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# pH-dependent promotion of phospholipid flip-flop by the KcsA potassium channel

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ABSTRACT

KcsA is a pH-dependent potassium channel that is activated at acidic pH. The channel undergoes global conformational changes upon activation. We hypothesized that the open–close conformational changes of the transmembrane region could promote the flip-flop of phospholipids. Based on this hypothesis, we measured the flip-flop of NBD-labeled phospholipids in KcsA-incorporated proteoliposomes. Both flip and flop rates of $C_6$NBD-PC were significantly enhanced in the presence of KcsA and were several times higher at pH 4.0 than at pH 7.4, suggesting that KcsA promotes the phospholipid flip in a conformation-dependent manner. Phospholipids were non-selectively flipped with respect to the glycerophospholipid structure. In the active state of KcsA channel, tetrabutylammonium locks the channel in the open conformation at acidic pH; however, it did not alter the flip rate of $C_6$NBD-PC. Thus, the open–close transition of the transmembrane region did not affect the flip-flop of phospholipids. In addition, the KcsA mutant that lacked an N-terminal amphipathic helix (M0-helix) was found to show reduced ability to flip $C_6$NBD-phospholipids at acidic pH. The closed conformation is stabilized in the absence of M0-helix, and thus the attenuated flip could be explained by the reduced prevalence of the open conformation. These results suggest that the open conformation of KcsA can disturb the bilayer integrity and facilitate the flip-flop of phospholipids.



## 1. Introduction

Phospholipid transbilayer movement (flip-flop) plays an essential role in the homeostasis of biological membranes. For example, phospholipids in the plasma membrane (PM) are asymmetrically distributed between the extracellular and cytoplasmic leaflets by means of aminophospholipid translocases and ATP-binding cassette (ABC) transporters [1,2]. Disruption of the asymmetry by phospholipid scramblases, accompanied by the externalization of phophatidylserine, is related to cell apoptosis and blood coagulation [3]. In the endoplasmic reticulum (ER), the newly synthesized phospholipids on the cytoplasmic surface need to be readily translocated to the luminal surface for maintaining the membrane integrity [4–6]. The phospholipid flip-flop in the ER is, therefore, extremely rapid, with $t_{1/2}$ value ranging from a few seconds to a few minutes [7,8]. This activity is sensitive to proteases or chemical reagents, indicating the involvement of proteins [8–10]. Furthermore, the process is energy-independent and bidirectional, and has a low specificity toward the headgroups of the lipids [8–10]. Although numerous studies have examined the energy-independent process of phospholipid flip-flop, the molecular entities that are responsible for the phospholipid flip-flop remain elusive [11,12]. In this regard, the investigation of the physicochemical mechanism of phospholipid flips is of great importance.

KcsA is a bacterial potassium channel that has been previously reported to enhance the phospholipid flip-flop [13]. Although KcsA is of prokaryotic origin, potassium channels are ubiquitous across the pro- and eukaryotic kingdoms. The architecture of the transmembrane domain and the conformational changes upon gating are believed to be universal for all potassium channels [14]. Among the structures of all potassium channels, the structure of KcsA channel has been the first to be crystallized [15], and its molecular gating mechanism has been extensively studied [16–18]. Thus, the investigation of the effects of KcsA on the flip-flop of phospholipids would provide valuable information for revealing the molecular mechanism of phospholipid flip-flop. Here we hypothesized that the open–close motion of the transmembrane region upon channel gating could induce the flip-flop of phospholipids.

*Abbreviations:* PM, plasma membrane; ER, endoplasmic reticulum; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; $C_6$NBD-PC, 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine; LUVs, large unilamellar vesicles; TBA, tetrabutylammonium

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The KcsA channel is activated when the cytoplasmic pH is acidic [19]. Upon gating, the transmembrane pore domain of the KcsA channel undergoes global conformational changes [16,17]. These changes include the twisting and untwisting of the transmembrane helical bundle around the axis of the pore [17], elongation and shortening of the longitudinal length of the transmembrane domain [20], and a revolving motion of the N-terminal amphipathic helix (M0-helix) at the membrane interface [21]. These global conformational changes were likely to perturb bilayer integrity at the channel-membrane interface and facilitate the flip-flop of phospholipids.

To verify our hypothesis, we measured the flip-flop rate in the KcsA-incorporated proteoliposomes by using fluorescently labeled phospholipids ($C_6$NBD-phospholipids) under different pH conditions. Additional measurements were performed by modifying the gating of the KcsA channel. Tetrabutylammonium (TBA) blocks the channel current and locks the ion channel in the open state [22]. In addition, the N-terminal amphipathic M0-helix substantially modifies the activation gate. Upon the removal of M0-helix, the closed conformation was stabilized, and the open probability was significantly reduced [21]. These gating-modifying tools may be helpful for elucidating the underlying mechanism of the KcsA channel on the flip-flop of phospholipids.

In the present study, we demonstrated that the effect of KcsA for enhancing the flip-flop of fluorescently labeled lipids at acidic pH is more significant than that at neutral pH. Furthermore, we found that the pH-dependent phospholipid flip-flop is affected by the open conformation, which may perturb the membrane integrity through hydrophobic mismatch, rather than by the open–close motion of the transmembrane region.

## 2. Material and methods

### 2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine ($C_6$NBD-PC), $C_6$NBD-phosphatidylglycerol ($C_6$NBD-PG), and $C_6$NBD-phosphatidylserine ($C_6$NBD-PS) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was obtained from Sigma Chemical Co. (St. Louis, MO). Tetra-*n*-butylammonium bromide was obtained from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals used were of the highest reagent grade.

The expression and purification process of wild-type (WT) KcsA with either an N-terminal or C-terminal His-tag and a mutant, lacking the N-terminal M0 segment (1–22) (KcsA ΔM0), was conducted by following the protocol described previously [22].

### 2.2. Sample preparation

Required amounts of chloroform–methanol solution of POPC and cholesterol were placed in a round-bottomed glass flask for preparing large unilamellar vesicles (LUVs). After the evaporation of organic solvents, the sample was dried overnight under vacuum. The lipid film was hydrated with a phosphate buffer (10 mM phosphate, 150 mM KCl, and 1 mM $NaN_3$; pH 7.4) or a citrate-phosphate buffer (4.5 mM citrate, 5.5 mM phosphate, 150 mM KCl, and 1 mM $NaN_3$; pH 4.0) with or without 10 mM tetra-*n*-butylammonium bromide. The suspension was freeze–thawed several times and extruded through a 100-nm pore polycarbonate filter using a LiposoFast low-pressure homogenizer (Avestin, Ottawa, Canada). The concentrations of POPC and cholesterol were determined by using the enzymatic assay kits for choline and cholesterol (Wako, Osaka, Japan), respectively.

Proteoliposomes were prepared as previously described [23] with minor modification. LUVs containing 0, 5, and 30% (in terms of mole%) cholesterol of the total lipids (POPC and cholesterol) were solubilized in the CHAPS detergent (Nacalai Tesque, Kyoto, Japan) at concentrations of 34, 34, and 150 mM, respectively (CHAPS at high concentrations was required for solubilizing vesicles with high cholesterol contents). After the mixture was incubated at 25 °C for 2 h, KcsA at a molar concentration (KcsA-tetramer/lipid) of 0.05 mol%, 0.025 mol%, or 0.0125 mol% was added to the mixture. After incubation at 25 °C for 20 min, the detergent was removed by dialysis at room temperature (r.t.) for 48 h. Thereafter, the obtained KcsA proteoliposomes were extruded through a 100-nm pore filter. Protein-free liposomes were prepared by using the same procedure without the addition of KcsA. The vesicle size of the suspension was confirmed to be ca. 110–150 nm by dynamic light scattering analysis using a FPAR-1000 particle analyzer (Otsuka Electronics, Osaka, Japan). The concentration of KcsA was determined by SDS-PAGE, where the band intensity of KcsA tetramer was densitometrically quantified using ImageJ (NIH, Bethesda, MD) and compared with that of detergent-solubilized KcsA used as a standard. The final lipid/protein ratio was determined based on the concentrations of KcsA and lipid, which were estimated as described above.

### 2.3. Flip assay

An ethanol solution of $C_6$NBD-phospholipid (PC, PG, or PS) at a molar concentration (fluorescent lipid/non-fluorescent lipid) of 0.2 mol% was added to KcsA proteoliposomes for incorporating the fluorescent lipids asymmetrically into the outer leaflet of the proteoliposomes. After incubation at 25 or 37 °C for several time periods (0.5–6 h) to allow the $C_6$NBD-phospholipids to translocate (flip) to the inner leaflet, sodium dithionite in 2 M Tris was added to 50 μM proteoliposome suspension to obtain a final dithionite concentration of 8 mM, and this led to the reduction (and fluorescence quenching) of $C_6$NBD-phospholipids in the outer leaflet of the proteoliposomes. After the addition of dithionite, the fluorescence of NBD was monitored on an F-2500 spectrofluorometer (Hitachi, Tokyo, Japan) for 500 s at the excitation and emission wavelengths of 460 and 534 nm, respectively.

The ratio of $C_6$NBD-phospholipids moved to the inner leaflet was calculated as $F/F_0$, where $F_0$ and $F$ are the fluorescence intensity before and after the addition of dithionite, respectively. The fluorescence quenching caused by dithionite displayed a biphasic decay model (Fig. S1 in the Supporting Material); the faster decay denoted the quenching of $C_6$NBD-phospholipids located in the outer leaflet, and the slower decay represented the quenching of $C_6$NBD-phospholipids that had been initially located in the inner leaflet but flopped after the injection of dithionite. Thus, to remove the influence of the second situation described above and to determine the real $F$ value, the fluorescence decay data from 400 to 500 s after the injection of dithionite were fitted with a single exponential function and extrapolated toward 0 s. The obtained $F/F_0$ values were plotted against the incubation time $t$ and fitted using the following equation:

$$F/F_0 = C_1 + (C_2 - C_1)\left\{1 - \exp\left(-k_{\text{flip}}t\right)\right\} \quad (1)$$

where $C_1$, $C_2$, and $k_{\text{flip}}$ were fitting parameters; $C_1$ and $C_2$ were the $F/F_0$ values at time points 0 and ∞, respectively, and $k_{\text{flip}}$ was the apparent flip rate constant. The initial flip rate of $C_6$NBD-phospholipids was determined as the first derivative of Eq. (1) at 0:

$$\text{Initial flip rate} = k_{\text{flip}}(C_2 - C_1). \quad (2)$$

### 2.4. Flop assay

Proteoliposomes, where the fluorescent lipids were symmetrically distributed, were prepared by adding $C_6$NBD-PC to the lipid mixtures in a chloroform–methanol solution before the LUV preparation. Sodium dithionite in 2 M Tris was added to the proteoliposome suspension to obtain the final dithionite and proteoliposome concentrations of 25 mM and 1.5 mM, respectively. This led to the reduction of NBD in

the outer leaflet and the production of proteoliposomes containing the fluorescent lipids only in the inner leaflet. After incubation for 5 min, sodium dithionite was removed on a spin column loaded with Superdex 200 (GE Healthcare UK, Buckinghamshire, UK). After incubation at 37 °C for several time periods (0.5–6 h) to allow $C_6$NBD-PC to translocate (flop) to the outer leaflet, the ratio of $C_6$NBD-PC flopped to the outer leaflet ($1–F/F_0$) was determined by the similar procedure used in the flip assay described above.

## 3. Results

In this study, we performed dithionite reduction assays for investigating the flip-flop of fluorescent lipids under both neutral and acidic conditions. Dithionite is known to function as a reductant at basic pH. We first determined whether this assay would be applicable to the solutions at pH 7.4 and 4.0. Dithionite in 2 M Tris was added to the protein-free LUVs, which were symmetrically labeled with $C_6$NBD-PC, to produce a final Tris concentration of 200 mM. $F/F_0$ was calculated to be 0.528 ± 0.028 and 0.519 ± 0.006 at pH 7.4 and 4.0, respectively. The nearly 50% quenching suggests that dithionite completely reduces NBD located in the outer leaflet under both pH conditions. This was also confirmed by the fluorescence intensity measurement that almost all NBD-phospholipids were incorporated into the liposomes irrespective of pH values, and this finding was in good agreement with that of the previous study [24] (data not shown). In addition, SDS-PAGE analysis verified that the reconstitution efficiency of KcsA was independent from the reconstitution conditions such as pH values and the presence of TBA or cholesterol (Fig. S2).

### 3.1. Promotion of phospholipid flip-flop by KcsA

Dithionite reduction assays were conducted for measuring the flip rate of $C_6$NBD-phospholipids (Fig. 1A). To distinguish the protein-mediated flip-flop from the intrinsic flip-flop of phospholipids affected by pH, we first measured the initial flip rate of $C_6$NBD-phospholipids in protein-free LUVs at different pH values (Fig. 1B–D, control). The influence of pH on flip-flop varied depending on the polar headgroups of NBD-phospholipids; the flip rate of $C_6$NBD-PC at pH 7.4 was similar to that at pH 4.0 (Fig. 1B), whereas the initial flip rates of $C_6$NBD-PG (Fig. 1C) and $C_6$NBD-PS (Fig. 1D) at pH 4.0 were higher than those at pH 7.4. The protonated forms of these lipids at acidic pH are considered responsible for enhancing their transbilayer movements [25]. As the flip of $C_6$NBD-PG at 37 °C was too rapid to be measured precisely (Fig. S3), the experiments of $C_6$NBD-PC and $C_6$NBD-PS were conducted at 37 °C, whereas the experiments of $C_6$NBD-PG were performed at 25 °C instead.

Next, dithionite reduction assays were conducted using proteoliposomes containing KcsA (with a C-terminal His-tag). At pH 7.4, the flip rates of $C_6$NBD-PC and $C_6$NBD-PS in the proteoliposomes with KcsA

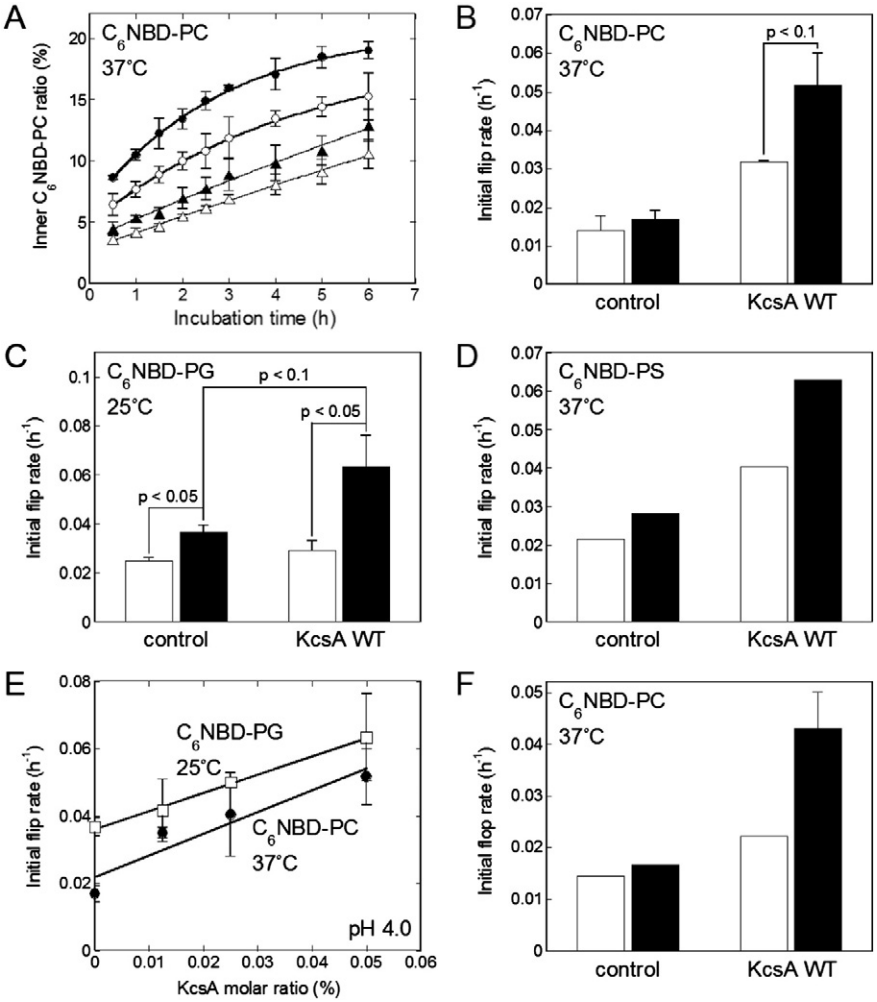


**Fig. 1.** (A) Flip assays of $C_6$NBD-PC in protein-free liposomes (triangles) or proteoliposomes (circles) containing 0.05 mol% WT KcsA (C-terminal His-tagged) at pH 7.4 (open symbols) or pH 4.0 (solid symbols). (B–D) Initial flip rates of $C_6$NBD-PC (B), $C_6$NBD-PG (C), and $C_6$NBD-PS (D) in protein-free liposomes (control) or proteoliposomes containing 0.05 mol% WT KcsA at pH 7.4 (open bars) or pH 4.0 (solid bars). (E) Initial flip rates of $C_6$NBD-PC (solid circles) and $C_6$NBD-PG (open squares) in proteoliposomes containing 0–0.05 mol% WT KcsA at pH 4.0. (F) Initial flop rates of $C_6$NBD-PC in protein-free liposomes (control) or proteoliposomes containing 0.05 mol% WT KcsA (N-terminal His-tagged) at pH 7.4 (open bars) or 4.0 (solid bars). Error bars represent standard deviations (SDs) of two or three experiments.

were 2.27 and 1.87 times higher, respectively, than those without KcsA (Fig. 1B and D). In contrast, the flip rate of $C_6$NBD-PG was slightly increased (1.16-fold) by KcsA (Fig. 1C). This result was consistent with a previous finding [13] that the flip rate of $C_6$NBD-PG was barely enhanced at the same protein/lipid ratio used in this study. More importantly, the effect of KcsA on the flip-flop was more significant under acidic conditions than that under neutral conditions, as the flip rates of $C_6$NBD-PC, $C_6$NBD-PG, and $C_6$NBD-PS at pH 4.0 were 1.62, 2.18, and 1.56 times higher, respectively than those at pH 7.4 (Fig. 1B–D). The increase in the flip rate with the decrease in pH was more notable in these proteoliposomes than that in the protein-free liposomes. Furthermore, the flip rates of $C_6$NBD-PC and $C_6$NBD-PG at pH 4.0 increased with the increase in the KcsA concentration in LUVs (Fig. 1E). The pH-dependent flip promotion by KcsA was also observed with the use of KcsA His-tagged at the N-terminus (Fig. S4), suggesting that the His-tag at either terminus has negligible effects on the flip-flop of phospholipids. In addition, the assay showed that KcsA (with an N-terminal His-tag) promoted the flop of $C_6$NBD-PC in a pH-dependent manner, and this was similar to its effect on the flip process (Fig. 1F). Overall, KcsA demonstrated pronounced effects of inducing the phospholipid flip-flop at an acidic pH, with a low specificity to the headgroups of lipids.

### 3.2. Effect of tetrabutylammonium on the flip-flop promotion by KcsA

Furthermore, we conducted flip assays in the presence of 10 mM TBA at pH 4.0 to reveal the mechanism of KcsA for promoting the phospholipid flip-flop at acidic pH. The binding of TBA to KcsA at acidic pH leads to an open-locked conformation of the channel, and the gating transition is abolished [17,22]. The flip rate of $C_6$NBD-PC in the protein-free LUVs remained unaffected regardless of the presence of TBA (Fig. 2), suggesting that TBA did not affect the intrinsic flip-flop of $C_6$NBD-PC. Importantly, TBA did not reduce the flip rate of $C_6$NBD-PC in the proteoliposomes. The gating blocker should have decreased the flip rate, if the open–close motion of the transmembrane domain could induce the flip-flop. Therefore, we concluded that the open–close motion of the transmembrane region does not affect the phospholipid flip-flop of phospholipids.

### 3.3. Effect of M0-helix on the flip-flop promotion by KcsA

We prepared proteoliposomes using a KcsA mutant that lacked the M0-helix (KcsA ΔM0). The mutated protein reconstituted in the artificial membranes retained the original channel activity, except for a reduction in the open probability [21]. At pH 7.4, the flip rate of $C_6$NBD-PC in the LUVs containing KcsA ΔM0 was almost the same as that in the LUVs containing WT KcsA (Fig. 3A). However, unlike the WT KcsA-containing LUVs, the KcsA ΔM0-containing LUVs did not show any increase in the flip rate at lower pH values. In other words, the decrease in pH did not promote the flip-flop in the proteoliposomes containing KcsA ΔM0. These results suggest that the presence of M0-helix is responsible for the enhancement of flip-flop at acidic pH. A similar tendency was observed in the flip process of $C_6$NBD-PG (Fig. 3B). The partial repression of flip-flop due to the absence of M0-helix suggests that the reduced open probability was accountable for the attenuated flip of $C_6$NBD-PG.

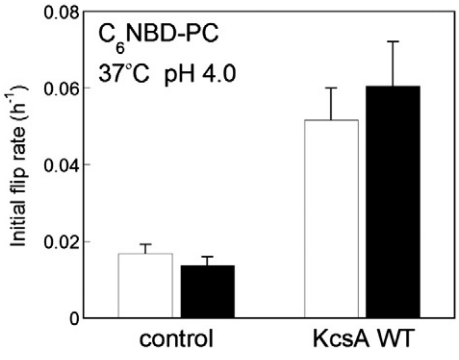


**Fig. 2.** Initial flip rates of $C_6$NBD-PC in protein-free liposomes (control) or proteoliposomes at pH 4.0 with (solid bars) or without TBA (open bars). Error bars represent SDs of two or three experiments.

### 3.4. Effect of cholesterol on phospholipid flip-flop

To assess the influence of different cholesterol contents in biological membranes on phospholipid flip-flop, cholesterol was added to both protein-free LUVs and proteoliposomes containing WT KcsA. LUVs containing 5% cholesterol were obtained for mimicking the cholesterol composition in the ER, while LUVs containing 30% cholesterol were used for mimicking the cholesterol composition in the PM. As shown in Fig. 4A, neither 5% nor 30% cholesterol affected the flip rate of $C_6$NBD-PC in the protein-free LUVs. In contrast, the flip of $C_6$NBD-PG was dramatically inhibited by cholesterol at 30% (Fig. 4B). Although these experiments were performed at different temperatures (37 °C and 25 °C for $C_6$NBD-PC and $C_6$NBD-PG, respectively), the inhibition of the flip of $C_6$NBD-PG by cholesterol was also observed at 37 °C (Fig. S5). This indicated that the distinct effects of cholesterol on $C_6$NBD-PC and $C_6$NBD-PG were not caused by the temperature difference. Moreover, the presence of cholesterol did not influence the flip of $C_6$NBD-PC in proteoliposomes (Fig. 4A). Although the flip of $C_6$NBD-PG in proteoliposomes was inhibited by cholesterol (Fig. 4B), the increase in the flip rate of the lipid by the incorporation of WT KcsA (i.e., KcsA-mediated flip) was comparable to that in the LUVs containing both 0% and 30% cholesterol (Fig. 4C). Taken together, our results indicate that cholesterol does not affect the KcsA-mediated flip-flop of phospholipids.

## 4. Discussion

In this study, we examined the effect of KcsA channel on the flip-flop of phospholipids by using fluorescently labeled $C_6$NBD-phospholipids. Although the previous study reported that KcsA induces phospholipid flip-flop at neutral pH [13], we hypothesized that the flip-flop could be even more enhanced at acidic pH due to the gating of the channel. In the present study, we demonstrated that KcsA substantially enhances the phospholipid flip-flop in a pH-dependent manner. Moreover, flip assays with TBA were performed for determining whether the open–close motion of the transmembrane region affects the flip-flop. At acidic pH, the TBA binding to the KcsA channel prevents the gating transitions, keeping the gate in the open conformation [17,22]. Therefore, the observation that TBA did not decrease the flip-flop rate indicates that the open conformation, rather than the open–close transition, facilitates the flip-flop of phospholipids. Conversely, the ΔM0 mutant exhibited the attenuated flip-flop promotional activity. A recent study demonstrated that the removal of the M0-helix stabilizes the closed conformation [21]. Thus, the attenuated flip-flop can be explained by the reduced probability of the open conformation at acidic pH, in good agreement with the TBA result.

How does the open conformation facilitate the flip-flop? Upon the opening of the gate, the helical bundle was untwisted, and the longitudinal length was shortened, as suggested by the reported crystal structures [16]; and these were recently proven under the membrane-embedded condition by means of atomic force microscopy [20]. By rearranging the helical bundle, the hydrophobic membrane surface of the transmembrane domain might not be substantially changed. In contrast, the shortening of the transmembrane domain alters the hydrophobic matching between the membrane and the channel protein.

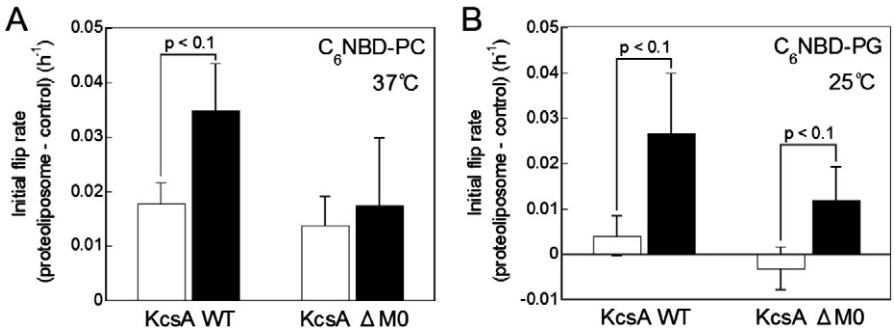


**Fig. 3.** Initial rates of KcsA-mediated flip of $C_6$NBD-PC (A) and $C_6$NBD-PG (B) in the proteoliposomes containing 0.05 mol% WT KcsA or KcsA ΔM0 (both C-terminal His-tagged) at pH 7.4 (open bars) or pH 4.0 (solid bars). The data are represented as the differences in the flip rates of $C_6$NBD-phospholipids in the proteoliposome and protein-free liposome. Error bars represent SDs of two or three experiments.

Thus, the deformation of the membrane adjacent to the channel could enhance the flip-flop rate (Fig. 5). It was recently revealed that the KcsA channel exhibits self-assembly and dispersion in the membrane in a gating-dependent manner [26]; the closed channels are clustered at neutral pH, and open channels are dispersed as singly isolated channels at acidic pH. This result suggests that the isolation of the channels increases the areas of the channel boundary regions, where the KcsA-mediated flip-flop takes place. (Fig. 5).

Cholesterol has been reported to reduce the flip-flop rate of phospholipids [25,27]. The presence of cholesterol increases the acyl chain packing order and thereby inhibits solute permeation [28]. Therefore, the reduced flip-flop rate could also be attributed to the increase in the packing order. Indeed, the flip of $C_6$NBD-PG was almost abolished in the presence of 30% cholesterol. As cholesterol did not reduce the flip rate of $C_6$NBD-PC, this rate was assumed to be the basal level at the experimental temperature. The flip rate of $C_6$NBD-PG at 30% cholesterol was 0.039 per hour at 37 °C, which was still higher than the basal level of $C_6$NBD-PC. Interestingly, we found that cholesterol hardly affected the KcsA-mediated flip-flop. In the case of 30% cholesterol, a cholesterol-rich, liquid-ordered phase and cholesterol-poor, liquid-disordered phase coexist in the lipid membranes [29]. As cholesterol did not affect the KcsA-dependent flip-flop, this finding indicates that KcsA is localized in the liquid-disordered phase and is unaffected by the ordering effect of cholesterol. This finding is consistent with the report that cholesterol did not affect the $Rb^+$ flux of the KcsA-incorporated liposome [30].

In conclusion, we demonstrated that the pH-dependent channel KcsA promoted the phospholipid flip-flop in a pH-dependent manner. The open conformation of the transmembrane region, rather than the open-close motion, is responsible for the increase in the flip-flop rate of phospholipids. Although KcsA is a bacterial cytoplasmic membrane protein, the flip-flop promotion mechanism presented here will provide useful clues to the regulation of energy-independent flip-flop in biological membranes.

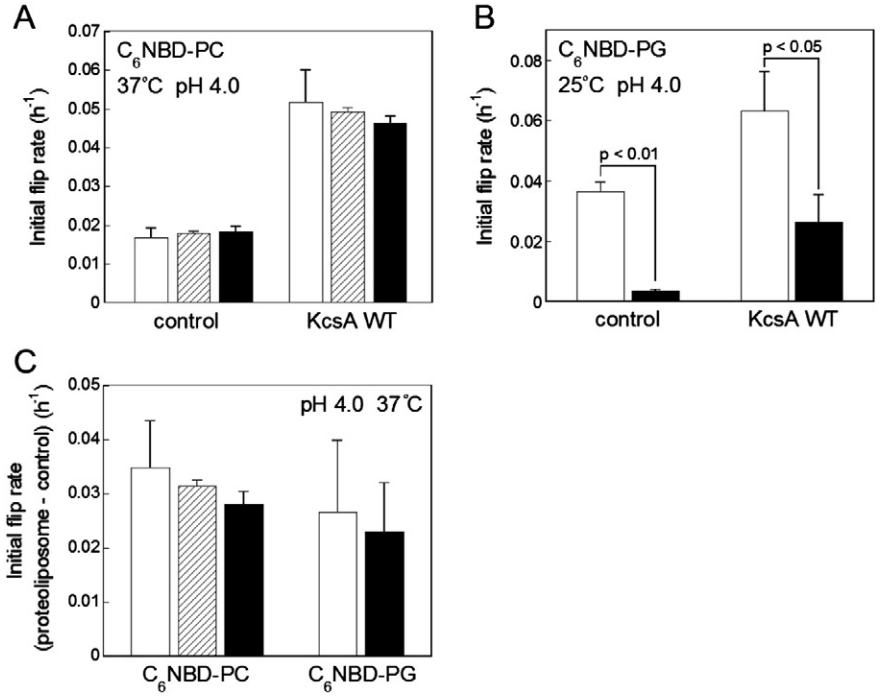


**Fig. 4.** Initial flip rates of $C_6$NBD-PC (A) and $C_6$NBD-PG (B) at pH 4.0 in protein-free liposomes (control) or proteoliposomes with 0% (open bars), 5% (striped bars), and 30% cholesterol (solid bars). (C) Initial rate of KcsA-mediated flip calculated by subtracting the initial flip rate of KcsA-incorporated proteoliposomes with that of control shown in (A) and (B). Error bars represent SDs of two or three experiments.

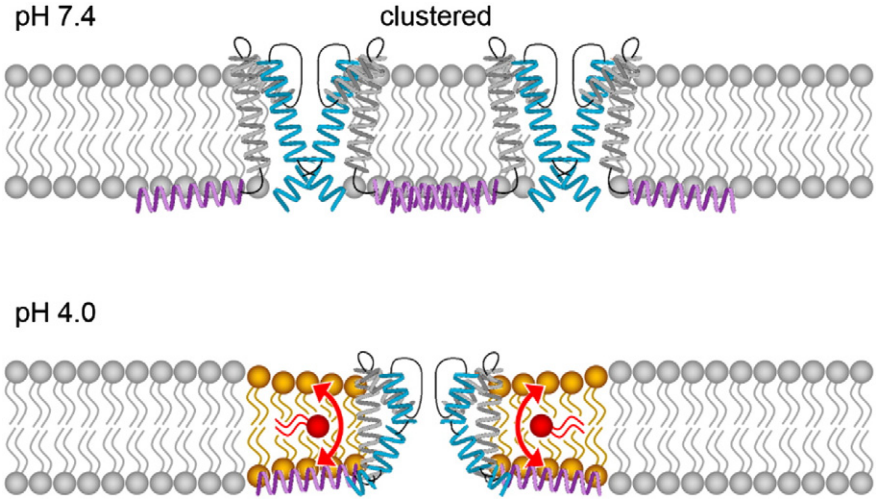


**Fig. 5.** Schematic representation of the conformation-dependent flip-flop promotion by KcsA. The closed channels are clustered at pH 7.4, and the longitudinal length of the transmembrane domain is shortened upon the opening of the gate at pH 4.0. Membrane deformation caused by the hydrophobic mismatch can promote the flip-flop of phospholipids. KcsA channels are dispersed as singly isolated channels, and this increases the areas of the boundary regions, where the KcsA-mediated flip-flop takes place.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.10.001.

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