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ARTICLE

Liposome chaperon in cell-free membrane protein synthesis: One-step preparation of KcsA-integrated liposomes and electrophysiological analysis by the planar bilayer method

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Chaperoning functions of liposomes were investigated using cell-free membrane protein synthesis. KcsA potassium channel-reconstituted liposomes were prepared directly using cell-free protein synthesis. In the absence of liposomes, all synthesized KcsA protein aggregated. In the presence of liposomes, however, synthesized KcsA spontaneously integrated into the liposome membrane. The KscA-reconstituted liposomes were transferred to the planar bilayer across a small hole in a thin plastic sheet and the channel function of KcsA was examined. The original electrophysiological activities, such as voltage- and pH-dependence, were observed. These results suggested that in cell-free membrane protein synthesis, liposomes act as chaperones, preventing aggregation and assisting in folding and tetrameric formation, thereby allowing full channel activity.

Introduction

Membrane proteins play a crucial role in various biological processes, such as ion transport, signal transduction, energy production and cellular communication. Therefore, gaining structural and functional insights into the molecular mechanisms of membrane proteins is important for understanding these biological phenomena[1–3] and for their use as nanodevices of advanced biomaterials in bioanalysis and drug delivery systems[4, 5]. In contrast to research into soluble proteins, advances in membrane protein science have been hampered by their hydrophobic nature, which predisposes these proteins to misfolding and aggregation. In general, structural and functional analyses of membrane proteins have been performed by reconstituting them into structures that mimic cellular membranes, such as liposomes[6]. Unfortunately, the reconstitution efficiency of membrane proteins as active forms is low using conventional preparation methods because a complex process involving solubilisation with detergents, isolation, purification and reconstitution into a lipid bilayer membrane is required[7, 8].

Recently, as one of the breakthroughs in membrane protein science, cell-free membrane protein synthesis has emerged as a powerful tool to overcome the problems of conventional cell-based preparation methods[9–11]. These cell-free protein synthesis systems are commercially available systems based on *Escherichia coli* extract[12], wheat germ extract[13,14], rabbit reticulocyte lysate[15], insect cell extract[16] or artificial systems (assembled entirely from reconstituted components, PURESYSTEM)[17].

We proposed a chaperoning system using liposomes in cell-free membrane protein synthesis[18]. We found that various expressed membrane proteins, such as apo-cytochrome b5[18], connexin 43[19,20] or bacteriorhodopsin[21] were incorporated directly into liposomes. The liposomes prevented the aggregation of water-insoluble membrane proteins and aided oligomerization in the liposome. We have demonstrated the novel drug delivery system comprising connexin 43-integrated proteoliposomes had the potential to transfer small molecules into the cytoplasm directly[19]. To investigate the versatility of the chaperoning function of liposomes, our system was applied to ion channel proteins, which are important target pharmaceutical proteins that have been studied extensively. We also report the application of the proteo-liposomes, prepared using our system, to the channel current measurement by the planar bilayer method,

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which is widely used in the functional analysis of various membrane channel proteins.

Streptomyces lividans potassium channel, KcsA, is one of the most well researched ion channels in structural and functional biology[22]. This channel protein includes two transmembrane domains, and exerts its potassium transport activities as a homo-tetramer[23]. Single-channel electrophysiology of KscA has been performed by reconstitution of proteins in a planer lipid membrane after the isolation of KcsA, using an *E. coli* protein expression system[24, 25]. Recently, cell-free membrane protein synthesis has been used to obtain KcsA[26]. Using the insect cell extract, which is included with the lipid membrane machinery, such as microsomes, KcsA was synthesized and directly reconstituted into the lipid membrane[27, 28]. The single-channel current of the potassium channel KcsA was measured by direct insertion into interdroplet lipid bilayers from microliters of a cell-free expression medium, based on an *E. coli* extract[29]. Here, we investigated the chaperoning functions of liposomes in KcsA membrane protein synthesis and the direct reconstitution to the liposome using an artificial cell-free system (PURESYSTEM) and avoiding the contamination risk associated with a cell extract system. Electrophysiological activity was also evaluated by transferring the KcsA-reconstituted liposome to a planar lipid membrane.

Experimental

Plasmid DNA construction

The His6-KcsA cDNA with codons optimized for *E. coli* use, was obtained as previously described[30]. pURE-His6-KcsA was constructed by inserting the polymerase chain reaction (PCR)-amplified DNA encoding His6-KcsA (forward primer: 5′-ACA TCA TAT GAG AGG ATC GCA TC-3′ and reverse primer: 5′-ATT AGA ATT CGA CCT AAC GAC GG-3′) into the *Nde*I/*Eco*RI site of the pURE1 vector (Cosmo Bio, Tokyo, Japan). pURE- His6-KcsA was amplified in the DH5α strain of *E. coli*. The plasmid DNA was purified using a QIAGEN Plasmid Maxi Kit (Qiagen Inc., Valencia, CA, USA).

Liposome preparation

Liposomes were prepared using the natural swelling method. In brief, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Nichiyu, Tokyo, Japan), dissolved in chloroform, was placed into a glass micro test tube and gently evaporated by rotation under flowing argon gas. To completely remove the chloroform, films were placed in a desiccator under a vacuum overnight. The lipid film was hydrated with 50 mM HEPES buffer (pH 7.5), incubated for 2 h at 37°C and vortexed lightly for 30 sec to remove the remaining lipid from the test tube. Following hydration, the liposome suspensions were extruded with a mini-extruder (Avanti Polar Lipids, Alabaster, AL, USA) equipped with a 0.1-µm pored polycarbonate membrane (Whatman, Maidstone, UK) at 37°C. The average size of the liposomes, large unilamellar vesicles, was measured by dynamic light scattering with a Zetasizer Nano ZS instrument (Malvern, Malvern, UK). The lipid concentration was measured using the Phospholipid C-Test (Wako, Osaka, Japan).

Preparation and purification of KcsA-integrated proteoliposomes

The *in vitro* protein synthesis of His6-KcsA was performed using PUREfrex®1.0 (GeneFrontier, Chiba, Japan), which comprises purified ribosomes and entirely reconstituted components that are responsible for synthesis, according to the manufacturer's instructions. In brief, the reaction mixture containing 4 ng/µL pURE- His6-KcsA was prepared with or without the final concentration of 10 mM DOPC liposomes. All samples were incubated without agitation for 4 h at 37°C in a heat block incubator. For purification, 40 µL samples were overlaid with 40 µL of 15% (w/v) sucrose solution and ultracentrifuged at 163,000 × *g*, 4°C for 2 h. The 60-µL upper layer of the supernatant was collected and the lower 20-µL fraction was collected as a pellet sample.

Western blot analysis

The samples were subjected to 10–20% gradient Laemmli sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 90 (W) x 83 (H)-mm mini-slab gel (ATTO, Tokyo, Japan) under reducing or nonreducing conditions, and proteins were transferred to a polyvinylidene difluoride (PVDF) membrane electrophoretically. The PVDF membrane was loaded onto a SNAP i.d. Protein Detection System (Millipore, Billerica, MA, USA). After blocking with Blockingone (Nacali tesque, Kyoto, Japan), the membrane was reacted with a mouse anti-His6 monoclonal antibody (1:2000; Roche Diagnostics, Mannheim, Germany) for 10 min at room temperature and subsequently incubated for 10 min at room temperature with goat anti-mouse IgG conjugated with horseradish peroxidase (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membrane was reacted with ECL Western Blotting Detection Reagents (GE Healthcare, Milwaukee, MI, USA) and bands were visualised using an LAS-4000 EPUV mini (FUJIFILM, Tokyo, Japan). The incorporation efficiency was calculated using the following equation: 100 x (chemiluminescence intensity of the band of the supernatant) x 60 / [(chemiluminescence intensity of the band of the supernatant) x 60 + (chemiluminescence intensity of the band of the pellet) x 20]. In the time-course experiment of KcsA synthesis, the amount of KcsA synthesized was calculated from western blotting bands, and normalized using separately prepared KcsA as the indicator.

Proteinase K treatment assay

After the cell-free protein synthesis of His6-KcsA in the presence of DOPC LUV, the supernatant samples were collected as mentioned above. The supernatant samples were incubated with the indicated concentrations of proteinase K (Promega, Madison, WI, USA) for 30 min at 37°C. After incubation, proteinase K was inactivated for 10 min at 95°C. The samples were subjected to 12.5% Tris-Tricine SDS-PAGE using a 106 (W) x 270 (H)-mm mini-long gel (Nihon Eido, Tokyo, Japan) under reducing conditions and

transferred to a PVDF membrane electrophoretically. Subsequently, western blot analysis was performed as described above.

Channel current recording

The currents of KcsA-integrated liposomes were measured by the planar bilayer method as previously described[30, 31] with some modifications. The side of the upper chamber was made of a glass tube and the bottom was made of a plastic sheet. A 0.1–0.2-mm-thick plastic sheet of polyvinyl chloride or polypropylene having a hole (100–250 μm in diameter) in the centre was glued to a glass tube (8-mm inner diameter, 10-mm height). The holes in the plastic sheet were handcrafted by the melt-and-shave method[32]. For the lower chamber, 35-mm plastic dishes were used. The lower and the upper chamber were filled with a recording solution (2–3 ml and 100–200 μL, respectively). Bilayers were then made by painting a lipid solution: 1-palmitoyl-2-oleoyl sn-glycero-3-phosphoethanolamine (POPE); 1-palmitoyl-2-oleoyl sn-glycero-3-[phospho-rac-(1-glycerol)] (POPG) 3:1 in n-decane across a small hole on a thin plastic sheet. Adding integrated 0.2 μl proteoliposomes to the upper solution incorporated channel proteins into bilayers by liposome-bilayer fusion. Currents were recorded in a symmetrical solution containing 200 mM KCl and 10 mM MES (pH 4.0). The pH-dependency of KcsA synthesized was measured as follows. First, the current was recorded in the upper solution (200 mM KCl and 10 mM Tris-Hepes (pH 7.0)) and the lower solution (200 mM KCl and 10 mM MES (pH 4.0)). After the current recording under the asymmetric pH condition, the lower solution was replaced with 200 mM KCl and 10 mM Tris-Hepes (pH 7.0). Subsequently, the current was recorded under symmetric pH conditions (pH 7.0). The lower solution was held at virtual ground such that the voltage at the upper solution connected to a patch-clamp amplifier (CEZ-2400, Nihon Kohden, Tokyo, Japan) by an Ag-AgCl electrode, which defined the membrane potential. Current data were collected with a patch-clamp amplifier, a digitizer (Digidata 1550, Molecular Devices, Union City, CA, USA) and pCLAMP software (Molecular Devices, Sunnyvale, CA, USA). Data were low-pass filtered at 2 kHz and sampled at 10 kHz. Single-channel records were analysed using Clampfit 10 (Molecular Devices) after digital filtering at 1 kHz. The average single-channel current, single-channel open time and open probabilities were calculated from current traces for 8 seconds using Clampfit software (n =4).

Statistical analysis

Differences were statistically evaluated using the Student's t-test. $P < 0.05$ was considered statistically significant.

Results and discussion

Chaperoning function of liposomes in cell-free KcsA synthesis

In our previous study, we demonstrated that the amount of incorporated membrane proteins into DOPC liposomal membrane was higher than that into anionic or zwitterionic liposomal membranes. Thus, we selected DOPC liposomes in this study[20]. Cell-free syntheses of KcsA were carried out for 4 h in the absence or presence of DOPC liposomes (170 nm). After the reaction, synthesised KcsA protein was analysed by western blotting. The reaction mixtures were ultracentrifuged to separate the pellets (aggregate) and supernatants (liposome fraction), and western blot analysis of each fraction was performed under reducing conditions (Fig. 1A). In the absence of liposomes, all synthesised KcsA was aggregated (detected in the pellet fraction). However, in the presence of liposomes, synthesised KcsA was also detected in the supernatant (liposome fraction). The amount of KcsA was approximately 44.2 ± 7.5%, and the amount of lipid was 50.6 ± 8.4% in the supernatant fraction. These results suggested that liposomes effectively prevented aggregation during the synthesis of KcsA. An experiment using plasmid DNA without DNA encoding KcsA in the cell-free system was carried out as a control. Lipids were also detected in the pellet fraction after ultracentrifugation. This may represent complex formation of liposomes with plasmid or components (proteins or ribosome) of the cell-free synthesis. In the case of the synthesis of KcsA, the pellet fraction may contain aggregates of KcsA including KcsA-containing liposomes and the aggregate of the lipid complex with various components.

The time-course of KcsA synthesis was estimated from western blot analysis using the separately prepared KcsA as an indicator (Fig. 1B). Both in the presence and absence of liposomes, KcsA synthesis was almost complete within 2 h. The amount of KcsA synthesised in the presence of liposomes was higher at all time points than in the absence of liposomes. This result suggested that the liposome assisted KcsA protein synthesis. In a previous study, the lipid supplementation-dependent increase in membrane protein synthesised was also observed in cell-free membrane protein synthesis[33]. KcsA is probably synthesised by a continuous translation reaction close to the liposome surface. The hydrophobic polypeptide or partly folded hydrophobic polypeptide of KcsA may be inserted into the liposomal membrane and aggregation of KcsA was prevented.

Characteristics of synthesised KcsA in the liposomal membrane

To further investigate the chaperon-like activities of liposomes, we confirmed the orientation of incorporated KcsA (Fig. 2). KcsA comprises two transmembrane domains and one intramembrane domain, with both N-terminal and C-terminal domains in the cytoplasm and two extracellular topological domains (Fig. 2A). Proteinase K was added to the exterior of KcsA-reconstituted liposomes (Fig. 2B). When the orientation of synthesised KcsA in the liposomal membrane was the same as that of a living system, KcsA was digested at the two extracellular topological domains (Fig. 2A arrow (a) or (b)). In the opposite orientation, KcsA was digested at the N- or C-terminal domains (Fig. 2A arrow (c) or (d)). After treatment, the two proteolytic fragments were detected at approximately 7 and 12 kDa. Detection of predicted

proteolytic fragments indicated that the synthesised KcsA was folded and inserted in the liposomal membrane. The 7-kDa proteolytic fragment possibly corresponded to a digested product (a, 6.7 kDa). Although the band of approximately 12 kDa did not distinguish whether the band derived from digested product (b, 10.6 kDa) or (c, 13.1 kDa), the chemiluminescence intensity of the 12-kDa band was apparently higher than that of the 7-kDa band. The ratio of the intensity of the 7- and 12-kDa bands was 5:95, suggesting that the N- and C-terminal domains of the 95% synthesised KcsA were located on the exterior surface of the liposomal membrane. In living systems, membrane machinery such as the translocons, regulate integration and topology of membrane proteins. It is worth noting that the orientation of reconstitution of KcsA into the liposome showed a preference in our system without the need for membrane machinery. One plausible explanation is that the more bulky water soluble outer membrane domain of KcsA could not easily penetrate the lipid bilayer and therefore was located on the outside of the membrane because protein synthesis proceeded only outside liposomes under these conditions.

In living systems, KcsA is self-assembled and forms a tetramer[34, 35]. To confirm the oligomerisation of KcsA in the liposomes (Fig. 3A), we performed western blot analysis of the supernatant fraction under non-reducing conditions (Fig. 3B) and only tetramer formation was confirmed. However, in the absence of liposomes, an incomplete complex, not tetrameric, but likely a dimer or trimer formation, was detected (Fig. 3C).

Functional analysis of synthesised KcsA using the planar bilayer method

To assess whether the functional activities of KcsA were exerted or not, we investigated its electrophysiology using the planar bilayer method[30]. In this study, we used horizontal bilayers to measure channel currents because the channel incorporation rate is higher than the vertical bilayer method (Fig. 4A). Bilayers were then made by painting a lipid solution POPE/POPG (3:1 molar ratio), the model membrane of *E. coli*[34, 35], in n-decane across a small hole on a thin plastic sheet at the bottom of the upper chamber. An aliquot of KcsA-integrated proteoliposome suspension (0.2 μL) was added to the upper solution directly above the pre-formed bilayer. If channels failed to appear within several minutes, the bilayer was ruptured and the procedure was repeated. The success rate of current recording was 33 % in this method. In addition, multiple channel analysis indicated that at least four KcsA channels had integrated into the planar bilayer (Fig. 4B). In open-gate conditions, the intense current by KcsA gating is frequently observed by applying a high positive potential to the cytoplasmic side[36]. To confirm whether the recorded channel current was derived from the open-gate condition of KcsA, we evaluated the voltage-dependency of KcsA. We physically reproduced the membrane potential gradient by changing the applied voltage at the upper chamber. Compared with the current response at +100 mV in the upper chamber (Fig. 4C left), the intense current by gating was recorded at −100mV, i.e., the lower chamber was the positive potential (Fig. 4C right), which was similar to observations in previous reports[36, 37]. The average single-channel current, single-channel open time and open probability at +100 mV were 8.58 ± 0.23 pA, 2.04 ± 0.56 ms and 0.07 ± 0.04, respectively. Those at −100 mV were −16.43 ± 1.50 pA, 1.06 ± 0.21 ms and 0.05 ± 0.03, respectively. Furthermore, the single-channel I-V plots clearly showed the voltage-dependency of KcsA (Fig. 4D). These results indicated that the cytoplasmic side of integrated KcsA in the lipid bilayer membrane was located within the lower chamber. Finally, we measured the pH-dependent response of KcsA prepared by this method (Fig. 5). The cluster of charged amino acids in KcsA is an important domain that functions as the main pH-sensor, and is located at the boundary between the membrane and the cytoplasm, and KcsA gating is shown when the cytoplasm has an acidic pH[38]. With the upper chamber at pH 7.0 and the lower chamber at pH 4.0, KcsA showed a current. In contrast, the current was hardly detectable when the upper chamber was at pH 7.0 and the lower chamber was at pH 7.0, indicating that the KcsA channel opened in response to an acidic pH[39]. Taken together, the electrophysiological experiments suggested that KcsA monomers self-assembled into tetramers as a functional form in the liposome, and that this direct-preparation method of KcsA-integrated liposomes is an effective method for the functional and structural analysis of membrane channels.

Conclusions

We demonstrated one-step preparation of KcsA-integrated proteoliposomes using cell-free protein synthesis/liposome systems. The synthesised KcsA spontaneously integrated into liposomal membranes in the correct conformation, and the liposomal membrane assisted the formation of functional KcsA tetramers. Liposomes act as chaperons for cell-free membrane channel protein synthesis. Furthermore, KcsA-integrated proteoliposomes displayed full electrophysiological activities. The processing time between protein synthesis and current recording was within 12 h. This method could also be applied to cell extract-based cell-free protein synthesis. The cell-free protein synthesis/liposome system is a promising method to achieve high throughput analysis of channel proteins.

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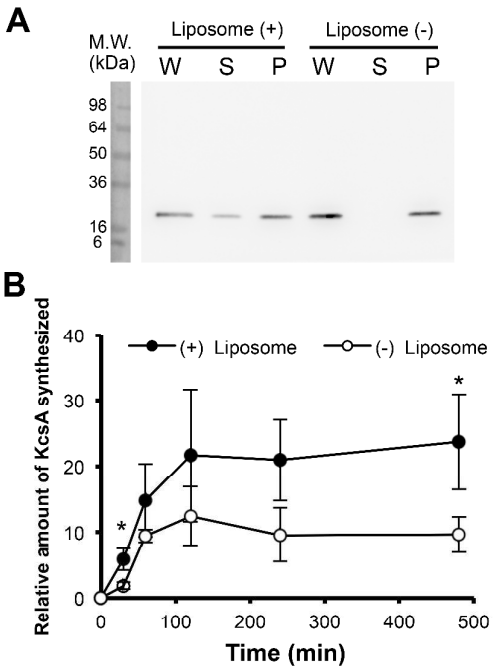


Fig. 1 Preparation of KcsA-integrated proteoliposomes by cell-free protein synthesis. Western blot analysis of KcsA protein expression after ultracentrifugation. (A) Cell-free protein synthesis was performed in the presence or absence of DOPC liposomes. The expressed whole sample (W) was ultracentrifuged and the supernatants (S) and pellets (P) were collected. Results are representative of three independent experiments. (B) Time-course of KcsA synthesis in the presence or absence of DOPC liposomes. Samples were collected at the indicated times. The relative amount of KcsA synthesized was calculated from western blotting bands, and normalized using the separately prepared KcsA as the indicator. Results are expressed as mean ± S.D. (n = 3). Asterisk (*) indicates a t-test result statistically different ($P < 0.05$) from the liposome null group at the same time point.

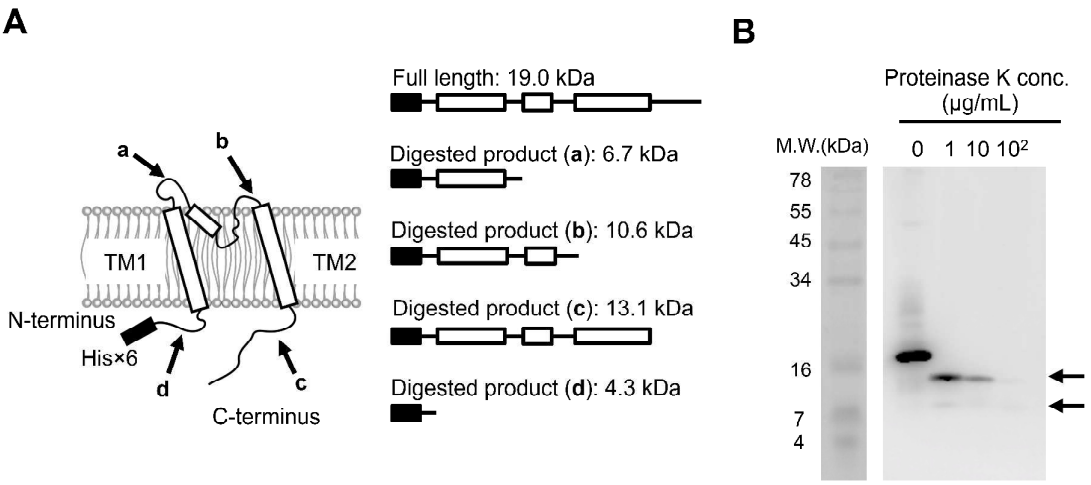


Fig. 2 Orientation of KcsA in the liposomal membrane. (A) Schematic illustration of a KcsA monomer in the lipid bilayer. The arrows indicate the proteinase K cleavable domains (a–d). The theoretical molecular weights of the digested products are represented. (B) Western blot analysis of proteolytic fragments of KcsA after proteinase K treatment. Results are representative of three independent experiments.

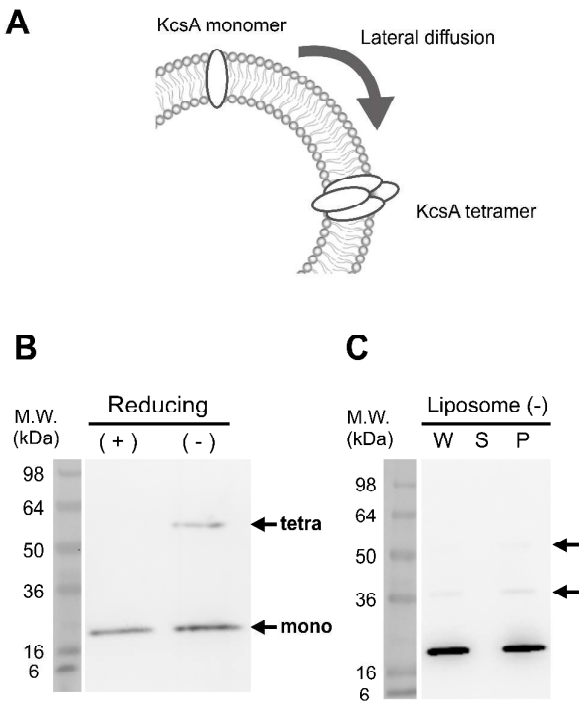


Fig. 3 Oligomerisation of KcsA in the liposomal membrane. (A) Schematic illustration of a cross-section of the KcsA-integrated proteoliposome. (B) Western blot analysis of KcsA-integrated liposomes was performed under reducing or non-reducing conditions. The arrows labelled mono and tetra indicate the KcsA monomer (apparent molecular weight: 21.3 kDa) and KcsA tetramer (apparent molecular weight: 57.3 kDa), respectively. Results are representative of three independent experiments. (C) Western blot analysis of ultracentrifugation samples under non-reducing conditions. Cell-free protein synthesis was performed in the absence of liposomes. The arrow indicates an incomplete complex of KcsA. Results are representative of three independent experiments.

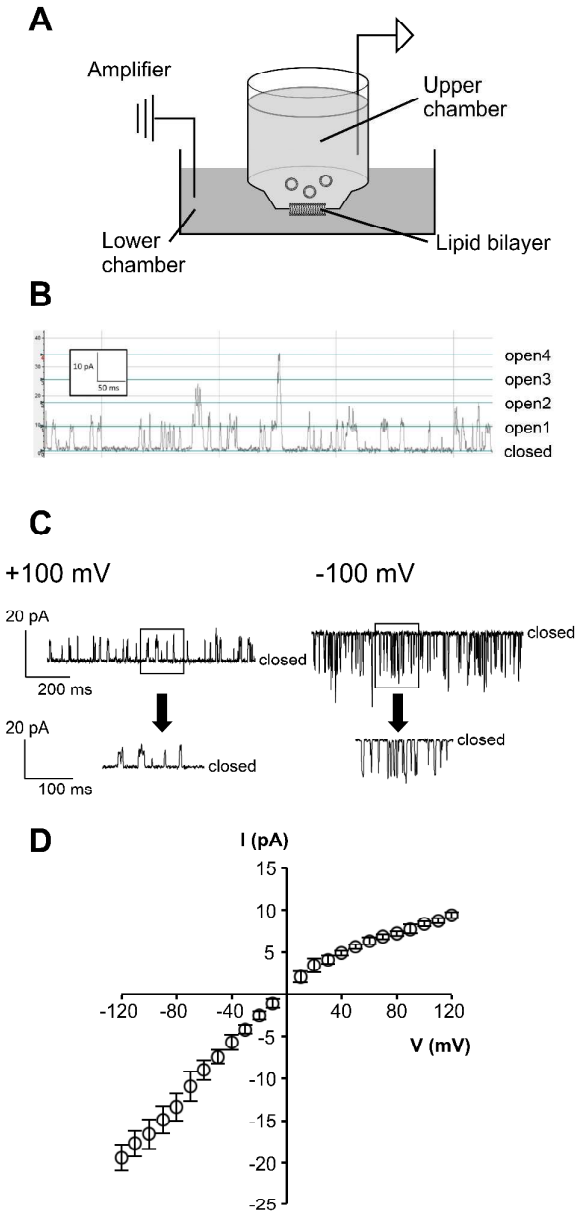


Fig. 4 Voltage dependence of KcsA channel activities. (A) Schematic illustration of the lipid bilayer method used in this study. A glass tube (8-mm inner diameter, 10-mm height) with a small hole on the plastic sheet at the bottom was used as the upper chamber. For the lower chambers, 35-mm plastic dishes were used. The lower and the upper chambers were filled with a recording solution (2–3 ml and 100–200 µL, respectively). (B) Multiple channel analysis was performed using Clampfit software. (C) The currents were recorded by the lipid bilayer method at +100 mV or −100 mV in symmetric pH conditions (both the upper and lower chambers were filled with 200 mM KCl and 10 mM MES (pH4.0)). Data were low-pass filtered at 2 kHz. (D) Single-channel I-V plots were calculated. The currents were recorded in symmetric pH conditions (both the upper and lower chambers were filled with 200 mM KCl and 10 mM MES (pH4.0)). Data were low-pass filtered at 2 kHz. Results are expressed as the mean ± S.D. (n = 4).

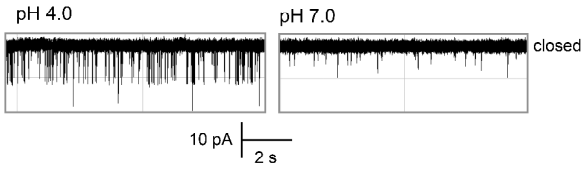
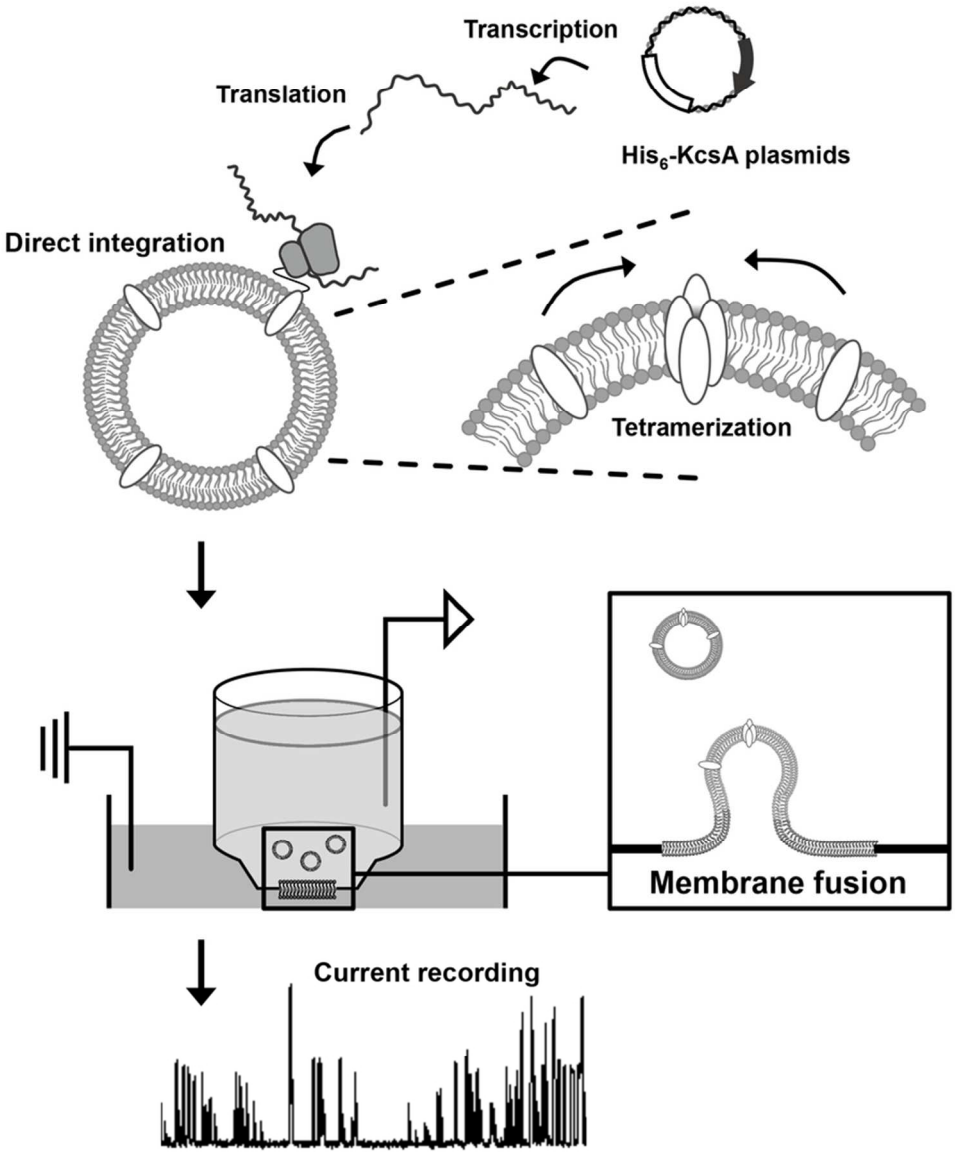


Fig. 5 pH dependence of KcsA channel activities. The currents were recorded by the lipid bilayer method at pH 4.0 and 7.0 at −60 mV. Data were low-pass filtered at 2 kHz.



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