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Conductance properties of des-Aib-Leu-des-Pheol-Phe-alamethicin in planar lipid bilayers

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An alamethicin analogue in which all the amino-isobutyric acid and the C-terminal Pheol residues were replaced with Leu and an amidated Phe, respectively, has been synthesized. The purpose was to remove the ambiguity of a partial 3_{10} helical character in alamethicin and thus, to study the conductance properties of a virtually full α helical rod modelling the natural voltage-dependent ionic channels. Macroscopic and single-channel experiments are consistent with the 'barrel-staves' model. The sequence of the conductance ratios of the sub-levels is very similar to the alamethicin one. The main difference lies in the very short-lived fluctuations displayed by the new analogue and is discussed in terms of helical conformation and length.

Alamethicin, an antibiotic peptide consisting of 20 amino acid residues, forms voltage-gated ionic channels in black lipid membranes [1]. Despite the multi-level behaviour of the single-channel fluctuations and the rather high unit-conductance amplitudes most frequently observed, this simple molecule can model some of the mechanisms found in excitable biological membranes. For example, macroscopic sodium and potassium conductances in squid giant axons both increase e-fold for a 4-5 mV change in membrane potential [2], just as for alamethicin in black lipid membranes [3]. Furthermore, the amplitude of the lowest conductance sub-level (presumably involving four monomers) observed with alamethicin [4] agrees well with the unit-conductance of the sodium channels [5].

Nevertheless, alamethicin (sequence shown in Fig. 1A) is rich in the non-coded amino-isobutyric acid (Aib), not found in the recently sequenced natural channels of interest in higher organisms [6]. Besides, due to the presence of these eight Aib residues, there still remains some ambiguity as to which precise helical conformation (mostly α but also maybe a few 3_{10} turns) applies to alamethicin in lipid bilayers [7]. Consequently we synthesized an alamethicin analogue replacing all the Aib by Leucine and leaving, in this first stage, the C-terminus (Phe) amidated (sequence shown in Fig. 1A). This should transform the eventual partial 3_{10} character of the peptide to a more complete α helical structure, as much as the prolines allow it. Preliminary results concerning macroscopic current-voltage characteristics and single-channel conductances exhibited by this product (Des-Aib-Leu-Des-Pheol-Phe-alamethicin), called hereafter alamethicin L1, are reported here.

The solid-phase technique [8], with a benzhydrylamino resin, was used to synthesize alamethicin L1. The protocol was essentially the same as summarized elsewhere [9]. After completion of the synthesis, the N-terminus was acetylated and the HF treatment left an amidated C-terminus peptide. The lyophilized raw product

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A

Alamethicin (Rf 30):

1 14 20

Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Pheol

Des-Aib-Leu-des-Pheol-Phe-alamethicin:

Ac-Leu-Pro-Leu-Ala-Leu-Ala-Gln-Leu-Val-Leu-Gly-Leu-Leu-Pro-Val-Leu-Leu-Glu-Gln-PheNH$_2$

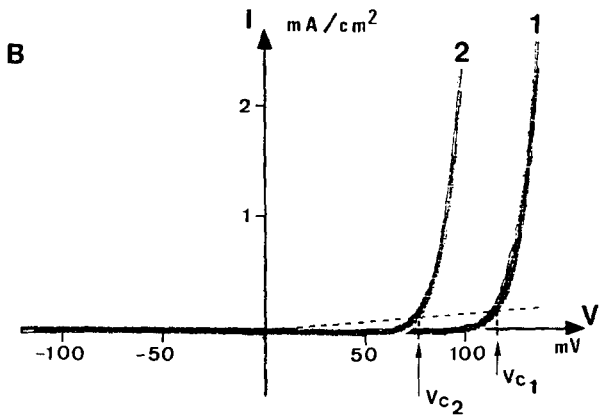

Fig. 1. (A) Comparison of the amino acid sequences of alamethicin and the newly synthesized des-Aib-Leu-des-Pheol-Phe-alamethicin (alamethicin L1). (B) Macroscopic current-voltage curves induced by alamethicin L1 in POPC/DOPE/DPPS/Chol (5:2:2:1) membranes. Both curves result from the superimposition of five voltage sweeps. Curve 1 is for an aqueous peptide concentration of $6 \cdot 10^{-8}$ M and curve 2 is for $1.2 \cdot 10^{-7}$ M. The dashed line represents a 3 nS conductance. Both compartments contain 1 M KCl; room temperature.

was then purified by HPLC on an inverse phase column under a gradient of methanol/water elution solvents. Fast atomic bombardment (FAB) mass spectrometry allowed the characterization of the purified peptide. The molecular mass was found to be 2 200 386 which, taking into account the isotopic profile, agrees with the expected 2 200 364. The experimental molecular envelope shown by the mass spectrum was identical to the theoretical one.

For macroscopic conductance experiments, virtually solvent-free planar lipid bilayers were formed by the apposition of two monolayers [10] on a 125 μm diameter hole in a Teflon film (25 μm thick) sandwiched between two glass half cells and pretreated with hexane/hexadecane (12:1, v/v). The lipid mixture * used was 1,2-POPC/DOPE/DPPS/cholesterol (5:2:2:1) dissolved (1%) in hexane/methanol (99:1, v/v). The rationale for this choice was that we wished to use the same lipids as in single-channel experiments and that a simpler mixture such as POPC/DOPE (7:3) resulted in current fluctuations at room temperature that were too rapid to be easily resolved even with the patch-clamp technique. Bilayer formation was monitored by the capacitance response and bare or doped membranes were tested with a triangular voltage waveform (at about 40 s/sweep). The voltage and current sign conventions are the usual ones and the electrolyte solution was 1 M KCl unbuffered. Fig. 1B shows the macroscopic current-voltage characteristics obtained for two alamethicin L1 aqueous concentrations. Note the asymmetry (no conductance development in the negative quadrant) as for alamethicin in bacterial PE membranes but also the much reduced hysteresis of the exponential branch between the rising and falling phases of the voltage sweep. The first point was expected since the new synthesized peptide was added to the *cis*- or positive-side and retains the negative charge near the

Abbreviations: 1,2-POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPPS, dipalmitoylphosphatidylserine; Chol, cholesterol; bacterial PE, *Escherichia coli* phosphatidylethanolamine.

The two former products were from Avanti Polar Lipids (Birmingham, AL, USA) and the three latter from Sigma (St. Louis, MO, USA).

C-terminus (Glu-18). As for the reduced hysteresis, it is indicative of short-lived channels as confirmed by the single-channel experiments to be described below. The crossing of the exponential branches with a reference conductance defines two characteristic voltages, V_{c_1} and V_{c_2} (see Fig. 1B). From V_e, the voltage increment producing an e-fold change in conductance and equal here to 6.75 mV, and from V_a, the V_c shift for an e-fold change in peptide aqueous concentration ($V_a = 54$ mV), a previously described analysis [11] allows the estimation of N, the apparent number of monomers per channel. For the new peptide, $N = 8$ and the apparent charge of one monomer during the gating event (product of the monomer charge by the intramembrane distance it moves) $\alpha = kT/NV_a = 0.46$, is not significantly different to values found for alamethicin and some other analogues in similar conditions [11].

For single-channel experiments, the same lipid mixture as above was used to form virtually solvent-free bilayers at the tip of patch-clamp pipettes [12]. High voltages (typically around 300 mV) were necessary to trigger single-channel activity even for slightly higher peptide concentrations than those usually sufficient with alamethicin. Multi-level events, compatible with the 'barrel-staves' model derived from alamethicin studies, were recorded (Fig. 2). It occurred in bursts with rather fast fluctuations within the bursts. In the recording taken at 9 °C and of which Fig. 2A presents a segment, there was a bursting activity 15% of the total time (10 s). Part B of the figure shows another example at a similar voltage but at 19 °C. Note that the mean open life-times of the sub-levels are larger than at the lowest temperature. This may be indicative of a phase separation in the membrane and a possible preference of the peptide for the most fluid lipid phase (see Ref. 13 for temperature studies of alamethicin activity in a single-phospholipid membrane).

The conductance histogram shown in Fig. 2C applies to the better resolved experiment (part B) but is in good agreement with three other trials. The absolute values of the sub-level conductances indicated on this histogram are slightly different

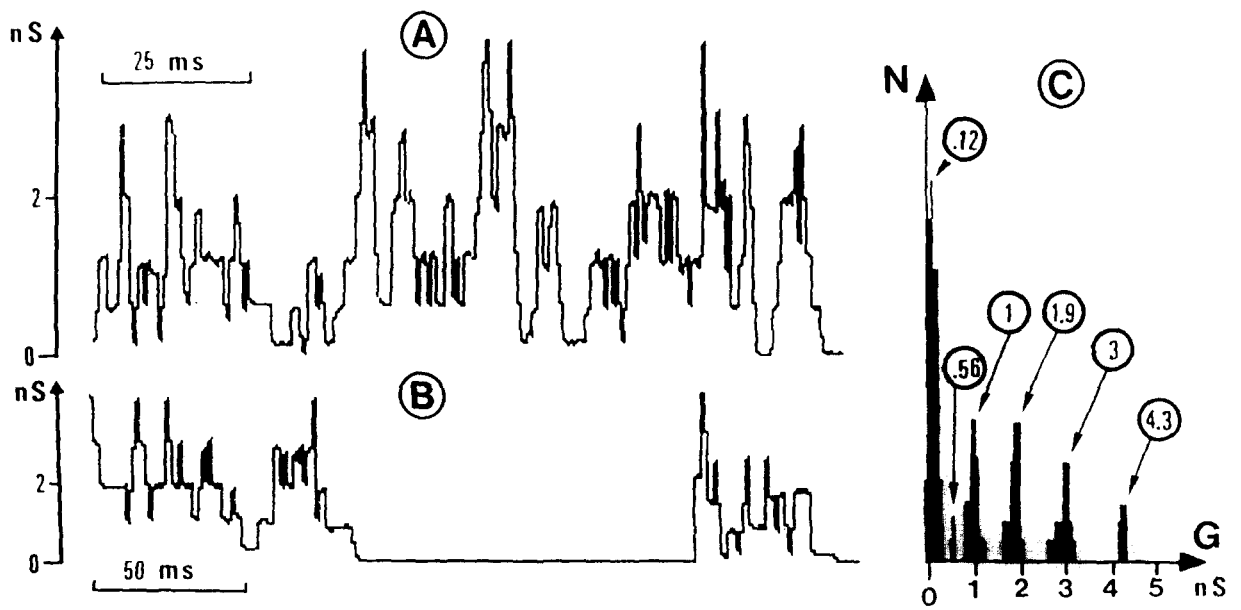

Fig. 2. Single-channel activity induced by alamethicin L1 in the same lipids as in Fig. 1B and with 1 M KCl. (A) At 9 °C and for a membrane potential of 290 mV. (B) At 19 °C and for 320 mV. The same peptide concentration ($2.5 \cdot 10^{-9}$ M) throughout. (C) Unit-conductance distribution of the sub-levels for a 10 s recording of experiment (B). The circled numbers give the conductance in nS of the different sub-levels.

from those displayed by alamethicin in similar conditions but the following sequence for the normalized ratios of conductances from one level to the next higher: 1: 4.6: 8.6: 13.8: 25.1: 35.6 agrees well, at least for the first five sub-levels, with the sequences found for alamethicin in various studies [4,14,15]. In the 'barrel-staves' model and if one assumes four monomers for the aggregate in the first substate [4,16] the two most probable sub-levels (1050 and 1900 pS) would consist of circular bunches of six and seven monomers. The macroscopic experiments giving an average number of eight, there should be at least one other sub-level (smaller than 120 pS and occasionally observed) if the two analyses are consistent. The main difference in the behaviour of the two analogues seems to lie in the kinetics of the channel fluctuations: the mean open life-time of the most frequent sub-level being 5–10-times smaller for the new peptide (alamethicin L1) than for natural alamethicin (Rf 30 fraction).

This situation is reminiscent of a recent study [17] with trichorzianines, another family of natural peptaibols closely related to alamethicin [18]. These peptides are one residue shorter (lacking Pro-2) and with serine in position 10 instead of glycine. 2D ^{1}H-NMR studies on these products [18] showed the coexistence of a few 3_{10} turns (at the N-terminus) with the predominant α helical structure whose residue length is smaller than for 3_{10} structure (1.5 vs. 2 Å, respectively). With alamethicin L1, the 3_{10} helical turns are replaced with α turns and for the same number of amino acids as alamethicin, the intramembrane helical part (from N-terminus up to Pro-14, see Ref. 19) should be shorter. Accordingly, as the results above show, the threshold voltage is higher and single-channel fluctuations faster. A longer analogue could possibly restore the usual alamethicin activity pattern. It may also be that the bulkier side chains of leucine modify the stabilization of the aggregates. Another alternative would be the role of Pheol (instead of the amidated Phe in the present derivative). Our current peptide synthesis programme aims at testing these hypotheses but it would appear that the new peptide, alamethicin L1, is an appropriate model to study the ionophore properties of α helices since the voltage dependence of its conductance (V_e) falls in the range found for alamethicin on the one hand [20] and for the voltage-dependent channels of natural excitable membranes on the other.

Recent studies on synthetic peptaibols analogs $(Ala\text{-}Aib\text{-}Ala\text{-}Aib\text{-}Ala)_n$ and without any proline, likely to adopt a fully α-helical structure, point to strongly voltage-dependent channels although the multistate single-channel behaviour is less well-resolved [21,22]. Our results clearly demonstrate that a leucine/Aib exchange does not alter the characteristic channel forming properties.

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