EFFECTS OF ANTIMICROBIAL ACTIVITIES ON D- AND L-BOMBININ USING ARTIFICIAL BACTERIA CELL-MEMBRANE

Y. Sekiya[1*], H. Watanabe[1], Y. Kitahashi[2], I. Kawamura[2], R. Kawano[1]
[1] Tokyo University of Agriculture and Technology, JAPAN and
[2]Graduate School of Engineering, Yokohama National University, JAPAN

ABSTRACT

Bombinins are one of antimicrobial peptides extracted from Yellow-bellied toed. The bombinins have mainly two sequences which have only difference of the single amino acid chirality in the second position of the N-terminus. Interestingly, they show the clear distinction of the antimicrobial activities. We has recently found that two bombinins mixture in a molar ratio of 1:1 has the highest antimicrobial activity against *E. coli*, but the mechanism has been still unclear. In this study, we prepared artificial bacteria model cell-membrane using micro-droplet system and analyzed the bombinin-membrane interactions in terms of the transmembrane current signals at the molecular level.

KEYWORDS: Bilayer Lipid Membranes, Antimicrobial Peptide, Bacteria Model Cell-membrane

INTRODUCTION

Most of animals and plants have antimicrobial peptides for the protection from invasion of bacteria into their bodies. Antimicrobial peptides bombinins are recently expected to become more effective therapeutic medicine for *Leishmaniasis*, which 12 million people infected all over the world. Bombinin have two different sequences, bombinin H2 [I-(L-Ile)-GPVLGLVGSALGGLLKKI-NH_2] and bombinin H4 [I-(D-allo-Ile) GPVLGLVGSALGGLLKKI-NH_2], which have L- or D- amino acid residue in the second position of the N-terminus [1]. Although this difference is only dependent on chirality in the single amino acid, the two types of bombinins show the different antimicrobial activities. Dr. I. Kawamura and co-workers have found that a mixture of H2 and H4 bombinin in a ratio of 1:1 (bombinin H2/H4 mix) has the highest antimicrobial activity against *E. coli*, and they have considered that some of the synergistic effect of H2 and H4 occurs at the molecular levels. Because of our previous studies, we trusted that several bombinin molecules form nanometer size pores, which leak cytoplasm through the pores in cell membrane. Therefore, we presumed that the mechanism of antimicrobial activities on bombinins are able to be clear in terms of the pore-forming activity in the membrane. In this study, we prepared artificial bacteria model cell-membrane with "droplet contact method" [2] and measured transmembrane current of reconstituted bombinin in membrane. Using the current conductance, we estimated the pore size and the association state of each bombinins.

EXPERIMENTAL

We used micro-droplet system for preparing artificial Bilayer Lipid Membranes (BLMs). Our device is shown in Figure 1A, the two micro-droplets were separated with hydrophobic film (paryleneC) which was processed to make a 100 μm diameter hole by MEMS technique. Two lipid monolayers contacted together to prepare a stable and reproducible BLM in the parylene film. This method has an advantage for prepare model cell-membranes, e.g. bacteria or mammalian cell membranes. We used some lipids for bacteria in the composition of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) / 1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycero (DOPG) (3:1, mol/mol) (Avanti Polar Lipids, USA). The both chambers contained the 2.4 μL of 10 mg/mL lipids mixture in decane and 4.7 μL of 100 μM each bombinin in the buffer consisting of 1 M KCl, 10 mM MOPS at pH 7. Besides, one of the side was applied the potential of +100 mV and the other side was connected to ground. Bombinin H2 and H4 were synthesized by microwave-assisted solid-phase peptide chemistry and purified by reverse phase HPLC on a C-18 column. Bombinin was reconstituted in the BLMs, and they allowed ions to pass through nanopore between chambers under the voltage gradient (Figure 1B). The channel current signals can be measured by a volt-

age clamping method using by Jet Patch Clamp Amplifier (Tecella, USA). We reconstituted each bombinins in BLMs and evaluated pore properties respectively. Bombinin pore diameters were calculated using Hill's equation from pore conductance [3]. In addition, we also calculated electric charge through pores per unit time (called "charge flux" [4]) to compare each bombinin antimicrobial activities.

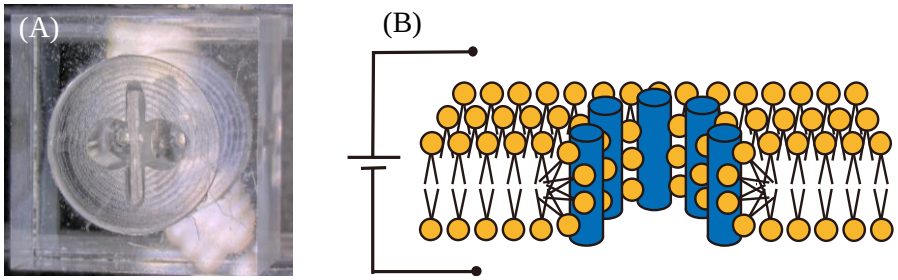

Figure 1: (A) A photograph of the device fabricated by MEMS technology for preparing the BLMs. (B) A pore-formation model of bombinin reconstituted in BLMs and ions pass through the nanopore under the potential gradient.

RESULTS AND DISCUSSION

In the current recordings of bombinin H2, bombinin H4 and bombinin H2/H4 mixture, the several levels of the amplitudes of current signals were observed. In these results, we found that bombinins form several types of pore-formation, and the typical multi-conductance signal is shown in Figure 2. This result suggests that the heterogeneous size of pores was formed by the oligomerization with various numbers of the monomer. However, the step-like current signals were occasionally appeared, suggesting the rigid pore-formation. Using this step-like signals and Hill's equation, we calculated the pore diameter of bombinin H2, bombinin H4 and bombinin H2/H4 mixture in the bacterial model membrane: 1.94 nm, 2.10 nm and 2.88 nm, respectively. In addition, we measured the charge flux in the case of the fluctuated signals shown in Figure 2. As the results of charge flux measurements with bacteria model membrane, H4 is the largest, followed by H2/H4 mix and H2. These results are the different from previous studies. We are going to study further bombinin antimicrobial activity in *Leishmania donovani* (a kind of *Leishmaniasis* germ) and mammalian erythrocytes model cell-membranes, then compare the difference of antimicrobial activities caused by membrane lipid composition.

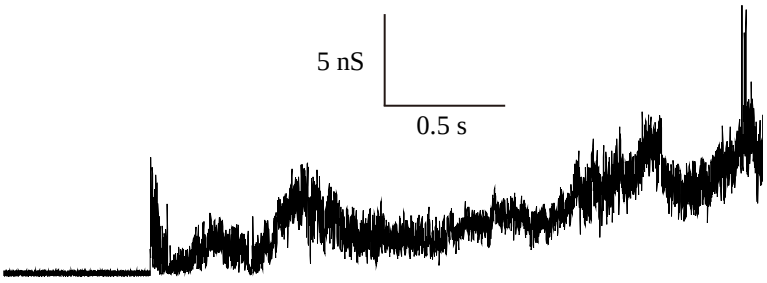

Figure 2: The typical current and time trace of the bombinin H4 nanopore at a voltage of +100mV. Bombinin formed heterogeneous sized and size-fluctuating nanopores in the DOPE/DOPG (3:1, mol/mol) membranes and the current increased according to the pore formation. The both chambers were filled with 100 µM bombinin H4 in buffer (1 M KCl, 10 mM MOPS).

CONCLUSION

We reconstituted each bombinin nanometer size pores in model cell-membranes and evaluated the properties of these pore-forming antimicrobial peptide. Bombinin H2, H4 and H2/H4 mix formed heterogeneous sized and size-fluctuating nanopore, which caused the increase of the channel current. Antimicrobial activities which were evaluated with charge flux suggested that H4 containing the D-amino acid residue had the largest charge flux activity. We believe that this result will lead to development of effective medicine against *Leishmaniasis*.

ACKNOWLEDGEMENTS

This work was partly supported by a Grant-in-Aid for Scientific Research on Innovative Areas (No. 24104002 and 26104513) and Scientific Research No. 26540160 and 25708024 of The Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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CONTACT

* Y. Sekiya e-mail: 50014251507@st.tuat.ac.jp