Constitutive boost of a K\textsuperscript{+} channel via inherent bilayer tension and a unique tension-dependent modality

Masayuki Iwamoto (岩本真幸) and Shigetoshi Oiki (老木成稔)

*Department of Molecular Physiology and Biophysics, Faculty of Medical Sciences, University of Fukui, 910-1193 Fukui, Japan

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Molecular mechanisms underlying channel-membrane interplay have been extensively studied. Cholesterol, as a major component of the cell membrane, participates either in specific binding to channels or via modification of membrane physical features. Here, we examined the action of various sterols (cholesterol, epicholesterol, etc.) on a prototypical potassium channel (KcsA). Single-channel current recordings of the KcsA channel were performed in a water-in-oil droplet bilayer (contact bubble bilayer) with a mixed phospholipid composition (azolectin). Upon membrane perfusion of sterols, the activated gate at acidic pH closed immediately, irrespective of the sterol species. During perfusion, we found that the contacting bubbles changed their shapes, indicating alterations in membrane physical features. Absolute bilayer tension was measured according to the principle of surface chemistry, and inherent bilayer tension was ~5 mN/m. All tested sterols decreased the tension, and the nonspecific sterol action to the channel was likely mediated by the bilayer tension. Purely mechanical manipulation that reduced bilayer tension also closed the gate, whereas the resting channel at neutral pH never activated upon increased tension. Thus, rather than conventional stretch activation, the channel, once ready to activate by acidic pH, changes the open probability through the action of bilayer tension. This constitutes a channel regulating modality by two successive stimuli. In the contact bubble bilayer, inherent bilayer tension was high, and the channel remained boosted. In the cell membrane, resting tension is low, and it is anticipated that the ready-to-activate channel remains closed until bilayer tension reaches a few millinewton/meter during physiological and pathological cellular activities.

bilateral tension | contact bubble bilayer | KcsA channel | single-channel current | stretch-activated channel

Fluidic and mosaic membranes undergo various changes that chemically and physically modulate the activity of membrane proteins. Among lipid constituents of the membrane, cholesterol is essential for regulating membrane physical properties, such as fluidity, stiffness, and thickness (1, 2), as well as chemical modification of membrane proteins (3, 4). Such diverse actions of cholesterol toward membranes and membrane proteins have been extensively studied, and ion channels comprise one such target of cholesterol action (5). For example, cholesterol enhances γ-aminobutyric acid–gated channel (GABAA) (6) and nicotinic acetylcholine receptor (nAChR) activity (7) but suppresses inwardly rectifying K\textsuperscript{+} (Kir) (8) and large-conductance voltage/Ca\textsuperscript{2+}-gated K\textsuperscript{+} (BK) channel (9) activity. In some cases, sterols bind specifically to the channel [e.g., nAChR (10), GABAA (6), Kir (11), BK (9)]; whereas in others, they alter membrane properties (volume-regulated anion channel) (12) or regulate channel recruitment from a cytosolic reservoir (Kv1.5) (13). In these studies, controlling cholesterol concentration in the cell membrane has been technically difficult owing to the highly hydrophobic nature of cholesterol and has been possible only by using methyl-β-cyclodextrin (14), which depletes membrane cholesterol. In contrast to biomembranes, lipid bilayers involving liposomes and planar lipid bilayers (15, 16) allow experiments under a controlled cholesterol concentration, but such approaches are still limited.

Here, we investigated the mechanism by which a sterol affected the KcsA potassium channel, whose activity we recently found to be attenuated by cholesterol (17). Although KcsA is a bacterial channel (18, 19) and never encounters cholesterol in its native membrane, sterol-like substances exist even in prokaryotic membranes (20). In this paper, various sterols including cholesterol, epicholesterol, ergosterol, and lanosterol (Fig. 1) were examined in a model membrane of mixed lipid composition (azolectin). The KcsA channel was reconstituted into a lipid bilayer using the contact bubble bilayer (CBB) method (21), which utilizes a water-in-oil droplet bilayer (22–24). In this system, highly hydrophobic sterols are delivered around membrane-embedded channels (membrane perfusion) (17), and immediate single-channel responses can be evaluated following rapid sterol concentration changes.

All tested sterols attenuated single-channel activities of the KcsA channel. Upon sterol perfusion, we found that the contacting bubbles changed their shapes, which led us to examine physical changes to the lipid bilayer. In the CBB, membrane physical properties, such as bilayer thickness and tension, are readily evaluated through principles of surface chemistry (25–27). Absolute bilayer tension at the resting lipid bilayer (inherent bilayer tension) was measured high and, surprisingly, all tested sterols reduced bilayer tension rather than increasing it. In parallel, ready mechanical manipulability of the CBB allowed for a reduction of bilayer tension, thus also closing the gate. Accordingly, the KcsA channel appears sensitive to bilayer tension via chemical and physical perturbations. In contrast, the resting channel never opened upon increased bilayer tension, indicating that the latter played a role only for ready-to-activate channels. This modality is distinct from conventional stretch activation and is instead an unprecedented example of tension-sensitive activation. In a lipid bilayer with high inherent bilayer tension,
Methods

Effect of sterol administration to the membrane on the KcsA channel

Effect of sterols on bilayer tension and thickness. Upon cholesterol perfusion, we found surprisingly that the bilayer membrane immediately expanded its area (Fig. 2A and Movie S1). Bubble shape changes with concomitant contact angle changes were rapid and reversible by switching the perfusion on and off. The bilayer area change itself is trivial for single-channel current recordings, whereas underlying membrane physical feature changes are crucially important for channel activity. Here, bilayer thickness and tension were measured in the presence of sterols (see Methods). In contrast to earlier studies (26, 29, 30) that utilized pure lipid bilayers, such as dipalmitoyl phosphatidylcholine and dimyristoylphosphatidylcholine, here, a biomembrane-mimicking azolectin membrane with mixed phospholipids was used to examine the effect of sterols on membrane physical features.

The KcsA channel activation gate was closed by all membrane sterols tested (cholesterol, epicholesterol, ergosterol, and lanosterol), although the effective concentration range varied substantially (Fig. 1F). As epicholesterol is a stereoisomer of cholesterol, the similarity of its effect to that of cholesterol indicates that the sterol effect is nonstereospecific.

Effect of Membrane Sterols on the Physical Properties of the Azolectin Membrane. Upon cholesterol perfusion, we found surprisingly that the bilayer membrane immediately expanded its area (Fig. 2A and Movie S1). Bubble shape changes with concomitant contact angle changes were rapid and reversible by switching the perfusion on and off. The bilayer area change itself is trivial for single-channel current recordings, whereas underlying membrane physical feature changes are crucially important for channel activity. Here, bilayer thickness and tension were measured in the presence of sterols (see Methods). In contrast to earlier studies (26, 29, 30) that utilized pure lipid bilayers, such as dipalmitoyl phosphatidylcholine and dimyristoylphosphatidylcholine, here, a biomembrane-mimicking azolectin membrane with mixed phospholipids was used to examine the effect of sterols on membrane physical features.

Fig. 1. Effect of sterol administration to the membrane on the KcsA channel current. (A) Chemical structures of sterols used in this paper. (B) Schematic of the membrane perfusion of sterols. Sterols dissolved in hexadecane were sprayed around the CBB and were immediately transferred into the membrane interior. (C) A typical single-channel current response to cholesterol perfusion. (D) An ensemble averaged trace upon perfusion of 3 mg/mL cholesterol with time 0 set at the onset of perfusion. (Inset) The residual current amplitude was obtained from the ensemble averaged current traces, and this current amplitude relative to that before cholesterol perfusion was defined as \( I_c \). (E) Ensemble averaged traces upon perfusion of various sterols. Each sterol was dissolved in hexadecane at the desired concentration and perfused at time 0. The number of raw traces for the ensemble average was 5–24. (F) Sterol concentration dependencies of \( I_c \). The error bar indicates the standard error of the mean (SEM) for the regression (n = 3–6).

The KcsA channel retains its constitutive activity (tension boosted). This modality complements our current understanding of channel-membrane interactions. We will discuss the physiological and pathological roles of channels under conditions that elicit changes in membrane tension via either chemical or mechanical perturbations.

Results

Effect of Sterols on the KcsA Channel in the Azolectin Membrane. To examine the effect of sterols on the KcsA channel and specifically on activation gating, we used the noninactivating E71A mutant (28). The channel was reconstituted into the CBB with azolectin, whose lipid composition was characterized by the predominance of phosphatidylcholine (PC) and phosphatidylethanolamine (see Methods). In a CBB, sterols could be perfused around the membrane-embedded KcsA channels under simultaneous current recording (see Methods) (17). Specifically, sterols dissolved in hexadecane were injected in the bulk hexadecane phase near the CBB, which was readily partitioned into the bilayer’s interior (Fig. 1B).

Single-channel current recordings of the E71A mutant revealed immediate gate closure upon cholesterol perfusion (Fig. 1C; 3 mg/mL cholesterol in hexadecane), indicating an inhibitory action of cholesterol toward the activation gate. Channel activity gradually recovered once perfusion ended because membrane cholesterol decreased through repartitioning back to the bulk oil phase. This indicates rapid cholesterol partitioning between the bulk oil phase and the bilayer interior. Notably, the effect of membrane cholesterol on activation gating was reversible. The perfusion method allows repeated on and off switchings of the bilayer cholesterol content, and the channel’s reversible action allows repeated recordings. An ensemble average of the single-channel response is shown in Fig. 1D, indicating the decaying time course and inhibition level of the steady current amplitude (\( I_c \)). Concentration-dependent ensemble current traces are shown in Fig. 1E. The KcsA channel closed almost completely at a cholesterol concentration of 3 mg/mL in hexadecane, whereas a lower concentration (<2 mg/mL) was insufficient to close the channel.

Fig. 2. Effect of sterols on bilayer tension and thickness. (A) Shape changes in the CBB in the absence or presence (3 mg/mL) of cholesterol. The bilayer area expanded, and the contact angle increased. The bar represents 50 μm. (B) Bilayer thickness as a function of sterol concentration. The dotted black line indicates thickness in the absence of sterol. (C) Bilayer tension as a function of sterol concentration. The dotted black line indicates bilayer tension in the absence of sterol. (D and E) Relationship between \( I_c \) values and bilayer thickness (D) or tension (E). The broken red line in (E) represents a Boltzmann fit (see Discussion). Cholesterol (green), epicholesterol (red), ergosterol (orange), and lanosterol (blue). The error bars indicate the SEM (n = 3–8).
Bilayer thickness (thickness of the membrane hydrophobic core) was evaluated by a conventional electrophysiological method in conjunction with the evaluation of the bilayer area (see Methods and SI Appendix, Fig. S1). In the absence of sterols, bilayer thickness was 3.00 ± 0.8 Å. Concentration-dependent changes in bilayer thickness for different sterols are shown in Fig. 2B. Addition of cholesterol at any of the tested concentrations failed to alter bilayer thickness. This is in contrast to earlier reports (31–34), demonstrating membrane thickening when cholesterol was added to membranes of pure lipid compositions.

Bilayer tension was evaluated based on the contact angle (see Methods). In the CBB, a leaflet of the bilayer is continuous to a bubble-lining monolayer. Viewing of the monolayer and bilayer transitions from the membrane’s tangential direction allows assessment of the contact angle (Fig. 2A) (26). Based on its changes as a function of the applied membrane potential, monolayer tension ($\gamma_{\text{mono}}$) can be evaluated according to the Young-Lippmann equation,

$$\gamma_{\text{mono}} = C_{\text{bi}} \frac{V_{\text{m}}^2}{4(\cos \theta_0 - \cos \theta_r)}$$  \[[1]\]

where $C_{\text{bi}}$ is the bilayer capacitance, $V_{\text{m}}$ is the membrane potential, $\theta_0$ and $\theta_r$ are the contact angles at 0 and $V_{\text{m}}$ mV, respectively. Eventually, bilayer tension was quantitatively evaluated using Young’s equation,

$$\gamma_{\text{bi}} = 2\gamma_{\text{mono}} \cos \theta_0.$$  \[[2]\]

Bilayer tension of the azolectin membrane was 4.8 ± 0.4 mN/m in the absence of sterols and decreased to 2.6 ± 0.2 mN/m in the presence of 3 mg/mL cholesterol (Fig. 2C). Experiments with a higher cholesterol concentration were not possible because a stable bilayer could not be formed, suggesting a high cholesterol content in the bilayer. Indeed, bilayer cholesterol content was estimated to be as high as 50% at 3 mg/mL cholesterol in hexadecane (SI Appendix, Fig. S3). All tested sterols were effective at decreasing bilayer tension.

**Bilayer Tension Rather than Thickness Affects Channel Gating.** A comparison of the physical effects of sterols observed in Figs. 1F and 2C revealed a similar pattern for concentration-dependent current amplitude and bilayer tension. Accordingly, channel activity was plotted as a function of bilayer tension (Fig. 2E) and revealed that the data for different sterols merged on a line. The KcsA channel fully opened at a bilayer tension above ~4 mN/m in the azolectin membrane without sterols, whereas it never opened below 1.5 mN/m in the presence of sterols. This indicates that channel activity was relevant to bilayer tension. Conversely, as shown in Fig. 2D, channel activity did not show any relationship as a function of bilayer thickness. To assess the possible occurrence of phase separation in the azolectin + cholesterol membrane, fluorescently labeled cholesterol in the membrane was imaged using total interference reflection fluorescence (TIRF) microscopy, and a homogeneous phase was observed (SI Appendix, Fig. S4).

**Response of the KcsA Channel Following Mechanical Manipulation of Bilayer Tension.** Bilayer tension was hitherto modified by membrane sterols. To confirm KcsA channel tension sensitivity, the bilayer tension was changed mechanically by exploiting the manipulability of the CBB system. Initially, bilayer tension was kept high (initial high tension) by applying high bubble-maintaining pressure. High pressure is defined as above 4 mN/m, which was deduced from the sterol experiment, exhibiting the saturating activity (Fig. 2E). Macroscopic KcsA current was measured while bilayer tension was changed (Methods and SI Appendix, Fig. S2). In this procedure, bilayer tension was evaluated postexperimentally, and current amplitude data with an initial tension below 4 mN/m were excluded from the evaluation. Fig. 3A shows a successful case with an initial high tension of 7.7 mN/m. Then, the bubble-maintaining pressure in both bubbles was reduced. The macroscopic current gradually decreased and reached a steady current level. As shown in Fig. 3B, the bilayer area increased, and the contact angle increased substantially. According to the Laplace equation, the monolayer tension should decrease as follows:

$$\gamma_{\text{mono}} = \Delta \rho / \left( \frac{1}{\gamma_1} + \frac{1}{\gamma_2} \right).$$  \[[3]\]

With a concomitant increase in the contact angle, the bilayer tension decreases according to Eq. 2. The bilayer tension was reduced to 1.4 mN/m, and the current amplitude dropped substantially. Lately, bilayer tension was increased again to 6.6 mN/m to confirm the reversibility of channel activity.

Similar experiments were performed in different membranes, and the effect of bilayer tension on channel current was evaluated as follows. Current amplitudes were normalized to those at high initial bilayer tension (>4 mN/m) where channels were fully activated (Fig. 2E) and a current amplitude at a different bilayer tension was expressed as a relative current amplitude. Fig. 3C shows the relative current amplitude as a function of bilayer tension ($\gamma_{\text{bi}}$), which followed the Boltzmann distribution,

$$I_{\text{rel}}(\gamma_{\text{bi}}) = \frac{1}{1 + \exp\left(\frac{\Delta G_0 - \gamma_{\text{bi}} \Delta A}{k_B T}\right)}.$$  \[[4]\]

where $\Delta G_0$ represents the free energy of the conformational change from the closed to the open form in the absence of bilayer tension, $\Delta A$ is the change in the cross-sectional area of the transmembrane part of the channel, $k_B$ is the Boltzmann factor,
and $T$ is the absolute temperature (35, 36). Fitting revealed a $\Delta A$ value of 7.33 ± 1.04 nm$^2$ with a $T_{1/2}$ value of 1.89 ± 0.14 mN/m, indicating expansion of the channel cross section upon opening. Thus, the KcsA channel is definitely tension sensitive. The curve was similar to those obtained by altering sterol concentrations (Fig. 2E) and thus confirmed that the action of cholesterol was mediated via bilayer tension. The wild-type KcsA channel also exhibited tension sensitivity (SI Appendix, Fig. S5).

The KcsA channel has an N-terminal amphiphatic M0 helix, which lies at the membrane interface and renders the KcsA channel membrane lipid sensitive (SI Appendix, Fig. S6A) (37). Interaction between the M0 helix and the lipids of the membrane’s inner leaflet suggests that M0 may mediate tension sensitivity. A mutant with a deletion of M0 was examined to assess tension sensitivity. Bilayer tension was reduced by either cholesterol or mechanical manipulation, and the channel immediately closed (SI Appendix, Fig. S6 B and C). Thus, the channel retains bilayer tension sensitivity even without M0, suggesting that the contribution of M0 was not discernible.

**Tension-Boosted Activation vs. Stretch Activation.** Throughout this paper, an intracellular pH was kept acidic for activating the KcsA channel. The question of whether resting channels at neutral pH could be activated by increased bilayer tension was finally examined in the CBB (Fig. 4). The KcsA channel macroscopic current decreased as the cytoplasmic pH became neutral through perfusion of a buffer solution (see Methods). The channel was never activated by increased bilayer tension up to 10.3 mN/m, indicating that it was not stretch activated, confirming a previous study (38).

**Discussion**

In this paper, we reveal an activation modality of the KcsA channel. The latter has been considered a pH-sensitive channel, but acidifying intracellular pH is not enough to fully open the channel. Instead, an additional stimulus mediated by bilayer tension with a half activation tension of ∼2 mN/m is necessary. This modality has been overlooked because the inherent bilayer tension of ∼5 mN/m has been neither evaluated nor appreciated as a means of channel activation. We called this modality tension-boosted activity in a lipid bilayer. Importantly, it offers a doubly controlled activation mechanism and opens possible roles for the channel in physiologically relevant situations in cell membranes.

The present lipid bilayer experiments using the versatile CBB method enabled the modification of the chemical and physical environments around the channels while measuring bilayer physical features and single-channel currents. Among them, measurements of absolute bilayer tension during channel recordings represent a prominent feature of this paper and were achieved by imaging the geometry and measuring bilayer capacitance of the CBB. The inherent bilayer tension of the CBB is comparable to those of conventional planar lipid bilayers (39) but has never been related to channel activity. Inherent bilayer tension is reminiscent of an issue encountered in patch-clamp recording whereby membrane stretching is steadily applied after a gigaseal formation with an estimated value of ∼0.5–4 mN/m (40). The additional tension imposed by applying negative pressure has been related to channel activation by mostly ignoring the inherent patch tension as “background.” Thus, the stretch activation evaluated in the current studies, using mostly patch clamp recording, depends on relative rather than absolute tension.

All experiments were performed in a mixed lipid bilayer of azolectin. TIRF microscopy revealed that the addition of cholesterol did not cause phase separation, which is in contrast with the prominent phase separation of PC + cholesterol membranes (SI Appendix, Fig. S4) (41). In the azolectin membrane, some sterols (epicholesterol and lanosterol) slightly increased membrane thickness (Fig. 2B), but lack of thickening by cholesterol was not expected. No correlation was found between bilayer thickness and channel activity (Fig. 2D). Nevertheless, changes in local thickness around the channels could not be excluded. Common changes to channel activity caused by all tested sterols (Fig. 1E) and the similarity between tension dependencies with sterols (Fig. 2E) and purely mechanical changes (Fig. 3C) strongly suggest that the action of sterols occurs via bilayer tension.

Bilayer tension in the cells’ resting state and the amount by which tension is increased upon certain physiological events have been examined. Even though KcsA is a bacterial channel, a comparison with eukaryotic cells can be made. The resting bilayer tension of eukaryotic membranes is 0.003–0.05 mN/m (42) and increases to over 10 mN/m when osmotic stress is applied (43). In parallel to these data, the activation tension for the Piez1 channel has been elucidated as 1–3 mN/m (44, 45). This value suggests that the cell membrane undergoes such tension changes during physiological and pathological conditions. Recent studies revealed local and global tension changes up to ∼0.5 mN/m upon cell migration and division (46, 47). In contrast, a bacterial MscS is activated at 3 to 4 mN/m (48, 49) and MscL channels above 10 mN/m (35, 48). The inherent bilayer tension of the bacterial membrane has been estimated at 1.6 mN/m (50, 51). Local strong curvatures of the membrane may render the bilayer tension even higher. This tension range corresponds to the range of KcsA channel modulation, and elucidating physiological processes leading to tension changes would provide insight into the hitherto unknown physiological function of KcsA in the bacterial membrane.

Given the substantial discrepancy between inherent lipid bilayer tension and resting cell membrane tension, the action of sterols on the KcsA channel should be considered differently in different membrane environments. The attenuating action of sterols on the channel in the lipid bilayer was clearly detected; however, this could not be the case at low tension where the channel remains closed. We nevertheless have experimental evidence suggesting a distinct action of sterols at low tension. We report a similarity between tension dependency via pure mechanical manipulation ($\Delta G_{\text{bilayer}}$ of 3.37 ± 0.37, $\Delta A$ of 7.33 ± 1.23 nm$^2$, and $T_{1/2}$ of 1.89 ± 0.14 mN/m) and sterol addition ($\Delta G_{\text{bilayer}}$ of 10.35 ± 4.78, $\Delta A$ of 14.69 ± 7.29 nm$^2$, and $T_{1/2}$ of 2.80 ± 0.12 mN/m). It should be noted, however, that the parameters differed substantially (Welch’s $t$ test $P$ value: $**P < 0.01$; Figs. 2E and 3C). The channel remained active at 2 mN/m in the absence of sterols but fully closed in the presence of sterols. This contrasting behavior suggests direct action of sterols on the
KcsA channel, which is intriguing and will be examined in future studies. The observation extends to a general issue of how membrane-dissolving substances could affect and be affected by membrane physical features. This issue has never been addressed but will be further examined.

Failure to open the channel by increased tension at neutral pH (nonstretch activation) suggests that conformational changes were “locked” in the resting conformation. Once activated at acidic pH, the channels became unlocked and underwent a change in open probability depending on bilayer tension. Tension sensitivity was retained even without the M0 helix (SI Appendix, Fig. S6). This indicates that bilayer tension is sensed mostly by the membrane-spanning domain, including M1 and M2 helices; nevertheless, further studies examining the contribution of M0 should be undertaken in the future. The tension-modulated activity would be structurally feasible were the resting and activated conformations substantially different in terms of flexibility (Fig. 5). The KcsA channel crystal structure revealed a more flexible structure for the open conformation, exemplified as distinctly higher B factors (or crystallographic temperature factor) (52) over that of the resting conformation, which was confirmed by molecular dynamics simulation (53). In addition to local structural fluctuations, softness of helices has been shown for the stretch-activated channel (54). With the crystal structure of the closed conformation of KcsA having a cross-sectional radius of ~25 Å, it was estimated that this could expand by ~4 Å upon the tension-modulated opening (from the Boltzmann fit ΔA = 7.3 nm²), which is in accordance with the radius of the open conformation (52, 53). The ΔA value is similar to those for the inherent stretch-activated channels, such as 15 nm² for MscL (45), 6–20 nm² for Piezo1 (55), and 8 nm² for MscS (56), indicating that the ready-to-activate channel has a similarly elevated flexible structure as those of the stretch-activated channels. We found the dramatic difference in tension sensitivity between the resting and the ready-to-activate channel, which brings to mind the tight and loose conformations proposed in allosteric transitions (57). Global conformation changes occur upon channel activation (58), whereas resting channels remain locked, likely as a result of electrostatic interactions. To summarize, we report the tension-modulated activity of the bacterial KcsA channel, which is distinct from stretch activation.

Previous screening has revealed the absence of inherent stretch activity in many channels, including the KcsA channel. Our paper opens the possibility for a form of activation based on tension modulation and thus a category of channels. It is plausible that nonstretch-activated but tension-modulated channels are predominant in both prokaryotic and eukaryotic cells. In tension-modulated channels, the likely mechanism comprises an activation process specific for each channel type, such as acidic pH for the KcsA channel, combined with additional tension modulation. This two-step process enables a logical conjunction of stimuli, yielding fine-tuning of channel activation in response to modification by local and subtle mechanical fluctuations of the cell membrane during variable physiological events.

Materials and Methods

Reagents. Phospholipids and sterols were obtained as follows: azolectin (1,2-phosphatidylcholine type IV-S), Sigma-Aldrich; cholesterol, Nacalai Tesque; ergosterol, Tokyo Chemical Industry; epicholesterol, Cambridge Isotope Laboratories; lanosterol, Nagara Science. Other chemicals were purchased from Nacalai Tesque. 1-palmityl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 25-[N-[7-nitro-2-1,3-benzoxadiazol-4-yl][methyl]amino]-27-norcholesterol (NB-d-cholesterol) were purchased from Avanti Polar Lipids.

Sample Preparation. Expression, purification, and reconstitution of KcsA channels into liposomes has been described elsewhere (59). Proteoliposomes were prepared by dilution as follows. First, liposomes, prepared using azolectin, were suspended in 200 mM KCl at a concentration of 2 mg/mL. Then, an aliquot of solubilized KcsA channel in 0.06% n-dodecyl-β-D-maltoside was diluted 50 times with the liposome solution. The lipid/weight ratio of the proteoliposome was 2000. The proteoliposome suspension was mixed with a small amount of concentrated buffer (pH 7.5 or 4.0) just before the experiments.

CBB Method. The CBB method has been described in detail elsewhere (21, 60). Liposome and proteoliposome solutions (2 mg/mL) were filled, respectively, in two bubble-forming pipettes (diameter at the tip, ~30 μm). The solution was blown from the tip of the pipettes into a hexadecane phase under observation with an inverted microscope (IX73; Olympus). Small bubbles (diameters of ~100 μm) coated with lipid monolayers were contacted by pipette manipulation, forming a CBB. The ionic current was measured using a patch-clamp amplifier (EPC800USB, HEKA, Lambrecht/Pfalz), which was stored in PC via a data acquisition (ALAMPS Scientific). pH of the bubble solutions was defined on the pH 7.5 side.

Bilayer Thickness. Bilayer thickness was evaluated from the electrical capacitance of the bilayer using an electrophysiological technique and the bilayer area measured from the microscopic image of the CBB. Details are provided in the SI Appendix, Supplementary Materials, Fig. S1. Each sterol was dissolved in hexadecane.

Bilayer Tension. Bilayer tension of the CBB was evaluated in the azolectin membrane according to the method reported by Taylor et al. (26). First, the tangential CBB image was taken at a membrane potential of 0 mV, and the contact angle was measured using Image J software (https://imagej.nih.gov/ij). Measurements of the contact angle were repeated as the membrane potential was changed up to ±200 mV with a step of ±25 mV. Values of 

$$\theta_B \approx \frac{\gamma_{SL} - \gamma_{SV}}{\gamma_{SL}}$$

were plotted against $\gamma_B$ (Eq. 1 and SI Appendix, Fig. S2B). Monolayer tension ($\gamma_{monolayer}$) was obtained from the slope (m) of the linear regression line using the following equation: $\gamma_{monolayer} = \frac{\gamma_{SV} - \gamma_B}{m}$. Finally, bilayer tension ($\gamma_B$) was obtained using Young’s equation (Eq. 2). The above experimental procedure takes a few seconds aside from the postexperimental current and image analyses, and bilayer tension is evaluated at the time of the current recordings.

**TIRF Microscopy.** Distribution of fluorescently labeled cholesterol in the azolectin or POPC membranes was observed by TIRF microscopy. Liposomes consisting of 50 mol% azolectin or POPC, 49 mol% cholesterol, and 1 mol% astolectin and cholesterol, Nacalai Tesque; ergosterol, Tokyo Chemical Industry; epicholesterol, Cambridge Isotope Laboratories; lanosterol, Nagara Science. Other chemicals were purchased from Nacalai Tesque. 1-palmityl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 25-[N-[7-nitro-2-1,3-benzoxadiazol-4-yl][methyl]amino]-27-norcholesterol (NB-d-cholesterol) were purchased from Avanti Polar Lipids.

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NBD-cholesterol were prepared and suspended in a 200 mM KCl solution (2 mg/mL). Then, a 1 μL droplet of the liposome suspension was immersed in hexadecane on the hydrophobized cover glass. Lipid monolayer membranes formed around the droplets were observed at the contacting face with the cover glass through a 100X objective (UAPON 100XOTIRF; Olympus) on an IX-71 inverted microscope equipped with an intensified high-speed camera (SV200i; Photron). The excitation source was a 440 nm laser (FVS-LD400-2; Olympus).

**Statistical Analysis.** Average values are expressed as means ± SEM. For comparison of means between categories, Welch’s t test (two tailed, no assumption of variance) was carried out, and P values were obtained from a t-value lookup table.

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