four alamethicins were coupled to a porphyrin template through their *N*-termini [18]. The channels were longer-lived but quite noisy, and again there was no evidence for voltage-dependence.

In our previous study, whereas the introduction of an alanine between two alamethicin motifs (in dimer II) was sufficient to allow enough flexibility for a high voltage-dependence [19], this was no longer the case with the tetramer prepared along a similarly designed template (TETRA-A3 in the present study). The analysis developed above ($\langle N \rangle \sim 3$) would imply that one of the alamethicin motifs may be 'left out' on the bilayer interface, whereas the other three would make the crossing. However, the main or most probable single-channel conductance level (at 500 pS in 1 M KCl) is outside the range expected for a trimeric bundle (and even more for a tetrameric bundle) and might be due to twinned trimers being allowed by the open or linear structure of the template.

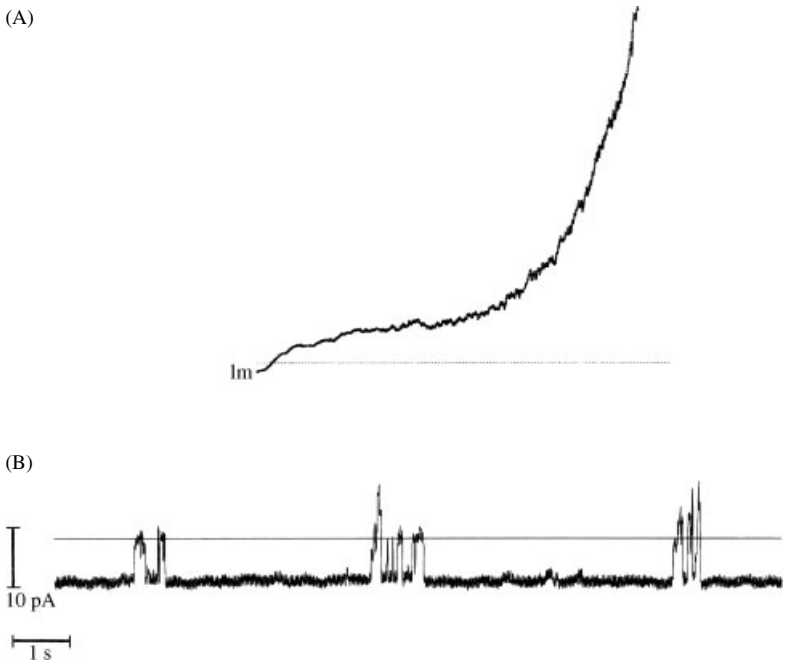

Figure 5 TETRA-A2-PG macroscopic and single-channel conductances. (A) Macroscopic current developing under a 0–110 mV voltage ramp and with 3 nM tetramer. The maximum current at the top of the exponential curve was 5 nA, i.e. a conductance of nearly 50 nS. (B) A representative single-channel trace (1 nM, 125 mV) with conductance averaging 60 pS.

Indeed, from studies on the neutral monomeric alamethicin (Rf 50) and in the same conditions as the present ones, the single-channel conductance values were 50, 250, 1100, 2250, 3650 and 5250 pS for substates 1–6, respectively [26]. In the same study, a fluorescein-labelled alamethicin yielded 60 pS (always in 1 M KCl). From our present work on TETRA-A2-PG, it is safe to infer that it faithfully reproduces the first conducting substate that was previously and indirectly ascribed to a tetrameric bundle [2,27]. As regards the limited channel stabilization encountered here, it might be another consequence of using a linear or open template, instead of the cyclic ones used in the above-mentioned studies where the open state lifetimes were larger [17,18]. Overall, the present study further stresses the influence of the template design [28] on the functional properties of well-known channel-forming and antimicrobial peptides.

Finally, as low efficient concentrations and high voltage-dependence are favourable features for antimicrobial activity, and possibly for limited side-effects, this set of template-assembled alamethicins might prove valuable antibiotics. Overall, these tetramers are 10–100 times more effective than monomers for inducing macroscopic conductance. Of the three tetramers analysed here, the first one (TETRA-K4) appeared the most efficient in terms of lytic concentration, although with ill-defined channel-like events which were more reminiscent of membrane defects. As regards the two other tetramers yielding resolved single-channel events, TETRA-A2-PG appeared more efficient than TETRA-A3 to elicit a given conductance. Indeed if Figures 4 and 5 are compared, the threshold for the development of macroscopic conductance induced by 3 nM of the former was smaller than with 7.5 nM of the latter. Antimicrobial and haemolytic assays are underway.

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REFERENCES

1. Woolley GA, Wallace BA. Model ion channels: gramicidin and alamethicin. *J. Membr. Biol.* 1992; **129**: 109–136.
2. Sansom MSP. Structure and function of channel-forming peptaibols. *Q. Rev. Biophys.* 1993; **26**: 365–421.
3. Cafiso DS. Alamethicin: a peptide model for voltage gating and protein–membrane interaction. *Annu. Rev. Biophys. Biomol. Struct.* 1994; **23**: 141–165.
4. Duclohier H, Wroblewski H. Voltage-dependent pore formation and antimicrobial activity by alamethicin and analogues. *J. Membr. Biol.* 2001; **184**: 1–12.
5. Fox RO, Richards FM. A voltage-gated ion channel model inferred from the crystal structure of alamethicin at 1.5 Å resolution. *Nature* 1982; **300**: 325–330.
6. Hanke W, Boheim G. The lowest conductance state of the alamethicin pore. *Biochim. Biophys. Acta* 1980; **596**: 456–462.
7. Laver DR. The barrel-stave model as applied to alamethicin and its analogs reevaluated. *Biophys. J.* 1994; **66**: 355–359.
8. Mutter J, Hersperger R, Gubernator K, Muller K. The construction of new proteins: V. A template-assembled synthetic protein (TASP) containing both a 4-helix bundle and beta-barrel-like structure. *Proteins* 1989; **5**: 13–21.
9. Tuchscherer G, Grell D, Mathieu M, Mutter M. Extending the concept of template-assembled synthetic peptides. *J. Pept. Res.* 1999; **54**: 185–194.
10. Grove A, Tomich JM, Iwamoto T, Montal M. Design of a functional calcium channel protein: inferences about an ion channel-forming motif derived from the primary structure of voltage-gated calcium channels. *Protein Sci.* 1993; **2**: 1918–1930.
11. Oblatt-Montal M, Buhler LK, Iwamoto T, Tomich JM, Montal M. Synthetic peptides and four-helix bundle proteins as model systems for the pore-forming structure of channel proteins. I. Transmembrane segment M2 of the nicotinic cholinergic receptor channel is a key pore-lining structure. *J. Biol. Chem.* 1993; **268**: 14 601–14 607.
12. Futaki S. Peptide ion channels: design and creation of function. *Biopolymers* 1998; **47**: 75–81.
13. Pawlak M, Meseth U, Dhanapal B, Mutter M, Vogel H. Template-assembled melittin: structural and functional characterization of a designed, synthetic channel-forming protein. *Protein Sci.* 1994; **3**: 1788–1805.
14. Scrimin P, Tecilla P, Tonellato U, Veronese A, Crisma M, Formaggio F, Toniolo C. Zinc(II) as an allosteric regulator of liposomal membrane permeability induced by synthetic template-assembled tripodal polypeptides. *Chemistry* 2002; **8**: 2753–2763.
15. Leplawy MT, Kociolek K, Slomczynska U, Stasiak M, Zabrocki J, Beusen D, Marshall GR. Covalently linked bundles of the 1–17 alamethicin as models for membrane channels. In *Peptides 1994* (Proceedings of the Twenty-Third European Peptide Symposium), Maia HLS (ed.). Escom: Leiden, 1995; 385–386.

16. You S, Peng S, Lien L, Breed J, Sansom MSP, Woolley GA. Engineering stabilized ion channels: covalent dimers of alamethicin. *Biochemistry* 1996; **35**: 6225–6236.
17. Matsubara A, Asami K, Akagi A, Nishino N. Ion-channels of cyclic template-assembled alamethicins that emulate the pore structure predicted by barrel-stave model. *Chem. Commun.* 1996; 2069–2070.
18. Wassner AJ, Hurt JA, Lear JD, Akerfeldt KS. Synthesis and ion conductance behavior of a tetrameric alamethicin ion channel. *Org. Lett.* 2002; **4**: 1647–1649.
19. Duclohier H, Kociolek K, Stasiak M, Leplawy M, Marshall GR. *C*-terminally shortened alamethicin on templates: influence of the linkers on conductances. *Biochim. Biophys. Acta* 1999; **1420**: 14–22.
20. Molle G, Duclohier H, Julien S, Spach G. Synthetic analogues of alamethicin: effect of *C*-terminal substitutions and chain length on the ion channel lifetimes. *Biochim. Biophys. Acta* 1991; **1064**: 365–369.
21. Leplawy MT, Kociolek K, Szewczuk Z, Lisowski M, Duclohier H. Template assembled channels derived from *C*-terminally modified alamethicin. Synthetic effort. *Pol. J. Chem.* 2003; in press.
22. Montal M, Mueller P. Formation of bimolecular membranes from monolayers and study of their electrical properties. *Proc. Natl. Acad. Sci. USA* 1972; **69**: 3561–3566.
23. Eisenberg M, Hall JE, Mead CA. The nature of the voltage-dependent conductance induced by alamethicin in black lipid membranes. *J. Membr. Biol.* 1973; **14**: 143–176.
24. Hall JE, Vodyanoy I, Balasubramanian TM, Marshall GR. Alamethicin: a rich model for channel behavior. *Biophys. J.* 1984; **45**: 233–247.
25. Béven L, Helluin O, Molle G, Duclohier H. Wróblewski. Correlation between antibacterial activity and pore sizes of two classes of voltage-dependent pore-forming peptides. *Biochim. Biophys. Acta* 1999; **1421**: 53–63.
26. Helluin O, Dugast J-Y, Molle G, Mackie AR, Ladha S, Duclohier H. Lateral diffusion and conductance properties of a fluorescein-labelled alamethicin in planar lipid bilayers. *Biochim. Biophys. Acta* 1997; **1330**: 284–292.
27. Hanke W, Boheim G. The lowest conductance state of the alamethicin pore. *Biochim. Biophys. Acta* 1980; **596**: 456–462.
28. Wong AK, Jacobsen MP, Winzor DJ, Fairlie DP. Template assembled synthetic proteins (TASPs). Are template size, shape, and directionality important in formation of four-helix bundles? *J. Am. Chem. Soc.* 1998; **120**: 3836–3841.

