Membrane Tension in Negatively Charged Lipid Bilayers under Osmotic Pressure and its Effects on their Membrane Dynamics

学 位 論 文 要 約

Summary of Doctoral Thesis

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Summary：

<Chapter 1>

Osmotic pressure (Π) induces membrane tension in cell membranes and the lipid bilayers of vesicles and plays an important role in the functions and physical properties of these membranes. The swelling of cells or vesicles comprising lipid bilayers due to Π induces a lateral tension in membranes (i.e., membrane tension). Membrane tension plays a vital role in various physiological functions and mechanical properties of cell membranes and lipid bilayers. Π greatly affect the function of membrane proteins and the interaction of peptides/proteins with biomembranes by changing the membrane tension of plasma membranes. Recently, a new method was established to measure quantitatively the membrane tension (σ_{osm}) of giant unilamellar vesicles (GUVs) under Π, in our lab and was applied to GUVs comprising electrically neutral dioleoylphosphatidylcholine (DOPC). This measurement was performed in water without salts and thus is of limited use in understanding the effect of Π on cells. However, it is well known that it is difficult to prepare GUVs comprising only neutral lipids in a buffer containing salts. This was problems for the role of Π on cells, since investigation on cells are performed in a medium carrying a physiological concentration of salts or ions, and all plasma membranes carry high concentrations of negatively charged lipids. In this thesis, to estimate quantitatively the σ_{osm} in GUVs under more physiological conditions, I examined the effect of Π on the physical properties of negatively charged GUVs comprising a mixture of DOPC and negatively charged dioleoylphosphatidylglycerol (DOPG) in a buffer containing a physiological concentration of ions. In such GUVs, electrostatic interactions due to surface charges play an important role in their mechanical properties. It is well known

that in the plasma membrane of eukaryotic and bacterial cells, electrostatic interactions due to surface charge play vital roles in various biophysical properties and physiological functions. By analyzing these results, I successfully determined the values of σ_{osm} in DOPG/DOPC-GUVs under Π. Based on these results, I also examined the effect of Π on antimicrobial peptide (AMP) magainin 2-induced pore formation in lipid bilayers. The main cause of antimicrobial activity of magainin 2 is the pore formation in bacterial plasma membrane. The purpose of this examination is to elucidate the mechanism for the effect of σ_{osm} on AMP-induced pore formation.

<Chapter 2>

In this chapter, I examined the effect of Π on DOPG/DOPC-GUVs in a buffer containing a physiological concentration of ions. The initial difference in osmolarity between the inside and the outside of GUVs (ΔC^0) determines the value of Π. First, to estimate the σ_{osm} of negatively charged GUVs quantitatively, I examined the rate constant (k_r) of constant tension-induced rupture of DOPG/DOPC (4/6)-GUVs under Π. After applying Π to a GUV, I applied constant tension (σ_{ex}) to the GUV by suction pressure due to aspiration by a micropipette and observed rapture of GUVs. Initially I did not observe any change in the GUV, but after some times I observed the aspiration of GUV. The GUV was completely aspirated into the micropipette at high speed (less than 1 s). The mechanism of this investigation can be described as follows. First, a pore is formed in the GUV membrane and then the radius of the pore increases under σ_{ex}, inducing rupture of the GUV, and finally the GUV is completely aspirated into the micropipette due to ΔP_m. I did the same experiment using 20 single GUVs ($n = 20$) and found that each GUV was aspirated completely into the micropipette at different times, indicating that stochastic rupture of single GUVs was occurred. The stochastic phenomenon was analyzed using a probability parameter, i.e., the fraction of intact GUVs among all the examined GUVs, $P_{intact}(t)$. $P_{intact}(t)$ values were obtained from this experimental result as a function of the duration of the application of σ_{ex}, t, and found that as time increased, $P_{intact}(t)$ decreased. I can analyze this time-course quantitatively using a theory describing a two-state transition from the un-raptured state to the ruptured state of a GUV. The values of k_r were obtained by the analysis of rupture of many GUVs. I obtained the dependence of k_r on σ_{ex} in GUVs under various values of Π and found that k_r increased with σ_{ex}. The total

membrane tension in GUVs under Π (σ_t) is the sum of σ_{ex} and σ_{osm}. From the comparison of σ_{ex} inducing the same values of k_r of GUVs in the presence of Π and in the absence of Π, I obtained the values of σ_{osm}. In this way, I also obtained σ_{osm} for different valuse of Π (different values of ΔC^0, concentration difference between inside and outside of GUVs) and dependence of ΔC^0 with σ_{osm}. The values of σ_{osm} increased as ΔC^0 increased. I also developed the theoretical estimation of σ_{osm} for DOPG/DOPC-GUVs in a buffer. The experimental values of σ_{osm} agreed with the theoretical values within the experimental error.

Next, I measured the Π-induced fractional volume change of DOPG/DOPC (4/6)-GUVs using the chamber transfer method where a GUV fixed at the tip of a micropipette in chamber A containing an isotonic solution is transferred to chamber B containing a hypotonic solution. When cells are transferred into a hypotonic solution (whose solute concentration is lower than that inside of the cells), water molecules enter the inside of cells from outside, and therefore, cell volume increases (i.e., swelling of cell) which generates lateral tension in membrane (i.e., membrane tension, σ_{osm}). First, PC/PG (6/4)-GUVs were prepared and the osmolarity of the GUV lumen, Cin0, was 388.0 mOsm/L. These GUVs were centrifuged (13000 rpm, 1200 s, 20.0° C) and then purified in buffer A carrying 93.0 mM glucose (i.e., 388.0 mOsm/L, same osmolarity inside GUV) at the rate 1 mL/min using the membrane filtering method (Tamba et al., 2011). Here I used 5.0 μm radius-nucleopore. As a result, some small size GUVs and some aggregates were removed by the flow of 93.0 mM i.e., 388.0 mOsm/L glucose about 60 min. Some small GUVs less than 5.0 μm radius and the purified GUV suspension was transferred into chamber A which was carried same solution inside GUV (i.e., isotonic solution). A single GUV in chamber A was held using a micropipette using σ_{ex} of 1.0 mN/m in the GUV for 120 s (Fig. 1A), and then transferred into chamber B filled with buffer A carrying a lower osmolarity of glucose solution. An air gap was present between the chamber A and B. When the GUVs were transferred without carrier the GUVs were raptured. If I used different osmolarity solution inside carrier the volume of GUVs was changed inside carrier. The adjustment of appropriate position between carrier and micropipette was necessary because without adjustment the GUVs were not transferred. A glass capillary

(internal radius of 0.5 mm) was used as a carrier which was carried the same solution as chamber A (same solution inside GUV i.e., isotonic solution) for transferring the GUV over the small air gap between the two chambers A and B. The equilibrium pressure of capillary was about 3.2 Pa. After holding a single GUV by micropipette (radius 5.0 μm) and waiting 120 s the GUV with micropipette was inserted by capillary (Fig. 1B). Then GUV was transferred chamber A to chamber B. During transfer of GUV only chamber was moving from right side to left side (Fig. 1 B, C) i.e., the position of GUV, micropipette and capillary was fixed. When the GUV was transferred to chamber B completely (Fig. 1 D, E), the capillary was removed from the GUV and finally GUV was transferred into the hypotonic solution (Fig. 2.1 E). I observed single GUV after waiting 120 s (i.e., from chamber A) about 360s. When GUV is transferred to chamber B small change of volume occurs. It is not possible to measure the small change by scale. For this case micropipette aspiration method is necessary because this method was accurate method for measuring volume change experiment of GUVs. When I applied negative pressure to a GUV in chamber A, the GUV was aspirated by micropipette. Some portion of GUV membrane exists into the micropipette which is called the projection length of GUV. When the GUV was transferred to chamber B projection length of GUV changes. By analyzing these results, I obtained the change in volume of the GUV, ΔV, and then the ratio of ΔV to the initial volume of the GUV in chamber A, V_0, i.e., $\Delta V/V_0$. $\Delta V/V_0$ also increased with time and after same times this value reached to an equilibrium value, $\Delta V_{eq}/V_0$. $\Delta V_{eq}/V_0$ increases as ΔC^0 increases. This result indicates that higher Π generates larger swelling of the GUVs. I also estimated the values of σ_{osm}, which agree with the theoretical values. The values of σ_{osm} for DOPG/DOPC (4/6)-GUVs were smaller than those for DOPC-GUVs under the same Π. Two factors, the solute concentration in a GUV suspension and the elastic modulus of the GUV membrane, can reasonably explain this difference based on the theory of σ_{osm}.

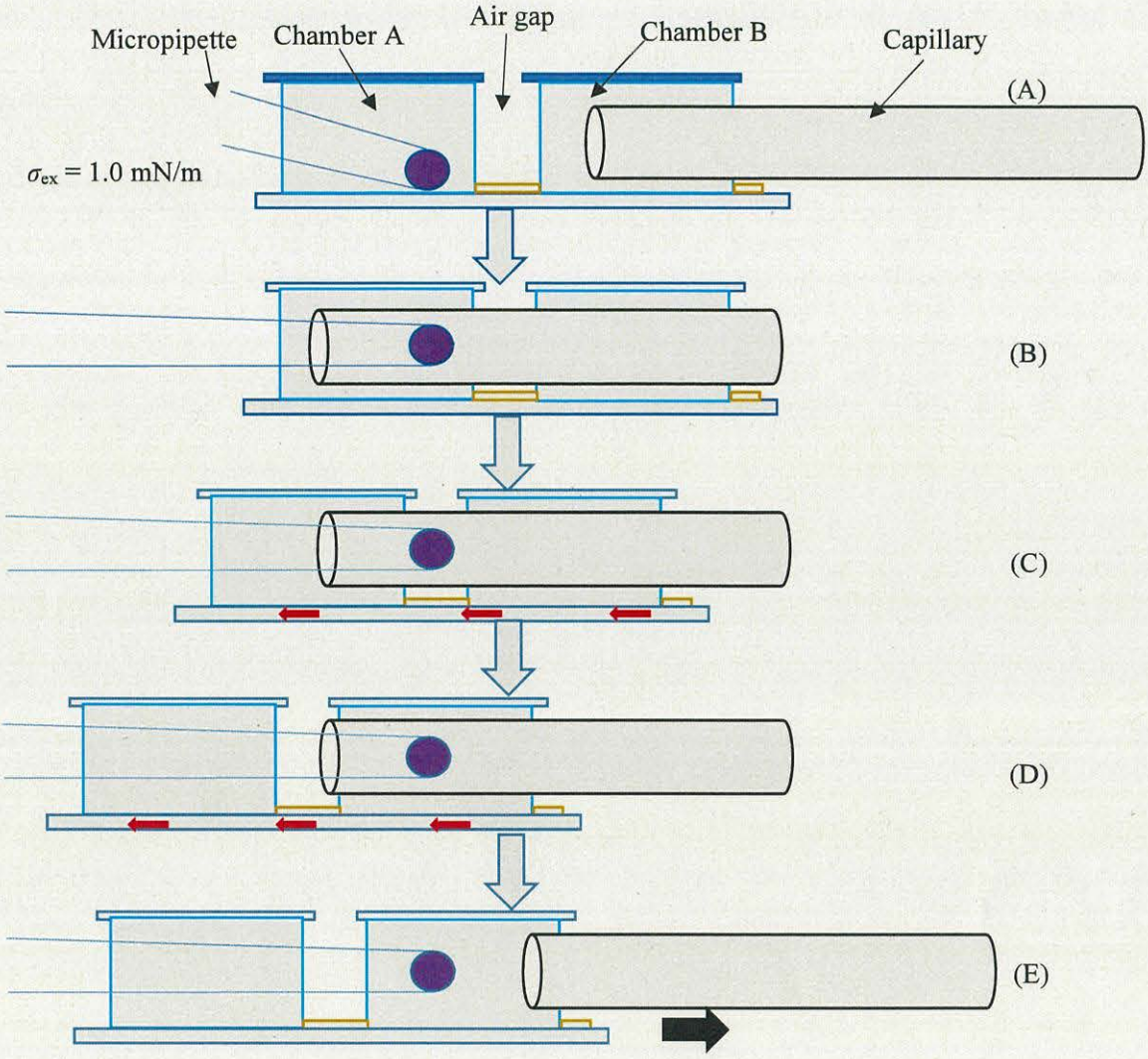

Figure 2.1: The chamber transfer method for transferring GUVs from chamber A to chamber B. (A) A single GUV carrying 388.0 mOsm/L sucrose solution was held by micropipette in chamber A which carries 388.0 mOsm/L glucose solution. (B) after waiting 120 s GUV with micropipette was inserted in capillary carrying 388.0 mOsm/L glucose. (C) (D) chambers were moved from right side to left side. (E) After transferring GUV to chamber B, Capillary was removed from the GUV.

Finally, I investigated the role of Π on the rate constant of the flip-flop of lipid molecules, k_{FF} in single GUVs. To monitor flip-flop of lipid molecules fluorescence probe labeled lipid is necessary. Here, I used NBD-PG. On the other hand, FI of a GUV membrane i.e., rim intensity (RI) due to NBD-PG was used to estimate the concentration of NBD-PG in the GUV membrane. On the other hand, lumen intensity (LI) i.e., FI of GUV lumen due to AF647, was used to detect pore formation in the membrane was monitored by released of AF647 from the GUV lumen. Based on these results, I discuss the σ_{osm} of DOPC/DOPG (6/4)-GUVs under Π and its role in the physical property of the lipid bilayers. The flip-flop or transbilayer movement of lipid molecules in a lipid bilayer or in biomembranes is one of the thermal motions of lipids using thermal energy. The flip-flop is the same as transbilayer diffusion of lipid molecules, where the charges of the hydrophilic segment must permeate through the hydrocarbon chain region of the membrane. At first, I investigated the role of Π on the rate constant of the transbilayer movement (k_{FF}) of the lipid, NBD-PG in single GUVs. For this purpose, I prepared GUVs in which NBD-PG molecules are distributed in the two monolayers asymmetrically (i.e., PC/PG/NBD-PG (59/40/1; inner monolayer)- PC/PG (59/40; outer monolayer)-GUVs, providing asymmetric PC/PG/NBD-PG-GUVs) by removing NBD-PG from the outer monolayer of symmetric PC/PG/NBD-PG (59/40/1)-GUVs using a method which is developed in our lab. First, symmetric GUVs carrying of PC/PG/NBD-PG (59/40/1; molar ratio) were prepared in buffer A carrying 6.0 μM AF647 and 100.0 mM sucrose using the same method described in Section 2.2.2. The osmolarity of the GUV lumen, Cin0, was 388.0 mOsm/L. These GUVs were centrifuged (13000 rpm, 1200 s, 20° C) and then purified in buffer A carrying 388.0 mOsm/L glucose (i.e., same osmolarity inside GUV) at the rate 1 mL/min using the membrane filtering method. Here I used 5 μm radius nucleopore membrane. As a result, some small size GUVs and some aggregates were removed by the flow of 93 mM glucose about 60 min. I obtained PC/PG/NBD-PG (59/40/1; inner monolayer)- PC/PG (59/40; outer monolayer)-GUVs (i.e., asymmetric PC/PG/NBD-PG-GUVs) by transferring NBD-PG molecules from the outer leaflet of symmetric PC/PG/NBD-PG (59/40/1)-GUVs to the new buffer using the membrane filtering method. Unstrapped fluorescent probe (AF647) was also removed in this step. Then I applied Π to the GUVs, and then measured the fluorescence intensity of the GUV

membrane due to NBD-PG using confocal laser scanning microscopy. I obtained the values of k_{FF} by analyzing these data. The values of k_{FF} increased with increasing Π, indicating that k_{FF} increased with σ_{osm} (Fig. 2.7). This result supports the existence of pre-pores in stretched lipid bilayers. Based on these results, I discussed the membrane tension of DOPG/DOPC (4/6)-GUVs under Π.

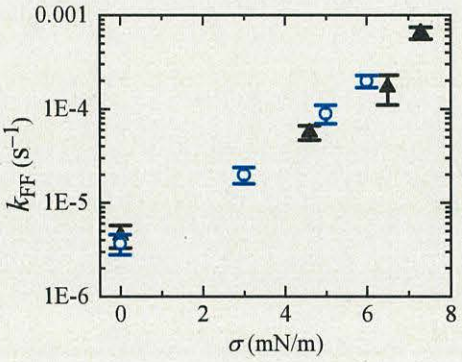

Figure 2.7: Role of Π on the flip-flop of NBD-PG in asymmetric PC/PG/NBD-PG (59/40/1; inner)- PC/PG (40/59; outer)-GUVs at 25.0 °C. Dependence of k_{FF} on σ_{osm} (▲). For comparison, the values of k_{FF} of the same asymmetric GUVs under σ_{ex} of micropipette are shown (○), which are taken from Hasan et al., 2018 with permission from the American Institute of Physics. Average values and SDs of k_{FF} for each σ_{ex} are shown.

<Chapter 3>

In this chapter, I examined the effect of Π on magainin 2-induced pore formation in DOPG/DOPC-GUVs. It is considered that Π greatly affects the function of membrane proteins and the interaction of membrane active peptides (i.e., antimicrobial peptides (AMPs)) or proteins with biomembranes by changing the membrane tension of plasma membranes. AMPs with bactericidal activity have been generated by various organisms, such as amphibians, invertebrates, plants, and mammals. The main mechanism for the antibacterial activity of AMPs is the AMPs-generated pore formation or damage in bacterial plasma membrane. AMPs are highly cationic peptides, and thus can bind strongly to the negatively charged outer monolayer of the bacterial cytoplasmic membrane via B. I applied the Π to GUVs using the same method described in Chap. 2. Based on the results of Chap. 2, the σ_{osm} of the GUVs can be estimated quantitatively. I examined the interaction of magainin 2 with single GUVs encapsulating calcein under Π using the single GUV method. Magainin 2 induced leakage of calcein from single GUVs stochastically. The statistical analysis of this phenomenon provided the values of the rate constant of magainin 2-induced pore formation in the GUV membrane (k_p). The k_p increased as Π increases. The k_r at ΔC^0 = 12 mOsm/L is ~3 times larger than that in the absence of Π. I can explain qualitatively that Π-generated stretching of the membrane enhances the rate of magainin 2-induced pore formation, which supports the concept of the stretch-activated pore of magainin 2. Based on these results, I discussed the mechanism of magainin 2-induced pore formation.