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Lysenin forms a voltage-dependent channel in artificial lipid bilayer membranes

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Abstract

Lysenin, a hemolytic protein derived from the body fluid of earthworm, was incorporated into artificial bilayer membranes. Upon insertion, it formed a voltage-dependent large conductance channel in asolectin bilayers in a sphingomyelin-dependent manner. The channel had low ion-selectivity. Single-channel conductance was calculated as approximately 550 pS in 100 mM KCl. The channel in asolectin bilayers closed when the membrane was held at a positive potential. In contrast, the channel showed no voltage dependency in membranes made of pure phosphatidylcholine and sphingomyelin, suggesting some lipid contents included in the asolectin membranes affected channel gating.

Keywords: Lysenin; Planar bilayer; Single-channel; Sphingomyelin

The body fluid of earthworms is known to contain various proteins that exhibit anti-bacterial, hemolytic, cytotoxic, and hemagglutinating activities [1–4]. Lysenin is a member of proteins derived from coelomic fluid of the earthworm *Eisenia fetida*, having 297 amino acids. It is known to specifically recognize sphingomyelin (SM) and induces cell lysis. Accordingly, it has been used as a probe for lipid rafts that are defined as sphingolipids and cholesterol rich clusters in the membrane [5–9]. Electron-microscopic studies and flux measurements from liposomes have shown that lysenin forms an oligomer in the membrane which has a large pore in the center of the complex [6]. This oligomer formation induces an increase in the membrane permeability and lysis of cells, although the detailed mechanism underlying lysenin-induced cell lysis has never been clarified.

Lysenin-induced increase in membrane permeability has been studied by measuring the lysis rate of living cells or by flux measurement with liposomes. To date, few electrophysiological studies examined the lysenin channel [10]. It is expected that detailed electrophysiological investigations on lysenin channels may unravel the mechanism of its physiological activities. For such a study, it is preferable to use the planar bilayer method because it is easy to control the lipid composition of the membrane. In this report, using this method, we measured the basic properties of the lysenin channel.

Materials and methods

Materials. Lysenin and asolectin were purchased from Sigma–Aldrich, diphytanoylphosphatidylcholine (DPhPC) and sphingomyelin (SM) from Avanti Plar Lipids, Inc. (Alabaster, USA), and *n*-decane from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were commercial products of an analytical grade.

Current recordings. Unless otherwise noted, electrophysiological recordings were performed with the planar bilayer current recording system in which a horizontal artificial bilayer membrane was formed across a small aperture in a plastic sheet [11,12]. The bilayer was made by painting a lipid solution in *n*-decane over the aperture. Formation of lipid bilayers were facilitated by applying membrane voltages (±75 mV) and checked

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with a bright field microscope. To form a bilayer at the tip of a glass pipette (tip diameter 5–30 μm), the pipette was plunged into a recording solution through a lipid layer which was made by applying a lipid solution on the aqueous solution. Lysenin was added to the bottom side of the bilayer membrane. The membrane voltage was defined as the voltage of the upper side of the membrane with respect to the bottom side which was held at virtual ground. Current fluctuations were recorded with a patch-clamp amplifier (CEZ-2400, Nihon Kohden), filtered, at 2 kHz and analyzed with pClamp9.02 (Axon Instruments).

Results

We found that lysenin molecules form voltage-dependent ion-channels in artificial lipid bilayer membranes. Current across a membrane began to increase stepwise just after the addition of lysenins to the solution, reaching a steady state value within several minutes. Fig. 1A is a current record taken after the current reached a steady plateau in the presence of 1.5 nM lysenin at the bottom of the artificial bilayer. These current records show that there were many channels incorporated into the bilayer. As shown later, the single-channel conductance was determined to be approximately 550 pS by single-channel recordings. Hence there were about 180 channels in this bilayer. In Fig. 1A, current traces recorded after the membrane potential jumped from −90 mV to the indicated values. As shown in this figure, multiple-channel current decreased slowly when the membrane was held at positive high voltages while no such decrease was seen at negative membrane potentials. Remaining current amplitudes were determined 30 s after the voltage-jump and plotted against membrane voltage in Fig. 1B.

Fig. 1C shows the closing process of the channel after changes in membrane voltage. In this figure, multi-channel currents shown in figure A were fitted with the sum of two exponential functions where the time constants, τ_1 and τ_2 ($\tau_1 < \tau_2$), are plotted against the membrane voltage. Being able to fit with two exponential functions means either at least two types of channels were formed by lysenin molecules or that there was a single-lysenin channel type with minimum two open states. As shown in the figure, the time constants decreased with discrete increases in membrane. The shorter time constant, τ_1, had a more pronounced dependency on the membrane voltage meaning the channel gating or speed of channel inactivation was dependent on the membrane voltage as well as the remaining current.

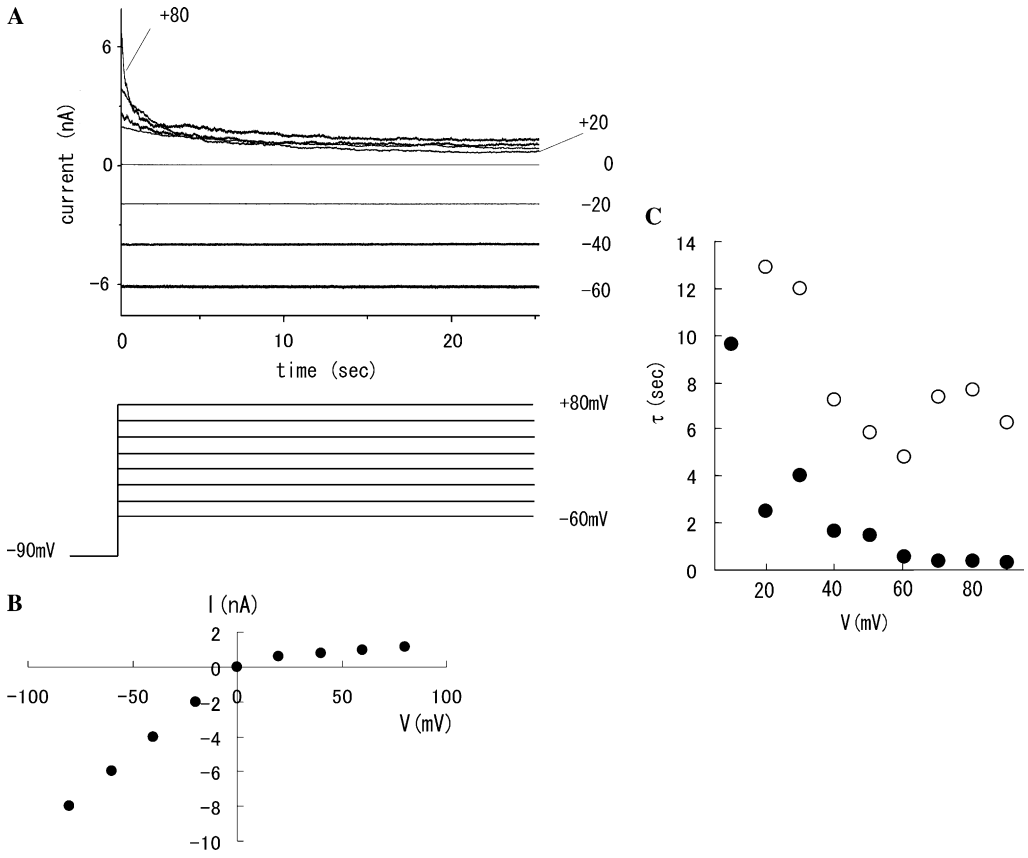

Fig. 1. Multi-channel recording of lysenin. (A) Lysenin (1.5 nM) was added to the bottom solution and the current across the membrane was recorded. As lysenin channels inserted into the membrane, the membrane conductance was increased and reached the stable current level within several minutes. Traces shown in this figure were recorded after saturation of current (more than 10 min after addition of lysenin). The membrane voltage was held at −90 mV for 2 min and then jumped to the indicated voltages. The recording solution was symmetrical across the membrane containing 100 mM KCl and 10 mM Hepes, pH 7.4. (B) Remaining current amplitudes determined at 30 s after voltage-jump are plotted against the membrane voltages. (C) Traces as shown in (A) were fitted by the sum of two exponential functions with the time constants, τ_1 and τ_2 ($\tau_1 < \tau_2$), are plotted against membrane voltage.

For further investigation, single-channel currents were recorded. As shown in Fig. 2A, insertion of each lysenin channel, or formation of channel in the artificial membrane, could be detected by a stepwise increase in current across the membrane when the lysenin concentration in the recording solution was low. Each step in Fig. 2A is the same in amplitude, showing that every lysenin oligomer had the same unitary conductance.

In Fig. 2B, 7.5 pM lysenin was added to the bottom solution allowing for current fluctuations across a membrane to be recorded upon detection of a single-step current increase. Current traces in this figure were taken in voltage-jump experiments from −90 mV to the indicated voltages. As expected from multi-channel experiments, at positive voltages, the channel closed faster as voltage increased while the channel remained open at negative voltages. Single-channel current amplitudes are plotted against membrane voltage in Fig. 2C. We analyzed current records in which only single step increases were observed at the beginning of the recording. From the slope of the line in Fig. 2C, the single-channel conductance of the lysenin channel, γ, was determined to be 552 pS. The mean value of γ was 548 ± 21 pS ($n = 10$). We found that making the solution asymmetric with respect to the membrane by adding concentrated KCl solution to one side does not shift the zero-current potential, showing the channel has no significant selectivity between potassium and chloride ions. Furthermore, the I–V curve in Fig. 2C is not changed by perfusing the bottom solution with the same concentration of NaCl solution, which indicates that there was no selectivity difference between potassium and sodium ions.

All the experiments described above were done with artificial bilayer membranes made of asolectin. We have found that the lysenin channel showed no voltage dependence when it was incorporated into DPhPC bilayer made of diphytanoylphosphatidylcholine and sphingomyelin. Fig. 3 shows single-channel records taken from the membrane into which a single-lysenin channel was incorporated. As shown in this figure, the channel was kept open during the recordings independent of membrane voltages. The single-channel conductance and ion-selectivity of the channel showed no significant difference from those

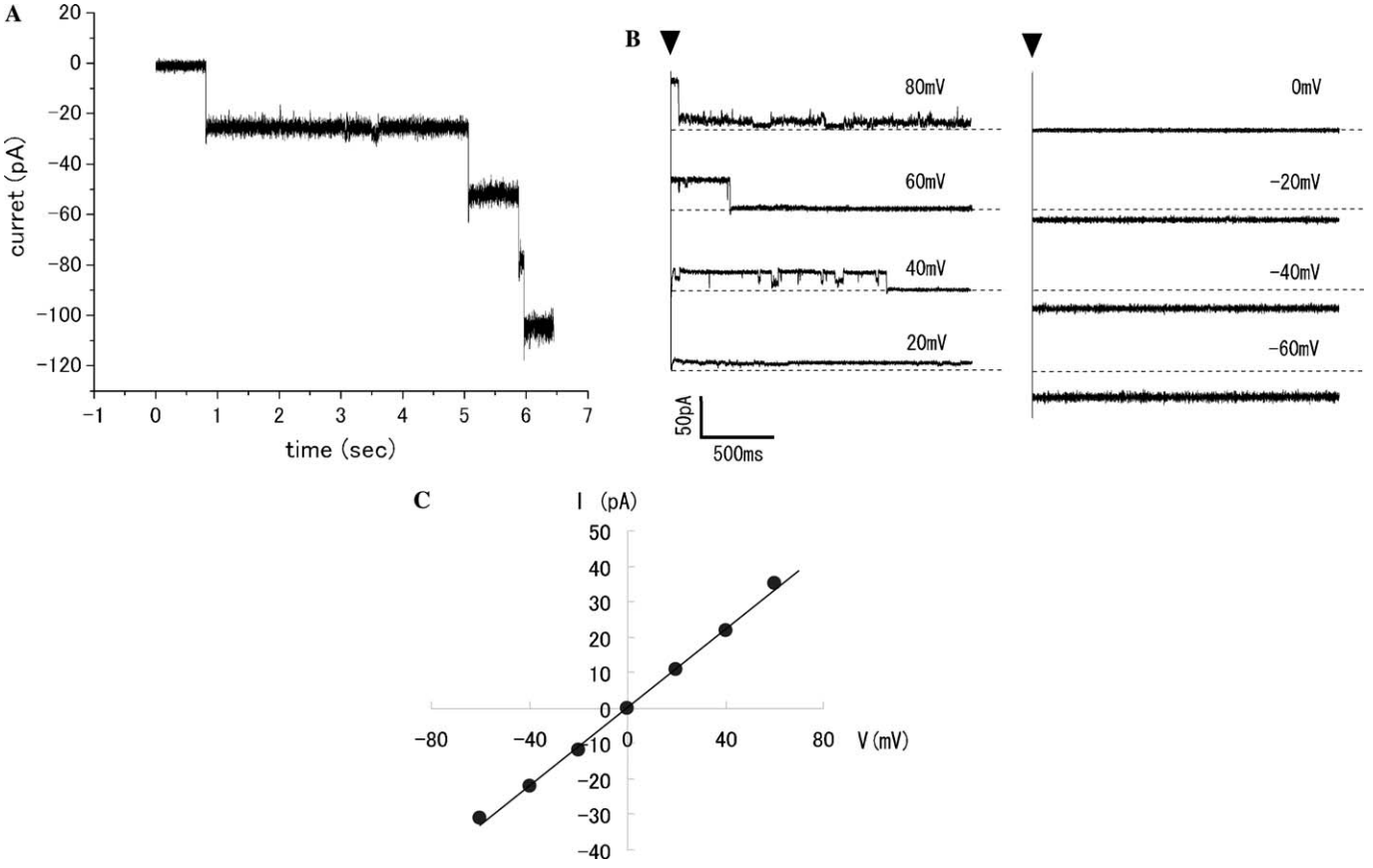

Fig. 2. Single-channel recording of lysenin channel. The recording solution was composed of 100 mM KCl and 10 mM Hepes, pH 7.4. The artificial bilayer was formed from lipid solution containing 20 mg/ml asolectin and 5 mg/ml SM in *n*-decane. (A) Within several minutes after the addition of 7.5 pM lysenin to the bottom of the solution, current across the membrane began to increase stepwise. The membrane voltage was held at −50 mV. (B) Single-channel current traces recorded at indicated voltages. Current records were taken after voltage-jumps from −90 mV to the indicated voltages at the arrowheads. To remove capacitive current of the membrane, current recorded before the addition of lysenin was subtracted from each trace. (C) I–V relationship of a single-lysenin channel. Open channel current amplitudes shown in (B) are plotted against the membrane voltage. The single-channel conductance is determined to be 552 pS from the slope of the line.

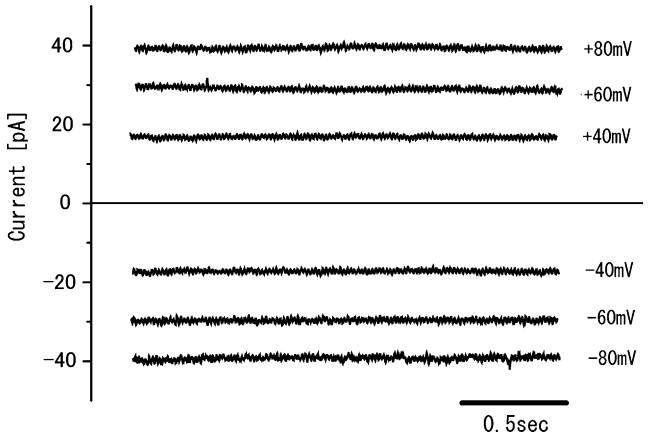

Fig. 3. Single-channel recording of lysenin channel in a DPhPC bilayer. The membrane was formed at the tip of a glass pipette from lipid solution containing DPhPC and SM (DPhPC:SM = 1:1). The recording solution was symmetrical across the membrane and consisted of 100 mM NaCl and 10 mM Mops (pH7.4). Lysenin (5 pM) was added to the bath solution. Each trace was recorded several minutes after the membrane was held at the indicated voltage.

determined with asolectin bilayer. Therefore, the lipid composition only affects the gating of the channel.

Discussion

Lysenin increases membrane permeability and, as its name indicates, induces lysis of cells. Although there has been ample speculation about the protein's affect on pores and channels in membranes, very little regarding the electrophysiological properties of the lysenin channel has actually been observed [10]. In this study, we investigated the properties of the channel using artificial planar bilayer membranes and found that lysenin incorporates into the membrane and forms a voltage dependent channel.

Kobayashi and co-workers recently used lysenin as a marker for lipid rafts composed of sphingolipid and cholesterol rich clusters in the membrane because lysenin is known to specifically recognize sphingomyelin-rich domains of membranes [5–9]. It had been expected that pore formation by lysenin is dependent on sphingomyelin content in the membrane and that lysenin does not form a channel in the absence of sphingomyelin. As expected, lysenin did not increase membrane permeability when the membrane was made only of DPhPC. In contrast, when the membrane was formed from a sphingomyelin-rich lipid solution, we observed stepwise increases in the membrane conductance within a minute after the addition of nM lysenin to the solution.

Multi-channel recordings as shown in Fig. 1 show the lysenin-induced conductance gradually decreased when the membrane is held at high positive voltages. There are two possibilities for this decay in conductance. One possibility is that lysenin oligomers deoligomerized or become unstable at positive potentials. The other possibility is that the intrinsic gate of the channel closes at positive membrane potentials. The robust nature of lysenin oligomers [6] and the results in our single-channel experiments, in which the channel closed once at high positive potentials but then instantaneously opened at negative potentials, suggest that the latter is more likely. Further detailed single-channel investigations are required to elucidate the mechanism of voltage-dependent gating.

As shown in Fig. 2, the lysenin channel is a low ion-selective channel that has a single-channel conductance of 550 pS in 0.1 M KCl. As described above, the *I–V* relationship showed that the channel has no difference in ion-selectivity among potassium, sodium, and chloride ions. The electron-microscopic study [6] shows that the oligomer formed by lysenin monomers has a pore of approximately 3 nm in diameter, which is about the same size as that of α-hemolysin channel, another channel with a large conductance pore.

Lysenin channels formed in a DPhPC (DPhPC:SM = 4:1) bilayer did not show voltage-dependent gating while channels formed in an asolectin bilayer (asolectin:SM = 4:1) did. Asolectin is a mixture of various types of lipids suggesting that one or a combination of these lipids affected the channel gatings. Recently, Yamaji-Hasegawa et al. reported that cholesterol in membranes changed the oligomerization of lysenins and the release of calcein from liposomes [6]. Therefore, cholesterol is a likely candidate for modulating the lysenin channel. As far as we investigated, change in SM content (10–50%) in the artificial bilayer showed no effect on the voltage dependency of the channel.

The results in this study suggest that some of the lipid components of the bilayer change the channel activity. Bruhn et al. have recently proposed that lysenin has SM-independent activities to kill bacteria as well as SM-dependent activities [10]. Further investigation of the effect of lipid content on channel activities should elucidate the physiological function of lysenin.

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References

[1] P. Valembois, P. Roch, M. Lassegues, P. Cassand, Antibacterial activity of the hemolytic system from the earthworm *Eisenia fetida Andrei*, J. Invert. Pathol. 40 (1982) 21–27.
[2] J. Vaillier, MA. Cadoret, P. Roch, P. Valembois, Protein analysis of earthworm coelomic fluid. III. Isolation and characterization of several bacteriostatic molecules from *Eisenia fetida Andrei*, Dev. Comp. Immunol. 9 (1) (1985) 11–20.
[3] P. Roch, Protein analysis of earthworm coelomic fluid: (1) polymorphic system of the natural hemolysin of *Eisenia fetida andrei*, Dev. Comp. Immunol. 3 (4) (1979) 599–608.

[4] P. Roch, M. Lassegues, P. Valembois, Antibacterial activity of *Eisenia fetida andrei* coelomic fluid: III–Relationship within the polymorphic hemolysins, Dev. Comp. Immunol. 15 (1-2) (1991) 27–32.
[5] A. Yamaji, Y. Sekizawa, K. Emoto, H. Sakuraba, K. Inoue, H. Kobayashi, M. Umeda, Lysenin, a novel sphingomyelin-specific binding protein, J. Biol. Chem. 273 (9) (1998) 5300–5306.
[6] A. Yamaji-Hasegawa, A. Makino, T. Baba, Y. Senoh, H. Kimura-Suda, S.B. Sato, N. Terada, S. Ohno, E. Kiyokawa, M. Umeda, T. Kobayashi, Oligomerization and pore formation of a sphingomyelin-specific toxin, lysenin, J. Biol. Chem. 278 (25) (2003) 22762–22770.
[7] E. Kiyokawa, A. Makino, K. Ishii, N. Otsuka, A. Yamaji-Hasegawa, T. Kobayashi, Recognition of sphingomyelin by lysenin and lysenin-related proteins, Biochemistry 43 (30) (2004) 9766–9773.
[8] R. Ishitsuka, A. Yamaji-Hasegawa, A. Makino, Y. Hirabayashi, T. Kobayashi, A lipid-specific toxin reveals heterogeneity of sphingomyelin-containing membranes, Biophys. J. 86 (2004) 296–307.
[9] R. Ishitsuka, T. Kobayashi, Lysenin: a new tool for investigating membrane lipid organization, Anat. Sci. Int. 79 (4) (2004) 184–190.
[10] H. Bruhn, J. Winkelmann, C. Andersen, J. Andra, M. Leippe, Dissection of the mechanisms of cytolytic and antibacterial activity of lysenin, a defence protein of the annelid *Eisenia fetida*, Dev. Comp. Immunol. 30 (7) (2006) 597–606.
[11] T. Ide, Y. Takeuchi, T. Aoki, T. Yanagida, Simultaneous optical and electrical recording of a single ion-channel, Jpn. J. Physiol. 52 (2002) 429–434.
[12] T. Ide, Y. Takeuchi, T. Yanagida, Development of an experimental apparatus for simultaneous observation of optical and electrical signals from single ion channels, Single Mol. 3 (2002) 33–42.