Single Giant Unilamellar Vesicle Method Reveals Lysenin-Induced Pore Formation in Lipid Membranes Containing Sphingomyelin

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ABSTRACT

Lysenin is a sphingomyelin (SM)-binding pore-forming toxin. To reveal the interaction of lysenin with lipid membranes, we investigated lysenin-induced membrane permeation of a fluorescent probe, calcein, through dioleoylphosphatidylcholine (DOPC)/SM, DOPC/SM/cholesterol (chol), and SM/chol membranes, using the single giant unilamellar vesicle (GUV) method. The results clearly show that lysenin formed pores in all the membranes, through which membrane permeation of calcein occurred without disruption of GUVs. The membrane permeation began stochastically, and the membrane permeability coefficient increased over time to reach a maximum, steady value, $P^s$, which persisted for a long time (100–500 s), indicating that pore concentration increases over time and finally reaches its steady value, $N_p^s$. The $P^s$ values increased as the SM/lysenin ratio decreased, and, at low concentrations of lysenin, the $P^s$ values of SM/DOPC/chol (42/30/28)-GUVs were much larger than those of SM/DOPC (58/42)-GUVs. The dependence of $P^s$ on the SM/lysenin ratio for these membranes was almost the same as that of the fraction of sodium dodecyl sulphate (SDS)-resistant lysenin oligomers, indicating that $N_p^s$ increases as the SDS-resistant oligomer increases. On the other hand, lysenin formed pores in GUVs of SM/chol (60/40) membrane in homogeneous liquid-ordered phase, indicating that the phase boundary is not necessary for the pore formation. The $P^s$ values of SM/chol (60/40)-GUVs were smaller than those of SM/DOPC/chol (42/30/28)-GUVs even though the SDS-resistant oligomer fractions was similar for both membranes, suggesting that not all of the oligomers can convert into a pore. Based on these results, we discuss the elementary processes of lysenin-induced pore formation.
Pore-forming toxins (PFTs) have been discovered in, and isolated from, a wide variety of organisms including bacteria and invertebrates.\textsuperscript{1-4} The target of most of these protein toxins is considered to be lipid membrane regions of plasma membranes; water-soluble toxins bind to the plasma membrane and then create pores in the membrane that induce cytolysis. For example, \(\alpha\)-hemolysin from \textit{Staphylococcus aureus},\textsuperscript{5} pneumolysin from \textit{Streptococcus pneumoniae},\textsuperscript{6} and equinatoxin II from Beadlet Anemone (\textit{Actinia equinae}),\textsuperscript{7} all bind a specific lipid and form a pore in the membrane. From the structural point of view, PFTs can be classified into \(\alpha\)-PFTs and \(\beta\)-PFTs; the pores of \(\alpha\)-PFTs and \(\beta\)-PFTs composed of \(\alpha\)-helices and a \(\beta\)-barrel, respectively.\textsuperscript{4} The pore-forming activity of PFTs has been investigated by analysis of their induced hemolysis of red blood cells (RBC) and by leakage of a fluorescent probe from small liposomes such as large unilamellar vesicles (LUV) using the LUV suspension method.\textsuperscript{4-5, 7-12} These PFTs are considered to form an oligomer in biomembranes, which then forms a pore. However, the detailed elementary processes and the mechanism of toxin-induced pore formation, including the relationship between oligomers and pores, and the characteristics of the pores such as membrane permeability, remain unclear.

Lysenin is a 33.4 kDa PFT secreted by earthworms (\textit{Eisenia foetida}), which specifically binds sphingomyelin (SM).\textsuperscript{8-10} Several experimental data indicate that lysenin molecules form oligomers in lipid membranes that contain SM. Transmission electron microscope images obtained using negative staining show that lysenin molecules form a hexagonal pattern in SM-containing membranes. The unit structure of this hexagon has a diameter of 10-12 nm, which corresponds to that of a lysenin oligomer.\textsuperscript{9} It is well established that the lysenin oligomers can be detected using SDS-PAGE since they are resistant to SDS solubilization.\textsuperscript{11} Lysenin can induce hemolysis of RBCs.\textsuperscript{8} However, preincubation of lysenin with SM-containing liposomes inhibits this hemolysis,\textsuperscript{11} and the presence of cholesterol in the SM-containing liposomes increases the efficiency of the inhibition of lysenin-induced hemolysis. A recent study showed that an increase in the amount of the SDS-resistant oligomer of lysenin in SM-containing liposomes increased the above-described
inhibition of hemolysis and also that lysenin monomers in lipid membranes can detach from the membrane and transfer to the RBC membrane, resulting in their hemolysis. These results show that the SDS-resistant oligomer of lysenin cannot either detach from the membrane or dissociate into monomers, indicating that the formation of an SDS-resistant oligomer is an irreversible process. It is considered that lipid domains containing SM molecules in plasma membranes or lipid membranes are sites of lysenin oligomerization.

Giant unilamellar vesicles (GUVs) of lipid membranes with diameters greater than 10 μm have been used for investigations of the physical and biological properties of vesicle membranes such as elasticity and shape change. Shape changes of a single GUV induced by substances such as peptides, proteins, and small molecules can be measured in real time. Based on the characteristics of these GUVs, we have recently developed the single GUV method to investigate functions and dynamics of biomembranes. Using this method, changes in the structure and physical properties of a single GUV that are induced by interactions with substances such as peptides are observed as a function of time and spatial coordinates. The same experiments are then carried out using many “single GUVs” and their results such as changes in the physical properties of a single GUV are statistically analyzed over many “single GUVs”. This single GUV method can reveal the details of elementary processes of individual events, and allow calculation of their kinetic constants. For example, we explain the single GUV-method studies of pore formation induced by the antimicrobial peptide, magainin 2, in lipid membranes. Using the technique of purification of GUVs containing water-soluble fluorescent probes, we developed the single GUV method to measure the leakage (or membrane permeation) of internal contents such as fluorescent probes using fluorescent microscopy. Then we applied this method to investigate the magainin 2-induced leakage, and successfully obtained the rate constants of magainin 2-induced pore formation in lipid membranes and also the rate constants of membrane permeation of fluorescent probes through the pores. These data provide information that is critical for elucidation of the mechanism for the magainin 2-induced pore formation. There are several advantages of the single GUV method over the LUV
suspension method and the hemolysis. First, it is easy to identify the cause of the substance-induced membrane permeation (or leakage) of fluorescent probes inside a vesicle. Generally, there are many factors inducing leakage of internal contents of liposomes such as fluorescent probes and leakage of hemoglobin from RBC (i.e., the hemolysis), which cannot be identified using the LUV suspension method and the hemolysis. For example, membrane fusion by virus induces hemolysis and polyethylene glycol-induced membrane fusion induces leakage of internal contents of liposomes. Rupture and fragmentation of liposomes or RBCs are also one of the factors of leakage and hemolysis. These factors such as membrane fusion and rupture can be easily detected using the single GUV method. Thereby, the detection of the leakage using the LUV suspension method and the hemolysis does not always indicate the pore formation in lipid membranes. Second, only the use of the single GUV method allows us to separate the step of the substance induced-pore formation in membranes from the step of membrane permeation of a fluorescent probe through the pores and to obtain the rate constants of these elementary steps. Analysis of the latter step can provide values of the membrane permeability coefficient of the fluorescent probe, from which we can get information on the number of the pores if the membrane permeability coefficient of single pore does not change in time. To elucidate the effects of mutation of proteins/peptides on the membrane permeation and the effects of lipid compositions or physical properties of lipid membranes on the membrane permeation, we need experimental data of the effects on both the kinetic constant of the pore formation and that of the membrane permeation through the pore.

The pore-forming activity of lysenin has been investigated by analysis of their induced hemolysis of RBCs and by leakage of a fluorescent probe using the LUV suspension method, which do not always indicate the pore formation, as described in the above section. In this report, to reveal the interaction of lysenin with SM-containing lipid membranes, we investigated lysenin-induced membrane permeation of fluorescent probes through dioleoylphosphatidylcholine (DOPC)/SM, DOPC/SM/cholesterol (chol) and SM/chol membranes using the single GUV method. We obtained detailed characteristics of lysenin-induced pore formation and the subsequent membrane
permeation of a probe through the pore. We also investigated the effects of lysenin concentration and the presence of cholesterol on lysenin-induced pore formation and membrane permeability. Based on the results, we discuss the relationship between the oligomerization of lysenin and lysenin-induced membrane permeability, and also the elementary processes of pore formation.

MATERIALS AND METHODS

Materials

DOPC, SM from brain, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG2K-DPPE), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)(NBD-DOPE) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). The Alexa Fluor 488 conjugate of the soybean trypsin inhibitor (AF-SBTI) was purchased from Invitrogen Inc. (Carlsbad, CA). Calcein was purchased from Dojindo Laboratory (Kumamoto, Japan). Bovine serum albumin (BSA) and cholesterol (chol) were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan). Lysenin was purchased from the Peptide Institute (Osaka, Japan).

Detection of lysenin-induced membrane permeation of fluorescent probes from single GUVs

GUVs composed of lipid membranes containing SM were prepared in PBS (1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl) by natural swelling of a dry lipid film at 60 °C using the PEG-lipid method. In this method, the addition of a small amount of PEG-lipid in lipid membranes enables to prepare GUVs of electrically neutral lipids such as DOPC and SM in a buffer containing high salt concentration. Here we used PEG-2K-DPPE as the PEG-lipid, and included 1 mol% PEG-2K-DPPE in all the GUV membranes. Two hundred microliters of 1 mM phospholipid (e.g., SM, DOPC, chol, and 1 mol% PEG-2K-DPPE) mixtures 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, 20 µL of water was added into this glass vial, and the mixture was incubated at 60 °C for 10 min (prehydration). The hydrated
lipid film was then incubated with 1 mL of 1 mM calcein (or 8 μM of AF-SBTI) in PBS containing 0.1 M sucrose for 2 to 3 h at 60 °C. To obtain a purified GUV suspension, untrapped calcein was removed using the membrane filtering method. The GUV suspension was centrifuged at 14,000 × g for 20 min at 20 °C to remove multilamellar vesicles. The supernatant was filtered through a nucleopore membrane with 12 μm diameter pores in PBS containing 0.1 M glucose for 1 h at a flow rate of 1 mL/min at room temperature (20~25 °C), and the suspension which was not passed through the filter was collected and used for following experiments as a purified GUV suspension.

The purified GUV suspension (300 μL) (0.1 M sucrose in PBS as the internal solution; 0.1 M glucose in PBS as the external solution) was transferred into a hand-made microchamber. A slide glass was coated with 0.1% (w/v) BSA in PBS containing 0.1 M glucose. The GUVs were observed using an inverted fluorescence phase-contrast microscope (IX-70, Olympus, Tokyo, Japan) at 37 ± 1 °C using a stage thermocontrol system (Thermoplate, Tokai Hit, Shizuoka, Japan). Phase-contrast and fluorescence GUV images were recorded using a high-sensitivity EM-CCD camera (C9100-12, Hamamatsu Photonics K.K., Hamamatsu, Japan) with a hard disk. Three neutral density filters (two ND6 and one ND12) were used to decrease greatly the intensity of the incident light to decrease the photobleaching of calcein. We did not observe any photobleaching of calcein at least for 10 min observation under the experimental conditions. 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 lysenin in PBS containing 0.1 M glucose were continuously added 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 approximately 70 μm. It is considered that this method of application results in the equilibrium lysenin concentration near the GUV being almost the same as that in the micropipet. The details of this method are described in our previous reports.

Detection of the SDS-resistant oligomers of lysenin by SDS-PAGE
Small unilamellar vesicles (SUVs) of various lipid membranes were prepared using the standard method. PBS was added to the dry lipid film prepared by the same method as described in section 2.2, and the suspension was then sonicated at 55 °C under a N₂-saturated atmosphere using a probe-type ultrasonicator (XL-2000, MISONIX Inc. NY), and was subsequently centrifuged to collect the supernatant. A lysenin solution (187 nM) was incubated with various concentrations of SUVs for various time at 37 °C. Immediately after the incubation, the samples were solubilized in a 2%(w/v) SDS solution containing 2-mercaptoethanol for 5 min at 95 °C and then applied to 12% gel. The proteins were detected by silver staining using the SilverQuest silver staining kit (Invitrogen Inc. CA). NIH ImageJ (ver. 1.45s) was used to obtain the area of the peak corresponding to the monomer or oligomer of lysenin in the gels, and the fraction of lysenin that was present as an SDS-resistant oligomer (= the area of the oligomer peak / (the area of the oligomer and the area of the monomer)) was calculated. Lipid concentrations in the SUV suspensions were determined by the Bartlett method.

Detection of phase separation using confocal microscopy

To detect phase separation between liquid-ordered and liquid-disordered phases, GUVs of DOPC/SM membranes and DOPC/SM/cholesterol membranes containing 1.0 mol% NBD-DOPE after purification using the membrane filtering method were observed using a confocal microscope (FV-1000, Olympus, Tokyo, Japan) at 37 ± 1 °C using a stage thermocontrol system (Thermoplate, Tokai Hit, Shizuoka, Japan).

RESULTS

Lysenin-induced membrane permeation of fluorescent probes in single SM/DOPC/chol (42/30/28)-GUVs

We first investigated lysenin-induced membrane permeation of the fluorescent probe, calcein (Stokes-Einstein radius, \( R_{SE} \), is 0.74 nm), from single SM/DOPC/chol (molar ratio: 42/30/28) -GUVs. The interaction of lysenin with single GUVs containing 1 mM calcein was carried out in PBS containing 0.1 M glucose at 37 °C, and was analyzed by fluorescence microscopy using the
single GUV method. A typical experimental result of the effect of the interaction of 40 ng/mL
lysenin with single GUVs on the calcein concentration within a GUV is shown in Fig. 1A. Prior to
lysenin addition, the GUV displayed high contrast in a phase-contrast microscopic image (Fig.
1A(1)) due to the difference in refractive index produced by the difference in the concentration 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 lysenin solution, the fluorescence
intensity inside the GUV remained almost constant over the first 50 s, following which the
fluorescence intensity decreased gradually (Fig. 1A(2)). Fig. 1B shows a semi-log plot (base 10) of
normalized fluorescence intensity, $FI (= I(t)/I(0))$, vs. time (s), where $I(t)$ and $I(0)$ are the
fluorescence intensity of the inside of the GUV at time, $t$, and that of the intact GUV before initiation
of membrane permeation of calcein, respectively. The rate of the decrease in fluorescence intensity
(i.e., the absolute value of the slope of the curve) first increased over time, and then remained
constant after achieving a maximum value at 170 s. This maximum rate did not change for up to 450
s. After 450 s, the fluorescence intensity inside the GUV ($I(t)$) had decreased to less than 10% of $I(0)$
although a phase-contrast image of the same GUV (Fig. 1A(3)) showed that the GUV structure was
still intact with no detectable breaks. During the leakage, association and membrane fusion of GUVs,
and large shape changes were not observed. As discussed in our previous report on the interaction of
magainin 2 with single GUVs,\textsuperscript{25-27} the decrease in fluorescence intensity occurred as a result of the
membrane permeation of calcein (i.e., calcein leakage) from the inside to the outside of the GUV
through lysenin-induced pores in the membrane. Thus, the time at which the fluorescence intensity
began to decrease corresponds to the time when the first pore was 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 decrease in the phase contrast of the GUV, indicating that, during the
membrane permeation of calcein, sucrose and glucose also passed through the same pores. When the
same experiments were carried out using many “single GUVs”, we observed that membrane
permeation of calcein from a GUV started stochastically, and that the time courses of the changes in the fluorescence intensity of single GUVs were almost the same; i.e., the rate of the decrease in fluorescence intensity first increased over time, and then leveled off at a maximum value (Fig. 1C). This result indicates that pores were formed stochastically, and that the rate of membrane permeation at first increased with time but ultimately leveled off at a maximum value.

The rate constant of the membrane permeation of the fluorescent probe from the inside to the outside of a GUV, $k_{mp}$, when membrane permeation starts at $t = 0$, is determined by the following experimental formula,

$$C^{\text{in}}(t) = C^{\text{in}}_0 \exp\left(-k_{mp}(t) \cdot t\right)$$

(1)

where $C^{\text{in}}(t)$ (mol/m$^3$) and $C^{\text{in}}_0$ (mol/m$^3$) are the concentration of the fluorescent probe inside of a GUV at time $t$ after, and at $t_0$ before, initiation of membrane permeation by lysenin addition, respectively. The normalized concentration of the fluorescent probe inside a GUV, $C^{\text{in}}(t)/C^{\text{in}}_0$, can be experimentally determined, because the probe concentration inside the GUV is roughly proportional to the fluorescence intensity of the GUV, $I(t)$, i.e., $C^{\text{in}}(t)/C^{\text{in}}_0 = I(t)/I(0) = FI$. The rate constant of membrane permeation $k_{mp}(t)$ is proportional to the absolute value of the slope of the curve in a semi-log plot (base 10) of $FI$ vs. time (s). The results in Fig. 1B indicate that $k_{mp}$ at first increased over time and leveled off at the maximum value, $k^*_\text{mp}$. Moreover, the membrane permeability coefficient of the fluorescent probe per unit area of a GUV membrane, $P(t)$ (m/s), is related with the rate constant of the membrane permeation of the probe $k_{mp}(t)$, as described in our previous reports.$^{25,27}$ Generally, the membrane permeation of a substance can be expressed by the flux of the substance per unit area of pores, $J$ (mol/m$^2$·s), which follows Fick’s law;

$$J = -P(t)(C^{\text{in}}(t) - C^{\text{out}}(t))$$

(2)

where $C^{\text{out}}(t)$ (mol/m$^3$) is the concentration of the substance outside of the GUV at time $t$. Thereby, the concentration inside the GUV can be described by $P(t)$ as follows (here we assume $C^{\text{out}} = 0$ for any time, because the volume of the outside the GUV is very large),
\[
\frac{4\pi r^3}{3} \frac{dC^{\text{in}}}{dt} = -4\pi r^2 P(t)C^{\text{in}}
\]
\[
\therefore \quad \frac{d}{dt} \left( \ln \frac{C^{\text{in}}(t)}{C_0^{\text{in}}} \right) = -\frac{3P(t)}{r}
\]  

(3)

where \( r \) (m) is the radius of each GUV. The comparison between eqs. (1) and (3) gives

\[
P(t) = r k_{\text{mp}}(t)/3
\]  

(4A)

At the steady state of the membrane permeation, \( P(t) \), becomes constant (\( = P^s \)), and thereby, the rate of membrane permeation of the fluorescent probe from a GUV at the steady state \( k^{s}_{\text{mp}} \) can be related to \( P^s \) as follows,

\[
P^s = r k^{s}_{\text{mp}}/3
\]  

(4B)

The result of Fig. 1B indicates that, after pore formation, the value of \( P \) increased with time and then reached a steady, maximum value of \( P^s \), which continued for a long time (280 s). To obtain \( k^{s}_{\text{mp}} \) values from Fig. 1, the region of the curve corresponding to the steady state of the membrane permeation was fitted by a following equation.

\[
FI(t) = B \exp \left\{ -k^{s}_{\text{mp}}(t-t_a) \right\}
\]  

(5)

where \( B \) and \( t_a \) are fitting parameters. Using the value of \( k^{s}_{\text{mp}} \) and that of the radius of the GUV used in this experiment, the \( P^s \) value for Fig. 1B was obtained (\( P^s = 2.0 \times 10^{-2} \mu\text{m/s} \)). We performed the same experiments using many “single GUVs”, and obtained the average value of \( P^s \) as \( (2.4 \pm 0.2) \times 10^{-2} \mu\text{m/s} \) (the number of the examined “single GUVs”, \( n \), is 14; i.e., \( n = 14 \)).

We then investigated the interaction of various concentrations of lysenin with single SM/DOPC/chol (42/30/28)-GUVs containing 1 mM calcein to obtain the dependence of \( P^s \) values on lysenin concentration. The time courses of the changes in \( FI \) of many single GUVs during their interaction with 200 ng/mL lysenin are shown in Fig. 1D. The absolute values of the slopes of the steady state of membrane permeation of all the curves in Fig. 1D were larger than those for 40 ng/mL lysenin. The average value of \( P^s \) was \( (6.5 \pm 0.7) \times 10^{-2} \mu\text{m/s} \) \( (n = 18) \). A decrease in the fluorescence
intensity of a GUV was not observed at or below 2 ng/mL lysenin. The dependence of $P_s$ on lysenin concentration, $C$, (Fig. 1E and Table 1) shows that $P_s$ increased with $C$. The average value of $P_s$ when 600 ng/mL lysenin was added ($(1.5 \pm 0.2) \times 10^{-1} \mu m/s$) was approximately 20 times larger than that of the $P_s$ value for 10 ng/mL lysenin ($(6.9 \pm 1.7) \times 10^{-3} \mu m/s$).

To estimate the size of the lysenin-induced pores, we investigated the interaction of lysenin with single SM/DOPC/chol (42/30/28)-GUVs containing AF-SBTI ($R_{SE} = 2.8$ nm). The interaction of 600 ng/mL lysenin with single SM/DOPC/chol (42/30/28)-GUVs did not induce a change in the fluorescence intensity of the inside of the GUV, which reflects the concentration of AF-SBTI inside the GUV, over 10 min ($n = 15$) (Fig. S1 in Supporting Information). This result indicates that membrane permeation of AF-SBTI through lysenin-induced pores did not occur. Based on these results, we conclude that the radius of the lysenin-induced pore is smaller than 2.8 nm (the $R_{SE}$ of SBTI), but larger than 0.74 nm (the $R_{SE}$ of calcein). This value agrees with the size of the lysenin-induced pores in sheep RBC, which were estimated as approximately 1.5 nm by assay of inhibition of lysenin-induced hemolysis by various sized substances.9

**Lysenin-induced membrane permeation of calcein in single SM/DOPC (58/42)-GUVs**

To elucidate the effect of cholesterol on the lysenin-induced membrane permeation, we investigated the interaction of lysenin with single SM/DOPC (58/42)-GUVs containing 1 mM calcein. The ratio of SM to DOPC in SM/DOPC (58/42)-GUVs is the same as that in the SM/DOPC/chol (42/30/28)-GUVs. Fig. 2A shows a typical experimental result of the effect of the interaction of 200 ng/mL lysenin with single GUVs on the calcein concentration within a GUV. As shown in Fig. 2A, the lysenin-induced change in the fluorescence intensity of single SM/DOPC (58/42)-GUVs was similar to those in SM/DOPC/chol (42/30/28)-GUVs. During the addition of the 200 ng/mL solution of lysenin, the fluorescence intensity inside the GUV was almost constant over the first 50 s, following which the fluorescence intensity started to decrease gradually (Fig. 2B). The rate of the decrease in fluorescence intensity first increased with time but ultimately leveled off at the maximum value at 100 s, and remained at this level for a long time. We made the same experiments
using many “single GUVs”, and obtained values of $P^s$ for each GUV (Fig. 2C). The average value of $P^s$ was $(3.0 \pm 0.3) \times 10^{-2} \, \mu\text{m/s} \, (n = 19)$.

We investigated the interaction of various concentrations of lysenin with single SM/DOPC (58/42)-GUVs containing 1 mM calcein to obtain the dependence of $P^s$ values on lysenin concentration. For example, Fig. 2D shows the time course of $FI$ of many single GUVs during the interaction of 40 ng/mL lysenin. The absolute values of the slopes of the steady state of membrane permeation of all the curves in Fig. 2D were much smaller than those for 200 ng/mL lysenin. The average value of $P^s$ was $(3.0 \pm 0.3) \times 10^{-3} \, \mu\text{m/s}) \, (n = 8)$. Even at 700 s the fluorescence intensities inside the GUVs $I(t)$ were almost 50% of $I(0)$, but a phase-contrast image of the same GUV showed that the sucrose had completely leaked out and that the GUV structure was still intact with no detectable breaks. This result indicates that the rate of membrane permeation of sucrose through the pore in these GUVs is much larger than that of calcein. No decrease in the fluorescence intensity of a GUV was observed at lysenin concentrations at or below 20 ng/mL. The dependence of $P^s$ in single SM/DOPC (58/42)-GUVs on lysenin concentration, $C$, (■ in Fig. 2E) shows that $P^s$ values greatly increased as the lysenin concentration was increased i.e., $P^s$ values increased as the SM/lysenin ratio decreased, since the SM concentration was the same for all GUVs. The lysenin concentration dependence of $P^s$ of the membranes without cholesterol (SM/DOPC (58/42)-GUVs) was much greater than that of $P^s$ of the membranes with cholesterol (SM/DOPC/chol (42/30/28)). Thus, at low concentrations of lysenin, the $P^s$ values of membranes containing cholesterol (SM/DOPC/chol (42/30/28)) were much larger than those of membranes without cholesterol (SM/DOPC (58/42)-GUVs), but at high lysenin concentrations these $P^s$ values were similar.

**Formation of SDS-resistant oligomers of lysenin in lipid membranes containing SM**

Several experimental data indicate that lysenin molecules form oligomers in lipid membranes containing SM.$^9,11$ It is well established that these oligomers can be detected using SDS-PAGE since they are resistant to SDS solubilization.$^9,11$ The result of SDS-PAGE analysis of a lysenin solution after incubation with SM/DOPC(58/42)-SUVs or SM/DOPC/chol(42/30/28)-SUVs is shown in Fig.
3. The apparent molecular weight of lysenin in PBS is 41 kDa on SDS-PAGE (lane 1 in Fig. 3A). After 5 min incubation with SM/DOPC(58/42)-SUVs at 37 °C, a new band with a molecular weight greater than 260 kDa corresponding to the SDS-resistant oligomer appeared on the gel. The molecular weight of this band is the same as that previously reported. The fraction of lysenin that was present as an SDS-resistant oligomer (i.e., the SDS-resistant oligomer fractions of lysenin) increased as the SM/lysenin molar ratio decreased, and its dependence on the SM/lysenin ratio was much greater in the absence of cholesterol (SM/DOPC (58/42)-SUVs) than in the presence of cholesterol (SM/DOPC/chol (42/30/28)) (Fig. 3B). Thus, at high SM/lysenin ratios, the SDS-resistant oligomer fractions of lysenin in SM/DOPC (58/42) membranes was much lower than that in SM/DOPC/chol (42/30/28) membranes, i.e., the presence of cholesterol increases the fraction of SDS-resistant oligomers, but at low SM/lysenin ratios, these values are similar. These results qualitatively agree with previous results obtained using membranes of different lipid composition; SM/DOPC(1/4) and SM/DOPC/chol (1/4/1.5). The SDS-resistant oligomer fractions of lysenin in the samples incubated at 37 °C for 2 min was similar to that in samples incubated for 30 min (Fig. 3C). This result indicates that the rate at which SDS-resistant oligomers of lysenin form in the lipid membrane is very fast and that most of the oligomerization processes occur within the first 2 min of incubation at 37 °C.

**Phase separation of SM/DOPC/chol-GUVs and SM/DOPC-GUVs**

It is well known that the phase separation between the liquid-ordered (lo)-phase domain and the liquid-disordered (ld) phase domain occurred in SM/DOPC/chol membranes. To check the phase separation, a small amount (1 mol% in the total membrane lipids) of a fluorescence probe, NBD-DOPE, were included in the SM/DOPC/chol-GUVs and SM/DOPC-GUVs. Fig. 4A shows confocal fluorescence microscopic images of a SM/DOPC/chol (42/30/28)-GUV in PBS. Clear large phase separation was observed, and the domains with high fluorescence intensity correspond to the ld phase. The size of the lo-phase domains is very large and the number of the domains was small. These results indicate that the fluorescence microscopy system we used here can reveal large phase
separation in SM/DOPC/chol (42/30/28)-GUVs clearly. In contrast, the fluorescence microscopic image of a SM/DOPC (58/42)-GUV (Fig. 4B) shows that the phase separation occurred and there were many small-sized lo-phase domains.

**Lysenin-induced membrane permeation of calcein in single SM/chol (60/40)-GUVs**

In the interaction of lysenin with SM/DOPC/chol (42/30/28)-GUVs, lysenin may preferentially produce pores in SM/chol-rich domains in the lo-phase, as demonstrated in the interaction of lysenin with cells. To elucidate the lysenin-induced pore formation in the homogeneous lo-phase membrane, we next investigated the interaction of lysenin with calcein (1 mM)-containing single GUVs composed of a SM/chol (60/40) membrane that is completely in the lo phase, under the same condition as that described above. Fig. 5A shows a typical experimental result of the effect of the interaction of 40 ng/mL lysenin with single GUVs on the calcein concentration within a GUV. During the addition of the 40 ng/mL solution of lysenin, the fluorescence intensity inside the GUV was almost constant over the first 140 s, following which the fluorescence intensity started to decrease gradually (Fig. 5A(2) and Fig. 5B). The rate of the decrease in fluorescence intensity increased with time, then became the steady, maximum value at 300 s. After 690 s, no fluorescence could be detected inside the GUV although a phase contrast image of the same GUV (Fig. 5A (3)) showed that the GUV structure was still intact with no detectable breaks. From the plot of FI vs. time, we obtained $P^s$. In the 40 ng/mL lysenin-induced pore, $P^s$ was relatively large (i.e., $P^s = (1.0 \pm 0.1) \times 10^{-2} \, \mu \text{ms}^{-1}$) ($n = 15$). When the same experiments were carried out using many “single GUVs”, we observed that the membrane permeation of calcein from a GUV started stochastically, but the time course of the fluorescence intensity of single GUVs were almost the same; i.e., the rate of the decrease in fluorescence intensity increased with time, then became the steady, maximum value (Fig. 5C).

We then investigated the interaction of various concentrations of lysenin with single SM/chol (60/40)-GUVs containing 1 mM calcein to obtain the dependence of $P^s$ values on lysenin concentration. The time courses of the changes in $FI$ of many single GUVs during their interaction
with 200 ng/mL lysenin are shown in Fig. 5D. The absolute values of the slopes of the steady state of membrane permeation of all the curves in Fig. 5D were larger than those for 40 ng/mL lysenin. The average value of \( P^s \) was \((1.7 \pm 0.2) \times 10^{-2} \mu \text{m/s} \) \((n = 18)\). A decrease in the fluorescence intensity of a GUV was not observed at or below 10 ng/mL lysenin. The lysenin concentration \((C)\) dependence of \( P^s \) in single SM/chol (60/40)-GUVs (▲ in Fig. 5E and Table 1) shows that \( P^s \) gradually increased with an increase in \( C \). Furthermore, the dependence of \( P^s \) on \( C \) was much smaller in SM/chol (60/40)-GUVs than in SM/DOPC/chol (42/30/28)-GUVs, and the \( P^s \) values of SM/chol (60/40)-GUVs were much smaller than those of SM/DOPC/chol (42/30/28)-GUVs at high concentrations of lysenin.

We also investigated the SDS-resistant oligomer formation in SM/chol (60/40) membranes. The result of SDS-PAGE analysis of a lysenin solution after incubation with SM/chol (60/40)-SUVs is shown in Fig. 6. The SDS-resistant oligomer fractions of lysenin in SM/chol (60/40) membranes gradually increased as the SM/lysenin molar ratio decreased, and its dependence on the SM/lysenin ratio was almost the same as that of SM/DOPC/chol(42/30/28) membranes. The SDS-resistant oligomer fractions of lysenin in the SM/chol (60/40) membranes incubated at 37 °C for 2 min was similar to that in samples incubated for 30 min (Fig. 6C). This result indicates that the rate at which SDS-resistant oligomers of lysenin form in the lipid membrane is very fast and that most of the oligomerization processes occur within the first 2 min of incubation at 37 °C.

**DISCUSSION**

**Correlation between lysenin-induced membrane permeation and the SDS-resistant oligomer fraction of lysenin**

The results obtained using the single GUV method clearly show that lysenin induced pores in three kinds of lipid membranes and the membrane permeation of calcein occurred through the pores without the disruption of the GUVs. It is reported that high concentrations of His-lysenin caused fragmentation of liposomes.\(^{38}\) If we would investigate the His-lysenin-induced leakage of a fluorescent probe using the LUV suspension method, we could not distinguish between the pore
formation and the fragmentation of liposomes. The membrane permeation of calcein began stochastically at the initial stage of the interaction of lysenin with a GUV. Subsequently, the membrane permeability coefficient increased over time to reach a maximum, steady value, $P^*$, which persisted for a long time (100–500 s). We succeed in determining $P^*$ values under various conditions. The results showed a strong correlation between lysenin-induced membrane permeability coefficient and SDS-resistant oligomerization of lysenin in SM/DOPC (58/42) and SM/DOPC/chol (42/30/28) membranes. The SDS-resistant oligomer fraction of lysenin and the $P^*$ values increased as the SM/lysenin ratio decreased, and the lysenin concentration dependence of the SDS-resistant oligomer fractions and that of the $P^*$ values of the membrane without cholesterol (SM/DOPC (58/42)-GUVs) were much greater than those of the membrane with cholesterol (SM/DOPC/chol (42/30/28)). Therefore, at low concentrations of lysenin, the SDS-resistant oligomer fraction and the $P^*$ values of SM/DOPC/chol (42/30/28) membranes were much higher than those of SM/DOPC (58/42) membranes, but these values were similar at high lysenin concentrations.

If we consider that lysenin molecules form a specific oligomer in lipid membranes containing SM and form pores all of which have the same diameter, the value of $P(t)$ of the lipid membrane is determined by the permeability coefficient of a single pore, $P_1$, the fraction of the open state of the pore (i.e., the probability of opening), $P_{\text{open}}$, and the pore concentration (i.e., the number of pores per unit area), $N_p(t)$, i.e., $P(t) = P_1 P_{\text{open}} N_p(t)$. We can therefore interpret the results of Figs. 1 and 2 as follows. The data in Figs. 1C and 2C indicate that a pore is first formed in the lipid membrane, following which $N_p$ increases over time and finally reaches a steady, maximum value, $N_p^s$. The data in Fig. 2E suggest that the value of $N_p^s$ increases as the lysenin concentration in the buffer increases, and that at low concentrations of lysenin $N_p^s$ values of membranes containing cholesterol (SM/DOPC/chol (42/30/28)) are much larger than those of membranes without cholesterol (SM/DOPC (58/42)-GUVs), but at high concentrations of lysenin these values are similar.
Based on the above discussion, we can reasonably conclude that $N_p$ increases as the SDS-resistant oligomer fraction of lysenin increases in lipid membranes. On the other hand, the $P_e$ of SM/chol (60/40)-GUVs was much smaller than that of SM/DOPC/chol (42/30/28)-GUV (Fig. 5E), even though the SDS-resistant oligomer fractions of lysenin were similar in both membranes (Fig. 6B). These results indicate that not all of the oligomers in the membrane can convert into pores. In both the membranes, concentration of oligomer (correctly speaking, concentration of irreversible oligomer, see the following section), $N_o(t)$, increased with lysenin concentration in buffer because the SDS-resistant oligomer fractions were similar irrespective of lysenin concentration (Fig. 6B), and thereby Fig. 5E indicates that $P_e$ increased with $N_o^s$, i.e., $N_o$ value at the steady state. Based on the above discussion, we can conclude that a part of the lysenin oligomers can convert into pores, and that the following equation can be held as an approximation of the relationship between $N_p^s$ and $N_o^s$;

$$N_p^s = \beta N_o^s$$

(where $\beta$ is a constant that depends on the physical property of the membrane), where the value of $\beta$ for SM/chol (60/40) membranes is smaller than that for SM/DOPC/chol (42/30/28) membranes. Moreover, we can conclude that the SDS-resistant oligomer fractions of lysenin represent both the oligomers which compose pores and the irreversible oligomers before conversion to pores, and thereby the results in this report provide the first experimental evidence that the SDS-resistant oligomer is not equal to the pore.

**Lysenin-induced pore formation in lipid membranes**

In this section we analyze the lysenin-induced pore formation more quantitatively. After starting the interaction of lysenin with single GUVs the fluorescence intensities inside the GUVs were almost constant over the first specific period, following which the fluorescence intensity started to decrease gradually and stochastically, indicating that the formation of the first pore in lipid membranes occurred stochastically. During the initial time when the fluorescence intensities inside the GUVs were almost constant, lysenin in buffer binds to SM molecules in the lipid membrane and lysenin monomers bound with SM form an oligomer, then a pore is formed. As demonstrated in our previous report, when estimating the rate constant of the pore formation, the time course of the
fraction of intact GUV, $P_{\text{intact}}(t)$, from which calcein did not start to leak among the population of GUVs examined, is important. When $P_{\text{intact}}(t)$ rapidly decreases with time, the rate of the pore formation is large. If the rate-determining step of the pore formation can be considered as a two-state transition (or conversion), $P_{\text{intact}}(t)$ decreases exponentially with time. In the case of magainin 2, we considered the two-state transition model from the binding state to the pore state, and thereby $P_{\text{intact}}$ of GUVs in the presence of various concentrations of magainin 2 can be well fitted by a single exponential decay function defined as follows,$^{25,26}$

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

(6)

where $k_p$ is the rate constant of the two-state transition and $t_{eq}$ is a fitting parameter. However, in other cases $P_{\text{intact}}$ may decrease pseudoexponentially with time.

We can estimate the rate of lysenin-induced pore formation by the time course of $P_{\text{intact}}(t)$. Fig. 7A shows that $P_{\text{intact}}$ of SM/DOPC/chol (42/30/28)-GUVs decreased with time in the presence of various concentrations of lysenin, which were almost fitted by a single exponential decay function defined by Eq. (6). The rate constant of the pore formation increased with increasing lysenin concentration: for the average value of $k_p$ for 600 ng/mL lysenin ($2.6 \pm 0.1 \times 10^{-1} \text{ s}^{-1}$) was about 70 times larger than that of $k_p$ for 10 ng/mL lysenin ($3.6 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$) (Fig. 7B). The $k_p$ values of SM/chol (60/40)-GUVs were much smaller than those of SM/DOPC/chol (42/30/28)-GUV, irrespective of lysenin concentrations (Fig. 7B). In contrast, in the case of SM/DOPC (58/42)-GUVs, several curves of the time course of $P_{\text{intact}}$ were not well fitted by a single exponential function. However, we can roughly compare the rate of the pore formation using the fraction of leaked GUV, $P_{\text{LS}}(t)$, i.e., the fraction of GUV in which leakage had already started, among all the examined GUV.$^{21,25}$ Thus: $P_{\text{LS}}(t) = 1 - P_{\text{intact}}(t)$. Fig. 7C shows $P_{\text{LS}}(120 \text{ s})$ for all the examined GUVs. At low concentrations of lysenin, the $P_{\text{LS}}(120 \text{ s})$ values of SM/DOPC/chol (42/30/28) membranes were much higher than those of SM/DOPC (58/42) membranes, but these values were similar at high lysenin concentrations. This result suggests that at low concentrations of lysenin, the values of the
rate of the pore formation of SM/DOPC/chol (42/30/28) membranes were much higher than those of SM/DOPC (58/42) membranes, but these values were similar at high lysenin concentrations.

Generally many kinds of schemes of the PFT-induced pore formation have been proposed. For example, PFT molecules in a buffer bind to a lipid membrane, and a monomer of the bound PFT can diffuse laterally in the membrane and collide each other to form an oligomer, and then a conformational change induces oligomer to pore conversion. In some PFTs insertion of protein segments may occur in the binding of monomer PFT, but in others insertion of protein segments occurs in the oligomer to pore conversion. For the lysenin-induced pore formation, we can consider several schemes involved in intermediate states (i.e., the oligomer states), but at present we cannot identify the right scheme due to limited experimental data. Here we consider a few models of the lysenin-induced pore formation in lipid membranes containing SM. A lysenin molecule in a buffer surrounding a single GUV can bind to the SM molecules in the lipid membranes specifically and the binding equilibrium is attained very rapidly. We call this lysenin as the monomer lysenin. Several monomer lysenin molecules in the lipid membrane form an oligomer lysenin, which induces a pore in lipid membranes. This lysenin-induced pore formation is composed of two elementary steps; one is the formation of lysenin oligomer (i.e., the oligomerization step) and the other is the oligomer-induced pore formation (i.e., the pore formation step). We can consider two cases of the reaction schemes; in the Case 1 the oligomerization step is rapid and the pore formation is the rate-determining step, and in the Case 2 the oligomerization step is the rate-determining one, and immediately after the oligomerization a pore is formed. For the Case 1, the scheme of the reaction of the pore formation can be represented as follows.

\[
\text{(Case 1)} \quad N \text{ (Monomer)} \Leftrightarrow \text{ (Reversible Oligomer)} \rightarrow \text{ (Irreversible Oligomer)} \rightarrow \text{ (Pore)}
\]

Here we assume the presence of reversible oligomer which can reversibly convert to the monomer, based on the general tendency of protein association, although we don’t have any experimental evidence on the existence of the reversible oligomer. We can reasonably consider that the lysenin irreversible oligomer which cannot convert to the monomer is the same as the pre-pore revealed in
the case of β-PFTs. As described in the above section, the SDS-resistant oligomer fractions of lysenin represent both the irreversible oligomers and the pores. In the Case 1, the conversion of irreversible oligomer (or pre-pore) to pore is slow and the rate-determining step. \( N_o(t) \) defined in the previous section is the concentration of the irreversible oligomer. The pore formation in the SM/chol (60/40) membrane may follow the scheme of the Case 1, because the results in this report indicate that \( N_o(t) \) is large but \( N_p(t) \) is small. If the rate of the irreversible oligomer formation from lysenin monomer in lipid membranes is very large and the conversion of irreversible oligomer to pore is very slow, the pore formation can be approximated as the two-state transition, and thereby \( k_P \) can be regarded as the rate constant of the conversion from the irreversible oligomer state to the pore state. In other cases, \( k_P \) may be obtained by a psuedoexponential decay of \( P_{\text{intact}} \) of GUVs. On the other hand, for the Case 2, the scheme of the reaction of the pore formation can be represented as follows.

\[
\text{(Case 2) } N (\text{Monomer}) \leftrightarrow (\text{Reversible Oligomer}) \rightarrow (\text{Irreversible Oligomer}) \Rightarrow (\text{Pore})
\]

Generally there are many pathways of the oligomerization of the monomer, and the rate of the oligomerization depends on the pathway. In the Case 2, the rate of the pore formation equals to that of the oligomerization, and thereby the rate constant \( k_P \) determined by the single exponential decay or the psuedoexponential decay of \( P_{\text{intact}} \) of SM/DOPC/chol (42/30/28)-GUVs (Fig. 7A) is that of the oligomerization. In other cases, a single exponential decay or a psuedoexponential decay of \( P_{\text{intact}} \) of GUVs may be observed. Irrespective the schemes of lysenin-induced pore formation, the results of Fig. 7B and C indicate that the rate of the pore formation increased with lysenin concentration depending on the lipid compositions of the membranes, and that at low concentrations of lysenin the values of the rate of the pore formation of SM/DOPC/chol (42/30/28) membranes were much higher than those of SM/DOPC (58/42) membranes, but these values were similar at high lysenin concentrations.

If we consider that, at equilibrium, a part of the lysenin monomers (\( aM_0 \), where \( M_0 \) is lysenin monomer concentration in lipid membrane before starting oligomerization) change into the irreversible oligomers (i.e., \( N_o = aM_0/N \), where \( N \) is the number of monomers which form an
oligomer). In the case where the formation of oligomer is irreversible as in the Case 1 and Case 2, eventually almost all the monomers change into the irreversible oligomers (i.e., $\alpha \approx 1$). Subsequently a part of the irreversible oligomers change ($\beta N_0$) into pores, the final pore concentration is $N_p^{eq} = \alpha \beta M_0 / N \cdot$ As described in the previous section, $P(t) = P_1 P_{open} N_p(t)$, and therefore the final equilibrium value of $P(t)$, $P^{eq}$, is proportional to $M_0$, because $P^{eq} = P_1 P_{open} N_p^{eq} = P_1 P_{open} \alpha \beta M_0 / N \cdot$ We can reasonably consider that the binding of lysenin with lipid membranes that contain SM molecules follows the Langmuir adsorption isotherm, and therefore $M_0$ is determined as follows,

$$M_0 = \frac{AK_B C}{1 + K_B C} = A \left(1 - \frac{1}{1 + K_B C}\right) \quad (7)$$

where $C$ is the lysenin concentration in buffer, $K_B$ is the binding constant of lysenin with SM, and $A$ is the concentration of lysenin binding sites composed of several SM molecules. It should be noted that in single GUV experiments the lysenin concentration around the GUV is always constant, and that this condition is used to derive the above Eq. (7). Therefore, $P^{eq}$ is determined by $C$ and $A$ as follows,

$$P^{eq} = \frac{P_1 P_{open} \alpha \beta}{n} M_0 = \frac{P_1 P_{open} \alpha \beta A}{n} \left(1 - \frac{1}{1 + K_B C}\right) \quad (8)$$

This relationship indicates that the dependence of $P^{eq}$ on the lysenin concentration follows the Langmuir isotherm type of equation. If the rate of oligomerization and that of conversion from an oligomer to a pore is rapid, $P^s$ can be approximated as $P^{eq}$.

The localization of SM molecules in the lipid membranes may play an important role in lysenin-induced pore formation. As shown in Fig. 4, a phase separation occurs in SM/DOPC/chol and SM/DOPC membranes; SM/chol-rich microdomains appear in SM/DOPC/chol membranes and SM-rich domains appear in SM/DOPC membranes. Atomic force microscopy also reveals the phase separations in SM/DOPC membranes of similar compositions. SM/chol-rich domains are in the $lo$ phase, and therefore the diffusion coefficients of lipid molecules are relatively high. Thus, lysenin molecules bound to SM molecules in SM/chol-rich domains can rapidly diffuse within this
domain and frequently collide with other lysenin molecules bound to SM molecules, resulting in a high rate of lysenin oligomerization and subsequent pore formation. In this situation, we can assume that the experimentally determined value of $P^s$ is almost equal to that of $P^eq$. The dependence of $P^s$ in single SM/DOPC/chol (42/30/28)-GUVs on $C$ (nM) (same data as in Fig. 1E) fitted well to Eq. (8) (○ in Fig. 8A). Based on this fitting, a lysenin-SM binding constant $K_B$ of 0.046 nM$^{-1}$ was obtained. Similarly, the dependence of $P^s$ in single SM/chol (60/40)-GUVs on $C$ (nM) also fitted well to Eq. (8) (Fig. 8B) and a lysenin-SM binding constant $K_B$ of 0.17 nM$^{-1}$ was obtained. These values of $K_B$ were almost the same as the $K_B$ value for SM/chol (50/50) membrane (i.e., $K_B = 0.12$ nM$^{-1}$). The other information obtained by this fitting was the value of $P_1P_{open}a\beta A/N$, which was 0.33 $\mu$m/s for SM/DOPC/chol (42/30/28), and 0.037 $\mu$m/s for SM/chol (60/40) membranes.

It is worth to try to obtain the value of the permeability coefficient of a single pore, $P_1$. Recent single channel recording measurement of lysenin-induced pore showed no observable closing events of the pore, indicating that $P_{open} = 1$. However, lysenin with a polyhistidine tag (lysenin-His) induced channel activity with closing events in other lipid membranes, indicating that $P_{open} < 1$. The difference between these results may be due to the presence of polyhistidine tag or different lipid composition. Here we approximate that the lysenin-induced pore is always in the open state, i.e., $P_{open} = 1$. If we assume that the oligomer of lysenin that is present in the lipid membranes is a hexamer (i.e., $N = 6$) and that 100% of the monomer changes into pores (i.e., $\alpha = \beta = 1$), then, $0.33 = P_1A/n$ in SM/DOPC/chol (42/30/28)-GUVs, the value of $P_1$ can be estimated as $1.7 \times 10^{-5}$ $\mu$m$^3$/s.

In contrast, the physical property of the SM rich domains in SM/DOPC membranes and the behavior of the SM-bound lysenin are not very clear. It is certain that the SM-rich domain contains some fractions of DOPC molecules from the point of view in physics of phase separation. To obtain the accurate value of SM (or DOPC) concentration in the SM rich domains after the phase separation, we need a phase diagram of temperature vs. SM concentration for SM/DOPC membranes, but it is not known. Moreover, differential scanning calorimetry revealed that a broad gel-to-liquid crystalline (Lα) phase transition with a maximum at 41 °C occurs in pure SM membrane.
due to the multicomponent mixture of hydrocarbon chains, indicating that some portions of SM molecules are in the l/d phase under the experimental conditions in this report. At present, we have a following hypothesis. The SM rich domains in SM/DOPC (58/42)-GUVs are in the solid-ordered (so) or the gel phase, and therefore the diffusion coefficients of the lipid molecules are low. In this case, the frequency of lysenin molecule collision due to lateral diffusion is low. However, at high concentrations of the bound lysenin monomer, these SM-bound lysenin molecules are close to each other in the membrane and therefore even a small movement or change in orientation can induce their oligomerization. Hence, at low concentrations of lysenin, the $P^s$ values were very small, but at high concentrations of lysenin, the $P^s$ values were very large, which were similar to those of SM/DOPC/chol (42/30/28)-GUVs. This may be the reason why the dependence of $P^s$ in single SM/DOPC (58/42)-GUVs on lysenin concentration did not fit to Eq. (8). The SM concentration in the SM-rich domains after the phase separation in SM/DOPC (58/42) membrane is larger than 58 mol%. We observed lysenin-induced membrane permeation of calcein with similar values of $P^s$ in SM/DOPC (90/10)-GUVs to those in SM/DOPC (58/42)-GUVs ($P^s = (1.7 \pm 0.3) \times 10^{-1} \mu \text{m/s} \ (n = 5)$, for 600 ng/mL lysenin) (Fig. S2 in Supporting Information). The confocal fluorescence microscopic images of SM/DOPC (90/10)-GUVs suggest no phase separation occurred in the resolution of optical microscopic image, although nano-scale phase separation may occur (Fig. S2 (D)). This SM/DOPC (90/10) membrane may have similar composition of the SM-rich domains in SM/DOPC (58/42)-GUVs, although it may have small amounts of l/d phase (i.e., the mixture of so and l/d phases). Thereby, this result may suggest that lysenin can form pores in the SM-rich domains.

At present we don’t have experimental evidence to explain the mechanism for the result that the $P^s$ of SM/chol (60/40)-GUVs was much smaller than that of SM/DOPC/chol (42/30/28)-GUV even though the SDS-resistant oligomer fractions of lysenin were similar in both membranes. We have a following hypothesis on the mechanism. For the formation of a pore, it is necessary that protein segments of lysenin insert into the lipid membrane to form a pore. The insertion of a pore composed of protein segments into lipid membrane increases the lateral pressure. Thereby further insertion of
protein segments is suppressed. Particularly for SM/chol (60/40) membranes, all of the membrane regions have a large area compressibility modulus, and therefore the rate of insertion of segments is low and also the number of the inserted segments or the number of the pores is small due to large lateral pressure. In contrast, in the case of SM/DOPC/chol (42/30/28) membranes, these membranes contain $l_d$ phase regions where the elastic modulus is small, and therefore a relatively large number of protein segments can enter into the membrane, which increases the number of the pores that can be formed.

**Biological implications**

The results in this report clearly show that low concentrations of lysenin can rapidly form pores in SM/DOPC/chol-GUVs using SM/chol-rich microdomains in the $l_o$ phase. Many cells have similar microdomains (or rafts) in plasma membranes that are composed of SM, cholesterol, and several proteins such as GPI-anchored proteins, which are in the $l_o$ phase, and they are considered to play important roles in signal transduction. Therefore, these microdomains in plasma membranes are very suitable targets for lysenin, because low concentrations of lysenin can rapidly form many pores in the plasma membrane to induce cytolysis and kill the cells. Sobota and his colleague found that during fractionation of Triton X-100 cell lysates SDS-resistant oligomers of lysenin with a glutathione-S-transferase (GST) tag associated with membrane fragments insoluble in Triton X-100, supporting the above conclusion.

It is considered that the phase boundary between the $l_o$ and $l_d$ phase plays an important role in pore formation of another PFT, equinatoxin II. However, our results clearly show that lysenin does form a pore in a complete, homogeneous $l_o$ phase membrane (i.e., SM/chol (60/40)) (Fig. 5), indicating that lysenin-induced pore formation can occur in the absence of the phase boundary. At present, we don’t know the structure of the lysenin-induced pore. However, to form a stable pore in lipid membrane, protein segments such as $\alpha$-helix or $\beta$-sheet must insert into lipid membranes. Thereby, the above results indicate that a protein segment can easily insert into a lipid membrane in the pure $l_o$ phase to form a pore. This possibility is supported by our previous results that lipids with
a single long hydrocarbon chain such as lysophosphatidylcholine can insert into a complete, homogeneous liquid phase membrane from an aqueous solution outside of single GUVs.23,24

**Advantages and an inherent problem in the present stage of the single GUV method**

Using the single GUV method, we succeeded in separating one elementary step (i.e., the lysoenin-induced pore formation) from the other elementary step (i.e., the membrane permeation of fluorescent probes through the pores), and in obtaining information on the rate constant or the rate of the pore formation and the membrane permeability coefficient of the fluorescent probe and its dependence on time.

However, there is a problem in the present stage of the single GUV method, i.e., the accuracy of the rate constants of the elementary steps. As shown in Table 1 and Figs. 2E and 5E, the standard errors of the membrane permeability coefficients are 10~20 % of the average values when the numbers of the examined single GUVs were 10~20. Thereby the membrane permeability coefficients have 2 significant digits. On the other hand, the standard errors of the rate constant of the lysoenin-induced pore formation are 10~67 % of the average values when 2 to 3 independent experiments were carried out for each lysoenin concentration using 10~20 single GUVs in each independent experiment (Fig. 7B). Thereby the rate constants of the lysoenin-induced pore formation have only 1 significant digit. Hence it is possible to compare only large differences of the rate constants of the pore formation. Recently inhomogeneity in lipid composition was observed in single nanoscale liposomes using fluorescent probe-labeled lipids.51 The inhomogeneity in lipid composition, i.e., the fluctuation of the number of constituent lipids, greatly decreased with an increase in diameter of liposomes, i.e., the total number of lipids in the membrane of single liposomes,51 as expected from the statistical physics.52 However, small inhomogeneity in the lipid composition exists in single GUVs of multicomponents. Fluctuation of lipid composition such as SM concentration in the membrane of single GUVs may be one of the important factors of large standard errors of the membrane permeability coefficients and the rate constants of the pore formation. In future we need more systematic study on the inhomogeneity of lipid compositions of single GUVs.
with diameters of 10–30 μm used in the single GUV method. Moreover, if we could improve the single GUV method to enable to examine much larger number of single GUVs, the significant digits of these rate constants would increase.

**Conclusion**

Using the single GUV method, we succeed in determining the rate constants of lysenin-induced pore formation in lipid membranes \( k_P \) and also the membrane permeability coefficient of a fluorescent probe through the lysenin-induced pores. We found that there was a strong correlation between the \( P^e \) values and the SDS-resistant oligomer fraction of lysenin in SM/DOPC (58/42) and SM/DOPC/chol (42/30/28) membranes, indicating that the pore concentration in lipid membranes increases with an increase in the SDS-resistant oligomer of lysenin. We also found that lysenin formed pores in GUVs of SM/chol (60/40) membrane in homogeneous liquid-ordered phase without disruption of GUVs, indicating that the phase boundary is not necessary for the pore formation. However, the results of the \( P^e \) values and the SDS-resistant oligomer fractions of lysenin of SM/chol (60/40) membranes indicate that not all of the oligomers in the membrane can convert into pores, i.e., there are irreversible oligomers (or pre-pores) before conversion to pores. These data provide us valuable information on the elementary processes of lysenin-induced pore formation in lipid membranes.

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**Supporting Information Available**
Experimental data of membrane permeation of AF-SBTI from single SM/DOPC/chol (42/30/28)-GUVs induced by lysenin and membrane permeation of calcein from single SM/DOPC(90/10)-GUVs induced by lysenin. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES


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Table 1: Membrane permeability coefficients at the steady state of the lysenin-induced membrane permeation of calcein in various membranes at 37 °C.

<table>
<thead>
<tr>
<th>Lysenin Conc. (ng/mL)</th>
<th>Membrane permeability coefficients at the steady state $P^s$ (µm/s)</th>
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<tbody>
<tr>
<td></td>
<td>SM/DOPC/chol (42/30/28)</td>
</tr>
<tr>
<td>600</td>
<td>$(1.5 \pm 0.2)\times10^{-1}$ $(n=19)$</td>
</tr>
<tr>
<td>200</td>
<td>$(6.5 \pm 0.7)\times10^{-2}$ $(n=18)$</td>
</tr>
<tr>
<td>100</td>
<td>$(4.2 \pm 0.4)\times10^{-2}$ $(n=16)$</td>
</tr>
<tr>
<td>40</td>
<td>$(2.4 \pm 0.2)\times10^{-2}$ $(n=14)$</td>
</tr>
<tr>
<td>20</td>
<td>$(1.5 \pm 0.2)\times10^{-2}$ $(n=13)$</td>
</tr>
<tr>
<td>10</td>
<td>$(6.9 \pm 1.7)\times10^{-3}$ $(n=8)$</td>
</tr>
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The average values and the standard errors of membrane permeability coefficients were determined from independent experiments ($n$ is the number of the examined “single GUVs”).
**Figure Captions**

Fig. 1: Membrane permeation of calcein from single SM/DOPC/chol (42/30/28)-GUVs induced by lysenin at 37 °C. (A) Fluorescence images (2) show that the calcein concentration inside the GUV progressively decreased during the addition of 40 ng/mL lysenin. The numbers below each image show the time in seconds after lysenin addition was started. Also shown are phase-contrast images of the GUV at time 0 s (1) and at 602 s (3). The bar corresponds to 20 μm. (B) Time course of the change in FI of the inside of the GUV shown in (A). (C) Other examples of the time course of the change in FI of single GUVs during the addition of 40 ng/mL lysenin. Each curve corresponds to a single GUV. (D) Time course of the change in FI of single GUVs following addition of 200 ng/mL lysenin. Each curve corresponds to a single GUV. (E) Dependence of the membrane permeability coefficient ($P_s$ (μm/s)) at the steady, maximum rate of membrane permeation on lysenin concentration ($C$ (ng/mL)). The bars indicate standard errors.

Fig. 2: Membrane permeation of calcein from single SM/DOPC (58/42)-GUVs induced by lysenin at 37 °C. (A) Fluorescence images (2) show that the calcein concentration inside the GUV progressively decreased during the addition of 200 ng/mL lysenin. The numbers below each image show the time in seconds after the lysenin addition was started. Also shown are phase contrast images of the GUV at time 0 s (1) and at 382 s (3). The bar corresponds to 10 μm. (B) Time course of the change in FI of the inside of the GUV shown in (A). (C) Other examples of the time course of the change in FI of several single GUVs during the addition of 200 ng/mL lysenin. Each curve corresponds to that of each GUV. (D) Time course of the change in FI of the inside of single GUVs during the addition of 40 ng/mL lysenin. Each curve corresponds to that of each GUV. (E) Dependence of $P_s$ (μm/s) on lysenin concentration ($C$ (ng/mL)). The relationship between $P_s$ and $C$ was plotted for SM/DOPC (58/42)-GUVs (■). The bars indicate standard errors. For comparison, the same relationship was also plotted for the SM/DOPC/chol (42/30/28)-GUVs (○) that are shown in Fig. 1E.
Fig. 3: SDS-resistant oligomerization of lysenin in the presence of lipid membranes containing SM.
(A) A lysenin solution (187 nM) was incubated with SM/DOPC (58/42)-SUVs or SM/DOPC/chol (42/30/28)-SUVs at various SM/lysenin molar ratios (20–20000 and 17–17000, respectively) for 5 min at 37 °C. The samples were then analyzed by SDS-PAGE (silver staining). The lysenin monomer and oligomer are indicated by M and O, respectively. (B) Quantification of the dependence of the SDS-resistant oligomer fraction on the SM/lysenin molar ratio shown in (A); SM/DOPC/chol (42/30/28)-SUVs (○) and SM/DOPC (58/42)-SUVs (■). (C) Quantification of the dependence of the SDS-resistant oligomer fraction on incubation time with SM/DOPC/chol (42/30/28)-SUVs (○) and SM/DOPC (58/42)-SUVs (■). SM/lysenin molar ratios for SM/DOPC/chol (42/30/28)-SUVs and SM/DOPC (58/42)-SUVs were 4000 and 2500, respectively. For (B) and (C), the SDS-resistant oligomer fraction was calculated as the percent of total lysenin that is present as an oligomer (oligomer fraction (%)), and the average values of the oligomer fraction among two independent experiments were obtained. The bars indicate standard errors.

Fig. 4: Phase separation of lipid membranes between lo and ld phases in SM/DOPC/chol (42/30/28)-GUVs (A) and SM/DOPC (58/42)-GUVs (B). The domains with high fluorescence intensity correspond to the ld phase. The bars correspond to 20 μm.

Fig. 5: Membrane permeation of calcein from single SM/chol (60/40)-GUVs induced by lysenin at 37 °C. (A) Fluorescence images (2) show that the calcein concentration inside the GUV progressively decreased during the addition of 40 ng/mL lysenin. The numbers below each image show the time in seconds after the lysenin addition was started. Also shown are phase contrast images of the GUV at time 0 s (1) and at 690 s (3). The bar corresponds to 10 μm. (B) Time course of the change in FI of the inside of the GUV shown in (A). (C) Other examples of the time course of the change in FI of the inside of single GUVs during the addition of 40 ng/mL lysenin. Each curve
corresponds to that of each GUV. (D) Time course of the change in $FI$ of the inside of single GUVs during the addition of 200 ng/mL lysenin. Each curve corresponds to that of each GUV. (E) Dependence of $P_s$ ($\mu$m/s) on lysenin concentration ($C$ (ng/mL)). The relationship between $P_s$ and $C$ is plotted for SM/chol (60/40)-GUVs (▲). The bars indicate the standard error. For comparison, the same relationship was also plotted for the SM/DOPC/chol (42/30/28)-GUVs (○) that are shown in Fig. 1E.

Fig. 6: SDS-resistant oligomerization of lysenin in the presence of lipid membranes containing SM. (A) A lysenin solution (187 nM) was incubated with SM/chol (60/40)-SUVs at various SM/lysenin molar ratios (25–25000) for 5 min at 37 °C. The samples were then analyzed by SDS-PAGE (silver staining). The lysenin monomer and oligomer are indicated by M and O, respectively. (B) Quantification of the dependence of the SDS-resistant oligomer fraction of lysenin on the SM/lysenin molar ratio shown in (A); SM/chol (60/40)-SUVs (▲) and SM/DOPC/chol (42/30/28)-SUVs (○). (C) Quantification of the dependence of the SDS-resistant oligomer fraction of lysenin on incubation time with SM/chol (60/40)-SUVs (▲). SM/lysenin molar ratio was 1900. For (B) and (C), the SDS-resistant oligomer fraction was calculated as the percent of total lysenin that is present as an oligomer (oligomer fraction (%)), and the average values of the oligomer fraction among two independent experiments were obtained. The bars indicate standard errors.

Fig. 7: Rate of the lysenin-induced pore formation in three kinds of membranes. (A) Time course of $P_{\text{intact}}$ of SM/DOPC/chol (42/30/28)-GUVs in the presence of various concentrations of lysenin: (Δ) 200, (▲) 40, and (○) 20 ng/mL lysenin. The solid lines represent the best fitted curves of Eq. (6). (B) Dependence of $k_P$ on lysenin concentration in PBS. (○) SM/DOPC/chol (42/30/28)-, and (▲) SM/chol (60/40)-GUVs. We made 2 or 3 independent experiments for a given lysenin concentration using 10-20 single GUVs in each experiment, and obtained average values and standard errors of $k_P$. 

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(C) Dependence of $P_{LS}(120 \text{ s})$ on lysenin concentration. (○) SM/DOPC/chol (42/30/28)-, and (■)
SM/DOPC (58/42)-, and (▲) SM/chol (60/40)-GUVs. The bars indicate the standard error.

Fig. 8: (A) Dependence of $P^s$ ($\mu$m/s) on $C$ (nM) for SM/DOPC/chol (42/30/28)-GUVs (○) and
SM/DOPC (58/42)-GUVs (■). Experimental data of SM/DOPC/chol (42/30/28)-GUVs were well
fitted using the Langmuir adsorption isotherm (Eq. (8)). (B) Dependence of $P^s$ ($\mu$m/s) on $C$ (nM) for
SM/chol (60/40)-GUVs (▲). Experimental data were well fitted using the Langmuir adsorption
isotherm (Eq. (8)).
Fig. 1

(A) 1 2 3
    0s 1s 50s 100s 240s 390s 550s 602s

(B) (C) (D) (E)

(10 100 1000) 1E-3
0.01 0.1 1

Lysenin conc. (ng/mL)
Fig. 2

(A) 1 2 3
0s 1s 30s 80s 120s 190s 323s 480s 382s

(B) (C) (D) (E)

Permeability coef. (μm/s)

Lysenin conc. (ng/mL)
Fig. 4

(A)

(B)
Fig. 5

(A) [Images of images and graphs]

(B) [Graphs showing changes over time]

(C) [Graphs showing changes over time]

(D) [Graphs showing changes over time]

(E) [Graphs showing changes over time]

Time (s) vs. FI

Permeability coef. (µm/s) vs. Lysenin conc. (ng/mL)
Fig. 6

(A) 

(B) 

(C)
Fig. 7

(A) Fraction of intact GUV vs. Time (s)

(B) Fraction of leaked GUV vs. Lysenin conc. (ng/mL)

(C) Fraction of leaked GUV vs. Lysenin conc. (ng/mL)
Fig. 8

(A) (B)

Permeability coeff. (μm/s)

Lysenin conc. (nM) 0 5 10 15 20

Permeability coeff. (μm/s)

Lysenin conc. (nM) 0 5 10 15 20
Single Giant Unilamellar Vesicle Method Reveals Lysenin-Induced Pore Formation in Lipid Membranes Containing Sphingomyelin

Jahangir Md. Alam, Toshihide Kobayashi, and Masahito Yamazaki