An Intersubunit Interaction between S4-S5 Linker and S6 Is Responsible for the Slow Off-gating Component in Shaker K⁺ Channels

Zarah Batulan, Georges A. Haddad, and Rikard Blunck

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From the Département de Physique and Groupe d’Étude des Protéines Membranaires, Université de Montréal, Montréal, Quebec H3C 3J7 Canada

Voltage-gated ion channels are controlled by the membrane potential, which is sensed by peripheral, positively charged voltage sensors. The movement of the charged residues in the voltage sensor may be detected as gating currents. In Shaker K⁺ channels, the gating currents are asymmetric; although the on-gating currents are fast, the off-gating currents contain a slow component. This slow component is caused by a stabilization of the activated state of the voltage sensor and has been suggested to be linked to ion permeation or C-type inactivation. The molecular determinants responsible for the stabilization, however, remain unknown. Here, we identified an interaction between Arg-394, Glu-395, and Leu-398 on the C termini of the S4–S5 linker and Tyr-485 on the S6 of the neighboring subunit, which is responsible for the development of the slow off-gating component. Mutation of residues involved in this intersubunit interaction modulated the strength of the associated interaction. Impairment of the interaction still led to pore opening but did not exhibit slow gating kinetics. Development of this interaction occurs under physiological ion conduction and is correlated with pore opening. We, thus, suggest that the above residues stabilize the channel in the open state.

The voltage dependence of ion channels is the basis for all electrical signaling in the central nervous system. In tetrameric voltage-gated K⁺ channels, each subunit is composed of six transmembrane α-helices (S1–S6), with S1–S4 forming the voltage sensing domain and S5–S6 of all four subunits forming the pore. The voltage-sensing domains are covalently connected to the S5 of the pore region by the S4–S5 linker. The intracellular gate is made up of the S6 C-terminal ends that cross each other, forming a bundle that occludes the pore when the channel is closed. Pore opening in voltage-gated K⁺ channels is controlled by the movement of the voltage sensor in which charged residues of the S4 respond to changes in membrane potential. During this conformational change, the charges are moved through the electric field, generating the transient gating currents (for review, see Ref. 1). Gating currents were first predicted by Hodgkin and Huxley and were first detected in sodium channels by Armstrong et al. (2, 3). The movement is transferred to the pore domain (electromechanical coupling) and subsequently leads to pore opening. Voltage sensor movement precedes pore opening so that the transitions the channel undergoes during electromechanical coupling are reflected in the gating currents.

Activation (on) and deactivation (off) gating currents for the non-conducting Shaker-IR channel, W434F (4–6) have been previously described (6–8). Briefly, on-gating currents rise and decay quickly after small depolarizations but rise more slowly and exhibit a more prolonged and complex decay kinetics after intermediate depolarizations and, finally, develop and decay rapidly after depolarizations large enough to activate all channels. In contrast, off-gating currents, which develop upon repolarization, exhibit the following salient features; (i) after partial activation from low depolarizations (e.g. pulsed up to −50 mV), currents are fast, representing backward transitions that return channels from partially activated back to resting states; (ii) after intermediate depolarizations (e.g. pulsed to between −40 and −30 mV) currents show a similar fast component seen at low depolarizations followed by a slow component, the latter indicating slow deactivation of a small number of fully activated channels; (iii) after high depolarizations (e.g. pulses >−30 mV), currents have a rising phase and slow decay, signifying the slow return from the fully activated state of all channels (see Fig. 2). The slow off-gating currents have been described early on (6–8). These characteristics of the off-gating currents have led others to suggest that the early transitions in the activation sequence represent quickly reversible events within one subunit, whereas the late transitions preceding the full activation of channels are slowly reversible and likely represent a concerted step involving all subunits (7, 9, 10). The slow off-gating component has also been proposed to be dependent on the permeant ion (6, 11, 12) and was thought to be linked to C-type inactivation (11). Other authors, however, view it rather as a stabilization of the open state (13). Although theoretical descriptions of off-gating currents have been proposed (14–16), the correlating structural determinants underlying these patterns remain unclear.

Several mutants of the Shaker K⁺ channel have been described previously to suppress the slow off-gating component (13, 17–19). In most cases, pore opening did not occur in the experimental range, confirming that the slow component is linked to pore opening (6, 14). This had first been demonstrated by preventing development of the slow component by application of 4-aminopyridine, which keeps the pore in the closed state.
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correlation (10, 14, 20–22). However, in other mutations, the slow component was diminished despite an open pore. The mutations were located in the C-terminal S6 (see "Discussion").

All of the voltage sensors of the Shaker K⁺ channel are thought to enter the activated state before the channel undergoes one final cooperative step that leads to pore opening (22–24). It has been suggested that the slow off-component is related to the final concerted step of the four voltage sensors (7–9). This gave us a hint where the responsible residues are to be found. Electromechanical coupling between voltage sensor movement and pore opening is mediated by interaction between the S4-S5 linker and the C-terminal S6 (25–33). Because the final opening transition is thought to be a cooperative step (7, 15, 19, 34), we looked for an interaction that couples together neighboring subunits of the tetrameric channels.

In this study we have mutated several conserved residues located at the interface between the S4-S5 linker and S6 of adjacent subunits to determine via analysis of the off-gating currents how interactions between these two regions influence electromechanical coupling and, in particular, whether the slow off-gating component is influenced. We have identified several key residues located at the C-terminal end of the S4-S5 linker and of the S6 whose interactions contribute to the rising phase and slow decay of the off-gating currents. We propose that interactions between these residues represent the last transition(s) in the activation pathway.

**EXPERIMENTAL PROCEDURES**

*Molecular Biology and Channel Expression—*All experiments were done using the pBSTA vector into which the Shaker IR ("inactivation-removed," containing an N-terminal deletion (Δ6–46) that removes its fast inactivation properties (4, 35)) was cloned. Point mutations were introduced using site-directed mutagenesis (QuickChange; Stratagene). Sequences were verified using automated DNA sequencing. cDNAs were linearized and *in vitro* transcribed using T7 RNA polymerase (T7 mMessage machine kit; Ambion). cRNAs (23 or 46 µl; 0.1–1.0 µg/µl) were injected into *Xenopus* oocytes, and currents were recorded 2–5 days after injection.

*Electrophysiology and Fluorescence Measurements—*With the cut-open voltage clamp epifluorescence technique using an Axioskop 2 FS upright microscope (Zeiss), CA-1B High Performance Oocyte Clamp (Dagan Corp.), and Photomax 200 Photodiode Detection System (Dagan Corp.) currents and fluorescence were measured as described previously (36). Before recording, oocytes were placed in a 3-compartment chamber containing an external solution (115 mM N-methyl-d-glucamine, 10 mM HEPES, 2 mM Ca(OH)₂) adjusted to pH 7.1 using MES,3 then permeabilized by exchanging external solution in the bottom chamber with 0.2% saponin. After permeabilization, saponin solution was replaced with an internal solution (10 mM HEPES, 2 mM EDTA, 115 mM N-methyl-d-glucamine (for gating current recording) or KOH (for ionic current recording)) adjusted to pH 7.1 using MES. The voltage electrode was filled with 3 M KCl. Recordings were done at room temperature.

Gating currents were recorded from oocytes expressing mutations introduced into a non-conducting (W434F) Shaker IR-pBSTA vector background. The relation between the gating charge (Q) as a function of voltage (V) was fit into a single Boltzmann equation,

\[
\frac{Q}{Q_{\text{max}}} = \frac{1}{1 + \exp\left(-\frac{ZF}{RT}(V - V_{1/2})\right)}
\]

(Eq. 1)

where \(Q/Q_{\text{max}}\) is the normalized charge obtained from integrating the on- or off-gating currents, \(V\) is Faraday's constant, \(V_{1/2}\) is the voltage at which 50% of the maximal gating charge has moved from one state to the other, \(R\) is the gas constant, \(T\) is the temperature, and \(dV = RT/ZF\).

Ionic currents were recorded in oocytes expressing mutations made in a conducting Shaker IR-pBSTA background, which has a cysteine substitution in the extracellular space above the S4 (A359C or M356C). Tagging these cysteines with a fluorophore (tetrathymethylrhodamine-5-maleimide; Invitrogen) enables the tracking of the movement of the S4 voltage sensor (37, 38) while simultaneously recording ionic current. For fluorescence experiments, oocytes were first labeled with 5 µm tetrathymethylrhodamine-5-maleimide dissolved in a solution containing 115 mM K⁺-MES, 10 mM HEPES, and 2 mM Ca²⁺-MES, pH 7.0, for 20 min at room temperature in the dark and washed twice in supplemented Barth solution before recording. For ionic current recordings, the steady state currents elicited from a series of depolarizations was used to calculate conductance (G) of each mutant channel. GV relations were also fit into a single Boltzmann equation,

\[
\frac{G}{G_{\text{max}}} = \frac{1}{1 + \exp\left(-\frac{ZF}{RT}(V - V_{1/2})\right)}
\]

(Eq. 2)

where \(G/G_{\text{max}}\) is the normalized conductance, and \(V_{1/2}\) is the voltage at which 50% of the maximal conductance has been reached.

Double Boltzmann distributions were fitted to

\[
\frac{G}{G_{\text{max}}} = \frac{1}{1 + \exp\left(-\frac{ZF}{RT}(V - V'_{1/2})\right)} \left(1 + \exp\left(-\frac{ZF}{RT}(V - V_{1/2})\right)\right)
\]

(Eq. 3)

according to a three-state sequential model closed ↔ acti-
vated (closed) $\leftrightarrow$ open with equilibrium at $V_{1/2}$ and $V_{\alpha}$ for the first and second transition, respectively. This follows after short derivation from Equation 4 below with voltage-dependent rate constants. It should be noted that this is not a superposition or multiplication of two single Boltzmann distributions.

For experiments evaluating the effect of 4-aminopyridine (4-AP) on-gating and ionic currents, the final molarities of the carrier ion (N-methyl-D-glucamine for gating and K$^+$ for ionic current) in internal solutions were adjusted to compensate for 10 mM 4-AP. After initial recording of currents in response to a series of increasing depolarizations, the channels were incubated with 10 mM 4-AP, and currents were repetitively acquired after the same saturating depolarizing pulse. Once 4-AP no longer had an effect on gating or ionic currents, currents were recorded in response to another series of depolarizations. Data acquisition and analysis programs were developed at the University of California, Los Angeles (Dept. of Anesthesiology).

Molecular Modeling—Energy minimization of Kv1.2 (PDB 2A79) S4-S6 domains (for wild type and mutant E327A (E395A in Shaker)) was conducted using Discover 3 forcefield and visualized with INSIGHT II software. Spherical areas of 8 Å from residue 327 in each subunit were subjected to forcefield conditions in a water box, whereas the rest of the molecule remained fixed.

Kinetic Modeling—We created a 5-state model (see Fig. 9B) and fitted it to the gating currents obtained from a series of depolarizing pulses using Matlab. The first two transitions were charge-carrying transitions with a ratio of 0.45 ($A_1 \leftrightarrow A_2$) to 0.55 ($A_1 \leftrightarrow A_2$) of the total charge. The subsequent two transitions were electro-neutral. We chose the model to describe the initial movement of the voltage sensor itself ($C \rightarrow A_2$) and the subsequent interactions developing during electromechanical coupling between voltage sensor and pore ($A_2 \rightarrow A_3$). We, thereby, abided by the paradigms that the on-rate constants were free to vary for all pulse voltages, whereas the off-rate constants were constant because they all occur at the same resting potential during deactivation. The initial conditions for the on-gating transition (start of the pulse) are given by the steady state of the rate constants at resting potential (off-rate constants), and for the off-gating transition the predicted values after the depolarizing pulse according to the on-rate constants were used. With this model the traces could be well fitted except for possible delays in the immediate onset of on-gating, which require additional steps in the charge movement. We, however, were interested in the transitions occurring after major charge movement.

In principle, rate constants should follow an exponential voltage dependence. However, we did not fit all voltages simultaneously and did, thus, not enforce the exponential voltage dependence. Therefore, transitions with low occupancy at a certain voltage range compensated for other transitions.

Steady State of a Sequential Model—One can derive from steady state probabilities that, in a sequential model $A_1 \leftrightarrow A_2 \leftrightarrow A_3 \ldots A_{n-1} \leftrightarrow A_n$ with forward rate constants $\alpha_1, \alpha_2, \alpha_3, \ldots \alpha_{n-1}$ and the backward rates $\beta_1, \beta_2, \beta_3, \ldots \beta_{n-1}$, the equilibrium occupancy of $A_1$ is given by

$$A_1(t \rightarrow \infty) = \frac{1}{1 + \sum_{i=1}^{n-1} \frac{\alpha_i}{\beta_i} \left(1 + \sum_{j=i+1}^{n-1} \frac{\alpha_j}{\beta_j} \left(1 + \sum_{k=j+1}^{n-1} \frac{\alpha_k}{\beta_k} \left(1 + \ldots \right) \right) \right)}$$

(Eq. 4)

Assuming $A_1 \leftrightarrow A_2$ is the charge carrying transition (or lumping all major charge carrying transitions into $A_1 \leftrightarrow A_2$), the normalized charge equals $Q/Q_{max} = 1 - A_1$. This means that, if $\beta_i \gg \alpha_i$, all subsequent transitions $j > i$ have only a minor influence on $A_1$. Also, the further apart from $A_1$ a transition is, the less it will influence the position of the $QV$.

It also follows immediately that $A_1 < \beta_1/(\alpha_1 + \beta_1)$ because the term in each of the parentheses is always larger than or equal to 1. This means that at $V_{1/2}$ of the first transition ($\alpha_1 = \beta_1$), $Q > 1/2$. Thus, the $V_{1/2}$ of the entire system will always be more negative than that of the charge carrying step(s), and the $QV$ will always be shifted to more negative potentials.

RESULTS

Using the crystal structure of the mammalian homolog of Shaker, Kv1.2 (29), as a template, we identified several amino acids whose side chains project out into the intersubunit space between the S4-S5 linker and S6 of adjacent subunits. In the presumed open, inactivated state of the Kv1.2 crystal structure, Tyr-485 at the C-terminal end of the S6 of subunit IV appears to interact with several residues on the neighboring subunit I (Fig. 1A). The closest contacts were with Arg-394 and Glu-395 on the S4-S5 linker of subunit I, whereas more distant contacts were with Val-476 on the S6 of subunit I and Leu-398 located on the N-terminal S5 of subunit I (Fig. 1A). To determine the importance of these residues for electromechanical coupling, we verified whether they were well conserved in different Kv channels. We aligned sequences spanning S4-S5 linker, N-terminal S5, and S6 regions in various related K$^+$ channels, including voltage-gated mammalian and Drosophila K$^+$ channels, a voltage-gated bacterial K$^+$ channel (KvAP), and a cyclic nucleotide-regulated bacterial ion channel (Mlo1K). The most conserved residues were Glu-395 (10/12) and Tyr-485 (11/12), with the latter found even in the distantly related Mlo1K. The basic amino acid Arg-394 was conserved in the Shaker/Kv1 family and both bacterial channels, KvAP and Mlo1K. In the other channels, it was replaced by another basic residue, lysine, in Shab and Shaw and by an amide, asparagine, in the mammalian homologs for Shab and Shaw. Val-476 and Leu-398 were conserved in 8 and 9 of 12 sequences, respectively. The fact that Arg-394, Glu-395, Leu-398, Val-476, and Tyr-485 are well conserved raises questions about their importance in proper channel function.

Mutations in Residues at the S4-S5 Linker/S6 Interface Affect Features of the Gating Current—We characterized the variation of gating current kinetics by several mutations in each of the positions identified above. Mutations were introduced into the non-conducting Shaker-IR channel, W434F (4–6). We mutated each residue to alanine but also studied more conservative mutations (R394Q, E395D, L398V, L398I, L398N, Y485F, Y485H) and charge reversal mutations (R394E, E395R) (Table 1). All mutants expressed functionally, except Y485F and L398A. We initially studied the effect of each mutation on
Voltage sensor movement by analyzing gating currents produced in response to membrane depolarization (Fig. 2). Variations were found in both the on- and off-gating currents. The on-gating currents were accelerated only in E395R, L398V, L398W, and Y485A, whereas they remained unchanged for all other mutants. The effects on off-gating currents were more pronounced, which may be due to the fact that during deactivation the channels have to traverse the later steps of electromechanical coupling before charge movement because these transitions occur between closing of the pore and movement of the voltage sensor to its resting position. Analysis of the off-gating currents will provide information on these late transitions, and thus, we concentrated on the off-gating currents in this manuscript.

The mutants can be classified into three groups on the basis of their effects on the off-gating currents (Fig. 2). The first is a group of mutants that completely removed the slow onset and slow decay kinetics typically seen in W434F when returning from high depolarizations (see the Introduction) and showed

### TABLE 1

Summary of gating current results in Shaker mutants

Q\textsuperscript{V} were fit into a single Boltzmann distribution as described under "Experimental Procedures." V\textsubscript{50} represents the voltage at which 50% of the maximal charge has been moved. dV represents the slope of the Boltzmann curve. ΔV\textsubscript{50} represents the difference in V\textsubscript{50} between the respective mutant and W434F non-conducting control. ND indicates that the off-gating curves could not be fit into a single Boltzmann function.

| Shaker construct | Q\textsubscript{ON} | I\textsubscript{gating} | Q\textsubscript{OFF} |
|------------------|------------------|------------------|------------------|
|                  | V\textsubscript{50} | dV               | ΔV\textsubscript{50} | V\textsubscript{50} | dV               | ΔV\textsubscript{50} |
| W434F            | –45.9 ± 0.8 mV    | 8.7 ± 0.7 mV     | –53.2 ± 0.7 mV   | 8.0 ± 0.6 mV |
| R394A            | –32.5 ± 0.7 mV    | 6.3 ± 0.6 mV     | 5.7 ± 0.5 mV     | +13.0 ± 5 mV |
| R394Q            | –47.9 ± 1.3 mV    | 8.4 ± 1.1 mV     | 7.8 ± 0.4 mV     | 16.3 ± 4 mV |
| R394E            | –60.0 ± 1.0 mV    | 7.7 ± 0.9 mV     | 9.2 ± 0.6 mV     | +11.0 ± 6 mV |
| E395A (V\textsubscript{50} = –90 mV) | –62.3 ± 0.5 mV | 7.8 ± 0.4 mV | 5.7 ± 0.5 mV | 16.3 ± 4 mV |
| E395A (V\textsubscript{50} = –120 mV) | –60.8 ± 0.7 mV | 11.6 ± 0.6 mV | 11.6 ± 0.5 mV | +11.0 ± 6 mV |
| E395D            | –76.2 ± 0.7 mV    | 9.1 ± 0.6 mV     | 8.3 ± 0.8 mV     | –29.2 ± 6 mV |
| E395R            | –48.8 ± 0.4 mV    | 9.7 ± 0.4 mV     | –58.3 ± 0.9 mV   | –5.1 ± 2 mV |
| L398A            | –38.9 ± 1.0 mV    | 12.1 ± 0.8 mV    | –37.2 ± 1.0 mV   | +16.0 ± 3 mV |
| L398N            | –28.0 ± 1.9 mV    | 11.0 ± 2.1 mV    | –27.5 ± 1.2 mV   | +25.7 ± 5 mV |
| L398V            | –94.5 ± 1.9 mV    | 24.4 ± 1.4 mV    | –52.5 ± 1.7 mV   | +16.1 ± 3 mV |
| L398I            | –59.4 ± 1.9 mV    | 24.4 ± 1.4 mV    | –69.9 ± 0.5 mV   | +16.7 ± 3 mV |
| L398W            | –69.5 ± 1.6 mV    | 9.1 ± 1.4 mV     | –69.9 ± 0.5 mV   | +16.7 ± 3 mV |
| V476A (V\textsubscript{50} = –90 mV) | –60.0 ± 0.7 mV | 7.6 ± 0.6 mV | 7.8 ± 0.4 mV | –16.7 ± 3 mV |
| V476A (V\textsubscript{50} = –120 mV) | –48.9 ± 0.6 mV | 10.8 ± 0.5 mV | 11.8 ± 0.3 mV | +2.9 ± 2 mV |
| Y485A            | –53.8 ± 0.4 mV    | 10.4 ± 0.4 mV    | –54.8 ± 0.4 mV   | +10.0 ± 3 mV |
| Y485F            | –45.7 ± 0.9 mV    | 7.6 ± 0.8 mV     | –52.2 ± 0.8 mV   | +1.0 ± 2 mV |
| Y485H            | –64.2 ± 0.4 mV    | 6.3 ± 0.3 mV     | –69.1 ± 0.5 mV   | +15.9 ± 6 mV |
| E395A/E395A      | –51.9 ± 0.6 mV    | 12.7 ± 0.6 mV    | –56.0 ± 0.4 mV   | +12.0 ± 4 mV |

Mutants in which gating currents were not detected.
almost symmetrical gating currents (E395D, L398V, L398I (data not shown), Y485A, Y485H). These mutants form an interaction stabilizing the activated (or open) state of the channel. As this interaction acts across subunits, it very likely has some implication in cooperative opening of the channel. The second group of mutants modified, i.e. slowed down (E395R), immobilized (E395A, V476A), or accelerated (R394A, L398W), the slow components of the off-gating currents and thereby modulated the underlying interaction, which indicates that the corresponding residues are part of or located in close proximity to the molecular determinants of the interaction. Different mutations of position Glu-395 and Leu-398 are represented in both the first and second groups, confirming that these two positions play a central role in the stabilization process. Mutations of the third group did not significantly alter the shape of the gating currents (L398N, R394Q, R394E (data not shown)).

When comparing the time courses of the gating currents (Fig. 2) with the amount of gating charge moved in response to membrane depolarization ($Q_{V}$, Fig. 3), it becomes evident that effects on time course and on $Q_{V}$ do not always correspond to each other. As expected from the gating current traces, $Q_{V}$ relations for the third group of mutants, i.e. R394Q, R394E, and L398N, were indistinguishable from those of W434F (Fig. 3). The first group of mutants, i.e. E395D, L398V and/or L398I, and Y485A and/or Y485H, which eliminated the slow component in the off-gating currents, all increased the slope factors ($dV$, shallower slope) of their $Q_{V}$s. They also had in common a reduced difference between the $Q_{ON}$ and $Q_{OFF}$, whereas their effects on the equilibrium voltage ($V_{1/2}$) varied (Table 1; Fig. 3). The difference between $Q_{ON}$ and $Q_{OFF}$ is likely caused by an immobilization of gating charges in the open state (see below). On the other hand, the second group of mutants all altered the $V_{1/2}$ of the $Q_{V}$ while leaving the $dV$ constant. The $V_{1/2}$ was increased when the slow off-gating component was accelerated (R394A) and decreased when the component was slowed down (E395R) or charges were immobilized (V476A, E395A), indicating that less energy was required to activate the voltage sensor in these three mutants. This is consistent with the idea that the acti-
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Neutralization of E395A Stabilizes the Activated State of the Voltage Sensor—Recall that E395D and E395A lead to almost opposing effects on the slow off-gating component. E395D removed the slow component and showed fast return of the voltage sensor, whereas in E395A the off-gating could not be detected anymore for depolarizations more positive than $-60 \text{ mV}$. In addition, neutralization of a charge in E395A led to a negative shift of the $QV$, indicating a strengthening of an interaction that stabilizes the open state. More specifically, for the different mutations at position 395, the $V_{1/2}$ and $dV$, in particular for mutants that remove the slow off-gating component, imply that two complementary effects occur. As both are not strictly correlated with each other, it is likely that they reflect two different transitions along the activation pathway.

Significant differences were observed in the $Q_{OFF}V$ relations. Whereas the $Q_{OFF}V$ of E395R, whose $Q_{ON}V$ was shifted the most, still followed a typical Boltzmann distribution (Fig. 3), the $Q_{OFF}V$ of E395A and V476A instead reached their maximum relative values after depolarizations between $-60 \sim -70 \text{ mV}$ (Fig. 3 and 4B) and tapered off at higher potentials. In both mutants, less $Q_{OFF}$ charge is measured compared with $Q_{ON}$ (Fig. 4A), signifying that for both mutants the voltage sensor is “immobilized” in the activated state and only very slowly returns to the resting position. This is confirmed by the fact that the $Q_{OFF}V$ of both mutants was restored by decreasing the resting potential to $-120 \text{ mV}$ (Fig. 4B). The effect was, thus, contrary to that of mutants of the first group. We, thus, not only established that Glu-395, Leu-398, and Tyr-485 form the open state stabilization (13), as we can remove the interaction by mutating either one of them, but we also, with E395A and/or E395R and V476A, have a way of increasing the interaction, which gives us the unique opportunity to investigate one of the later transitions in electromechanical coupling over the background of the charge movement.

Neutralization of E395A Stabilizes the Activated State of the Voltage Sensor—Recall that E395D and E395A lead to almost opposing effects on the slow off-gating component. E395D removed the slow component and showed fast return of the voltage sensor, whereas in E395A the off-gating could not be detected anymore for depolarizations more positive than $-60 \text{ mV}$. In addition, neutralization of a charge in E395A led to a negative shift of the $QV$, indicating a strengthening of an interaction that stabilizes the open state. More specifically, for the different mutations at position 395, the $V_{1/2}$ of the $QV$ became more negative in the order E395D ($+5 \text{ mV}$), E395E (wild type), E395A ($-16 \text{ mV}$), and E395R ($-30 \text{ mV}$).
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following the electrostatic charge of the residues. The diminished off-gating charge in E395A cannot neither by explained by the negative shift of the $Q_{V}$, as both E395R (Fig. 3) and W434F with a holding potential of $-70$ mV (data not shown) returned “normally” to the voltage sensors resting position, nor by destabilization of a transition before charge movement. More likely, an additional mechanism is responsible for the slow return of the voltage sensors, affecting a transition later in the activation pathway, prompting us to further investigate the mechanism underlying the effects of Glu-395 mutations and, thus, the nature of the stabilization of the activated voltage sensor.

For pulses to potentials at which the off-gating currents develop, the slow component in W434F, the $Q_{OFF}/Q_{ON}$ ratios of E395A showed an $\sim97\%$ loss in charge movement (Fig. 4A), suggesting that the voltage sensors only return very slowly to resting potential, and accordingly, the slow off-gating component was recovered when the holding potential was reduced from $-90$ to $-120$ mV, effectively providing energy to overcome the additional interaction and return the voltage sensors (Fig. 4, B and C). The kinetics of the E395A off-gating currents were still significantly slower than that of W434F (Fig. 4C). In contrast, $Q_{OFF}/Q_{ON}$ gating relations of E395A were unaltered by changes in holding potential (data not shown). The $Q_{OFF}$ $V$ gating relations of E395A were shifted to more negative potentials by $\sim18$ mV with respect to W434F (Fig. 4B, Table 1). Because the charges returned too slowly to detect the voltage sensor movement in the gating currents, we tracked it using voltage-clamp fluorometry. We labeled the channels at an additionally introduced cysteine (A359C) in the S3-S4 linker. Fluorescence changes from this position had been shown to monitor voltage sensor movement (36–38). We compared the mutant E395A/W434F/A359C with its control, W434F/A359C. The return of the voltage sensor of E395A from depolarizations of $> -60$ mV was significantly slower (Fig. 5A). When the resting potential was decreased from $-90$ to $-120$ mV, the fluorescent decay was accelerated as before the off-gating currents (Fig. 4D) but was still significantly slower than W434F. Fitting the decay curves into two exponentials showed that E395A had an additional, 4-fold higher, slow component compared with control ($\tau = 190 \pm 17$ ms; Fig. 5A). This additional slower component was the major fraction of charge movement in E395A, whereas in wild type the faster time constant was the dominant component.

The slow kinetics of the return of the voltage sensor from the immobilized state at negative holding potential ($-90$ mV) was also determined by subjecting the channel to a dou-
able pulse protocol (Fig. 5B). The gating charge available for a second depolarizing test pulse (+20 mV) recovered to 98% only after 300 ms in E395A (τ = 70 ms). In contrast, W434F control was fully recovered after 30 ms (τ = 7 ms; Fig. 5B), similar to the time needed for the off-gating current to decay fully to zero. We also varied the length of a −60-mV depolarizing pulse to determine the speed with which the mutant channel enters the immobilized state (Fig. 5C). Charges became immobilized with a time constant of τ = 61.4 ms at −60 mV.

An Interaction between Arg-394 and Tyr-485 Causes Voltage Sensor Immobilization in the E395A Mutant—The delayed return of the voltage sensors characterized above is caused by an interaction that forms when the charge at position Glu-395 is neutralized. Yet the effect of mutations at position 395 is dependent on the electrostatic charge where the neutral residue (Ala in E395A) leads to the strongest interaction. This suggests that the residue is not directly responsible for the additional interaction energy. To identify the underlying molecular determinants, the region surrounding Glu-395 was modeled based on the Kv1.2 crystal structure (29). The structures of both wild type Kv1.2 and of the E395A mutant (E327A in Kv1.2) were compared after an energy minimization protocol that allowed residues in the surrounding of Glu-395 to move freely, whereas the rest of the molecule remained fixed. When glutamate 395 was replaced by alanine, a structural reorientation in the side chains of Tyr-485 and Arg-394 was observed (Fig. 5D). This adjustment reduced the distance between the hydroxyl group of Tyr-485 and the guanidinium group of Arg-394, implying a stronger interaction between these oppositely charged groups. The stronger bond may account for the voltage sensor immobilization seen experimentally with E395A.

To verify that the strong bond indeed results in voltage sensor immobilization, we neutralized both
that both mutations (E395A and V476A) lead to open state
analysis (39). However, our fluorescence measurements show
reduced the energy needed to activate the voltage sensor and
 pore was still intact because the slow component would
not be able to develop if the mutant channel does not open. To
this end, we elicited ionic currents in parallel with fluorescent
 traces that tracked the movement of the voltage sensor (A359C)
in conducting wild type and mutant channels (E395A and/or
E395D, Y485A) in response to a series of depolarizations. Sim-
ilar to the QV gating relations (Fig. 3), FV relations of E395A/
A359C were negatively shifted compared with control, con-
firming activation of its voltage sensor at lower depolarizations
(Fig. 7A, Table 2). The GV was also shifted to more negative
potentials ($V_{1/2} = -52$ mV). The mutation Y485A/A359C
yielded FV relations not significantly different from control
(Fig. 7A, Table 2), a finding similar to Y485A QV relations.
In Y485A/A359C, a shift to more positive potentials and a shal-
lower slope of the GV curve compared with A359C was
observed. QV and GV were, thus, further separated than in wild
type, and the voltage dependence was less steep. The fact that
the pore opening occurs at higher potentials suggests that the
intersubunit bridge in the wild type channel pulls the pore into
the open state. This energy is missing for opening the pore in
Y485A. Nevertheless, because no slow off-gating component
developed even at depolarizing pulses to voltages where the
channel opened (100 mV), we can conclude that suppression of
the slow off-gating component by the alanine replacement of
Tyr-485 is not caused by interruption of electromechanical
coupling but by direct disruption of the interaction, giving
rise to the slow component. For E395D, a similar effect was
observed (Fig. 7A).

The GV curves of both Y485A and E395D did not follow
simple Boltzmann relations but had to be fitted to double Bolt-
zmann curves (Y485A: $V_{1/2,1} = -17.3$ mV ($dV_1 = 11$ mV);
$V_{1/2,2} = 14.8$ mV ($dV_2 = 38$ mV); E395D: $V_{1/2,1} = -14.9$ mV
($dV_1 = 11$ mV); $V_{1/2,2} = 21.6$ mV ($dV_2 = 50$ mV); see “Experi-
mental Procedures”). This indicated that pore opening is gov-

Arg-394 and Glu-395 simultaneously (R394A/E395A (RE-
AA)). In RE-AA, the slow off-gating components are
preserved (Fig. 6A), and unlike E395A, no voltage sensor immo-
bilization was observed. The RE-AA double mutant resembles
the W434F control in both gating phenotype and energetics
(Fig. 6, A and B). In contrast, the gating currents of the E395A/
Y485A (EY-AA) double mutant lost the slow off-gating compo-
nents (Fig. 6A), as was seen in the single mutation Y485A (Fig.
2), and exhibited QV relations similar to Y485A (Fig. 6B). Thus,
both in double mutants YY-AA and RE-AA, the strong stabili-
zation of the activated state developing in the absence of a
charge at position 395 was eliminated, confirming that in
E395A a strong bond develops between Tyr-485 and Arg-394.
Stabilization of the activated state in the RE-AA double mutant
is formed by a bond between Tyr-485 and Leu-398, and this
interaction gives rise to the slow off-gating currents.

In addition, the V476A mutation, as in the case of E395A,
reduced the energy needed to activate the voltage sensor and
delayed its recovery when repolarizing to resting potential (Fig.
4, A and B). In Kv4.2, V476A and E395A had been suggested to
interact with one another supported by double mutant cycle
analysis (39). However, our fluorescence measurements show that
both mutations (E395A and V476A) lead to open state
stabilization. Thus, they add an interaction that occurs else-
where (namely, between Arg-394 and Tyr-485). Inspection of
the Kv1.2 crystal structure indicates that Val-476 is located
close to Glu-395 of the adjacent subunit. We assume that Val-
476 helps to properly position Glu-395 in the context of an
RELY intersubunit interaction. Replacement of Val-476 with
alanine allowed Glu-395 to swing away, allowing the strong
bond between Arg-394 and Tyr-485.

Pore Opens in Y485A in the Absence of Slow Component—
The occurrence of the slow component has previously been
associated with pore opening (6, 11, 14). Thus, we had to ascer-
tain that the electromechanical coupling between voltage sen-
or and pore was still intact because the slow component would
not be able to develop if the mutant channel does not open. To
this end, we elicited ionic currents in parallel with fluorescent
traces that tracked the movement of the voltage sensor (A359C)
in conducting wild type and mutant channels (E395A and/or
E395D, Y485A) in response to a series of depolarizations. Sim-
ilar to the QV gating relations (Fig. 3), FV relations of E395A/
A359C were negatively shifted compared with control, con-
firming activation of its voltage sensor at lower depolarizations
(Fig. 7A, Table 2). The GV was also shifted to more negative
potentials ($V_{1/2} = -52$ mV). The mutation Y485A/A359C
yielded FV relations not significantly different from control
(Fig. 7A, Table 2), a finding similar to Y485A QV relations.
In Y485A/A359C, a shift to more positive potentials and a shal-
lower slope of the GV curve compared with A359C was
observed. QV and GV were, thus, further separated than in wild
type, and the voltage dependence was less steep. The fact that
the pore opening occurs at higher potentials suggests that the
intersubunit bridge in the wild type channel pulls the pore into
the open state. This energy is missing for opening the pore in
Y485A. Nevertheless, because no slow off-gating component
developed even at depolarizing pulses to voltages where the
channel opened (100 mV), we can conclude that suppression of
the slow off-gating component by the alanine replacement of
Tyr-485 is not caused by interruption of electromechanical
coupling but by direct disruption of the interaction, giving
rise to the slow component. For E395D, a similar effect was
observed (Fig. 7A).

The GV curves of both Y485A and E395D did not follow
simple Boltzmann relations but had to be fitted to double Bolt-
zmann curves (Y485A: $V_{1/2,1} = -17.3$ mV ($dV_1 = 11$ mV);
$V_{1/2,2} = 14.8$ mV ($dV_2 = 38$ mV); E395D: $V_{1/2,1} = -14.9$ mV
($dV_1 = 11$ mV); $V_{1/2,2} = 21.6$ mV ($dV_2 = 50$ mV); see “Experi-
mental Procedures”). This indicated that pore opening is gov-
erned by two different transitions with different voltage dependence. A double Boltzmann relation occurs if two (or more) voltage-dependent transitions in series have to be passed before the observable (conductance) may change. In most cases the result would be similar to a single Boltzmann relation. It is only when the two slopes of the voltage dependences are very different that a significant influence on the observable (here $GV$) is noted. The fits of Y485A and E395D $GV$s show that the second transition represented in the double Boltzmann has become very broad. This is likely due to the lack of open state stabilization (see Fig. 9C) when eliminating the interaction responsible for the slow component in Y485A and E395D. In contrast, voltage sensor movement ($QV$) is left unaltered, indicating that the final open transition does not influence the state of the voltage sensor.

**The RELY Interaction Also Develops under Physiological Ion Permeation**—We have demonstrated that in the non-conducting mutant W434F an interaction develops between the residues $GV$ and $FV$ relations were fit into a single Boltzmann distribution as described under “Experimental Procedures.” $V_1/2$ represents the voltage at which 50% maximal conductance has been reached. $dV$ represents the slope of the Boltzmann curve. $\Delta V_{1/2}$ represents the difference in $V_1/2$ between the respective mutant and A359C conducting control.

| Shaker construct | $V_1/2$ (mV) | $dV$ (mV) | $\Delta V_{1/2}$ (mV) | $n$ | $\Delta F/F_{max}$ (mV) | $dV$ (mV) | $\Delta F_{1/2}$ (mV) | $n$ |
|------------------|-------------|---------|----------------------|-----|------------------------|---------|---------------------|-----|
| A359C            | $-21.0 \pm 1.2$ | $15.9 \pm 1.0$ | 5 | $-39.6 \pm 2.7$ | $20.9 \pm 2.3$ | 4 |
| E395A/A359C     | $-52.1 \pm 0.6$ | $7.4 \pm 0.5$ | $-31.1$ | 8 | $-58.4 \pm 1.3$ | $11.1 \pm 0.1$ | $-18.8$ | 4 |
| E395D/A359C     | $-14.9 \pm 1.8$ | $11.0 \pm 1.8$ | 6 | $-34.8 \pm 0.3$ | $12.9 \pm 0.3$ | 6 |
| Y485A/A359C     | $+21.6 \pm 1.7$ | $50.0 \pm 2.5$ | 6 | $-33.9 \pm 0.6$ | $15.1 \pm 0.6$ | $+5.7$ | 8 |
| A359C/W434F     | $-17.3 \pm 1.8$ | $11.1 \pm 1.4$ | 6 | $-33.9 \pm 0.6$ | $15.1 \pm 0.6$ | $+5.7$ | 8 |
| E395A/A359C/W434F | $+14.8 \pm 1.8$ | $38.0 \pm 1.8$ | 6 | $-33.9 \pm 0.6$ | $15.1 \pm 0.6$ | $+5.7$ | 8 |

$GV$ and $FV$ relations were fit into a single Boltzmann distribution as described under “Experimental Procedures.” $V_1/2$ represents the voltage at which 50% maximal conductance has been reached. $dV$ represents the slope of the Boltzmann curve. $\Delta V_{1/2}$ represents the difference in $V_1/2$ between the respective mutant and A359C conducting control.
dues Arg-394, Glu-395, Leu-398, and Tyr-485, which we will abbreviate as RELY interaction, stabilizing the activated state of the channel. Nevertheless, concerns that the slow off-gating component may not develop at physiological conditions need to be addressed as it had been suggested that the non-conducting phenotype of W434F Shaker channels is due to C-type inactivation and that the slow off-gating component arises from this feature (40, 41), although results of other authors are more consistent with an open state stabilization than with C-type inactivation (11, 13). In addition, it had been proposed that different permeant ions variably alter the kinetics of the off-gating currents (11, 12, 42).

To confirm that the RELY interaction also develops under physiological conditions of ion permeation, we carried out fluorescence voltage-clamp measurements of the conducting mutants as well as their respective wild type controls labeled at an additionally introduced cysteine at position A359C or M356C. Using fluorescence to trace the movement of the voltage sensors at physiological conditions obviates the need to vary the permeating ion or use non-physiological ion concentrations. We can, rather, follow the movement during normal ion conduction that prevails under physiological function of the channel.

We first compared the off-decay of the fluorescence during repolarization from varying depolarizations of the wild type channel to the non-conducting mutant W434F and the conducting mutant T449Y, which very slowly develops C-type inactivation (43). To this end an additional cysteine was introduced at position M356C of each channel. No difference in the fluorescence signal between wild type and T449Y was observed. Comparison of the fluorescence decay to W434F revealed, however, that wild type fluorescence initially returned significantly faster than W434F fluorescence (Fig. 8C). The fast initial decay was followed by a slower component, which is comparable with (or slower than) W434F/M356C. Accordingly, we found two time constants each for wild type and W434F. In W434F, the time constants were slower in comparison to wild type and T449Y (Fig. 8A). At the same time, the amplitude of the slower component (~60 ms) only accounted for a small fraction of total charge movement (10–15%) of the entire fluorescence intensity decay of W434F compared with 40% in wild type (Fig. 8B). The time constants found in the off-gating currents of W434F were consistent with the major faster component found in the inactivation of the RELY interaction (Y485A, E395D) and slow voltage sensor movement (E395A with wild type in the conducting mutant, we observed an accelerated decay of fluorescence traces tracking the voltage sensor during deactivation. This is consistent with the absence of the slow off-gating component (Fig. 7B). Thus, also the fast off-gating decay seen in Y485A and E395D is preserved under physiological ion conduction and that this immobilization is independent of C-type inactivation.

When comparing the fluorescence signals of Y485A and E395D with wild type in the conducting mutant, we observed an accelerated decay of fluorescence traces tracking the voltage sensor during deactivation. This is consistent with the absence of the slow off-gating component (Fig. 7B). Thus, also the fast off-gating decay seen in Y485A and E395D is preserved under physiological ion permeation.

The above results indicate that the slow component is still found in the conducting wild type channel, although it is accelerated in comparison to W434F as it was proposed earlier (11, 12, 42). Both acceleration of voltage sensor movement by elimination of the RELY interaction (Y485A, E395D) and slow voltage sensor return by strengthening the stabilization (E395A) are observed in the conducting channel as in the W434F mutant. The slow off-gating components and the RELY interaction do not seem to be specific to W434F or related to C-type inactivation.

The RELY Interaction Correlates with Channel Opening—It still remains to be established whether the RELY interaction is formed in the open state or whether it is maintained from an earlier transition. We, therefore, tested the effect of the K+
Origin of Slow Off-gating

channel blocker 4-AP on gating currents (W434F) and ionic currents (wild type) in E395A and Y485A. 4-AP has been described to stabilize channels in an “activated-not-open” state because 4-AP blocks the final transition of the intracellular activation gate (10, 14, 20–22). In both mutants and wild type, ionic current was blocked by the addition of 4-AP. In the gating current traces of the W434F control, 4-AP eliminated the slow off-gating components (Fig. 9A), as shown previously (10). 4-AP also altered the off-gating kinetics of E395A, as a slow component was observed similar to W434F but faster than E395A in the absence of 4-AP (Fig. 9). 4-AP facilitated, thus, a faster return of the off-gating charge, indicating that in the E395A mutant an interaction still developed between Arg-394 and Tyr-485 even in the presence of 4-AP, although this interaction is not as strong as in the absence of 4-AP. This shows that the RELY interaction partners are close enough to develop an (electrostatic) interaction if the channel is in the activated, not-

open state but are prevented from assuming their native configuration required to develop the open state stabilization observed in wild type. The final opening step, therefore, seems closely related to the development of the RELY interaction.

4-AP did not change the gating patterns of Y485A (Fig. 9A) or EY-AA (data not shown). Fast off-gating decay was still observed in both mutants, confirming that in Y485A the last transition associated with the slow component is not rate-limiting.

Structural Interactions of Tyr-485 Represent Late Closed States in the Activation Pathway; a Five-state Activation Model—Three different effects have been observed when disrupting the RELY interaction; (i) the modulation of the “strength” of the stabilizing interaction; (ii) alteration of the QVs, and (iii) GV's that follow a double Boltzmann characteristic. These effects have to be explained. We, therefore, fitted the gating current traces to a five-state sequential model (Fig. 9B, left). The model contains two initial transitions that carry charge. These are followed by two further transitions representing the later steps of electromechanical coupling. With this model we were able to well fit all gating currents at different voltages (supplemental Fig. 1, see “Experimental Procedures” for details).

The rate constants showed the basic features that explain the occurrence of the slow component in the gating currents. For W434F, we found two voltage-dependent transitions with $V_{1/2}$ of $-25.1$ mV ($A_1$) and $-83.4$ mV ($A_1$ to $A_2$) (supplemental Fig. 1). The last transition ($A_2$ to $A_3$) also showed a voltage dependence with a $V_{1/2}$ of $-50.0$ mV. Two features are of interest here; (i) the first transition has a higher $V_{1/2}$ than the second one, which means that the second transition pulls the first transition open (thus, $V_{1/2}$ of the QV is lower than $-25.1$ mV, see “Discussion”); (ii) the last transition seems to show a voltage dependence, which may arise from a genuine voltage dependence of the opening step but which may also hint at cooperative interaction. With increasing probability of the neighboring subunits to dwell in the activated state, the rate constant of entering the open stabilized state increases as well.

We compared these values with fits of the two mutants Y485A and E395A, which represent the two extremes of eliminating and strengthening the open state stabilization.
Origin of Slow Off-gating

In E395A, we found two differences compared with W434F; first, the last transition from $A_3 \rightarrow A_5$ was much stronger (ratio forward to backward rate higher), and the forward rate always remained larger than the backward rate, which itself corresponded to the value of W434F. Second, the $V_{V_2}$ of the $C \rightarrow A_1$ transition was shifted to $-38$ mV. The $V_{V_3}$ of the second transition ($A_1 \rightarrow A_2$) could not be determined because, also in this case, the forward rate remained larger than the backward rate within the experimental voltage range. During return of the voltage sensors to the resting state, the movement is, therefore, driven by the first transition only. This first transition has to pull the sensors against the subsequent transitions, which all favor the activated position. This led to the negative shift of the $QV$ and the slow return of the voltage sensors.

In Y485A, the $C \rightarrow A_1$ transition was, although slightly accelerated, similar to W434F, with a $V_{V_2}$ of $-23.6$ mV. The second transition was similar to W434F as well, which explains that the $QV$ is not significantly shifted with respect to wild type. However, the last transition has a very fast backward rate, which is always faster than the forward rate. The dwell time in the last state is, thus, very short. This reflects the missing activated (open) state stabilization due to lack of Tyr-485. Although E395A stabilizes the final open state ($A_5$) strongly in Y485A, the rate constants suggest that the state is never entered, confirming a lack of some of the structural determinants. Both results are in accordance with the RELY intersubunit interaction.

DISCUSSION

The interface between the S4-S5 linker and S6 plays a key role in coupling between voltage sensor and pore domain in voltage-gated $K^+$ channels. In the Kv1.2 crystal structure (29), portions of these two $\alpha$-helical regions lie in close proximity to one another. Furthermore, past experiments have identified specific residues on the S4-S5 linker and S6 C terminus as critical for pore opening by the voltage sensor (electromechanical coupling) (26–28, 31–33). In the present study we identified an interaction in Shaker $K^+$ channels (RELY interaction) involving residues located at the junction of the C-terminal S4-S5 linker and the S5 (Arg-394, Glu-395, Leu-398) of one subunit and the C-terminal S6 of the adjacent subunit (Y485A). The RELY interaction brings about the open state stabilization of Shaker $K^+$ channels, which has previously been suggested to represent the final transition from the last closed state to the open state in the activation pathway (5–8, 10, 44). The fact that the identified interaction acts across different subunits suggests immediately itself that this last transition is involved in the concerted opening of the pore. Evidence for the significance of the three residues Glu-395, Leu-398, and Tyr-485 is their high conservation among voltage-dependent $K^+$ channels (Fig. 1B). We demonstrated using voltage clamp fluorometry that the RELY interaction develops under physiological ion permeation, although the slow component is, in the presence of ionic current, accelerated with respect to W434F in accordance to previous results (11, 12, 42).

Our results are consistent with previous reports. Two of the residues involved, Glu-395 and Leu-398, fall within the region mapped by Soler-Llavina et al. (18) to disturb electromechanical coupling. Tyr-485 is part of the YFYH$^4$ motif suggested by Lu et al. (27) to couple S6 to the S4-S5 linker. Two mutants other than the ones in this manuscript have been described earlier to abrogate the slow off-gating component while leaving electromechanical coupling intact. Y485C (30) and F481W (17) lacked the slow off-gating components without interrupting electromechanical coupling. The results of mutation Y485C confirm the key role of Tyr-485 for the open state stabilization that we suggest here, in particular, as the authors describe that the $V_{V_3}$ of the GV was not shifted in this channel, thus verifying that the electromechanical coupling is not interrupted by mutating Tyr-485 (30). As for Phe-481, it is located directly above Tyr-485 such that it is likely that the bulky tryptophan influences the RELY interaction.

Although the slow component was not related to C-type inactivation or specific to the W434F mutation, it was related to pore opening. In the two mutants Y485A and E395D, both of which remove the slow off-gating component, the pore still opened voltage-dependently, indicating that electromechanical coupling remained intact. However, the final pore opening transition was governed by (at least) two sequential voltage-dependent transitions. Because of the lack of open state stabilization, the last transition has a shallower voltage dependence, leading to the double Boltzmann GV’s of Y485A and E395D, although the GV remained the same.

The different effects of the Y485A and E395D mutations on $QV$ and GV are explained by the different dependences of charge movement and pore opening on the late transitions of electromechanical coupling. The major charge-carrying transitions occur during the movement of the S4 helix. It is, therefore, unlikely that mutations at the C-terminal S4-S5 linker or S6 would directly influence the “electrical transition energy.” Rather, the “mechanical load” on the S4 movement is varied either by pulling the sensor into a more stable position (open state stabilization) or by preventing such a transition. We, thus, cannot directly alter the slope of the charge carrying (early) transitions because the slope is determined by the contributing gating charges. Nevertheless, the $QV$ of Y485A features a decreased slope but was hardly shifted, whereas the GV is far shifted and very shallow. These effects may be caused by altering the later transitions (non-charge carrying) in our proposed model. To illustrate it better, we combine the first two states such that only one charge-carrying transition remains (Fig. 9B, right). In this case, the gating charge is according to Equation 4 given by,

$$Q = 1 - \frac{1}{1 + \gamma \left( \frac{1 + \frac{\alpha}{\beta}}{1 + \frac{\gamma}{\delta}} \right)^{\frac{\alpha}{\delta}}}$$

(Eq. 5)

whereas the conductance will be given by $A_S$,

$$G = A_S = \frac{1}{1 + \frac{\phi}{\epsilon} \left( \frac{1 + \frac{\delta}{\gamma}}{1 + \frac{\beta}{\gamma}} \right)^{\frac{\beta}{\gamma}}}$$

(Eq. 6)

$^4$ H486A of the YFYH motif did not have any effect.
Origin of Slow Off-gating

As outlined under “Experimental Procedures,” subsequent transitions cannot shift the equilibrium (and, thus, the $\Delta V$) to more positive potentials than the equilibrium of the charge carrying transition ($V_{52}, \alpha = \beta$) or any other transition ($A_2 \rightarrow A_3$) occurring earlier in the sequence. On the other hand, stabilization of a subsequent transition will shift the equilibrium (and, thus, the $\Delta V$) to more negative potentials. The most positive $V_{52}$ values observed were $-28$ and $-32$ mV for L398I and R394A, respectively. A value close to these potentials should, thus, be smaller or equal to the “native” $V_{52}$ of the charge-carrying transition. The value corresponds well to our fitting results for the E395D and Y485A mutants. A value close to these potentials should, thus, be smaller or equal to the “native” $V_{52}$ of the charge-carrying transition. The value corresponds well to our fitting results for the first transition $C \rightarrow A_1$ ($-25.1$ mV W434F; $-23.6$ mV Y485A). A destabilization of the last state (shifting it to more positive potentials), as we proposed for Y485A, would, thus, leave the $V_{52}$ of the $\Delta V$ unaltered if the transition was already more positive than any of the previous ones. One would, however, observe a slight decrease of the slope. The $\Delta V$, in contrast, is controlled primarily by the last transition. According to Equation 6, an earlier transition ($C \rightarrow A_2, A_2 \rightarrow A_3$) can never shift the $\Delta V$ to more negative potentials than the last transition. A destabilization of the last state would, therefore, strongly shift the $\Delta V$ to more positive potentials. Fig. 9C, right, shows the results for the $\Delta V$ and $\Delta V$ if the last transition is shifted to the values of $V_{52}$ and $dV$ observed for the GV of Y485A (red) while leaving the first two transitions untouched. Just by changing the subsequent transitions of electromechanical coupling, we are, thus, able to explain the opposing effects on $\Delta V$ and $\Delta V$ in the E395D and Y485A mutants.

In this context, the fact that mutating position Glu-395 leads to an increasing shift of the $V_{52}$ of the $\Delta V$s following the electrostatic charge of the residue (E395D, +5 mV; E395E, 0 mV; E395A, $-16$ mV; E395R, $-30$ mV) suggests that a more positive charge at this position stabilizes the activated state of the voltage sensor. Mutations at this position had a strong influence on the $\Delta V$ and $dV$ observed for the $\Delta V$ of Y485A (red) while leaving the first two transitions untouched. Just by changing the subsequent transitions of electromechanical coupling, we are, thus, able to explain the opposing effects on $\Delta V$ and $\Delta V$ in the E395D and Y485A mutants. In E395A, one of the last transitions of electromechanical coupling (little or no gating charge) has been shifted to more negative potentials. That will pull the voltage sensor into the activated state, leading to a negative shift of the $\Delta V$, which is, according to Equation 5, allowed. It is possible that two different effects occur in the E395A mutant. An electrostatic interaction might occur in the electromechanical coupling sequence before the actual open state stabilization. Considering that the Y485F mutant did not express, which indicates an important role for the Tyr-485 hydroxyl group, it is likely that the electrostatic interaction between Glu-395 and Arg-394 and the polar hydroxyl group of Tyr-485 is responsible for the dependence on the electrostatic charge of Glu-395. Accordingly, in the EY-AA double mutant no significant shift of the $\Delta V$ was observed.

For the positions Leu-398 and Arg-394, it appears that size and atomic structure of the residue rather than the electrostatic charge are decisive for its influence on gating charge movement. R394A shifts the $\Delta V$ to more positive potentials. Although a charge is neutralized in R394A, R394E (charge reversal) or R394Q (neutralization) do not have the same effect as R394A. However, arginine, glutamine, and glutamate, all longer than alanine, have in common an NH$_2$ or OH group at the C$\delta$ position, suggesting at this position the interaction with the tyrosine occurs. One likely possibility would be a hydrogen bond with the hydroxyl group of Tyr-485. This is supported by the fact that Arg-394 forms a very strong interaction with Tyr-485 in the E395A mutant, which disappears in both the RE-AA and the EY-AA double mutants.

A similar dependence on the size and structure of the residue is found for mutations at position Leu-398. The $V_{52}$ of the Leu-398 mutants is shifted with respect to wild type in the order L398I ($+18$ mV), L398V ($+7$ mV), L398L ($0$ mV), L398N ($-3$ mV), and L398W ($-11$ mV); of these mutations only L398I and L398V eliminated the slow off-gating component. The single difference between isoleucine and leucine is a methyl group (C$\delta_2$ in leucine) attached to the C$\beta$ instead of C$\gamma$. Asparagine, in contrast, has the identical basic structure as leucine but has polar groups attached to C$\gamma$. The replacement of C$\delta_2$ by NH$_2$ in asparagine has no significant influence on the binding to the tyrosine. The group at position C$\delta_2$, thus, appears to interact with Tyr-485 to stabilize it in the open state. The position of Leu-398 in the Kv1.2 crystal structure suggests formation of a CH–π interaction (45–47) between the aromatic ring of Tyr-485 and C$\delta_2$. Neither isoleucine nor valine contain a C$\delta_2$ group and, thus, are not positioned to develop the π interaction. Therefore, no slow component develops in these mutants. In the mutant L398W it is more likely that the bulky tryptophan residue disturbed the packing in this region (18).

From the above analysis of the results of the single positions and their interrelations emerges a picture of the mechanism of open state stabilization. After (not necessarily with a temporal delay) the movement of the voltage sensor, the electrostatic charges of Glu-395 and Arg-394 interact with the hydroxyl group of Tyr-485. This interaction leads to a conformational change, during which Tyr-485 enters a pocket formed by Arg-394, Glu-395, and Leu-398 (and possibly Val-476; Fig. 1A) where Tyr-485 is stabilized by short range interactions. The resulting conformational change is linked to pore opening. The proposed mechanism is in accordance to our results obtained with the blocker 4-AP. Although the long-reaching electrostatic interaction still develops and slows down the return of the voltage sensors to resting position in E395A, development of the interaction Leu-398–Tyr-485 is prevented by 4-AP. Thus, the open state stabilization is an integral mechanism in the final steps of pore opening in both conducting and non-conducting channels and may be linked to the concerted opening.

CONCLUSION

In this study we demonstrated the physiological role of the RELY interaction between the S4–S5 linker of one subunit with the S6 of the adjacent subunit. We established that the RELY intersubunit interaction develops open state stabilization in a functional channel under physiological conditions, giving rise to the slow off-gating component. Studying the process of elec-

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5 This would be the equilibrium of the charge carrying transition in the absence of any other transition; thus, the voltage at which forward and backward rate constants are equal (for details, see “Experimental Procedures”).
tromechanical coupling provides not only invaluable insight into the fundamentals of ion channel function but also practical understanding of certain diseases linked to mutations in voltage-gated channels. For instance, the mutation E395D, which abrogates the open state stabilization, has been described to cause a neurological disorder, episodic ataxia type I (48), that manifests in sporadic loss of motor control. This suggests that an impaired concerted opening event due to disrupted interactions at the interface of S4–S5 linker and S6 underlies one type of this disease.

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REFERENCES

1. Bezanilla, F. (2008) Nat. Rev. Mol. Cell Biol. 9, 323–332
2. Armstrong, C. M., Bezanilla, F., and Rojas, E. (1973) J. Gen. Physiol. 62, 375–391
3. Bezanilla, F., and Armstrong, C. M. (1974) Science 183, 753–754
4. Hoshi, T., Zagotta, W. N., and Aldrich, R. W. (1990) Science 250, 533–538
5. Zagotta, W. N., and Aldrich, R. W. (1990) J. Gen. Physiol. 95, 29–60
6. Perozo, E., MacKinnon, R., Bezanilla, F., and Stefani, E. (1993) Neuron 11, 353–358
7. Bezanilla, F., Perozo, E., and Stefani, E. (1994) Biophys. J. 66, 1011–1021
8. Stefani, E., Toro, L., Perozo, E., and Bezanilla, F. (1994) Biophys. J. 66, 996–1010
9. Taglaatela, M., Kirsch, G. E., VanDongen, A. M., Drew, J. A., Hartmann, H. A., Joho, R. H., Stefani, E., and Brown, A. M. (1992) Biophys. J. 62, 34–36
10. Loboda, A., and Armstrong, C. M. (2001) Biophys. J. 81, 905–916
11. Chen, F. S., Steele, D., and Fedida, D. (1997) J. Gen. Physiol. 110, 87–100
12. Varga, Z., Rayner, M. D., and Starkus, J. G. (2002) J. Gen. Physiol 119, 467–485
13. Kanevsky, M., and Aldrich, R. W. (1999) J. Gen. Physiol. 114, 215–242
14. McCormack, K., Joiner, W. J., and Heinemann, S. H. (1994) Neuron 12, 301–315
15. Zagotta, W. N., Hoshi, T., and Aldrich, R. W. (1994) J. Gen. Physiol 103, 321–362
16. Sigk, D., Qian, H., and Bezanilla, F. (1999) Biophys. J. 76, 782–803
17. Hackos, D. H., Chang, T. H., and Swartz, K. J. (2002) J. Gen. Physiol 119, 521–532
18. Soler-Llavina, G. J., Chang, T. H., and Swartz, K. J. (2006) Neuron 52, 623–634
19. Schoppa, N. E., and Sigworth, F. J. (1998) J. Gen. Physiol. 111, 313–342
20. Armstrong, C. M., and Loboda, A. (2001) Biophys. J. 81, 895–904
21. del Camino, D., Kanevsky, M., and Yellen, G. (2005) J. Gen. Physiol. 126, 419–428
22. Pathak, M., Kurtz, L., Tombola, F., and Isacoff, E. (2005) J. Gen. Physiol. 125, 57–69
23. Zagotta, W. N., Hoshi, T., Dittman, J., and Aldrich, R. W. (1994) J. Gen. Physiol. 103, 279–319
24. Ledwell, J. L., and Aldrich, R. W. (1999) J. Gen. Physiol. 113, 389–414
25. Slesinger, P. A., Jan, Y. N., and Jan, L. Y. (1993) Neuron 11, 739–749
26. Lu, Z., Klem, A. M., and Ramu, Y. (2001) Nature 413, 809–813
27. Lu, Z., Klem, A. M., and Ramu, Y. (2002) J. Gen. Physiol. 120, 663–676
28. Labro, A. J., Raes, A. L., Grottesi, A., Van Hoornick, D., Sansom, M. S., and Snyders, D. I. (2008) J. Gen. Physiol. 132, 667–680
29. Long, S. B., Campbell, E. B., and MacKinnon, R. (2005) Science 309, 897–903
30. Ding, S., and Horn, R. (2003) Biophys. J. 84, 295–305
31. Chen, J., Mitcheson, J. S., Tristani-Firouzi, M., Lin, M., and Sanguinetti, M. C. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 11277–11282
32. Decher, N., Chen, J., and Sanguinetti, M. C. (2004) J. Biol. Chem. 279, 13859–13865
33. Tristani-Firouzi, M., Chen, J., and Sanguinetti, M. C. (2002) J. Biol. Chem. 277, 18994–19000
34. Tytgat, I., and Hess, P. (1992) Nature 359, 420–423
35. Zagotta, W. N., Hoshi, T., and Aldrich, R. W. (1990) Science 250, 568–571
36. Cha, A., and Bezanilla, F. (1998) J. Gen. Physiol. 112, 391–408
37. Mannuzza, L. M., Moronne, M. M., and Isacoff, E. Y. (1996) Science 271, 213–216
38. Cha, A., and Bezanilla, F. (1997) Neuron 19, 1127–1140
39. Barghaan, J., and Bähring, R. (2009) J. Gen. Physiol. 133, 205–224
40. Starkus, J. G., Kuschel, L., Rayner, M. D., and Heinemann, S. H. (1997) J. Gen. Physiol. 110, 539–550
41. Yang, Y., Yan, Y., and Sigworth, F. J. (1997) J. Gen. Physiol. 109, 779–789
42. Starkus, J. G., Kuschel, L., Rayner, M. D., and Heinemann, S. H. (1998) J. Gen. Physiol. 112, 85–93
43. Ogilvieska, E. M., Zagotta, W. N., Hoshi, T., Heinemann, S. H., Haab, I., and Aldrich, R. W. (1995) Biophys. J. 69, 2449–2457
44. Hoshi, T., Zagotta, W. N., and Aldrich, R. W. (1994) J. Gen. Physiol. 103, 249–278
45. Umezawa, Y., Tsuboyama, S., Takahashi, H., Uzawa, J., and Nishio, M. (1999) Bioorg. Med. Chem. 7, 2021–2026
46. Coquière, D., de la Lande, A., Marti, S., Parisel, O., Prange, T., and Reinaud, O. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 10449–10454
47. Okumura, A., Sano, M., Suzuki, T., Tanaka, H., Nagao, R., Nakazato, K., Iwai, M., Adachi, H., Shen, J. R., and Enami, I. (2007) FEBS Lett. 581, 5235–5258
48. Imbrici, P., D’Adamo, M. C., Kullmann, D. M., and Pessia, M. (2006) Eur. J. Neurosci. 24, 3073–3083