Hysteresis of KcsA potassium channel's activation–deactivation gating is caused by structural changes at the channel's selectivity filter

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Mode-shift or hysteresis has been reported in ion channels. Voltage-shift for gating currents is well documented for voltage-gated cation channels (VGCC), and it is considered a voltage-sensing domain's (VSD) intrinsic property. However, uncoupling the *Shaker* K^+ channel's pore domain (PD) from the VSD prevented the mode-shift of the gating currents. Consequently, it was proposed that an open-state stabilization of the PD imposes a mechanical load on the VSD, which causes its mode-shift. Furthermore, the mode-shift displayed by hyperpolarization-gated cation channels is likely caused by structural changes at the channel's PD similar to those underlying C-type inactivation. To demonstrate that the PD of VGCC undergoes hysteresis, it is imperative to study its gating process in the absence of the VSD. A back-door strategy is to use KcsA (a K^+ channel from the bacteria *Streptomyces lividans*) as a surrogate because it lacks a VSD and exhibits an activation coupled to C-type inactivation. By directly measuring KcsA's activation gate opening and closing in conditions that promote or halt C-type inactivation, we have found (*i*) that KcsA undergoes mode-shift of gating when having K^+ as the permeant ion; (*ii*) that Cs^+ or Rb^+, known to halt C-inactivation, prevented mode-shift of gating; and (*iii*) that, in the total absence of C-type inactivation, KcsA's mode-shift was prevented. Finally, our results demonstrate that an allosteric communication causes KcsA's activation gate to "remember" the conformation of the selectivity filter, and hence KcsA requires a different amount of energy for opening than for closing.

hysteresis | KcsA | potassium channels | mode-shift | C-type inactivation

Hysteresis is a phenomenon in which the energy required for a system to transition between two states is different for the forward versus the backward reaction. In other words, the system has "memory," and it remembers its starting point (1) (Fig. 1*A*). Hysteresis of ion channels is linked to an ever-growing number of human physiological processes, among them normal heartbeat (2), stable rhythmic firing of pacemaking neurons, synaptic integration (3), regulation of cell excitability (4, 5), and temperature sensitization of transient receptor potential channels (6, 7).

In ion channels, this phenomenon has been reported and is known as "mode-shift." Voltage-shift for QV curves (gating charge vs. voltage) has been well-documented for voltage-gated ion channels: hyperpolarization-gated cation channels (HCN) (2), sodium (8, 9), potassium (5, 10–12), calcium (13, 14), and proton channels (15). Initially, the mode-shift of voltage-gated ion channels was considered a voltage-sensing domain's (VSD) intrinsic property. (16). However, it was shown that uncoupling the VSD from the pore domain (PD) effectively halts the mode-shift of the *Shaker* K^+ channel (17). Based on these experimental observations, it was suggested that, in the *Shaker* channel, the PD imposes a mechanical load on the VSD that manifests as a mode-shift of its QV curves, mostly due to an energetic stabilization of the PD open-state (17).

Interestingly, HCN's mode-shift of QV and GV (conductance-voltage) curves can be explained by assuming the existence of a four-kinetic-state model that includes two gating modes ($C_1 \leftrightarrow O_1$ and $C_2 \leftrightarrow O_2$) with different voltage dependences (2). At physiological external K^+ concentration ($[K^+]_o$), upon hyperpolarization, channels undergo rapid activation through mode 1 (2). After being in the O_1 state for more than 100 ms, channels switch to mode 2 through the transition $O_1 \rightarrow O_2$, which causes hysteresis (2). However, increasing the $[K^+]_o$ delays the shift between the two modes, which consequently prevents hysteresis (2). The $[K^+]_o$ dependence of HCN's hysteresis is similar to the one found in K^+ channels undergoing C-type inactivation (18). C-type inactivation is a time-dependent cessation of the ion channel function (19), likely caused by structural changes at the selectivity filter (SF) (20), and eventually leading to the SF structural collapse (21) (Fig. 1*B*).

Because both processes—open-state stabilization and C-type inactivation—occur at the channel's PD, we reasoned that, to precisely quantify the contribution of the PD to the mode-shift of voltage-dependent K^+ (Kv channels), the PD's gating mechanism should be studied in structural and functional isolation, i.e., in the absence of the VSD. Given the high degree of functional and structural conservation between KcsA (a K^+ channel from the bacteria *Streptomyces lividans*) and voltage-gated cation channels, we decided to use KcsA as the logical structural surrogate for this study because it lacks a VSD and contains all of the structural elements required for ion selectivity and permeation as well as for activation and C-type inactivation gating (21–26). In KcsA, the

Significance

Hysteresis in hyperpolarization-gated ion channels determines a normal heartbeat, a stable rhythmic firing of pacemaking neurons, and synaptic integration. Hysteresis in ion-selectivity changes of the two-pore K^+ channels regulates dynamically cell excitability, and recently it was shown that Kv7-channel's hysteretic gating mediates the Retigabine (an anticonvulsant)-dependent reduction of neuronal excitability. Given the ever-growing significance of this process in human physiology, it is imperative to determine its underlying structural bases. In this work, we have identified the molecular determinants of a hysteretic behavior in the prototypical ion channel, KcsA. Our results indicate that hysteresis or molecular "memory" in tetrameric cation-selective channels can arise from an allosteric coupling between the channel's activation gate and selectivity filter.

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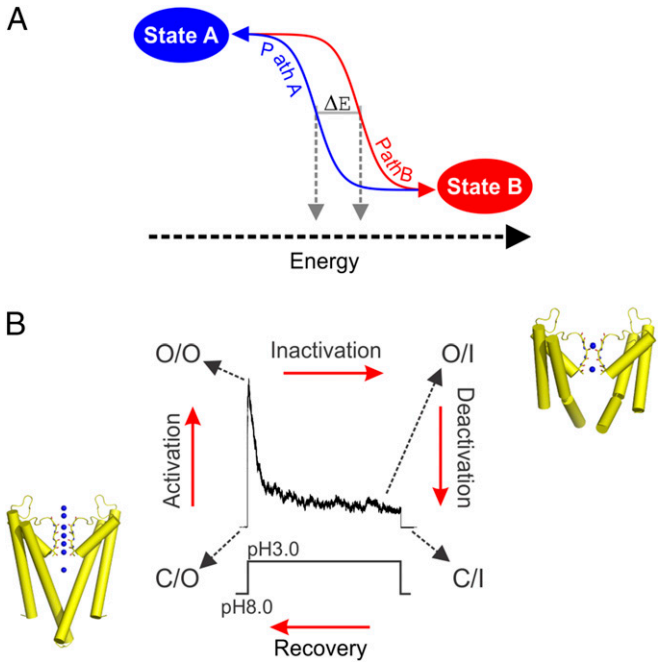

Fig. 1. Graphical depiction of mode-shift of gating or hysteresis and a minimal kinetic cycle for KcsA. (*A*) An idealized two-state system that requires an amount of energy for the forward transition between states A → B different from for the reverse one (B→A). This process is known as hysteresis and implies that the system has "molecular memory" because it remembers its initial state. In ion channels, this phenomenon has been known as the mode-shift of function. (*B*) A schematic representation of KcsA's minimal kinetic cycle. The permutation of two structural gates, the AG and SF or inactivation gate, into two different conformations, an AG open or closed and a SF conductive or collapsed, yields a gating cycle with at least four well-defined kinetic states: C/O, closed-AG/conductive-SF; O/O, open-AG/conductive-SF; O/I, open-AG/collapsed-SF; and C/I, closed-AG/collapsed-SF and the system reset to the initial state, C/O, closing the gating cycle. At the center of the kinetic cycle is a typical KcsA macroscopic current evoked by rapidly changing the intracellular pH from 8 to 3 in symmetrical 200 mM KCl. Dashed arrows indicate the most likely kinetic state of KcsA that will explain a given region of the macroscopic current. (*Insets*) Cartoon representations of KcsA's C/O (*Lower Left*) and O/I states (*Upper Right*).

functional permutation of two structural gates [activation gate (AG) and SF or inactivation gate (Fig. 2*A*)] in two different conformations—an AG open or closed and a SF conductive or collapsed—yields a gating cycle with at least four well-defined kinetic states (21): C/O, closed-AG/conductive-SF →O/O; open-AG/conductive-SF →O/I; open-AG/collapsed-SF →C/I; and closed-AG/collapsed-SF. Then the system resets to the initial state, C/O, closed-AG/conductive-SF, closing the gating cycle (Fig. 1*B*).

We investigated whether the PD of a K^+ channel in isolation undergoes mode-shift of gating and the molecular basis underlying this phenomenon within the PD of cation-selective channels. To this end, we systematically studied KcsA pH-dependent gating by site-directed mutagenesis, patch-clamp liposome recording, site-directed spin label (SDSL), and continuous-wave electron paramagnetic resonance (CW-EPR) spectroscopy to track directly the structural changes of the AG associated with activation–deactivation gating. This multipronged experimental approach allowed us to identify a mode-shift of KcsA's gating. We also found that structural changes at the SF associated with C-type inactivation (i.e., collapsing of the SF) cause the mode-shift of gating in KcsA. Finally, our experimental results strongly suggest that KcsA's mode-shift is caused by an allosteric communication between KcsA's activation gate and its selectivity filter that allows the former to "remember" the conformation of the latter (conductive or C-type–inactivated), and hence the channel requires different amounts of energy for opening than for closing.

Results

Structural Assessment of Mode-Shift of Gating or Hysteresis in KcsA. In Kv channels, mode-shift or hysteresis has been evidenced by measuring the voltage dependence of ionic currents (GV curves) or gating currents (QV). Gating currents of Kv channels are a direct measurement of a voltage-dependent conformational change at their VSD. This is a very powerful methodology that reports on conformational changes at this specific structural domain. To dissect the contribution of the PD to mode-shift in Kv channels, it is imperative to measure the structural changes associated with activation and deactivation gating at the activation gate in the absence of their VSD (Fig. 2*A*), as recently done for the isolated VSD (27).

A backdoor strategy to determine if the Kv channel PD undergoes mode-shift is to use the prototypical K^+ channel PD, KcsA. Toward this end, we applied both SDSL and CW-EPR spectroscopy to measure the pH-dependent conformational changes of KcsA's AG associated with gating. A spin label attached at position G116C is a faithful reporter that directly tracks KcsA's AG pH-dependent conformational changes (25) (Fig. 2*A*) and hence can report about mode-shift during activation and deactivation gating.

We conducted steady-state measurements of KcsA spin-labeled at position G116C reconstituted in Asolectin liposomes. The CW-EPR spectral-line shape of spin label (SL) attached to this position reports on the effect of local steric constraints restricting the mobility of the spin label and therefore on the opening and closing of the activation gate (25, 28). Additionally, the CW-EPR spectral-line shape broadens due to dipolar coupling between spin labels close in space. When KcsA's AG is closed at basic pHs, the SLs at position 116 are in close proximity, which produces a strong dipolar coupling that manifests in the broadening of the CW-EPR spectrum.

As the $[H^+]$ at the channel's intracellular side is increased, the AG opens and the SLs attached at position 116 move away from each other, resulting in a decrease on the spectrum broadening as a consequence of a lessening of the dipolar coupling (28–30). To measure the pK_a for KcsA's activation gating, five independent KcsA-containing liposome samples were prepared at pH 7 (at this pH the KcsA's AG is closed); next, the pH in each tube was decreased as indicated, 7, 6, 5, 4, and 3, until full channel activation was achieved and a complete set of CW-EPR spectra was obtained. A clear and progressive pH-dependent change of the spectral-line shape was observed (Fig. 2*B*).

We also measured KcsA's pH-dependent deactivation. To this end, five independently and freshly made KcsA-G116-SL–containing liposomes were pelleted at pH 7 and then resuspended in a high-capacity buffer at pH 3 (at this pH, KcsA's AG is open) and allowed to equilibrate for 10 min. Next, the six liposome samples equilibrated at pH 3 were spun down, and deactivation gating was assessed by resuspending the pellets in buffers with decreasing $[H^+]$ and by tracking the movement of the channel's AG using CW-EPR spectroscopy (Fig. 2*C*). The central resonance line amplitude of the normalized CW-EPR spectra (normalized by the number of spin labels) vs. $[H^+]$ was plotted, and the Hill equation was fitted to the data (Origin, OriginLab) (Fig. 2*D*).

KcsA deactivation pK_a shifted to a more basic pH, a pH-shift (ΔpH) of ∼1, indicating that less energy is needed for opening than for closing the channel's AG. This result demonstrates the existence of a mode-shift of gating or hysteresis in KcsA; i.e., the channel's gating energetics depend on the previous holding pH and/or kinetic state. The pK_a (the proton concentration at which half of the channels are open) for activation and deactivation gating were estimated to be ∼4.3 ± 0.05 and 5.3 ± 0.02, respectively. Fig. 2*D* shows that the pH difference for opening KcsA's

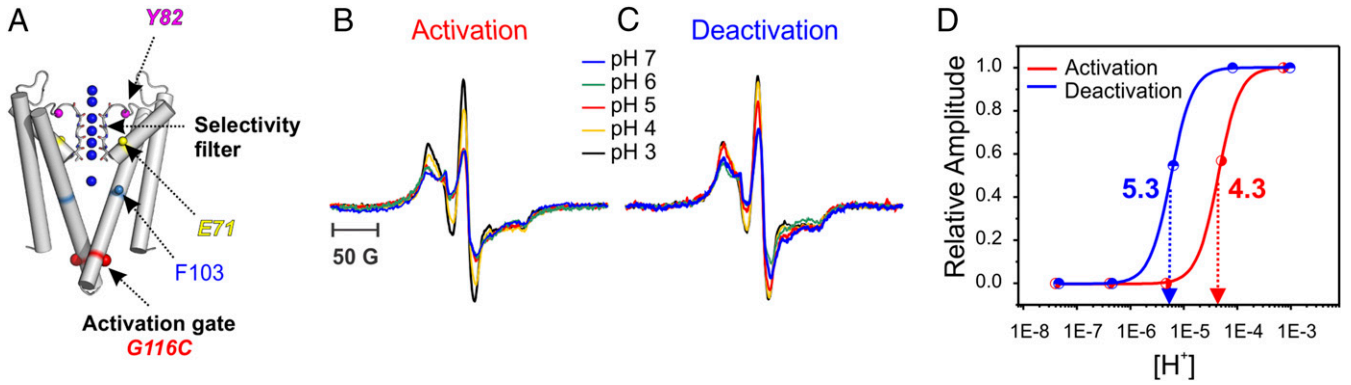

Fig. 2. A structural assessment of KcsA mode-shift of gating or hysteresis by SDSL and CW-EPR spectroscopy. (*A*) A cartoon representation of KcsA (only two subunits are shown for clarity) highlighting the channel's AG and its SF. KcsA's amino acid positions known to regulate C-type inactivation gating are indicated by colored spheres: Y82 (pink), E71 (yellow), F103 (blue), and G116C (red), which were used to attach the spin-label probe at the channel AG to report pH-dependent conformation changes associated with activation–deactivation gating (G116C). (*B*) CW-EPR spectra datasets were collected from samples with a spin label attached at position G116C while undergoing activation gating, from pH 7 to pH 3, or (*C*) deactivation gating, from pH 3 to pH 7. (*D*) The amplitude of the normalized CW-EPR spectrum's central resonance line (normalized by the number of spin) was plotted versus the proton concentration $[H^+]$, and the Hill equation was fitted to the data. The pK_a for KcsA wild-type activation and deactivation gating were 4.3 ± 0.05 and 5.3 ± 0.02, respectively.

AG (~1.7 pH units) is less than for closing it (~2.3 pH units). This experimental observation can be reconciled with the larger thermodynamic stability of the KcsA's O/I state (31). In the O/O states, the SF is intrinsically unstable (31, 32) and the opening of the AG drives it to a very deep collapsed/inactive state (21). It follows that more energy is required to close KcsA's AG with a collapsed SF than for opening it. Consequently, the energy required for KcsA's deactivation is greater than for activation, which causes KcsA's hysteretic behavior.

Structural changes at the HCN channels' SF, similar to those characteristics of C-type inactivation gating, seem to cause mode-shift (2). Therefore, we hypothesized that hysteresis in KcsA could originate from an allosteric coupling between its AG and SF (AG↔SF coupling) that underlies C-type inactivation gating (21, 31).

In the *Shaker* K^+ channel replacing a Threonine with Alanine at position 449 accelerates the rate and depth of the C-type inactivation process (18). In KcsA, Y82 is the equivalent position to *Shaker*-T449 (Fig. 2*A*) and substituting it for the smaller Alanine residue (Y82A) produced a substantial increase of both the rate and the depth of C-type inactivation (Fig. 3*A*) (32). The pH-dependent macroscopic current recordings from patches containing the Y82A mutant displayed extremely fast and deep-inactivation kinetics (Fig. 3). We then reasoned that, in the fast and deep-inactivating mutant, Y82A, the mode-shift or hysteresis could be augmented. In other words, the ΔpK_a between activation and deactivation gating should be larger compared with the wild-type channel. An evaluation of the mode-shift of the spin-labeled Y82A-G116C was performed by CW-EPR spectroscopy as indicated before. The activation and deactivation pK_a's for this mutant were 4.0 ± 0.07 and 5.0 ± 0.1, respectively, with a ΔpK_a of ~1 pH unit (Fig. 3*C*). This result indicates that the rate of inactivation (the transition rate from O/O to O/I, Fig. 3*B*) is not the determining factor for KcsA exhibiting mode-shift of gating but rather the C-type inactivation process itself (Fig. 3*B*). To substantiate this idea, we measured the mode-shift of another KcsA fast-inactivating mutant, F103Y. The ΔpK_a between activation and deactivation gating was indistinguishable from that of the Y82A mutant (Fig. 3*D*), which validates that KcsA's mode-shift of gating does not depend on the rate of C-type inactivation.

Interestingly, mode-shift in HCN channels has been linked to structural changes at the SF similar to those underlying C-type inactivation (2) because it is highly dependent on the external $[K^+]_o$, which is a hallmark of C-type inactivation in K^+ channels (18). Furthermore, extracellular Cesium, known to impair C-type

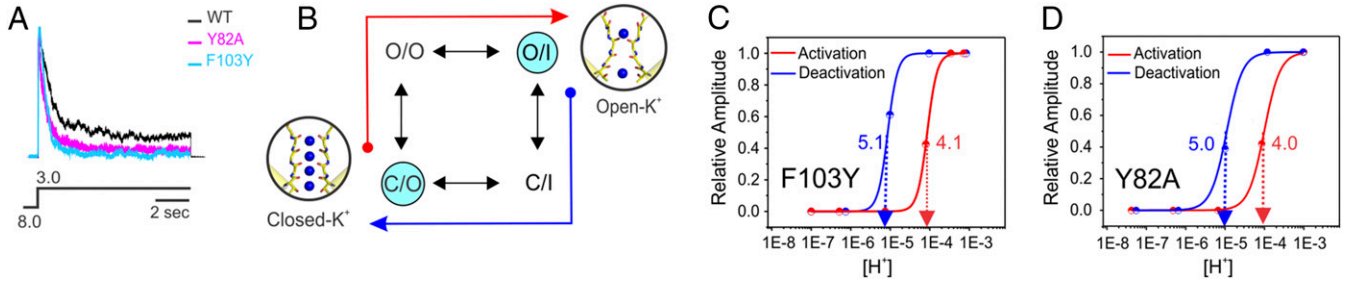

Fig. 3. Assessment of the mode-shift of gating in the fast-inactivating mutants, Y82A and F103Y. (*A*) Representative macroscopic current recordings were elicited by rapidly changing the pH from 8 to pH 3 on the intracellular side in symmetrical 200 mM KCl. Y82A and F103Y caused significant increases of the C-type inactivation rate compared with the wild-type channel. (*B*) A four-kinetic-state cycle for KcsA wild type is the result of the permutation between two conformations of the channel's activation gate, closed (C) or open (O), and the selectivity filter, conductive (O) or inactive (I). The red arrow indicates the transition of KcsA through two different kinetic reactions: C/O→O/O (activation) followed by O/O→O/I (inactivation). The blue arrow indicates the transition between the O/I→C/I states (deactivation) followed by a repriming reaction C/I→C/O, which closes the kinetic cycle. (*C* and *D*) pH-dependent Y82A and F103Y activation and deactivation curves were obtained by CW-EPR spectroscopy measurements of a spin label attached to position G116C. The amplitude of each CW-EPR spectrum normalized by the number of spins was plotted against $[H^+]$, and the Hill equation was fitted to the data. The pK_a's for activation and deactivation gating were 4.0 ± 0.07 and 5.0 ± 0.1 for Y82A and 4.0 ± 0.01 and 5.1 ± 0.02 for F103Y.

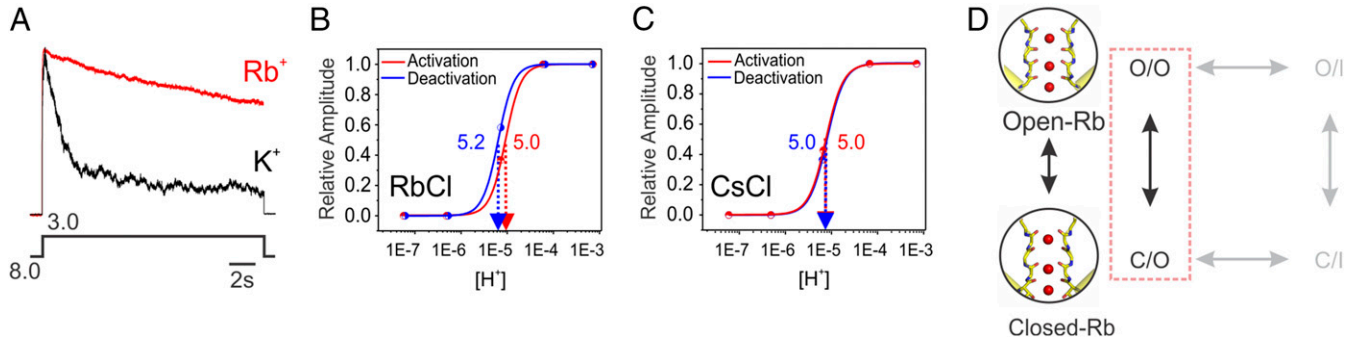

Fig. 4. The effect of the permeant ion on KcsA's C-type inactivation and mode-shift of gating. (*A*) The permeant ion effect on C-type inactivation was evaluated by measuring KcsA macroscopic currents evoked by pH jump experiments (pH 8–3) in symmetrical 200 mM KCl (black trace) or 200 mM RbCl (red trace). Rb^+ ions effectively impaired the C-type inactivation rate. (*B*) The normalized CW-EPR spectrum of a spin label attached at position G116C was plotted against $[H^+]$ in the presence of 200 mM RbCl and (*C*) 200 mM CsCl. By fitting the Hill equation to the data, the pK_a's for activation and deactivation gating were 5.2 ± 0.04 and 5.0 ± 0.03 for Rb^+ ions and 5.0 ± 0.05 and 5.0 ± 0.01 for Cs^+ ions. In the presence of these permeant ions KcsA's mode shift was effectively prevented. (*D*) A KcsA four-state-kinetic cycle highlighting the most probable transitions that occur when the channel's selectivity filter contains Rb^+ or Cs^+ as a permeant ion instead of K^+.

inactivation (33), prevented mode-shift (2). To explain this behavior, a four-state kinetic model was proposed that involved two gating modes ($C_1 \leftrightarrow O_1$ and $C_2 \leftrightarrow O_2$) with different voltage dependences. After being in the O_1 state for more than 100 ms, channels switch to mode 2 through the following transition, $O_1 \rightarrow O_2$, which causes hysteresis (2).

In KcsA, the combination of the structural changes at its AG and SF results in a four-kinetic-state model (Fig. 1*B*), and, as in most K^+ channels, its C-type inactivation can be halted by changing the permeant ion to Rb^+ or Cs^+ (33, 34). Hence, we decided to study the effect of different permeant ions on KcsA's mode-shift.

Cs^+ or Rb^+ Ions Prevented KcsA C-Type Inactivation and Mode-Shift. KcsA macroscopic currents were recorded in the presence of symmetrical 200 mM KCl or 200 mM RbCl. When K^+ was the permeant ion, KcsA displayed typical C-type inactivation (19, 32). In contrast, in the presence of 200 mM of Rb^+ ions, the C-type inactivation process was significantly halted (Fig. 4*A*).

To test whether Rb^+ ions can avert KcsA's mode-shift as it halted C-type inactivation, the KcsA-G116C spin-labeled protein was reconstituted in Asolectin liposomes, and activation and deactivation gating pK_a's were measured by CW-EPR spectroscopy in the presence of Rb^+ ions (Fig. 4*B*). Our results demonstrated that, in the presence of Rb^+ ions, activation and deactivation gating became almost iso-energetic processes, reducing significantly KcsA's mode-shift (ΔpH of ∼0.2).

In the presence of 200 mM Cs^+, KcsA's activation and deactivation gating were iso-energetic with a ΔpH of ∼0, and consequently KcsA's mode-shift was averted (Fig. 4*C*). These results strongly suggest that hysteresis in KcsA is caused by the energetic difference between two different conformations of KcsA's SF, i.e., the conductive conformation with four bound K^+ ions (26) and the collapsed or C-type inactivated one with only two K^+ ions (Fig. 3*B*) (21, 26).

In the absence of C-type inactivation, KcsA transitions between two well-defined kinetic states, C/O↔O/O (Fig. 4*D*, red dashed box). Under these experimental conditions, activation and deactivation gating were iso-energetic because no energy was used to reset the SF, and consequently KcsA's mode-shift was prevented.

A C-Type Inactivation-Deficient Mutant Prevented KcsA's Mode-Shift of Gating. The E71A mutant is a noninactivating channel (32) (Fig. 5*A*) that, when opening and closing, transitions exclusively between the C/O↔O/O states (Fig. 5*B*). We evaluated by CW-EPR spectroscopy whether this mutant undergoes mode-shift of gating in 200 mM KCl, a physiologically relevant ionic condition.

The E71A exhibited iso-energetic activation and deactivation, as revealed by the pK_a measurements, 4.0 ± 0.02 and 4.0 ± 0.05,

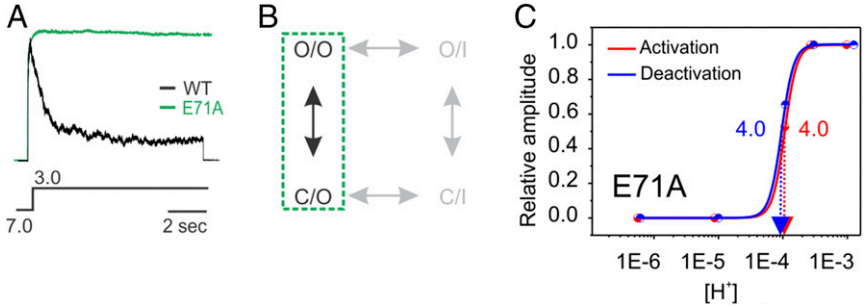

Fig. 5. Measuring KcsA mode-shift or hysteresis in the K^+-selective and C-type-inactivation–deficient E71A mutant. (*A*) Macroscopic current recordings of the wild-type channel and the E71A mutant were elicited by rapidly changing the intracellular pH from 8 to 3 in symmetrical 200 mM KCl. In contrast to the wild-type channel, the E71A did not exhibit C-type inactivation gating in a near-physiological concentration of K^+ ions. (*B*) A KcsA's kinetic cycle highlighting the more probable kinetic transition (dashed green box) that the E71A mutant experiences during activation/deactivation gating. In this C-type-inactivation–deficient mutant, during opening and closing, the structural changes are strictly limited to the transmembrane segment of the channel with little or no effect at the channel's selectivity filter. (*C*) CW-EPR spectroscopy measurements of the KcsA-E71A mutant mode-shift of gating revealed that, in the absence of C-type inactivation, KcsA mode-shift of gating was prevented. The E71A mutant activation and deactivation pK_a's were 4.0 ± 0.02 and 4.0 ± 0.05 (SEM), respectively.

PHYSIOLOGY

respectively (Fig. 5*C*). This result demonstrates that structural changes at the channel's SF, associated with C-type inactivation, are the determining factors for KcsA's mode-shift of gating in KcsA.

Discussion

The biological function of a protein system relies on the intramolecular communication between structural motifs that can act as a "sensor," harvesting the energy necessary to do work, and as an "effector," which is the structural motif that actually does the work. A network of energetically coupled amino acid residues largely mediates the communication between these structural motifs (35). This process is known as "allosteric coupling," and in the pore domain of K^+ channels is responsible for the functional and structural coupling of the channel's AG and its SF (24, 35–40). This allosteric coupling underlies a process known as C-type inactivation coupled to activation gating in K^+ channels (24), and recently we have started to understand this mechanism at the molecular level (31, 38, 39).

Mode-shift of function or hysteresis refers to a system that, when transitioning between different kinetic states, requires different amounts of energy for the forward and the backward reactions. In other words, the system has memory and remembers the starting state of the transition (41).

In cation-selective ion channels, mode-shift of gating has been argued to be an intrinsic property of the pore domain (2, 10, 17), and it is believed to be caused by structural changes at the channel's SF similar to those associated with C-type inactivation. In this work, we have demonstrated from a structural point of view that the archetypal pore domain of a K^+ channel, KcsA, undergoes mode-shift of gating. By directly measuring the pH dependency of the structural changes at KcsA's activation gate with CW-EPR spectroscopy, we showed that KcsA pK_a's for activation and deactivation differed by ∼1 pH unit. This experimental result demonstrates that the PD of K^+ channels can undergo mode-shift of gating as does the voltage-sensing domain of voltage-gated ion channels (15, 16, 27). Follow-up questions that derive from this study are the following: Why do the PD and the VSD of Kv channels display mode-shift of gating? Do the PD and VSD mode-shifts cooperate in the context of the whole Kv-channel function? Or do these two hysteretic structural domains work independently, perhaps in a different timescale or under different cellular conditions? These questions need to be addressed in the near future.

We have also shown that mutations or ionic conditions known to modulate C-type inactivation gating correspondingly regulated the mode-shift of gating in KcsA. Interestingly, in the well-known fast-inactivating mutant (Y82A) (32), there was no change in the magnitude of the mode-shift. This strongly suggests that the rate of C-type inactivation is not the determining factor eliciting KcsA mode-shift but rather the transition itself from the open-conductive to the open and C-type–inactivated states. This type of hysteretic gating behavior is known as "rate-independent hysteresis" in which the velocity of the transition between two states does not affect the hysteresis of a system. The memory of the system persists irrespective of the rate of the transition between its initial and final states. This distinct hysteretic functional behavior is reminiscent of rate-independent hysteresis, which has been reported before for gap junctions (42), TRPV3 channels (6, 7, 43), and two-pore domain K^+ channels (4).

In contrast, when KcsA's mode-shift was measured in the presence of Rb^+ or Cs^+ as permeant ions, activation and deactivation processes became iso-energetic, which strongly suggests that ion-induced removal of C-type inactivation effectively prevents KcsA mode-shift. This result is in agreement with the documented effect of the type of permeant ions on the mode-shift of the HCN channels (2).

To understand how Rb^+ or Cs^+ prevent the K^+-dependent mode-shift of KcsA, we have to consider that ion selectivity is dynamically controlled by variations in the coordinating groups in the SF, as the permeant ions (Rb^+, Cs^+, or K^+) replace water molecules with the backbone carbonyl groups of the SF (44).

It follows that K^+ interacts differently with the SF than Cs^+ and Rb^+ do, as evidenced in KcsA's single-channel recordings (45, 46) or in crystal structures solved in the presence of these permeant ions (47).

In the presence of K^+, KcsA's filter displays the canonical 1,3 and 2,4 ion configurations (26) and undergoes C-type inactivation. However, crystal structures of KcsA solved in the presence of Cs^+ or Rb^+ (ions that prevent C-type inactivation in K^+ channels and avert hysteresis in KcsA) characteristically are missing an ion in the second K^+ binding site (47). These experimental observations inspired a provocative idea in which the occupancy of the second K^+-binding site is required for channel inactivation. This hypothesis was just elegantly demonstrated (48) and provides an explanation to the permeant-ion dependence of hysteresis and C-type inactivation in KcsA. Rb^+ and Cs^+ have a stronger interaction with the channel SF than K^+, reducing significantly the single-channel conductance (46, 49). Therefore, these two permeant ions prevent C-type inactivation and hysteresis by interacting strongly with the channel SF and preventing its structural collapse (38).

To reinforce the notion that C-type inactivation provides the open-state stabilization linked to hysteretic gating (17), we decided to measure mode-shift of gating in the noninactivating mutant KcsA-E71A (32). This mutant has become the paradigm of a noninactivating, highly selective K^+ channel (50) and provides us with a back-door strategy to study KcsA's mode-shift in the total absence of inactivation gating while having K^+ ions as permeant ions. Upon intracellular acidification (activation gating), the KcsA-E71A mutant transitions from the C/O to the O/O state, and it does not inactivate (Fig. 5*B*). When the channel returns to the resting conformation by decreasing the $[H^+]$ (deactivation gating), it transitions backward from O/O to the C/O state (Fig. 5*B*). Under such a functional regime, the activation and deactivation processes require the same amount of energy, displaying identical pK_a values. The noninactivating KcsA-E71A mutant, as we predicted, prevented the mode-shift of gating in KcsA.

Altogether, our results strongly suggest that mode-shift of gating in KcsA is caused by the difference in energy between two known conformations of the channel SF, conductive and C-type–inactivated, the latter being more stable when the channel has its AG open (31). Because at an acidic pH the KcsA's O/I conformation is energetically more stable than the C/O conformation at a basic pH, the activation pathway C/O→O/I requires less energy than the deactivation pathway O/I→C/O, resulting in a mode-shift of gating or hysteresis. In the *Shaker* K^+ channel, the same open-state stabilization seems to be responsible for its mode-shift of gating (17).

Our experimental approach demonstrates two very important aspects of mode-shift of gating in K^+ channels: (*i*) the isolated pore domain of a K^+ channel undergoes mode-shift of gating in the absence of a VSD and (*ii*) this phenomenon is caused at least in KcsA, and perhaps in all K^+ channels that undergo C-type inactivation, by the energetic difference between two conformations of the channel's selectivity filter, the conductive (noncollapsed) and the nonconductive (collapsed) conformations.

Finally, a clearer understanding of the molecular events responsible for the mode-shift or hysteresis of the PD and the VSD in the voltage-gated cation channel family will help us design newer and safer conformation-specific therapeutic drugs to target and correct physiological disorders in which the enhancement of an ion channel's hysteresis is beneficial and can be mediated by kinetic-state–specific drugs (5).

Materials and Methods

KcsA cloned in pQE-70 was expressed in *Escherichia coli*, and membrane was extracted with 1.5% (wt/vol) Triton X-100, spin-labeled, and reconstituted in Asolectin polar extract liposomes by incubation with bio-beads (Bio-Rad) for 2 h (30). Samples were harvested by centrifugation at 100,000 × *g* for 1 h. CW-EPR spectra were recorded using a dielectric resonator (ER 4123D) with 2 mW incident power, 100 kHz modulation frequency, and 1 G field-modulation amplitude (28, 29). Liposomes patch-clamp measurements were done in 200 mM KCl, 20 mM 3-(*N*-morpholino)propanesulfonic acid at the specified pH (51). Detailed descriptions are provided in *SI Materials and Methods*.

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