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Channel Current Analysis for Pore-forming Properties of an Antimicrobial Peptide, Magainin 1, Using the Droplet Contact Method

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This study describes the pore-forming properties of magainin 1 in planar lipid bilayers. These bilayers were prepared by the droplet contact method, which was executed on a microfabricated device for a high-throughput study. We arrayed four droplet chambers parallelly in the single device, and the current measurements were carried out simultaneously. Using this system, we measured the channel current conductance of magainin 1. We determined the pore size and the number of assembling monomers in magainin pores in mammalian and bacterial model membranes. This system is a powerful tool for analyzing transmembrane peptides and their antimicrobial activities.

Keywords Droplet, lipid bilayers, electrophysiology, magainin 1, antimicrobial peptide

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Introduction

Magainins are antimicrobial peptides (AMPs), which were discovered in the skin of the African clawed frog *Xenopus laevis*. This frog can remain infection-free in nature;[1-4] this is believed to be mainly because of the presence of Magainins. Magainins have 23 amino acid residues and demonstrate broad-spectrum antibacterial and antifungal activities.[5] To apply a clinically useful antimicrobial therapeutic agent, extensive studies, such as NMR,[6,7] Raman spectroscopy,[8] fluorescence,[3,9] and electrophysiological studies,[1,10] have been performed to analyze the antibacterial mechanism. Among them, an electrophysiological method that uses lipid bilayers is an integral tool for analyzing lipid-peptide interactions and pore formations. However, the electrophysiological experiment requires technical skills for preparing planar lipid bilayers, and is time consuming. Therefore, efficient electrophysiological methods are required.

We recently proposed stable and reproducible planar lipid bilayer formation using microdroplets in a "droplet contact method" (DCM).[11] A planar lipid bilayer can be spontaneously formed at the interface between contacting microdroplets in an oil/lipid mixture solution.[12,13] In this method, membrane stability is improved by reducing the contact area. The long-term stability of the membrane is notable, and remains for more than 300 h. Moreover, we constructed a droplet array for high-throughput measurements.[11] Using bilayer lipid membranes (BLMs), we have been measuring various membrane protein and biological nanopores, *e.g.*, single molecule detection using DNA aptamers,[14] high-throughput ion channel measurements at the single-protein level,[11,15] solution-exchange experiment in the microdroplet system,[16] and the development of portable nanopore sensors.[17]

In this paper, we report on the pore-forming properties of magainin 1 (GIGKFLHSAGKFGKAFVGEIMKS) from channel current conductance measurements using the high-throughput DCM. Both magainin 1 and magainin 2 are isolated from *Xenopus laevis*, and have a similar amino-acid sequence. It has been known that magainin 2 has higher antibacterial activity than magainin 1. Although mgainin 2 is a popular AMP and there are extensive studies, molecular mechanism of magainin 1 is still in challenge. Magainins and many AMPs form transmembrane pores by assembling monomers. This study focuses on determining the pore size and the number of assembling monomers. Thus, we mathematically estimate this number from the current conductance and a polygonal model. Our methods have significant potential for AMP analysis and in clinical applications.

Experimental

Reagents and chemicals

The reagents used in this study were as follows: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, Avanti Polar Lipids, Alabaster, AL); 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, Avanti Polar Lipids); 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (DOPG, Avanti Polar Lipids); *n*-decane (Sigma-Aldrich, St. Louis, MO); 3-morpholinopropane-1-sulfonic acid (MOPS, Nacalai Tesque, Kyoto, Japan); and KCl (Nacalai Tesque). Buffered electrolyte solutions were prepared from ultrapure water, which was obtained from a Milli-Q system (Millipore, Billerica, MA). Magainin 1 (LKT Laboratories Inc., St. Paul, MN) was obtained as a monomer

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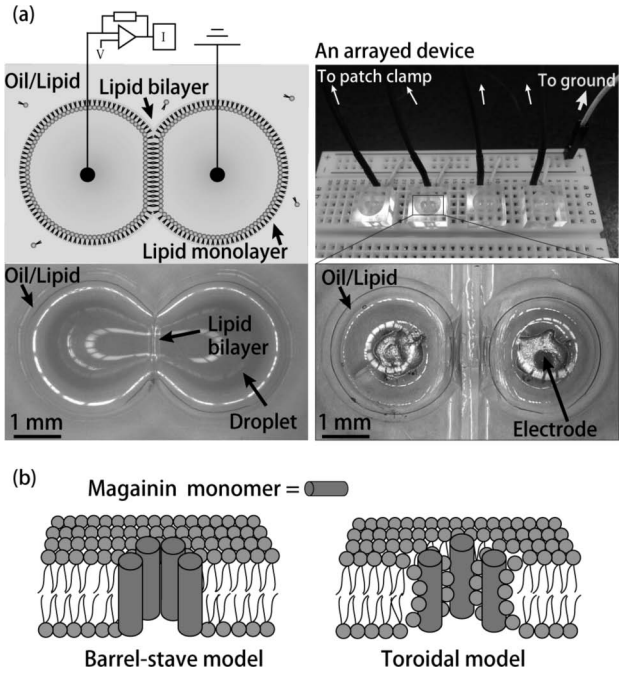

Fig. 1 (a) Lipid bilayer prepared by the droplet contact method, and a photograph of the device for measuring channel currents. (b) Magainin reconstituted in BLM forms barrel-stave and toroidal pore by assembling monomers.

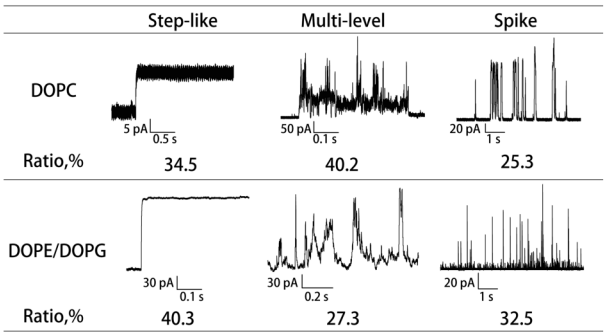

Fig. 2 Representative current signals in the PC and PE/PG membranes. The signals were classified into three types: step-like, multi-level, and spike signals. The percentages of each types of signals represent the ratio of each signals in all of signals.

polypeptide isolated from African frogs in the form of a powder, and dissolved at a concentration of 1 mM in ultrapure water. For use, samples were diluted to 10 μM using a buffered electrolyte solution and stored at 4°C.

Lipid bilayer preparation and reconstitution of magainin 1

BLMs were prepared using an arrayed device, which has four chambers fabricated by microfabrication.[11,15] Four individual BLMs can be formed simultaneously in this device, which allowed for a higher-throughput measurement compared to the conventional system. Using this device, the DOPC or DOPE/DOPG (3:1 mol/mol) mixture (lipids/*n*-decane, 10 mg/mL) solution (2.3 μL) was poured into each chamber. Next, the buffer solution (4.7 μL) was poured into both chambers. The same buffer solution (200 mM KCl, 10 mM MOPS, pH 7.0) was used for both droplets in this study. Magainin 1 was dissolved in an aqueous droplet at a 10 μM concentration to one side (voltage applying side). Within a few minutes of adding the buffer solution, two lipid monolayers connected and formed BLMs, and magainin 1 formed nanopores by reconstitution in the BLM. When the BLM ruptured during the process, we reformed the BLM by tracing with a hydrophobic stick at the interface of the drops.

Channel current measurements and data analysis

Channel currents were monitored using a Jet patch clamp amplifier (Tecella, Foothill Ranch, CA) connected to an Ag/AgCl electrode in each chamber. These electrodes were in each droplets when we added the solution into the chambers. The *trans* side was grounded, and a constant voltage of +100 mV was applied to the *cis* side. Reconstituted magainin in BLMs allowed ions to pass through nanopores under the voltage gradient, and we obtained the channel current signals. The signals were detected using a 4-kHz low-pass filter at a sampling frequency of 20 kHz. Analysis of channel signal was performed using pCLAMP Ver. 10.5 (Molecular Devices, Sunnyvale, CA).

We classified the current signals into three types in reference to the shape of the time–current traces. First, the step-like signals were the signals where the current increased sharply and plateaued. We counted one step of the current increase as one signal. Second, we regarded the current increase fluctuating continuously as multi-level signals and counted from the start to finish of the current increase as one signal. The end of the current increase means the return to 0 pA or an increase larger than the measurement limit. Third, the spike signal was a short current increase that momentarily returns to 0 pA, and we counted a series of events at a similar amplitude as one signal.

Results and Discussion

The pore-forming activities of many AMPs is different between mammalian and bacteria model membranes.[3] AMPs usually show a stronger activity in a negatively charged membrane (*e.g.*, DOPE/DOPG) because of the greater interaction between lipid head groups and peptides at the surface of the membrane. Thus, we used different compositions of BLMs of DOPC (PC) or DOPE/DOPG (PE/PG) as mammalian or bacteria model membranes.

Figure 2 shows a representative example of current recordings in the PC and PE/PG membrane systems. Several types of current signals appeared in both system. We classified these signals by reference to Fyles group's study.[18] The three types of signals, *i.e.*, step-like, multi-level, and spike signals, obtained reflect the several pore-forming modes of magainin 1. Previous studies have proposed[19] that magainin and many other AMPs form peptide helices and associate to form a bundle with a central lumen, similar to a barrel made of helical peptides, which is the "barrel-stave model." However, in the "toroidal model," the peptides are always associated with the lipid head groups, even when they are perpendicularly inserted into the lipid bilayer. During the formation of the pore structure in the toroidal model, the lipid monolayer bends continuously from top to bottom to form a toroidal hole. The toroidal pore is lined by both peptides and lipid head groups. In channel current recordings, the obtained current signals would reflect these models, as proposed in a previous study.[19] The step-like signals correspond to a barrel-stave-model pore where the rigid-ring pore shows a stepwise transition to the upstairs direction. In contrast, the multi-level or spike signals indicate a toroidal-

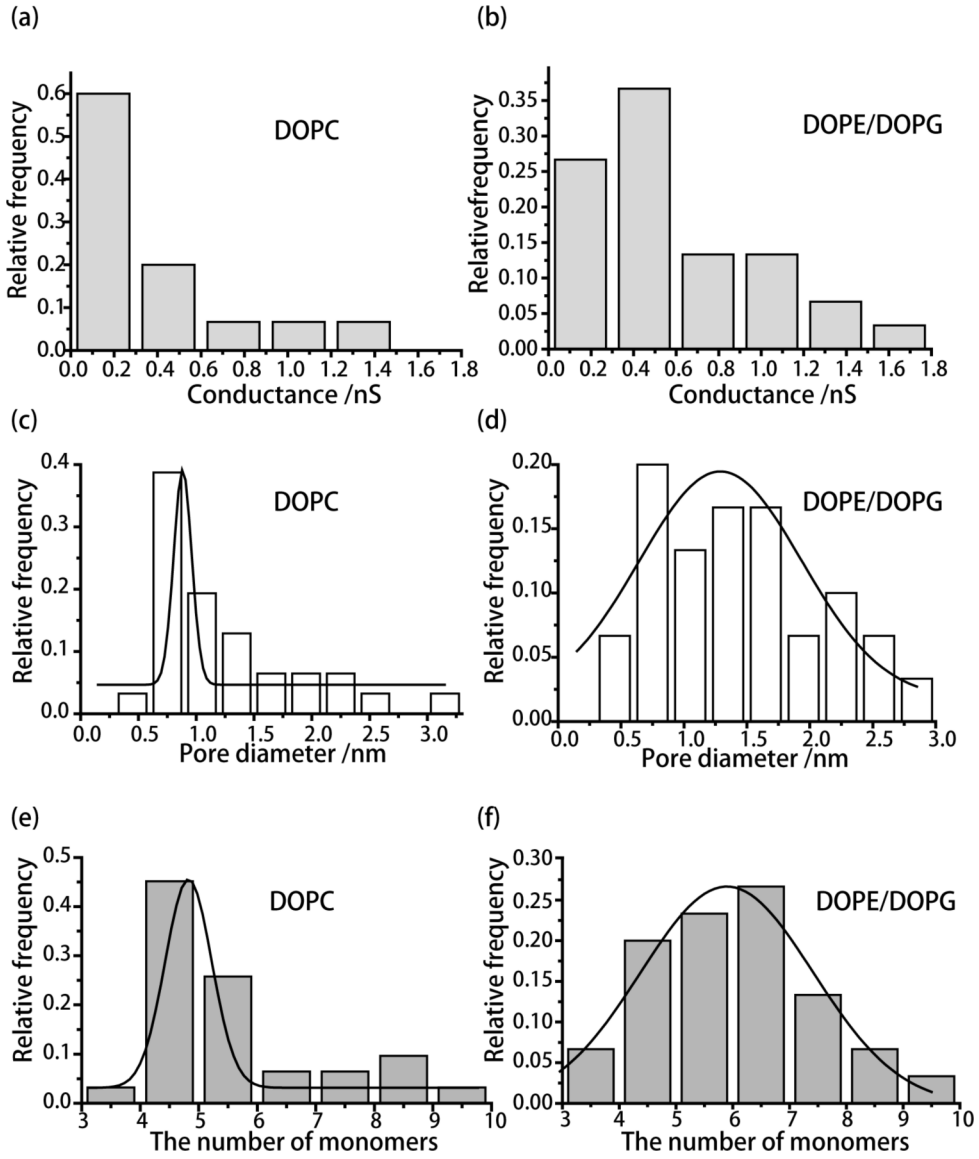

Fig. 3 Histograms of conductance of channel-current (a, b), pore diameter (c, d) and the number of monomers assembling to form a nanopore (e, f) fitted by a Gaussian distribution. Magainin formed heterogeneous-sized nanopores in BLMs.

model pore or other instantaneous membrane defects. These fragile pores vary in size dynamically because lipids can diffuse laterally from the pore to lipid membranes. Consequently, the current signals in these cases were fluctuating or spike signals. Using all current signals, we determined the rate of occurrence for each signal, as shown in Fig. 2. The orders of occurrence were "multi-level > step-like > spike" and "step-like > spike > multi-level" in the PC and PE/PG systems, respectively. This result implies that magainin 1 should be predisposed to form rigid pores in the negatively charged membrane (PE/PG). When compared with neutral lipids, magainin 1 reconstituted in negatively charged lipid bilayers is likely to follow the barrel-stave model because of electrostatic repulsion.

To estimate pore formation in terms of the pore size and monomer assembling state of magainin 1, we analyzed the current conductance of the "step-like" signals. Figures 3a and 3b shows the histograms of the conductance. In these signals, magainin 1 forms pores following the barrel-stave model as mentioned above. Therefore, we can ignore the lipid-mixed pores such as those formed through the toroidal model. The Hille equation[20] was used in pore diameter estimation:

$$R = \left(l + \frac{\pi r}{2} \right) \frac{\rho}{\pi r^2}, \quad (1)$$

where r is the pore radius, ρ is the resistivity of the buffered solution, l is the length of the pore (7 nm from the thickness of the BLM), and R is the resistivity of the pore. R is calculated as V/I, where I is the current through the magainin pore and V is the applied voltage between two chambers (100 mV). Several pore sizes can be obtained from several levels of current amplitudes. Histograms of the pore diameters in the PC and PE/PG systems are presented in Figs. 3a and 3b. The mean pore diameters fitted by a Gaussian distribution were 0.88 nm in the PC system and 1.29 nm in the PE/PG system. Magainin 1 formed larger pores in the PE/PG system when compared with the PC system because of the negatively charged lipids. This result is consistent with that of the antimicrobial activity of

magainin 2 and its analogs in human red blood and bacteria cells.[21]

Using the pore diameter and the monomer size from the Protein Data Bank (PDB), the number of assembling monomers was mathematically calculated by

$$d = a\left(\frac{1}{\sin(\pi/n)} - 1\right) \quad (2)$$

where d is the pore diameter, a is the width of a peptide monomer from the PDB, and n is the number of assembling monomers. Figures 3c and 3d show histograms of the number of monomers that assembled to form pores. From the histograms, 4 – 10 monomers formed a pore. Considering the previous results of a mean of seven monomers of magainin 2 per channel,[22] these assembling numbers are reasonable. In addition, the assembling number in the PE/PG system was also larger than that of the PC system. The mean assembling numbers were 4.9 and 5.9 in the PC and PE/PG systems, respectively. Using our DCM, the pore-forming properties of magainin 1 were analyzed from the channel current conductance. These results can reflect antimicrobial activities in living cells without *in vitro* lipid bilayer experiments.

Conclusions

In summary, we measured the channel current conductance of magainin 1 using BLMs prepared by DCM. Three types of current signals were observed: step-like, multi-level, and spike signals. The appearance of these signals depended on the lipid content. The step-like signal was mainly observed in the PE/PG system as the bacterial model membrane. We estimated the pore diameter and the number of assembling monomers, and showed that magainin 1 uses 4 – 10 monomers to assemble a pore of 0.5 – 3 nm in diameter.

Our DCM can readily prepare lipid bilayers and analyze pore-forming properties in a high-throughput study. This method will be applied to the extensive analysis and estimation of pore-forming properties of AMPs.

Acknowledgements

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