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Rapid report

Antiamoebin can function as a carrier or as a pore-forming peptaibol

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Abstract

Antiamoebin is a 16-residue polypeptide whose crystal structure and lytic activity in membrane vesicles have recently been reported. It is a bent helical molecule and a member of the peptaibol family of antibiotics. Under conditions which produce voltage-dependent conductance activity by other members of the family, no single-channel conductance was detected for antiamoebin, and a carrier-like mechanism was put forward to account for its mode of action. We now present evidence for pore formation that is largely voltage-insensitive, with large amplitude single-channel events on top of a background conductance that may account for the previously proposed carrier-like activity. Thus, antiamoebin may be the first instance of a peptide which can function both as an ion carrier and a pore former.

Keywords: Peptaibol; Conductance; Planar lipid bilayer; Membrane transport; Crystal structure; Alamethicin

Antiamoebins are polypeptide antibiotics, rich in α-amino isobutyric acid (U in the single letter code), isolated from *Emericellopsis* and *Cephalosporium* strains of fungi. They are 16 residues long and the main fraction, antiamoebin I (hereafter referred as AAM) has the sequence [1]:

AcF U U U J G L U U O Q J O U P Fol

where J is isovaline, O is hydroxyproline, and Fol is phenylalaninol. It has three imino acids that result in a deep bend in the centre of the molecule, connecting two helical segments [2]. It is highly homologous, especially in its C-terminal region, with Leu-zervamicin [3], another 16-residue member of the peptaibol family, which has been shown to form channels in bilayer lipid membranes [4]. Preliminary reports suggested that AAM had some membrane-modifying properties (calcium flux in liposomes and mitochondrial uncoupling) [5] and indicated a non-standard behaviour in macroscopic conductance measurements [6]. It was presumed it would function in a highly voltage-dependent pore-forming manner similar to that of the longer peptaibols, by forming a barrel-stave arrangement of helices that produce multistate behaviour, such as been proposed for the best-characterised member of the family, alamethicin [7–10].

In a recent structure/function study of AAM [2], we reported that while the polypeptide was seen to induce ion permeability in lipid vesicles, we found no evidence for channel-like activity under a variety of conditions, including ones that produced channels of Leu-zervamicin or alamethicin. Due to the exponential decay observed, a hypothesis for a carrier mechanism accounting for membrane transport function of AAM was put forward. We now report that AAM

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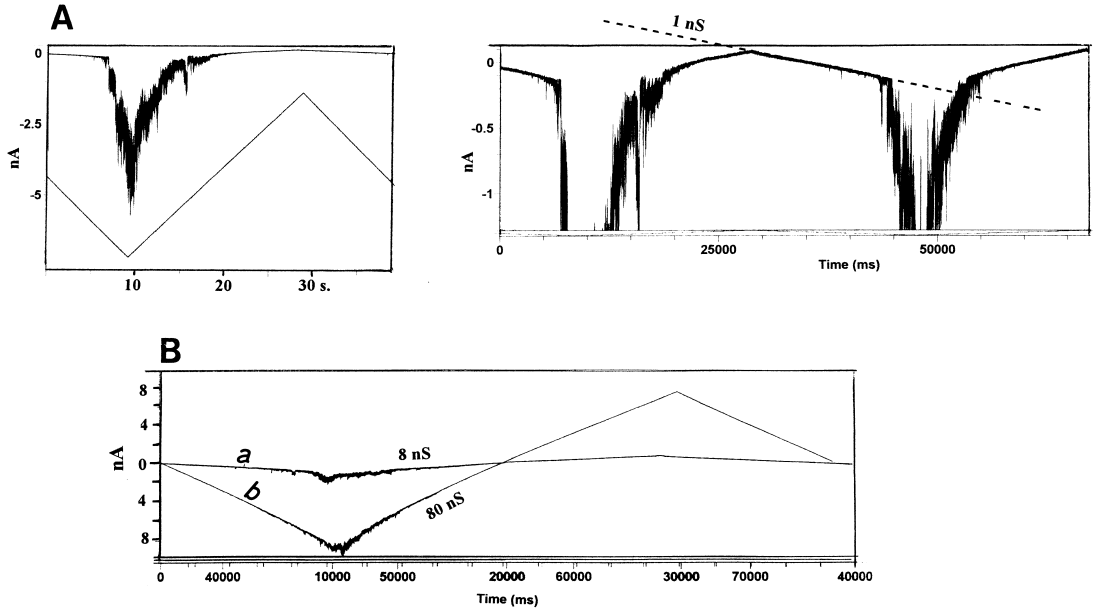

Fig. 1. Macroscopic current–voltage curves induced by AAM in Montal–Mueller solvent-free bilayers. Electrolyte: 0.5 M KCl, pH 7.4 both sides. Current responses to slow voltage ramps of ±100 mV and with increasing AAM concentrations: (A) 100 nM, $V_e = 20$ mV; enlarged on the right to highlight the 1 nS slope voltage-independent conductance), and (B) at 200 and 400 nM (curves a and b).

can, under some conditions, form pores in planar bilayers, albeit in an atypical way, especially as regards voltage sensitivity and conductance distribution, when compared to other well-studied peptaibols.

AAM (Sigma, UK) was purified by HPLC following the protocol of Das et al. [11]. The identity and purity of the peptide was checked by mass spectrometry. For recording macroscopic current–voltage (I–V) curves and single-channel events in some experiments, virtually solvent-free planar lipid bilayers were 'folded' [12] over a 200 μm diameter hole in a PTFE film pretreated with 4% hexadecane in hexane. The lipids used to form monolayers on top of either 0.5 or 1 M KCl, 5 mM HEPES (pH 7.4) in the two compartments were a mixture of palmitoyloleoylphosphatidylcholine (POPC) and dioleoylphosphatidylcholine (DOPE) (7/3), from Avanti Polar Lipids (Alabaster, AL). For most of single-channel experiments, bilayers of these same lipids were formed at the tip of patch pipettes [13] and a standard patch-clamp apparatus was used.

Relatively high AAM concentrations (of the order of 100 nM, i.e. about five times that typically used to form alamethicin channels) had to be used to induce significant macroscopic conductances. In most experiments, voltage-independent or slope conductance developed with amplitudes strongly sensitive to AAM bulk concentrations (Fig. 1A). Out of three sets of three experiments where the concentrations were progressively doubled between 100 and 400 nM and on applying the relation $G_j/G_i = (C_j/C_i)N$, where G_i is the macroscopic (slope or background) conductance associated with the concentration C_i [14] yielded $N = 4$ as the apparent mean number of monomers per conducting aggregate. Only at the end of responses were fluctuations reminiscent of channel activity observed. The voltage-dependent quasi-exponential branch shown in Fig. 1B that is typical of alamethicin, zervamicins and other peptaibols, was very rarely encountered (twice out of 10 experiments) and it was not possible to record two such curves at different concentrations on the same bilayer. The voltage-dependent parameter V_e (the voltage increment producing an e-fold change in conductance) is a modest 20 mV (when compared to alamethicin and derivatives values of 5–6 mV).

Fig. 2 shows channel-like activity obtained on the same type of bilayers as above but for reduced AAM concentrations and higher voltages. Part A is a 1.5 s recording at the start of the experiment. Medium-sized fast switching channels evolve towards a pat-

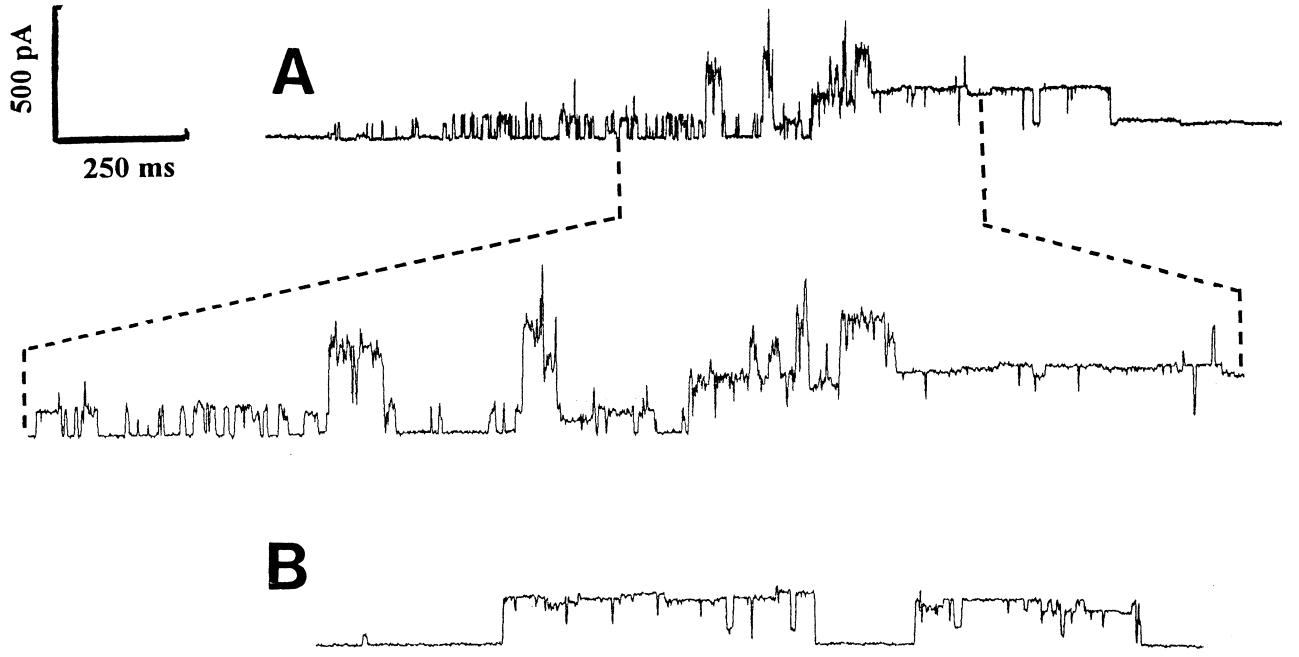

Fig. 2. Single-channel events in Montal–Mueller bilayers. Low AAM concentration (25 nM, *cis*-side) and high applied voltage: 160 mV. Electrolyte: 0.5 M KCl, pH 7.4 both sides. (A) Fast fluctuations to a single open state around 480 pS evolve towards pseudo multi-state behaviour (1.6 nS) and stabilisation of longer duration events with a conductance which is twice the initial one (960 pS). (B) Same bilayer later on, the same pseudo-stabilised level is retrieved with smaller substates on top.

tern that is reminiscent of alamethicin before quickly stabilising at upper levels. Another stretch of high conductance states and long duration channels is shown in Fig. 2B (same bilayer, later on). As it allows a better resolution of small and fast channels (through a reduction of noise associated with bilayer capacitance and area), the tip-dip method was implemented. With this method, smaller levels could be recorded as shown in Fig. 3. In 1 M KCl, two events of 90 pS are superimposed on top of a significant background or leak (trace A). On reducing the applied voltage (from 90 to 75 mV), only one single level of similar conductance (90 pS) but of shorter duration was observed (trace B). Thus, there seems to be some voltage-sensitivity at the single-channel level of investigation as well. When pooled together from a number of experiments and normalised for 1 M KCl, the different single-channel conductances are thus 90 pS (as in Fig. 3), 480, 960 and 1600 (Fig. 2), a further level at 3200 pS being also more rarely observed. When normalised to the smaller level, these conductances levels follow a sequence (1:5.3:10.6:18:36) which does not obey the regular geometric progression that is typical for a dynamic barrel-stave of amphipathic helices [15]. Indeed, the macroscopic and single-channel activities reported here for AAM are quite different from those of other peptaibols so far studied, whether of the long sequence class (alamethicin, trichorzianins [7,16,17]) or short ones such as harzianins [18] and the homologous zervamicins [4].

The question then arises as to why AAM behaves so differently from the highly homologous Leu-zervamicin. Both polypeptides are composed of the same number of amino acids, have acetylated N-termini and phenylalaninol at their C-termini, have an abundance of α-amino acids and three imino acids. There are, however, a few significant differences between AAM and Leu-zervamicin. AAM has a larger bend angle in the centre of the molecule [2], which has two structural consequences: (1) AAM is slightly shorter than Leu-zervamicin, and (2) its dipole moment differs in direction and magnitude. The length is unlikely to be the reason for differences, as it is less than 1 A shorter than zervamicin and even in the thin phosphatidylcholine membranes under identical conditions used for zervamicin, AAM is not active as a channel. However, the differences in dipole mo-

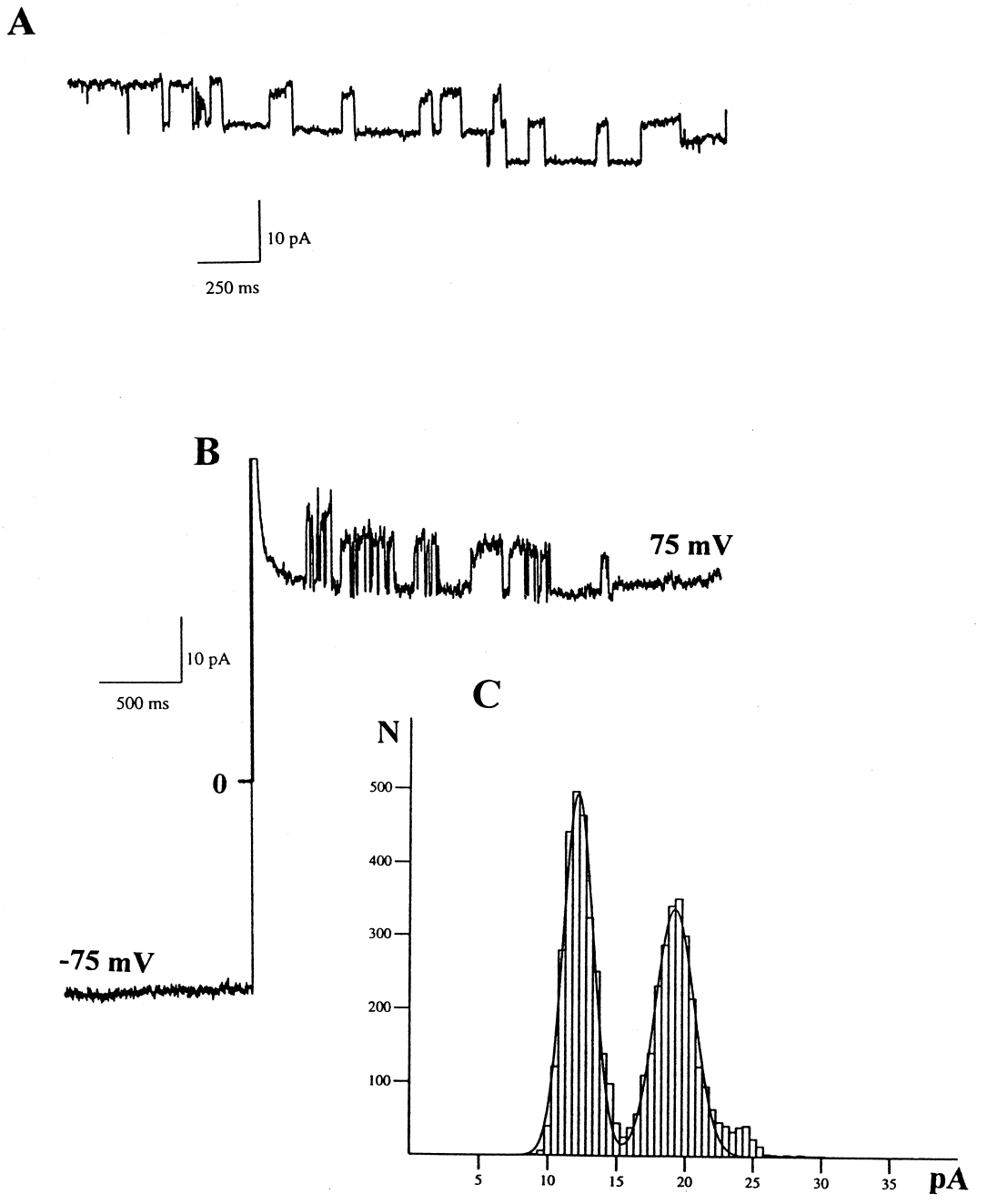

Fig. 3. Single channel events induced by AAM in neutral bilayers formed at the tip of patch pipettes. Electrolyte both sides is 1 M KCl, 5 mM Hepes buffered to pH 7.4. AAM concentration was 100 nM (*cis*-side). (A) 90 mV applied, the current fluctuates between an apparent closed state and two open levels of 90 pS each. Note that there was a significant leak (or background current, of about 40 pA at 90 mV, possibly accounting for the slow rippling of the trace. (B) on another bilayer which was silent at −75 mV, pulsing to 75 mV induce a transient activation of fast events whose conductance (see the distribution or histogram in C) is also 90 pS.

ments may be important. For AAM, the dipole moment direction is at more of an angle to the helix axis than is zervamicin's (which lies nearly parallel to the helix axis). Perhaps the most significant difference between the molecules, however, is that Leu-zervamicin has polar Gln and Thr residues at positions 3 and 6, whereas these are non-polar residues in AAM. It is noteworthy that these two polar residues lie on the

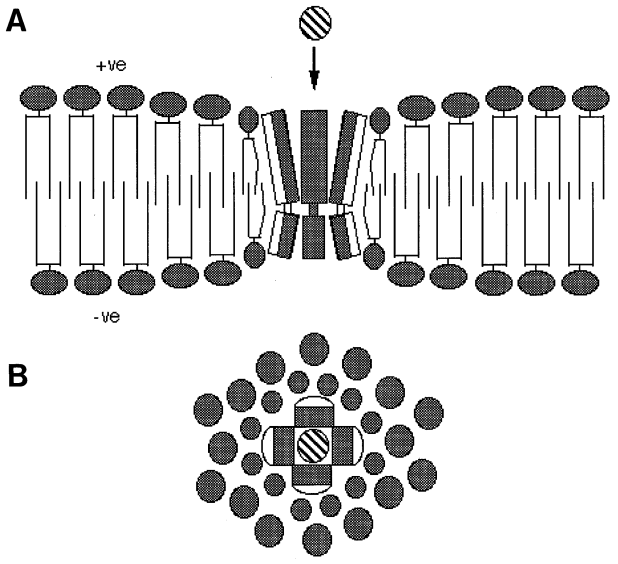

Fig. 4. Schematic diagram of proposed channel formation by AAM. The grey areas indicate hydrophilic areas. (A) A slice through the centre of the channel. (B) View looking down onto the bilayer.

same face of the helix, and thus may play a role either in helix bundle stabilisation via inter-helices H-bonds [19,20] or in the formation of the conductance pathway [2]. Conductance measurements on the synthetic zervamicin analogue Zer-AI-16 [4] in which these polar residues are replaced with apolar residues, indicate this molecule also does not form such stable channels as the zervamicin parent.

Finally, why does AAM form channels in DOPE/POPC but not in membranes made of glycerol monooleate (formed either with hexane or hexadecane) or diphytanoyl-phosphatidylcholine, or amoeba lipid extracts, as used in the previous study [2]? It is relatively unlikely to be due to the lipid chain lengths, as the original membrane thicknesses used should bracket the membrane thickness used in the present study. However, it may be significant that the present study employed lipids with positively charged headgroups which also favour hexagonal phase formation to some extent. In addition, and perhaps more importantly, the present studies found conductance only at much higher concentrations of peptide. Thus, it may be that the channels detected constitute a minor population of the total peptide present and that the voltage-independent background conductance is due to the carrier conformation which is the major membrane-active component in these samples, or to a non-specific leakage as found for lytic peptides. The lower (and single) value of N and the difference in voltage-dependence suggest that the mechanism of channel formation may be somewhat different from that of alamethicin, which recruits additional monomers into its barrel staves to produce multiple conductance levels. We propose the transient formation of discrete pores at high concentrations (Fig. 4) which are in equilibrium with the type of carrier complexes proposed earlier [2]. This appears to be the first instance of a peptide endowed with such a dual functioning on membranes.

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