Supporting Information

Channel Current Analysis Estimates the Pore-formation and the Penetration of Transmembrane Peptides

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Reagents and chemicals

In this study, we used the following reagents: 1,2-dioleoyl-*sn*-glycero-3-phosphoethnolamine (DOPE; Avanti Polar Lipids, AL, USA); 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (DOPG; Avanti Polar Lipids); 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC; Avanti. Polar Lipids); *n*-decane (Wako Pure Chemical Industries, Osaka, Japan); 3-morpholinopropane-1-sulfonic acid (MOPS, Nacalai Tesque, Kyoto, Japan); and potassium chloride (KCl; Nacalai Tesque). Buffered electrolyte solutions were prepared from ultrapure water, which was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). L4K3, L8K6, L12K9 and L16K12 were synthesized by solid-phase peptide synthesis and purified by reverse phase HPLC (TOF-Mass data) and dissolved in ultrapure water and stored at −20 °C. For use, samples were diluted at 10 μM in buffer solution including 150 mM KCl and 10 mM MOPS, pH 7.0. Magainin1 (LKT Laboratories Inc., MN, USA) was dissolved at 10 μM in buffer solution including 200 mM KCl and 10 mM MOPS, pH 7.0. Alamethicin (Sigma-Aldrich, USA) was dissolved at 25 nM in buffer solution including 1 M KCl and 10 mM MOPS, pH 7.0.

All chemicals and solvents for peptide synthesis were of reagent or HPLC grade and were used without further purification. HPLC was performed on a GL-7400 HPLC system (GL Sciences, Tokyo, Japan) using an Inertsil ODS-3 column (10 × 250 mm; GL Science) for preparative purification, with a linear acetonitrile/0.1% trifluoroacetic acid (TFA) gradient at a flow rate of 3.0 mL/min. The peptides were analyzed using MALDI-TOF MS on an Autoflex III (Bruker Daltonics, Billerica, MA, USA) mass spectrometer with 3,5-dimethoxy-4-hydroxycinnamic acid as the matrix. Amino acid analysis was carried out using an Inertsil ODS-2 column (4.6 × 200 mm; GL Science) after samples were hydrolyzed in 12 M HCl at 110°C for 48 h in a sealed tube and labeled with phenyl

isothiocyanate.

Preparation of a high-throughput pBLM device

The multi-channel device was fabricated by machining a 6.0 mm thick, 10 × 10 mm polymethyl methacrylate (PMMA) plate (Mitsubishi Rayon) using a CAD/CAM 3-D modeling machine (MM-100, Modia Systems). Then, two wells and a chase between the wells were manufactured on the PMMA plate. Each well had a through-hole in the bottom and Ag/AgCl electrodes set into this hole. The two wells were 2.0 mm in diameter and 4.0 mm in depth. A polymeric film made of parylene C (polychloro-p-xylylene) with a thickness of 5 μm was patterned as a single pore (100 μm in diameter) by a conventional photolithography method and then placed between PMMA film sheets (0.2 mm thick) using an adhesive bond (Super X, Cemedine Co., Ltd.). The films, including the parylene film, were inserted into the device to separate the wells. Ag/AgCl electrodes of each device set into a solderless breadboard (E-CALL Enterprise Co., Ltd.) were connected to a Jet patch clamp amplifier (Tecella, Foothill Ranch, CA, USA) using a jumper wire.ref
We used a microdroplet system for preparing an artificial lipid bilayer using an arrayed device, which has eight chambers fabricated by microfabrication (Figure 1c and 1d). Then, two lipid monolayers were contacted together to prepare a stable and reproducible lipid bilayer in the parylene film. This method is advantageous for preparing model cell-membranes; e.g., bacteria or mammalian cell membranes.

Synthesis of the Peptides

The peptides were synthesized manually on TentaGel S RAM resin by Fmoc solid phase peptide synthesis with Fmoc-AA-OH (10 eq.), 2-(1Hbenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, Watanabe Chemical Industries, Hiroshima, Japan) (10 eq.), 1-hydroxybenzotriazole (HOBt, Watanabe Chemical Industries) (10 eq.) and diisopropylethylamine (DIEA, 15 eq.).[1, 2] Side-chain protection was t-butoxycarbonyl (BOC) for Lys. After assembly of all peptides, peptides were cleaved from the resins and sidechain protection was removed by incubating the peptideresin for 2 h in TFA (Watanabe Chemical Industries)/H_2O/triisopropylsilane (Wako Pure Chemical Industries, Tokyo, Japan) (20:1:1, v/v). The peptides were precipitated by the addition of cold diethyl ether and collected by centrifugation. The peptides were purified by RP-HPLC (**Fig. S1**) and characterized by amino acid analysis and MALDI-TOF MS: L4K3 m/z 855.5 ($[M+H]^+$ calcd. 855.2); L8K6 m/z 1692.5 ($[M+H]^+$ calcd. 1692.3); L12K9 m/z 2529.2 ($[M+H]^+$ calcd. 2529.5) and L16K12 m/z

3366.7 ([M+H]$^+$ calcd. 3366.6). The peptides were dissolved in MilliQ water to about 1 mM, and the concentration was determined by amino acid analysis. The peptide solutions were stored at 4°C.

Circular Dichroism (CD) Spectroscopy

CD spectroscopy was performed at room temperature using peptides. A J-820 spectropolarimeter (JASCO Corporation) with a thermoregulator and a quartz cell with a 0.2 cm path length was used. Molecular ellipticities represented mean residual values calculated by the number of peptide residues (7 for L4K3, 14 for L8K6, 21 for L12K9 and 28 for L16K12). The helical content was calculated from the molecular ellipticities at 222 nm as previously described.[3]

Liposome preparation

20× liposome stock solution (DOPE:DOPG=3:1 (molar ratio) in 10 mM MOPS, pH 7.0) was made gentle hydration method.[4] DOPE 279 μL and DOPG 99.6 μL (10 mg mL^{-1} in $CHCl_3$) were mixed in vial bottle. $CHCl_3$ was vaporized by N_2 gas flow and vacuum desiccator for 1 hour to form lipid film in the bottom of the bottle. The buffer was added to the vial bottle and the solution was incubated at 4°C for 24 hr. After the incubation, the solution was used for CD spectroscopy.

Planner bilayer lipid membrane preparation and reconstitution of peptides

The planner bilayer lipid membranes (pBLMs) were prepared using an arrayed device, which had eight chambers on the breadboard. Eight individual lipid bilayers could be formed simultaneously in this device, which allowed for a higher-throughput measurement compared to the conventional system. First, the phospholipid solution (lipids/*n*-decane, 10 mg mL^{-1}) solution 2.4 μL was poured into all chambers. Next, 4.7 μL of the buffer solution was poured into voltage applied chambers, then 4.7 μL of the buffer solution with peptide was poured into grounded chambers. After a few minutes of adding the buffer solution, the two lipid monolayers connected and formed a lipid bilayer, and peptide molecules formed nanopores by reconstitution in the lipid bilayer and/or penetrated the lipid bilayer. L4K3, L8K6, L12K9, L16K12 and magainin1 were reconstituted in DOPE:DOPG = 3:1 (molar ratio) membrane. Alamethicin was reconstituted in DPhPC membrane. When the lipid bilayer ruptured, we reformed the lipid bilayer by tracing with a thin hydrophobic stick between two droplets.

Channel current measurements and data analysis

Channel current was monitored by using a JET patch clamp amplifier connected to each chamber. Ag/AgCl electrodes were in each droplet when we added the solution into the chambers. A constant voltage of +100 mV (LK peptide and magainin1) or +200 mV (alamethicin) was applied to the one side, with the other side being grounded. The reconstituted peptide pore in the lipid bilayers allowed ions to pass through the nanopore under the voltage gradient so that we obtained the channel current signals. All measurements were conducted at room temperature (ca. 23 ^{o}C). The signals were detected using a 4 kHz low-pass filter at a sampling frequency of 20 kHz. Analysis of channel current signals was performed using pCLAMP ver. 10.5 (Molecular Devices) and Excel (Microsoft) software.

Signal classification

We discriminated the pore-forming current signals. The single pore formation in case of the step, multi, and spike signals can be recognized because their currents raise up from the base level. After that, the next pore formation is able to be observed. This type of pore formation should be single. However, in the case of erratic signals, the single pore formation may be observed at the initial stage, but there are complex overlapping with the second pore formation.

Four different current signals were recognized by estimating the shape, initially. And then, we performed more precise classification using the definition, as shown in Fig. S4.

Table S1. The sequence of magainin 1, alamethicin, and LK peptides.

Peptide	Sequence
L4K3	H-LKKLLKL-NH_2
L8K6	H-LKKLLKLLKKLLKL-NH_2
L12K9	H-LKKLLKLLKKLLKLLKKLLKL-NH_2
L16K12	H-LKKLLKLLKKLLKLLKKLLKLLKKLLKL-NH_2
Magainin1	H-GIGKFLHSAGKFFVGEIMKS-NH_2
Alamethicin	Ac-UPUAUAQUVUGLUPVUUEQ-Phl

Ac: acetyl, U: 2-aminoisobutyric acid, Phl: phenylalaninol

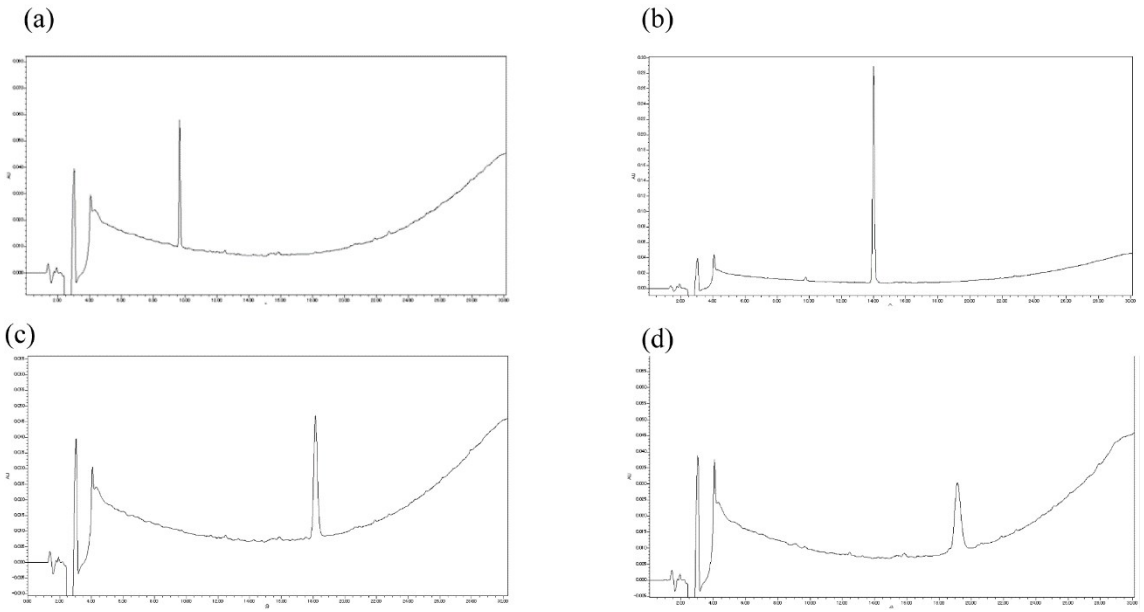

Figure S1. HPLC for purified L4K3 (a), L8K6 (b), L12K9 (c), L16K12 (d) separated on an ODS column (4.6×150 mm) with MilliQ water (containing 0.1% TFA) using a gradient from 5% to 95% acetonitrile (containing 0.08% TFA) over 30 min, 1.0 mL/min; detection at 220 nm.

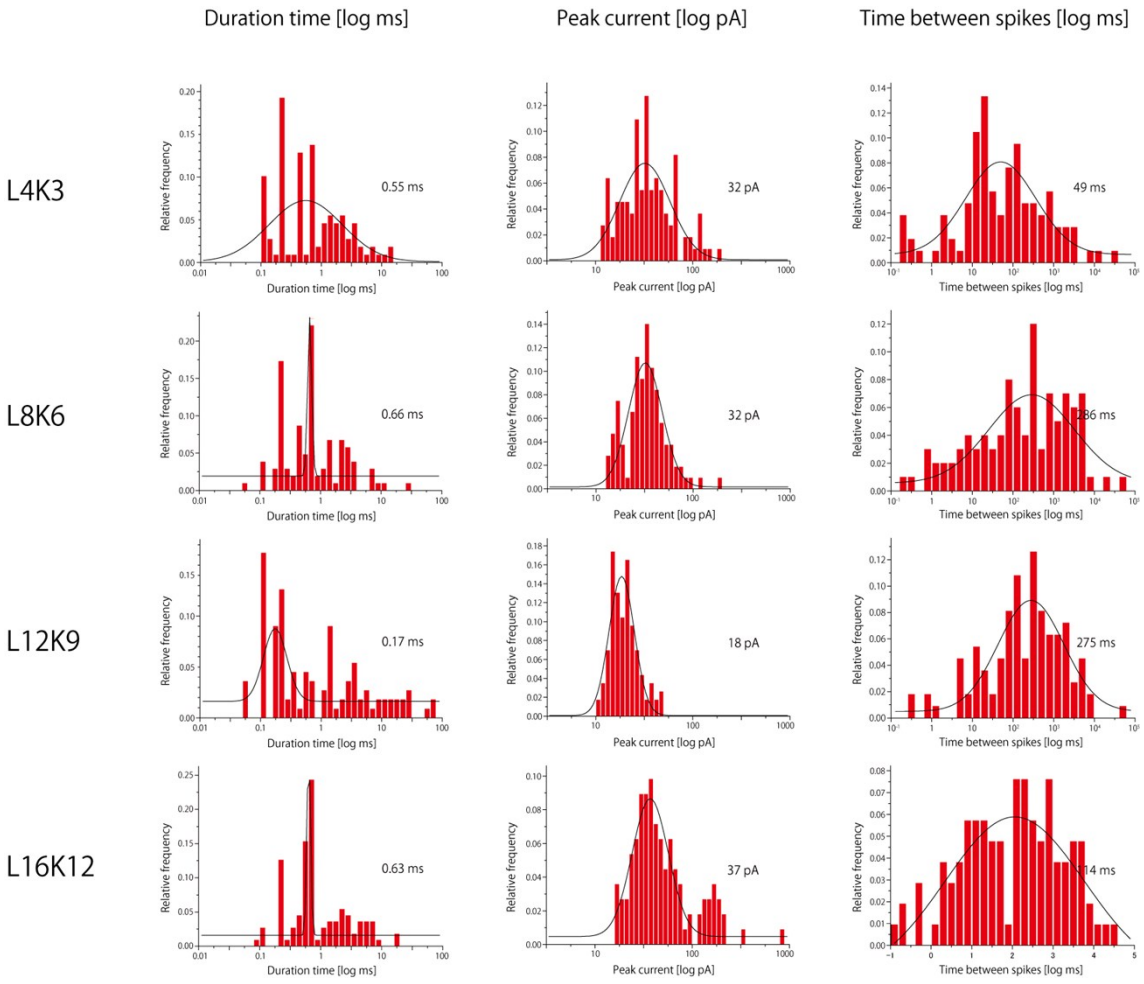

Fig. S2. Histograms of the duration time, the current amplitudes, and the time between peaks. The fitting with normal distribution gives the most probable values and depicted in these figures.

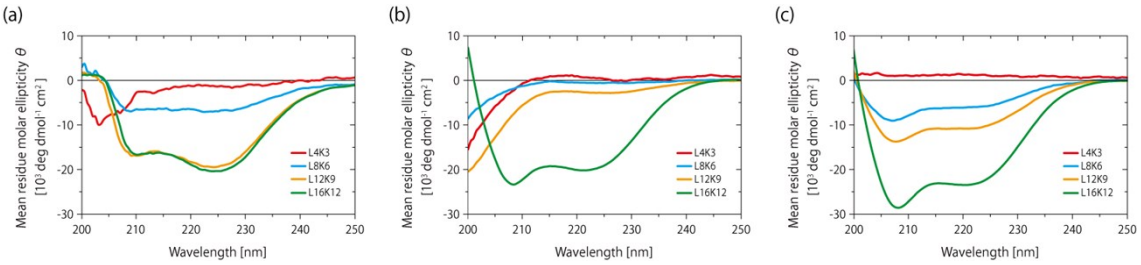

Fig. S3. CD spectra of LK peptides in aqueous, liposome, and TFE solution. (a) [peptide]=20 μM, [liposome]=0.5 mM in 10 mM MOPS Buffer (pH 7.0), Liposome: DOPE/DOPG=3/1 (mol/mol). (b) [peptide]=20 μM in 10 mM MOPS Buffer (pH 7.0). (c) [peptide]=20 μM in 20 mM Tris-HCl Buffer (pH 7.0), 20 ％TFE.

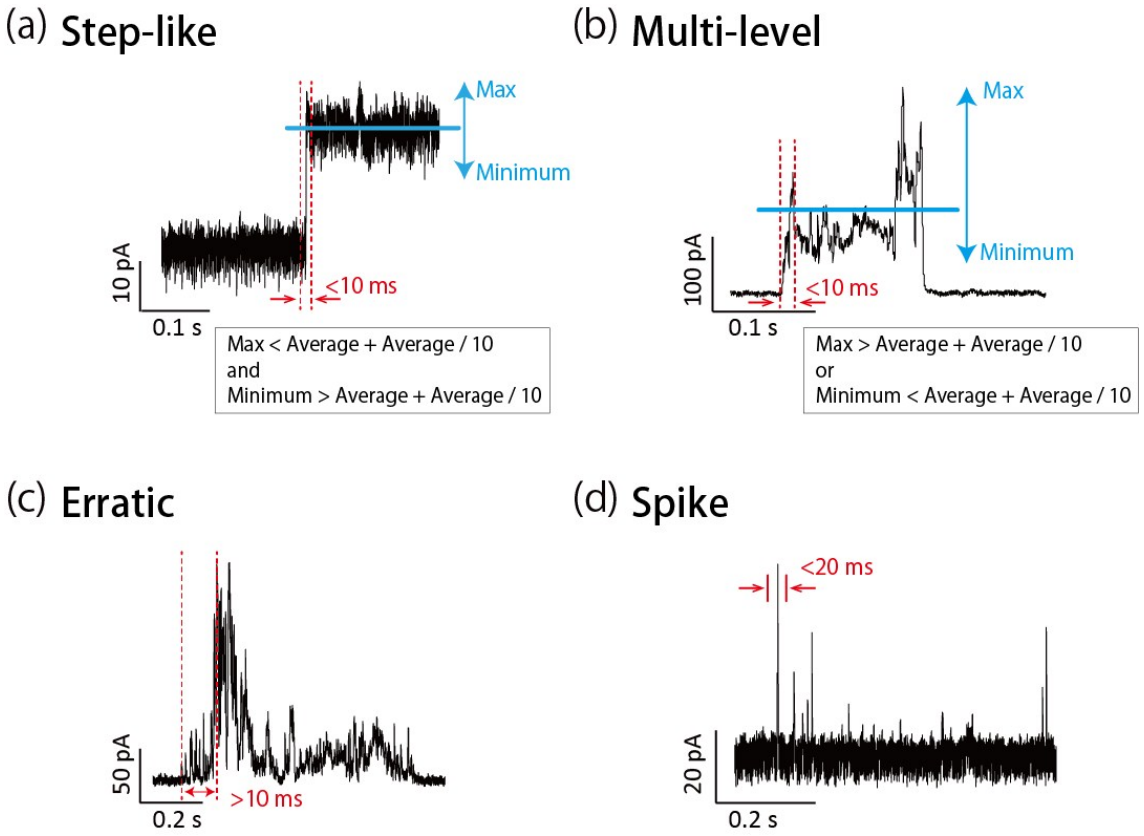

Fig. S4. Definition of the signal classification. We performed the classification of four different current signals according to this definition.

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