Discrimination of Conductance of Lower and Higher Oligomeric Alamethicin Pores

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Abstract: Experimental studies of antimicrobial peptides having a pore-forming mechanism of action have recently attracted increasing interest due to their broad spectrum of activity and numerous clinical applications. Alamethicin has been widely studied as an antimicrobial peptide and a model for ion channel-forming proteins. However, the lack of simple analytical tools for studying mechanisms of interaction of pore-forming peptides and biological membranes at very low peptide concentrations remains a problem. Here in a short report, we describe an experimental approach for performing time-dependent discrimination of transmembrane current induced by fractions of lower- and higher-order oligomeric alamethicin pores using mitochondria.

Keywords: Alamethicin, Pore formation, Potassium transmembrane current, Rat liver mitochondria.

INTRODUCTION

Antimicrobial peptides (AMPs) have been extensively studied in recent years due to increasing interest in their medical use and the development of new pharmaceuticals to combat emerging antibiotic resistance. It is well known that many AMPs interact with microbial cell membranes by permeabilizing them, thus forming conducting pores [1, 2].

Alamethicin is a 19-residue channel-forming peptide, a member of the family of peptaibols (containing 8 α-aminoisobutyric residues), isolated from the fungus *Trichoderma viride*, which have been intensively studied [1, 3]. This peptide can insert into membranes and form voltage-dependent self-organized transmembrane channels of various conductivity and different diameter depending on its oligomerization [3, 4]. The smallest alamethicin pores [5] are impermeable for hydrated $Tris^+$ and Ca^{2+} cations (the latter having diameter 4.12 Å [6]), but permeable for K^+ (hydrated diameter 2.32 Å) [7]). The largest channels are permeable to ATP (hydrated diameter 20Å [8]). The lowest conductance state of alamethicin induces fluctuations of conductivity more sensitive to pH-dependent variation of the membrane surface potential and modulators of membrane dipole potential [9]. Direct analysis of the high-order oligomeric structure of alamethicin pores [10] visualized its protein-like nature by the barrel-stave model [1, 3] that has been described in numerous studies [4, 11].

A direct approach for investigation of the relationship between degree of alamethicin channel oligomerization and transmembrane conductivity using dimers and tetramers cross-liked by means of a flexible linker (attempts to stabilize the lowest conductance sub-states of this peptide in bilayer lipid membrane (BLM)) did not give clear results [12]. This shows the need for indirect approaches.

Here we have used tightly coupled mitochondria and a sensitive oximetric cell as a biosensor to determine transmembrane cationic current induced by alamethicin.

MATERIAL AND METHODS

Tightly coupled intact rat liver mitochondria (RLM) were isolated by the modified method of Weinbach using the heavy mitochondrial fraction (centrifugation: 5500g for 10 minutes), while all fractions of these organelles are sedimented at 12,000g [13]. RLM protein concentration was determined by a modification of the Bradford method [14]. Rates of succinate oxidation of RLM (v) were determined under isothermic conditions (25ºC) with a Clark-type electrode exhibiting a half-time of response of 20 s [15] in the main Tris monocationic isotonic incubation medium (pH 7.2) containing 4 μM horse cytochrome *c*, 35 mM tris (hydroxymethyl) aminomethane (Tris), 1.5 μM rotenone, (all Serva, Germany), 209 mM sucrose

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(Merck, Germany, 10 mM succinic acid, 1 mM phosphoric acid, 6 mM $MgCl_2$, 2 mM ethylenediaminetetraacetic acid (all chemically pure Reakhim, Russia). Oximetric effects of alamethicin were normalized by the initial rate of RLM respiration (v_0) to minimize errors associated with accuracy of addition of RLM preparations. We also used medium with decreased content of $MgCl_2$ (2 mM) and other components as in the main Tris monocationic isotonic incubation medium. In addition we used medium with increased content of Tris (70 mM), decreased content of sucrose (174 mM), 2 mM $MgCl_2$ and other components as in the main Tris monocationic isotonic incubation medium. In the course of the experiment, 110 nM alamethicin from *Trichoderma viride* (Fluka, Switzerland) and KCl of special pure grade (Reakhim, Russia) were used. Alamethicin and rotenone (the latter was added just before addition of RLM to the incubation medium) were added using concentrated stock solutions in ethanol.

RESULTS AND DISCUSSION

Earlier, it was shown that the transmembrane cationic current induced in RLM by various inducers of permeability depends linearly on the degree of activation of the RLM respiration [16]. Figure **1A** shows that the first steady-state phase of RLM respiration activation by alamethicin is absent in Tris monocationic incubation medium; it appeared only in the presence of added KCl.

In model experiments on bilayer lipid membranes formed from bacterial phosphatidylethanolamine/-hexane solution, single pore conductance fluctuations induced by alamethicin have been measured [5]. Figure **1B** demonstrates a scheme of such conductance fluctuations and their values in 1 M KCl and in 1 M Tris-HCl (the upper and the lower scheme, accordingly) based on the data from Table **1** [5]. The lowest conductance sub-state of fluctuation (19 pS, attributed to cationic current through an alamethicin pore with the lowest oligomerization degree) was found in 1 M KCl but not in 1 M Tris-HCl [5]. Comparison of these data with the results obtained in our experiments using RLM preparations (Figure **1A**) showed that the results are consistent with each other. The time course of RLM v_0 activation by alamethicin in the presence of KCl in the main Tris monocationic isotonic incubation medium is biphasic (Figure **1A**). The first phase of RLM v_0 activation can be determined by the cationic current through an alamethicin pore with the lowest oligomerization degree only (Figure **1A**). In our experiments, it could be potassium transmembrane current. Theoretically, the absence of this first phase of RLM v_0 activation without addition of KCl can be related to the low $Tris^+$ concentration. Figure **2**. shows that increasing the $Tris^+$ concentration in the medium from 35 to 70 mM (values physiologically possible and safe for RLM) did not promote the appearance of the first phase of dependences.

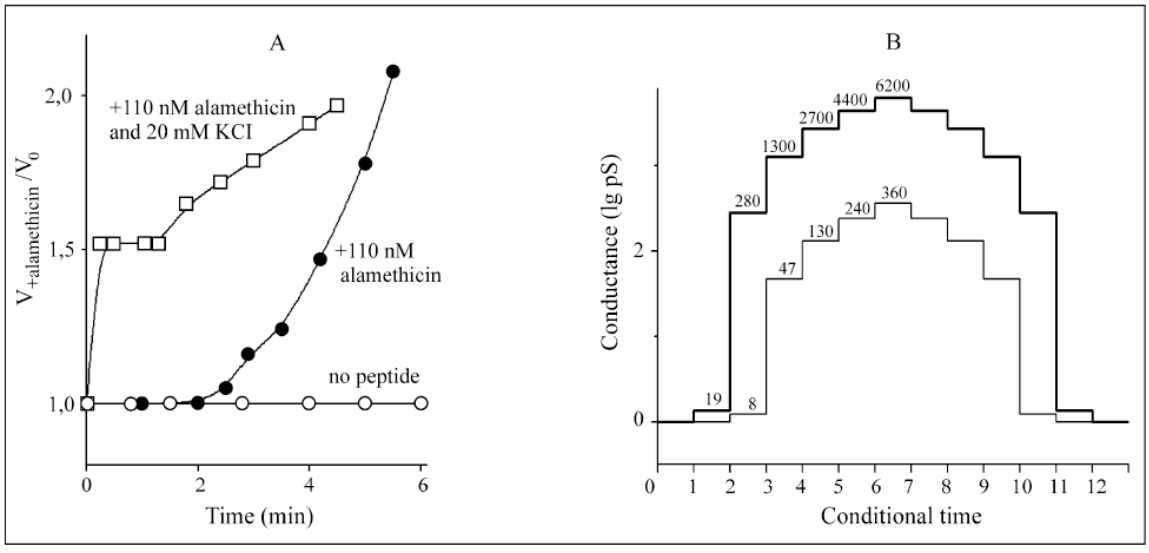

Figure 1: A Time course of degree of activation of RLM v_0 respiration (v_0) by 110 nM alamethicin ($v_{+alamethicin}$) in Tris monocationic medium in the presence and in the absence of 20 mM KCl (**A**). Plots are representative of three different RLM preparations. B. Scheme of fluctuations of conductivity in lipid bilayer membrane induced by alamethicin in 1M KCl (upper diagram) and 1 M Tris-HCl (lower diagram), time is conditional (**B**). Conductance values (lg pS) for the different sub-states are adopted from the Table **1** [5]. Non-transformed conductance values in "pS" are presented at each respective "step" (**B**).

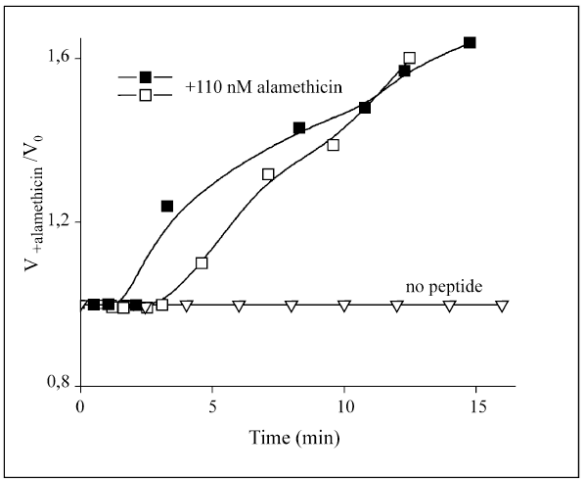

Figure 2: Time course of degree of activation of RLM respiration (v_0) by 110 nM alamethicin ($v_{+\ alamethicin}$) in Tris monocationic media with 2 mM $MgCl_2$ containing lower (35 mM) and higher (70 mM) concentrations of $Tris^+$ (upper -■- and lower -□- curves, respectively). Plots are representative of three different RLM preparations.

However, the slope of the curve of the second phase increased. Apparently, $Tris^+$ transmembrane current through lower-oligomeric alamethicin pores has not appeared, but this current through higher-oligomeric pores has increased.

CONCLUSION

Low conductance values of the low-oligomeric alamethicin pores (Figure **1B**) make it impossible to examine the conductance of fractions of such pores against the background of high-oligomeric pores on BLM or liposomes. Our experimental approach may be useful to perform time-dependent discrimination of transmembrane current induced by fractions of lower and higher-order oligomeric alamethicin pores in inner mitochondrial membrane using RLM. This approach will enable exploration of the details of the interaction of alamethicin with mitochondrial membranes and the mechanism of the initial phase of permeabilization. In addition, it is of interest to compare the low conductance values for alamethicin analogues and derivatives of this peptide with substitutions of key amino acids.

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