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Magainin 2-Induced Pore Formation in Membrane Depends on its Concentration in Membrane Interface

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ABSTRACT

Antimicrobial peptide magainin 2 forms pores in lipid membranes to induce leakage of internal contents of cells, which is a main cause of its bactericidal activity. However, the conditions and the mechanism of its pore formation remain unclear. In this report, to reveal the effect of the surface charge density of membranes on magainin 2-induced pore formation, we investigated the interaction of magainin 2 with giant unilamellar vesicles (GUVs) composed of a mixture of electrically neutral dioleoylphosphatidylcholine (DOPC) and negatively charged dioleoylphosphatidylglycerol (DOPG) in various ratio, using the single GUV method. We found that magainin 2 induced pores in the membranes of all kinds of single GUVs. For GUVs with the same charge density, the rate of the pore formation increased with magainin 2 concentration. The magainin 2 concentrations in a buffer required to induce the same rate of the pore formation greatly increased with a decrease in the surface charge density; e.g., the magainin 2 concentration required for the pore formation in 30%DOPG/70%DOPC-GUVs were 50 times higher than those in 60%DOPG/40%DOPC-GUVs. However, after we converted the magainin 2 concentration in the buffer into that in the membrane interface, \(X^\text{mag}_b\), we found that \(X^\text{mag}_b\) mainly determine the rate of the pore formation in various GUVs. These data support our model of two-state transition from the binding state to the pore state of the GUV for magainin 2-induced pore formation.

Key words: antimicrobial peptide, pore-forming peptide, single GUV method, giant liposome, leakage of internal contents, electrostatic interaction
1. Introduction

Antimicrobial peptides with bactericidal and fungicidal activity have been discovered in, and isolated from, a wide variety of organisms including amphibians, invertebrates, plants, and mammals.\textsuperscript{1,2} The target of these peptides is believed to be lipid membrane regions of bacterial and fungal biomembranes. Among these antimicrobial peptides, magainin 2, which was first isolated from the African clawed frog \textit{Xenopus laevis},\textsuperscript{3,4} has been extensively investigated. The interaction of magainin 2 with lipid membranes has previously been investigated using a suspension of many small liposomes (LUVs).\textsuperscript{5-7} However mechanistic details of the interactions remain unclear.

Recently, giant liposomes or giant unilamellar vesicles (GUVs) of lipid membranes with diameters greater than 10 $\mu$m have been used for investigations of the physical and biological properties of vesicle membranes such as elasticity and shape change.\textsuperscript{8-13} The shape of a single GUV and its physical properties in water can be measured in real time. Based on the characteristics of these GUVs, we have proposed the single GUV method to investigate functions and dynamics of biomembranes.\textsuperscript{14-18} Using this method, changes of structures and physical properties of single GUVs induced by the interactions with substances such as peptides can be observed as a function of time and spatial coordinates. We make the same experiments using many “single GUVs” and analyze statistically the changes of the physical properties of a single GUV over many “single GUVs”. For example, measurement of the leakage of internal contents (such as small fluorescent probes) from LUV suspensions (the LUV suspension method) has been extensively used to investigate liposome interaction with various substances including drugs, antibacterial substances, and fusogens.\textsuperscript{16-18} A large amount of leakage indicates that the substance strongly interacts with lipid membranes, thereby inducing instability in the structure of the vesicles and lipid membranes. However, the LUV suspension method does not allow determination of the main cause of the leakage, because there are many factors that could be involved in the leakage. In our previous study,\textsuperscript{16} we investigated the interaction of magainin 2 with lipid membranes using the single GUV method. Before that study, many researchers had reported that magainin 2 induced a gradual leakage of a fluorescent probe,
calcein, from liposomes over a 10 to 20 min period, based on leakage experiments using the LUV suspension method. Using the single GUV method, we found that low concentrations (3–10 μM) of magainin 2 induced a rapid leakage of calcein from the inside of single GUVs composed of a mixture of electrically neutral dioleoylphosphatidylcholine (DOPC) and negatively charged dioleoylphosphatidylglycerol (DOPG) in a 1:1 molar ratio. This leakage occurred without disruption of the liposomes or changes in their membrane structure, indicating that magainin 2 formed pores in the membrane through which the calcein leaked. Rapid leakage of the fluorescent probe from a GUV started stochastically, and was completed rapidly, within 1 min. This result indicates that the pore formation, rather than leakage through the pore, is the rate-determining step. These data also suggest that the previously observed gradual increase in the leakage from a suspension of many LUVs over time was caused by an increase in the fraction of leaked liposomes over time. In other words, the magainin 2 induced-calcein leakage was all-or-none (i.e., in some LUVs all their fluorescent probes leaked while in other LUVs no leakage occurred), not graded (i.e., in all the LUVs their internal fluorescent probe leaked gradually). Statistical analysis of pore formation in single GUVs over many single GUVs gave the rate constants of pore formation. These results clearly show that the single GUV method is considerably more useful than the conventional LUV suspension method for analysis of the interaction of magainin 2 with lipid membranes.16,18 Later, other groups used similar methods to investigate interactions of toxin proteins and peptides with lipid membranes.19,20

On the basis of these results, we have proposed the following two-state transition mechanism of the magainin 2-induced pore formation.16 The first state (Bex state) of a single liposome is defined as the binding state of magainin 2 to the membrane interface of the external monolayer of the liposome. The second state (P state) of the liposome represents the pore state, in which internal contents in the liposome can leak through the pores in the membrane. As the concentration of magainin 2 that is bound to the membrane interface of the external monolayer of the liposome increases, the surface pressure and elastic energy of the external monolayer increases. As a result, the transition from the
B_{ex} to the P state occurs, and the rate of pore formation increases as the magainin 2 concentration in the membrane interface is raised.

One of the main factors that mediates the binding of magainin 2 to lipid membranes is the electrostatic attraction between the positively charged magainin 2 and the negatively charged lipid membranes. Therefore it should be possible to control the magainin 2 concentration in the membrane interface by modulation of the surface charge density of lipid membranes. To validate our model of a two-state transition mechanism of the magainin 2-induced pore formation, we investigated the effect of the surface charge density of lipid membranes on the magainin 2-induced pore formation. Surface charge density was modulated by using GUVs composed of a mixture of negatively charged DOPG, and electrically neutral DOPC in which the concentration of DOPG (mol%) in the membrane was controlled.

2. Experimental Methods

2.1. Materials and peptide synthesis

DOPC and DOPG were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Calcein was purchased from Dojindo Laboratory (Kumamoto, Japan). Bovine serum albumin (BSA) was purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan). Texas Red-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Texas Red-DHPE) was purchased from Invitrogen Inc. Peptides (magainin 2 and F5W-magainin 2) were synthesized by the FastMoc method using a 433A peptide synthesizer (PE Applied Biosystems, Foster City, CA). The sequence of magainin 2 (23-mer) is GIGKFLHSAKKFKAVGEIMNS and that of F5W-magainin 2 is GIGKWLHSAKKFKAV-GEIMNS, and both peptides have an amide-blocked C terminus. The methods for purification and identification of the peptides were described in our previous paper. The purified peptides were analyzed by ion-spray ionization mass spectrometry using a single quadrupole mass spectrometer (API 150EX, PE SCIEX, PE Applied Biosystems, Foster City, CA).
The measured mass of magainin 2 and F5W-magainin 2 was 2465.1 ± 0.3 Da and 2504.6 ± 0.3 Da, respectively, which correspond to the molecular mass calculated from their amino acid composition.

2.2. Experiments using the single GUV method

GUVs were prepared in a buffer by the natural swelling of a dry lipid film at 37 °C as follows. One hundred µl of 1 mM phospholipid mixtures (e.g., DOPG and DOPC) in chloroform were placed in a glass vial (5 ml) and dried under a stream of N2 gas to produce a thin, homogeneous lipid film. The solvent was completely removed by placing the bottle containing the dry lipid film in a vacuum desiccator connected to a rotary vacuum pump for more than 12 h. Next, 10 µl water was added into this glass vial, and the mixture was incubated at 45 °C for 10 min (prehydration). The hydrated lipid film was then incubated with 1 ml of 1 mM calcein in buffer A (10 mM PIPES, pH 7.0, 150 mM NaCl, and 1 mM EGTA) containing 0.1 M sucrose for 2 to 3 h at 37 °C. To get purified GUV solution, untrapped calcein was removed as described in our previous report. The GUV suspension was centrifuged at 14,000 × g for 10 min at 20 °C to remove multilamellar vesicles. The supernatant was collected and passed through a Sephadex G-75 column with buffer A containing 0.1 M glucose, and the fractions containing GUVs were collected. In these fractions, there are a few kinds of vesicles such as GUVs, oligolamellar giant vesicles, and GUVs containing smaller liposomes. In the single GUV experiments, as GUVs we selected the vesicles of which the contrast of the membranes was very low. When we observed these vesicles containing a small percentage of fluorescent probe such as Texas-Red-DHPE using fluorescence microscopy, the fluorescence intensity of GUVs was the minimum among that of all kinds of vesicles and was much lower than that of oligolamellar vesicles We did not use highly unstable GUVs with a very large fluctuation of their membranes, but the population of these unstable DOPG/DOPC-GUVs were very low under this condition (less than 10% of all the GUVs).
300 µl of the purified GUV solution (0.1M sucrose in buffer A as the internal solution; 0.1M glucose in buffer A as the external solution) was transferred into a hand-made microchamber.\textsuperscript{15,16} A slide glass was coated with 0.1 % (w/v) BSA in buffer A containing 0.1 M glucose. The GUVs were observed using an inverted fluorescence phase contrast microscope (IX-70, Olympus, Tokyo) at 26 ± 1 °C using a stage thermocontrol system (Thermoplate, Tokai Hit, Shizuoka, Japan). Phase contrast and fluorescence images of GUVs were recorded using a high-sensitive fluorescence camera EM-CCD camera (C9100-12, Hamamatsu Photonics K.K., Hamamatsu, Japan) with a hard disk. Three ND filters were used to decrease the intensity of the incident light. Thereby, we could decrease the photobleaching of calcein compared with that in our previous report.\textsuperscript{16} The fluorescence intensity inside the GUVs was determined using the AquaCosmos (Hamamatsu Photonics K.K., Hamamatsu, Japan), and the average intensity per GUV was estimated. Various concentrations of magainin 2 in buffer A containing 0.1 M glucose were added slowly in the vicinity of a GUV through a 20 µm diameter glass micropipet positioned by a micromanipulator. The distance between the GUV and the tip of the micropipet was ~70 µm. The details of this method are described in our previous reports.\textsuperscript{15-18}

To obtain $P_{ls}(6 \text{ min})$ and $k_P$ for various GUVs, 2-4 independent experiments were carried out for each magainin 2 concentration using 30-40 single GUVs in each experiment for analysis of 40%DOPG/DOPC-, and 30%DOPG/DOPC-GUVs, and using 10-20 single GUVs in each experiment for analysis of 60%DOPG/DOPC-GUVs. Average values and standard deviations of were calculated

\section*{2.3. Measurement of the binding constant of F5W-magainin 2 to DOPG/DOPC-membranes}

To determine the binding constant of F5W-magainin 2 binding to DOPG/DOPC membranes, the change in fluorescence intensity of the peptide on binding was monitored. DOPG/DOPC-LUVs in buffer A were prepared by the extrusion method using 200-nm pore membranes.\textsuperscript{16} A Hitachi F7000 spectrofluorimeter was used for fluorescence measurements. Fluorescence intensities of
samples in a black micro cell were measured at 25 °C using a temperature-controlled holder. The excitation wavelength was 280 nm, and the emission wavelength was 336 nm. The excitation and emission band-passes were 2.5 nm and 5.0 nm, respectively. First, the solution background intensities of LUV suspensions (composed of various lipid concentrations) were measured. Then, 1000 μL of these LUV suspensions were mixed with 10 μL of a 10 μM solution of F5W-magainin 2 in buffer A (final concentration; 0.1 μM), and the mixtures were incubated for 1 h at room temperature (~25 °C). Their fluorescence intensities were then measured and the background intensity of the same LUV suspension was subtracted. We prepared the same samples 2 or 3 times and measured their fluorescence intensities in order to obtain average values. The lipid concentration in the samples was determined by the Bartlett method.

3. Results and Discussion

3.1. Induction of calcein leakage from 60mol%DOPG/40mol%DOPC (60%DOPG/DOPC) -GUVs by magainin 2

We first investigated the interaction of magainin 2 with single 60%DOPG/DOPC-GUVs containing 1 mM fluorescent dye, calcein. The interaction was carried out in buffer A containing 0.1 M glucose at 26 °C and was analyzed by fluorescence microscopy using the single GUV method. Fig. 1A shows a typical experimental result of the effect of the interaction of 3 μM magainin 2 with single GUVs on the calcein concentration within a GUV. The magainin 2 solution was continuously added into the vicinity of a GUV through a micropipet. Thereby, the equilibrium magainin 2 concentration near the GUV, \( C_{eq}^{mag} \), is considered almost the same as that in the micropipet.\(^{15,16} \) Prior to magainin 2 addition, the GUV had a high contrast in a phase contrast microscopic image (Fig.1A(1)) due to the difference in concentrations of sucrose and glucose between the inside (0.1 M sucrose) and the outside (0.1 M glucose) of the GUV. A fluorescence microscopic image of the same GUV (Fig.1A (2)) showed a high concentration of calcein inside the GUV at this time. During the
addition of the 3 μM solution of magainin 2, the fluorescence intensity inside the GUV was almost constant over the first 174 s, following which the fluorescence intensity decreased rapidly (Fig.1A(2) & Fig.1B). After 226 s, no fluorescence could be detected inside the GUV although a phase contrast image of the same GUV (Fig.1A(3)) showed that the GUV structure was still intact with no detectable breaks. As discussed in our previous report, the rapid decrease in fluorescence intensity occurred as a result of calcein leakage from inside the GUV to the outside through a magainin 2-induced pore (or pores) in the membrane. Thus the time at which the fluorescence intensity began to rapidly decrease corresponded to the time when pores were formed in the membrane. Furthermore, a comparison of the phase contrast images in Figs.1A(1) and 1A(3) also showed that there was a substantial loss in the phase contrast of the GUV, indicating that, during the leakage of calcein, sucrose and glucose also passed through the same pores. When the same experiments were carried out using many “single GUVs”, we observed the rapid leakage of calcein from a GUV started stochastically. This result indicated that pores were formed stochastically, and that leakage was complete within ~30 s (Fig.1C).

As demonstrated in our previous report, when estimating the leakage from single GUVs, the probability that a single GUV, among the population of GUVs examined, did not contain calcein molecules is important. To calculate this probability, we define $P_{LS}(t)$ as the probability that leakage had already started in a GUV, or that leakage had been completed in a GUV, among the population of GUVs examined, at any given time $t$ during the interaction between magainin 2 and the GUV. Therefore, $P_{LS}(t)$ represents the fraction of GUV in which leakage had already started, called the fraction of leaked GUV. At any given time $t$, $P_{LS}(t)$ is exactly related to the fraction of intact GUV from which calcein did not leak, called the fraction of intact GUV, $P_{intact}(t)$. Thus: $P_{LS}(t) = 1 - P_{intact}(t)$. Therefore, $P_{LS}(t)$ is an important parameter for the estimation of pore formation under a given condition. For the interaction of 3 μM magainin 2 with GUVs, $P_{LS}$ increased with time after magainin 2 addition (Fig.1D).
We also investigated the interaction of other concentrations of magainin 2 with single 60\%DOPG/DOPC-GUVs. Low concentrations (1–5 \(\mu\)M) of magainin 2 caused rapid leakage of calcein from single GUVs, directly showing that magainin 2 induced pores in the membrane. For each magainin 2 concentration, \(P_{LS}\) increased with time (Fig.1D). Fig.1E shows the dependence of \(P_{LS}\), measured at 6 min after magainin 2 addition was started, \((P_{LS}(6\ \text{min}))\), on the magainin 2 concentration. \(P_{LS}(6\ \text{min})\) increased as the magainin 2 concentration increased. At a concentration of \(\leq 0.5\ \mu\)M, magainin 2 did not induce the leakage \((P_{LS}(6\ \text{min}) = 0)\); at 1 and 2 \(\mu\)M concentrations of magainin 2 \(P_{LS}(6\ \text{min})\) was 0.07 \(\pm\) 0.05 and 0.4 \(\pm\) 0.1 respectively, and at \(\geq 5\ \mu\)M, \(P_{LS}(6\ \text{min})\) was 1.0.

3.2. Effect of surface charge density on magainin 2-induced pore formation

Next, we investigated the interaction of magainin 2 with single GUVs of DOPG/DOPC membranes containing various concentrations of DOPG (30-50\%) that had lower surface charge densities than 60\%DOPG/DOPC membrane. In all the cases, we observed a similar rapid leakage of calcein from single GUVs without disruption of the GUVs, and \(P_{LS}\) increased with time. Therefore, in all kinds of DOPG/DOPC-GUVs containing 30-60\% DOPG, the pore formation is the rate-determining step, and in other words the leakage type was all-or-none, as described in the introduction. Fig.2A shows the dependence of \(P_{LS}(6\ \text{min})\) on the magainin 2 concentration in solution for various GUVs with different surface charge density. From these results (Fig.2A), it is evident that higher concentrations of magainin 2 were required in the buffer to induce leakage of calcein from single GUVs (pore formation) with decreasing surface charge density. The magainin 2 concentration at \(P_{LS}(6\ \text{min}) = 0.5\) increased with a decrease in the surface charge density; 2.3, 4.5, 25, and 80 \(\mu\)M for 60\%DOPG/DOPC-, 50\%DOPG/DOPC-, 40\%DOPG/DOPC-, and 30\%DOPG/DOPC-GUVs, respectively.

To determine the effect of electrostatic interactions between magainin 2 and the DOPG/DOPC membrane on pore formation, we examined the dependence of pore formation on the salt
concentration of the buffer. For this purpose, we investigated the interaction of magainin 2 with a single 30% DOPG/DOPC membrane in buffer B (10 mM PIPES, pH 7.0, and 1 mM EGTA) (i.e., in the absence of NaCl). Lower concentrations of magainin 2 (2–7 μM) induced a similar rapid leakage of calcein to that in the presence of 150 mM NaCl (i.e., buffer A) (Fig. 2B). The magainin 2 concentration at $P_{LS} = 0.5$ was 5 and 80 μM in the presence of 0 mM and 150 mM NaCl, respectively.

It is well known that electrostatic interactions due to the surface charges of lipid membranes increase with increasing surface charge density or with decreasing salt concentration. These results shown in Fig. 2A and 2B clearly indicate that electrostatic interactions play an important role in pore formation. It is reasonable to consider that, with the same magainin 2 concentration in the buffer, the magainin 2 concentration in the membrane interface of the GUVs increases with an increase in the surface charge density, or with a decrease in salt concentration, due to enhanced electrostatic attraction of magainin 2 to the lipid membranes. Therefore, these results strongly indicate that the probability of pore formation induced by magainin 2 in the membrane greatly increases as the magainin 2 concentration in the membrane interface increases.

### 3.3. Two-state transition model for the magainin 2-induced pore formation

In this section we analyze the magainin 2-induced pore formation more quantitatively. We can obtain the time course of the fraction of intact GUV, $P_{intact}(t)$, which does not have pores in its membrane, from the time course of $P_{LS}(t)$ such as Fig. 1D, since $P_{intact}(t) = 1 - P_{LS}(t)$. Fig. 3A and 3B show that $P_{intact}$ of 60% DOPG/DOPC-GUVs and 40% DOPG/DOPC-GUVs decreased with time for each magainin 2 concentration. As shown in Fig. 3A, all the curves of the time course of $P_{intact}$ of 60% DOPG/DOPC-GUVs in the presence of various concentrations of magainin 2 were well fitted by a single exponential decay function defined by eq. 1 as follows,

$$P_{intact}(t) = \exp\left\{-k_P(t-t_{eq})\right\}$$

where $k_P$ and $t_{eq}$ are fitting parameters. In this fitting, we neglected a few unstable GUVs whose leakage occurred easily. We made 2-4 independent experiments similar to Fig. 3A to obtain $k_P$, and
then calculated the average value of $k_P$ using the results of all the independent experiments. The rate constant increased with increasing magainin 2 concentration, and the average value of $k_P$ for 5 µM magainin 2 (0.05 ± 0.01 s$^{-1}$) was about 30 times larger than that of $k_P$ for 2 µM magainin 2 (0.0017 ± 0.0007 s$^{-1}$) (Fig.3C). In the case of 40%DOPG/DOPC-GUVs, all the curves of the time course of $P_{\text{intact}}$ in the presence of various concentrations of magainin 2 were well fitted by a single exponential function (Fig.3B). The rate constant increased with an increase in magainin 2 concentration, and the average value of $k_P$ for 80 µM magainin 2 (0.048 ± 0.007 s$^{-1}$) was about 10 times larger than that of the $k_P$ for 25 µM magainin 2 (0.0035 ± 0.0009 s$^{-1}$) (Fig.3C). As shown in Figs.3A and 3B, $t_{eq}$ increased with a decrease in magainin 2 concentration.

There may be several models which can be expressed by eq.1. Here we consider the model of the two-state transition from the $B_{\text{ex}}$ state of a single GUV (i.e., the initial binding state of magainin 2 to the membrane interface of the external monolayer of an intact GUV) to the P state of the GUV (i.e., the pore state in the membrane in which the calcein leaks) for the magainin 2-induced pore formation, which we have proposed in our previous paper$^{16}$. In the $B_{\text{ex}}$ state, magainin 2 forms an α-helix that lies parallel to the membrane interface, and we assume that magainin 2 in the membrane interface exists as a monomer, not as an oligomer. In the P state, magainin 2 inserts into the membrane perpendicularly to the membrane interface and form pores in the membrane. In the interaction of magainin 2 with a single GUV, magainin 2 in solution binds to the membrane interface of the external monolayer of the GUV due to electrostatic attraction and then inserts deeply into the membrane interface due to the high interfacial hydrophobicity.$^{16}$ With an increase in the magainin 2 concentration in the membrane interface, $X_b^{\text{mag}}$, the surface pressure and elastic strain energy of the external monolayer increase. Hence, the free energy of the $B_{\text{ex}}$ state increases, causing a decrease in the activation energy $E_p$. As a result, above a critical $X_b^{\text{mag}}$, many magainin 2 molecules insert perpendicularly to the membrane cooperatively and rearrange rapidly to form the pores in the membrane (the P state). In this process, the rate determining step is the insertion of the magainin 2.
As a structure of the pore composed of α-helix peptides, we can consider two kinds of models; the barrel-stave model and the toroidal (wormhole) model. Although the toroidal model where the monolayer membrane bends in a toroidal fashion to create a pore is generally considered for the magainin 2-induced pore, we cannot determine which structure is suitable for the magainin 2-induced pore on the basis of only the results using the single GUV method in this report. The analysis and the conclusion derived from the results in this report do not depend on the structure of the magainin 2-induced pore.

On the basis of the two-state transition model, we can determine the rate constant of the transition from the Bex to the P state of single GUVs, i.e., the rate constant of pore formation. Individual events of the two-state transition occur stochastically, which has been well demonstrated by the transition between open and closed state of single ionic channels and by the force-induced unfolding of proteins. If we assume that the pore formation occurred by the irreversible two-state transition from the Bex to the P state, the fraction of the Bex state equals to Pintact(t), and thereby we can conclude that kP in eq.1 is the rate constant of the transition from the Bex to the P state of single GUVs. We can reasonably consider that teq in eq.1 is the time when the binding equilibrium of magainin 2 to the GUV was attained, and thereby the state of the GUV became the Bex state at teq, which can well explain the above results.

Fig.3C shows the dependence of kP on the magainin 2 concentration in solution for various GUVs with different surface charge density. kP greatly depended on the DOPG concentration in the GUV membrane; the magainin 2 concentrations required to induce the same rate constant kP greatly increased with decreasing DOPG concentrations, i.e., with decreasing surface charge density of the GUV membrane. For example, the magainin 2 concentration required to induce the same rate of pore formation in 30%DOPG/DOPC-GUVs were 50 times higher than those in 60%DOPG/DOPC-GUVs. kP also depended on the salt concentration. For 30%DOPG/DOPC-GUVs, the rate constants in the absence of NaCl (i.e., buffer B) were much larger than those in the presence of 150 mM NaCl (i.e.,
buffer A) (data not shown). These data concerning $k_P$ provide support to the conclusion drawn based on the dependence of $P_{LS}$ on electrostatic interactions due to the surface charge of the membranes.

3.4. A similar magainin 2 concentration in the membrane interface induced pore formation in various DOPG/DOPC-GUVs

Next, we calculated the magainin 2 concentration in the membrane interface, $X_{b}^{\text{mag}}$ and examined the dependence of magainin 2-induced pore formation on $X_{b}^{\text{mag}}$ in a more quantitative manner. Due to the electrostatic attraction between magainin 2 and DOPG/DOPC membranes, magainin 2 concentration close to the membrane interface is higher than that in the bulk solution (i.e., far from the membrane interface). Based on the Gouy-Chapman theory,\textsuperscript{21} the magainin 2 concentration immediately above the membrane interface, $C_{M}^{\text{mag}}$ is determined by the equilibrium peptide concentration in the bulk solution, $C_{eq}^{\text{mag}}$ and the surface potential of the membrane, $\Psi_0$, as follows:

$$C_{M}^{\text{mag}} = C_{eq}^{\text{mag}} \exp(-ze\Psi_0 / kT)$$

(2)

where $z$ is the net electric charge of magainin 2 under this condition ($z = 3.8$),\textsuperscript{28} $e$ the electronic charge, $k$ Boltzmann’s constant, and $T$ absolute temperature. At low magainin 2 concentration, the magainin 2 concentration in the membrane interface, which is expressed by the molar ratio of the magainin 2 bound to the membrane interface to total lipid in the solution, $X_{b}^{\text{mag}}$ (mol/mol), is proportional to $C_{M}^{\text{mag}}$ as follows:\textsuperscript{28}

$$X_{b}^{\text{mag}} = K_{\text{int}}^{\text{mag}} C_{M}^{\text{mag}} = K_{\text{int}}^{\text{mag}} \exp(-ze\Psi_0 / kT)C_{eq}^{\text{mag}}$$

(3)

where $K_{\text{int}}^{\text{mag}}$ is the intrinsic binding constant of magainin 2 to lipid membrane. We also considered the binding of Na$^+$ to DOPG with an intrinsic binding constant of 0.6 M$^{-1}$, which follows a Langmuir
adsorption isotherm.\textsuperscript{29,30} The relationship between surface charge density of the lipid membrane, $\sigma$, and $\psi_0$ is given by the Graham equation\textsuperscript{21} as follows:

$$\sigma = \sqrt{2000\epsilon_0\epsilon R T \frac{N_a}{c_{eq}} \cdot 2 \sinh(e\psi_0 / 2kT)}$$ \hspace{1cm} (4)

where $\epsilon_0$ is the dielectric constant of a vacuum, $\epsilon$, the relative dielectric constant of the medium, and $c_{eq}^{Na}$ the equilibrium Na\textsuperscript{+} concentration in the bulk solution. In our calculation, we neglected the contribution of magainin 2 because of its low concentration in the buffer. We assumed $c_{eq}^{Na} = 0.17$ M since we included buffer molecules as NaCl. On the other hand, $\sigma$ is determined by the DOPG concentration and the binding of magainin 2 and Na\textsuperscript{+} in the membrane interface as follows:

$$\sigma = \frac{\epsilon}{A} (-X_{PG} + X_{b}^{Na} + 3.8 X_{b}^{mag})$$ \hspace{1cm} (5)

where $X_{PG}$ is the mole fraction of total DOPG in the membrane, $X_{b}^{Na}$ the mole fraction of DOPG bound by Na\textsuperscript{+}, $A$ the cross-sectional area of a lipid molecule in the membrane interface, and we assume that $A$ of the DOPG molecule is the same as $A$ of DOPC ($70 \times 10^{-20}$ m\textsuperscript{2}).

To determine $K_{int}^{mag}$, we used fluorescence spectroscopy. The fluorescence intensity of a fluorescent analogue of magainin 2, F5W-magainin 2, increases when it binds to the lipid membrane interface, because the dielectric constant of the membrane interface is lower than that of water.\textsuperscript{31} The fluorescence intensity of F5W-magainin 2 in buffer A was very small, but it increased after the 30\%DOPG/DOPC-LUV suspension was added into the peptide solution (Fig.4A). Fig.4B indicates that the fluorescence intensity of F5W-magainin 2 increased with increasing lipid concentration and reached a saturated value at high lipid concentrations (above 40 $\mu$M). These data clearly indicate that at high lipid concentrations almost all of the peptides are bound to the membrane interface. We obtained $X_{b}^{mag}$ and $c_{eq}^{mag}$ from the normalized fluorescence intensity, $F_N$, at each lipid concentration ($C_{T}^{Lipid}$) using the following equations:
where \( C_T^{\text{mag}} \) is the total magainin 2 concentration in buffer in the absence of the LUVs. For these calculations we considered that magainin 2 binds only to the external monolayer. Using the obtained values of \( X_b^{\text{mag}} \) and \( C_{\text{eq}}^{\text{mag}} \), we determined \( \Psi_0 \) and \( \sigma \) using Eqs. 4 and 5. Fig.4C shows the binding isotherms of magainin 2 to lipid membrane, which indicates the relationship between \( X_b^{\text{mag}} \) in the external monolayer of LUVs and \( C_{\text{eq}}^{\text{mag}} \). These isotherms were well fitted by eq.3, and we obtained the best-fit value of \( K_{\text{int}}^{\text{mag}} \). The average values of \( K_{\text{int}}^{\text{mag}} \) for 30\%DOPG/DOPC- and 40\%DOPG/DOPC-LUVs was 1100 ± 100 and 1200 ± 100 respectively, based on 2 independent experiments. We could not get a good fit for 50\%DOPG/DOPC- or for 60\%DOPG/DOPC-LUVs, probably because a strong interaction between the peptide and the membrane occurred locally and an equilibrium condition could not be obtained.

Using the estimated values of \( K_{\text{int}}^{\text{mag}} \), we obtained \( X_b^{\text{mag}} \) and \( \Psi_0 \) for various \( C_{\text{eq}}^{\text{mag}} \) using Eqs.3-5. Employing these values we transformed the graph of the dependence of \( P_{\text{LS}}(6 \text{ min}) \) on \( C_{\text{eq}}^{\text{mag}} \) for 30\%DOPG/DOPC- and 40\%DOPG/DOPC-GUVs (Fig.2A) into a new graph of the dependence of \( P_{\text{LS}}(6 \text{ min}) \) on \( X_b^{\text{mag}} \) (Fig.5A). This graph clearly shows that for both GUVs magainin 2-induced pore formation began at a similar \( X_b^{\text{mag}} \) and occurred in a highly cooperative manner (\( X_b^{\text{mag}} = 60 \) and 70 mmol/mol for 30\%DOPG/DOPC- and for 40\%DOPG/DOPC-GUVs, respectively). The similar dependence of \( P_{\text{LS}}(6 \text{ min}) \) on \( X_b^{\text{mag}} \) for both of the GUVs indicates that \( X_b^{\text{mag}} \) is the main determinant of the rate of the magainin 2-induced pore formation.

We also calculated the dependence of \( k_P \) on \( X_b^{\text{mag}} \) (Fig.5B) by replotting the graphs of the dependence of \( k_P \) on \( C_{\text{eq}}^{\text{mag}} \) (Fig.3C) using the estimated values of \( K_{\text{int}}^{\text{mag}} \). The dependence of \( k_P \) on
\(X^\text{mag}_b\) for 40\%DOPG/DOPC- and 30\%DOPG/DOPC-GUVs in buffer A was almost the same. These data further support the conclusion derived from the dependence of \(P_{LS}(6 \text{ min})\) on \(X^\text{mag}_b\) (Fig.5A), that is, that \(X^\text{mag}_b\) determines the rate of magainin 2-induced pore formation. Small shifts in the curve of the \(P_{LS}\) plotted against \(X^\text{mag}_b\) and in the curve of the \(k_P\) plotted against \(X^\text{mag}_b\) of the 30\%DOPG/DOPC-GUV to a lower \(X^\text{mag}_b\) were observed (Fig.5A, B). At present, we don’t know the reason for these shifts.

To date, it has been considered that, in the interaction of antimicrobial peptides such as magainin 2 with lipid membranes, it is the peptide to lipid ratio (P/L, where P and L are the total concentration (amounts) of peptides and lipids in a suspension), that is the key parameter that determines leakage and pore formation.\(^5\text{-}^7,\text{20}\) The ratio P/L corresponds to \(C^\text{mag}_T / C^\text{Lipid}_T\) in eq. 6. It has also been shown that the pore state is stable at high P/L (>1/30) based on X-ray and neutron diffraction experiments of a magainin 2/lipid mixture.\(^25\) However, the results in this report clearly indicate that the key parameter of pore formation is not P/L and \(C^\text{mag}_p\), but \(X^\text{mag}_b\). The binding of magainin 2 to the lipid membrane interface greatly depends on the membrane composition and solution conditions, and therefore, P/L alone cannot determine \(X^\text{mag}_b\) and pore formation.

The data in this report also support our model of a two-state transition from the B\(_{ex}\) to the P state of a GUV as follows. In all kinds of GUVs with different surface charge density, the transition occurred in the same kinetics expressed in eq.1. Moreover, the results in this report clearly indicate that \(X^\text{mag}_b\) is the main determinant of the rate of the magainin 2-induced pore formation. With an increase in \(X^\text{mag}_b\), the surface pressure and elastic energy of the external monolayer increases, inducing instability of the B\(_{ex}\) state. This is the main driving force of the transition.

Very recently, Almeida and his colleagues indicated that magainin 2 caused all-or-none leakage from LUVs of 50\%-1-palmitoyl-2-oleoylphosphatidylglycerol(POPG)/50\%-1-palmitoyl-2-oleoyl-
phosphatidylcholine (POPC) membrane and 30%POPG/70%POPC-LUVs using LUV suspension method.\textsuperscript{32} Their results support our conclusion in this report.

4. Conclusion

Using the single GUV method, we investigated the interaction of magainin 2 with DOPG/DOPC membranes composed of different DOPG concentrations and therefore having a different surface charge density. We found that the magainin 2 concentration in a buffer required to induce pore formation in the membrane greatly depended on the surface charge density of the membrane and the salt concentration in the buffer. However, our quantitative analysis indicates that almost the same magainin 2 concentration in the membrane interface induced pore formation. This result strongly supports our hypothesis of a two-state transition model for the magainin 2-induced pore formation.

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**Figure Captions**

Fig.1: Leakage of calcein from single 60%DOPG/DOPC-GUVs induced by 3 μM magainin 2. (A) Fluorescence images (2) show that the calcein concentration inside the GUV progressively decreased after the addition of magainin 2. The numbers above each image show the time in seconds after the magainin 2 addition was started. Also shown are phase contrast images of the GUV at time 25 (1) and at 255 s (3). The bar corresponds to 10 μm. (B) Time course of the change of the fluorescence intensity of the GUV shown in (A). (C) Other examples of the change in fluorescence intensity of single GUVs over time under the same conditions as in (A). (D) Time course of $P_{LS}$ of 60%DOPG/DOPC-GUV in the presence of various concentrations of magainin 2: (○) 5, (□) 3, (▲) 2.5, and (●) 1 μM. 10-20 single GUVs were used for each experiment. (E) Dependence of $P_{LS}(6 \text{ min})$ on magainin 2 concentration. We made 3-4 independent experiments for a given magainin 2 concentration using 10-20 single GUVs in each experiment, and obtained average values and standard deviations of $P_{LS}(6 \text{ min})$ using the results of all the independent experiments.

Fig.2: Dependence of the fraction of leaked GUV on magainin 2 concentration in buffer $C_{eq}^{mag}$. (A) Dependence of $P_{LS}(6 \text{ min})$ on $C_{eq}^{mag}$ in buffer A at 26 °C. (■) 60%DOPG/DOPC-, (○) 50%DOPG/DOPC-, (●) 40%DOPG/DOPC-, and (□) 30%DOPG/DOPC-GUVs. (B) Dependence of $P_{LS}(6 \text{ min})$ of 30%DOPG/DOPC-GUVs on $C_{eq}^{mag}$ at 26 °C. (▲) in the absence of NaCl, and (■) in the presence of 150 mM NaCl. For 40%DOPG/DOPC-, and 30%DOPG/DOPC-GUVs, we made 2-3
independent experiments for a given magainin 2 concentration using 30-40 single GUVs in each experiment, and obtained average values and standard deviations of $P_{LS}$ (6 min). For 50%DOPG/DOPC-GUV, we made 2-4 independent experiments for a given magainin 2 concentration using 10-20 single GUVs in each experiment, and obtained average values and standard deviations of $P_{LS}$ (6 min).

Fig.3: Dependence of the rate constant of pore formation $k_P$ on magainin 2 concentration in buffer A, $C_{eq}^{mag}$. (A) Time course of $P_{intact}$ of 60%DOPG/DOPC-GUV in the presence of various concentrations of magainin 2: ($\circ$) 5, ($\square$) 3, ($\oplus$) 2.5, and ($\bullet$) 1 μM. The solid lines represent the best fitted curves of eq. 1. The obtained values of $k_p$ were 0.059, 0.017, and 0.0095 s$^{-1}$ for 5, 3, and 2.5 μM magainin 2, respectively. (B) Time course of $P_{intact}$ of 40%DOPG/DOPC-GUV in the presence of various concentrations of magainin 2: ($\Box$) 80, ($\square$) 30, ($\circ$) 25, and ($\uparrow$) 5 μM. Solid lines represent the best fitted curves of eq. 1. The obtained values of $k_p$ were 0.041, 0.014, and 0.0051 s$^{-1}$ for 80, 30, and 25 μM magainin 2, respectively. (C) Dependence of $k_P$ on $C_{eq}^{mag}$. ($\blacksquare$) 60%DOPG/DOPC-, ($\circ$) 50%DOPG/DOPC-, ($\bullet$) 40%DOPG/DOPC-, and ($\square$) 30%DOPG/DOPC-GUVs. We made 2-4 independent experiments for a given magainin 2 concentration using 30-40 single GUVs in each experiment for 40%DOPG/DOPC-, and 30%DOPG/DOPC-GUVs, and using 10-20 single GUVs in each experiment for 60%DOPG/DOPC-, and 50%DOPG/DOPC-GUVs, and obtained average values and standard deviations of $k_P$.

Fig.4: Measurement of the binding constant of magainin 2 to lipid membranes. (A) Fluorescence spectrum of F5W-magainin 2 in buffer A in the presence of various concentrations of 30%DOPG/DOPC-LUVs. (B) Normalized fluorescence intensity at 336 nm of F5W-magainin 2 in buffer A plotted against lipid concentration (30%DOPG/DOPC-LUVs). Fluorescence intensity of magainin 2 in buffer A containing the LUVs was normalized based on the following conditions: $F_N =$
0 when all the magainin 2 is in buffer A, and $F_N = 1$ when all of the magainin 2 are bound to the membrane. (C) The binding isotherms of magainin 2 to lipid membrane indicates the relationship between $X_{b}^{\text{mag}}$ in the external monolayer of LUVs and $C_{eq}^{\text{mag}}$. (●) 40%DOPG/DOPC-, and (□) 30%DOPG/DOPC-LUVs. The best fit gave values of $K_{\text{int}}^{\text{mag}}$ of 1100±100 for 30%DOPG/DOPC-LUV and 1130 ± 50 for 40%DOPG/DOPC- LUV.

Fig.5: Dependence of the rate of the pore formation on magainin 2 concentration in the membrane interface, $X_{b}^{\text{mag}}$. (A) Dependence of $P_{LS}(6 \text{ min})$ on $X_{b}^{\text{mag}}$. Fig.5A was prepared by replotting the graph of the dependence of $P_{LS} (6 \text{ min})$ on $C_{eq}^{\text{mag}}$ (Fig 2A) using the estimated values of $K_{\text{int}}^{\text{mag}}$. (B) Dependence of $k_P$ on $X_{b}^{\text{mag}}$. Fig.5B was prepared by replotting the graphs of the dependence of $k_P$ on $C_{eq}^{\text{mag}}$ (Fig.3C) using the estimated values of $K_{\text{int}}^{\text{mag}}$. (●) 40%DOPG/DOPC-, and (□) 30%DOPG/DOPC- GUVs.
Fig. 1

(A) Images of GUVs at different times.

(B) Fluorescence intensity over time for different concentrations.

(C) Fluorescence intensity over time for different concentrations.

(D) Fraction of leaked GUVs over time for different concentrations.

(E) Fraction of leaked GUVs over time for different concentrations.
Fig. 2

(A) Fraction of leaked GUV vs. Magainin 2 conc. (µM) for different conditions.

(B) Fraction of leaked GUV vs. Magainin 2 conc. (µM) for another set of conditions.
Fig. 3
Fig. 4

(A) Fluorescence intensity vs. emission wavelength (nm)

(B) Fluorescence intensity vs. lipid concentration (µM)

(C) $C_{eq}$ vs. $C_{eq}^{imp}$ (µM)
Fig. 5

(A) Fraction of leaked GUV

(B) $k_p$ (s$^{-1}$)