AN INTERSUBUNIT INTERACTION BETWEEN S4-S5 LINKER AND S6 IS RESPONSIBLE FOR THE SLOW OFF-GATING COMPONENT IN SHAKER K+ CHANNELS

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running title : origin of slow OFF-gating

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; while 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 R394, E395 and L398 on the C-termini of the S4-S5 linker and Y485 on the S6 of 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.

INTRODUCTION

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 comprised of 6 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, which 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 Bezanilla (1)). Gating currents were first predicted by Hodgkin and Huxley and were first detected in sodium channels by Armstrong and Bezanilla (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 following small depolarizations, but rise more slowly and exhibit 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.
pulsing 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. pulsing 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 (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, while 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, rather view it as a stabilization of the open state (13).

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 conformation (10;14;20-22). However, in other mutations, the slow component was diminished in spite of 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). Since 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 in order 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 (Quikchange; 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). Prior to recording, oocytes were placed in a 3-compartment chamber containing an external...
solution (115 mM N-methyl-D-glucamine (NMDG), 10 mM HEPES, 2 mM Ca(OH)2) adjusted to pH 7.1 using MES, 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 NMDG [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:

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

(1)

Here, \(Q/Q_{\text{max}}\) is the normalized charge obtained from integrating the ON- or OFF-gating currents, \(z\) is the valence, \(F\) Faraday’s constant, \(V_{1/2}\) the voltage at which 50% of the maximal gating charge has moved from one state to the other, \(R\) the gas constant, \(T\) 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 (tetramethylrhodamine-5-maleimide [TMRM], 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 TMRM dissolved in a solution containing 115 mM KMES, 10 mM HEPES, and 2 mM CaMES2 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:

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

(2)

\[
= \frac{1}{1 + \exp\left(-\frac{V - V_{1/2}}{V_{1/2}}\right) / (dV)}
\]

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

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

(3)

according to a 3-state sequential model \(\text{closed} \leftrightarrow \text{activated(closed)} \leftrightarrow \text{open}\) with equilibrium at \(V'_{1/2}\) and \(V_{1/2}\) for the first and second transition, respectively. This follows after short derivation from eq. (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 (NMDG 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 wildtype 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
that the ON-rate constants were free to vary for all conditions for the ON-gating transition (start of the potential during deactivation). The initial constant because they all occur at the same resting potential for the OFF-gating transition the predicted values of the voltage sensor itself (C $\rightarrow$ A2) and the subsequent interactions developing during electromechanical coupling between voltage sensor and pore (A2 $\rightarrow$ A3). We, thereby, abided by the paradigms that the ON-rate constants were free to vary for all pulse voltages, while 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), 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 thus did 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:

$$A_1(t \rightarrow \infty) = \frac{I}{1 + \frac{\alpha_1}{\beta_1} \cdot (1 + \frac{\alpha_2}{\beta_2} \cdot (1 + \frac{\alpha_3}{\beta_3} \cdot \ldots (1 + \frac{\alpha_{n-1}}{\beta_{n-1}}) \ldots))}$$

(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 $Q/Q_{\text{max}} = 1 - A_1$.

This means that, if $\beta_i > \alpha_i$, all subsequent transitions $j > i$ have only 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 $A_1 < \beta_1/\beta_i$ 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, Y485 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 arginine (R) 394 and glutamate (E) 395 on the S4-S5 linker of subunit I, while more distant contacts were with valine (V) 476 on the S6 of subunit IV and leucine (L) 398 located on the N-terminal S5 of subunit I (Fig. 1A). In order 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 (MloTik). The most conserved residues were E395 (10/12) and Y485 (11/12), with the latter found even in the distantly related MloTik. The basic amino acid R394 was conserved in the Shaker / Kv1 family and both bacterial channels, KvAP and MloTik. 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. V476 and L398 were conserved in 8 and 9 out of 12 sequences, respectively. The fact that R394, E395, L398, V476 and Y485 are well-
conserved suggests 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 studied also more conservative mutations (R394Q, E395D, 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/W 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, which completely removed the slow onset and slow decay kinetics typically seen in W434F when returning from high depolarizations (see Introduction), and showed 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 E395 and L398 are represented in both the first and second groups, confirming that these two positions play a central role in the stabilization process. Thirdly, some mutations 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 (QV; Fig. 3), it becomes evident that effects on time course and on QV do not always correspond to each other. As expected from the gating current traces, QV 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/I and Y485A/H – which eliminated the slow component in the OFF-gating currents – all increased the slope factors (dV, shallower slope) of their QVs. They also had in common a reduced difference between the QON- and QOFF while their effects on the equilibrium voltage (V½) varied (Table 1; Fig. 3). The difference between QON and QOFF 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 V½ of the QV, while leaving the dV constant. The V½ was increased when the slow OFF-gating component was accelerated (R394A), and decreased when the component was slowed down (E395R) or charges 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 activated states later in the sequence are additionally stabilized, which “pulls” the sensors into the activated state and thus shifts the QV to more negative potentials.

L398W shifted the QONV to more negative potentials and increased the dV to 24 mV (Table 1, Fig. 3). Yet, L398W only developed a slow OFF-gating component at potentials higher than +60 mV, and even these were considerably accelerated in comparison to W434F (Fig. 2). The bulky tryptophan may have caused more severe changes, however. The varying effects on V½ 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 ~ -70 mV (Fig. 3 & 4B) and taper off at higher potentials. In both mutants, less Q_{OFF} charge is measured compared to 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 mV (Fig. 4B). The effect was thus contrary to that of mutants of the first group. We thus not only established that E395, L398 and Y485 are forming the open state stabilization (13) – as we can remove the interaction by mutating either one of them – but we also, with E395A/R 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 –A 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 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 mV), -E (=WT), -A (-16 mV) and -R (-30 mV), following the electrostatic charge of the residues. The diminished OFF-gating charge in E395A can neither be explained by the negative shift of the QV - 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 prior to 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 E395 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 ~97% 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. 4B & C). The kinetics of the E395A OFF-gating currents were still significantly slower than that of W434F (Fig. 4C). In contrast, Q_{ON}V gating relations of E395A were unaltered by changes in holding potential (data not shown). The Q_{OFF}V (at V_h = -120 mV) was shifted to more negative potentials by ~ -18 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 > -60 mV was significantly slower (Fig. 5A). When the resting potential was decreased from -90 mV to -120 mV, the fluorescent decay was accelerated as before the OFF-gating currents (Fig. 4D) but still significantly slower than W434F. Fitting the decay curves into two exponentials showed that E395A had an additional, 4-fold higher, slow component compared to control (τ = 190 ± 17 ms; Fig. 5A). This additional slower component was the major fraction of charge movement in E395A whereas in wildtype the faster time constant was the dominant component.

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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 double 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 in order 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 R394 and Y485 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 E395 is neutralized. Yet, the effect of mutations at position 395 is dependent on the electrostatic charge where the neutral residue (A in E395A) leads to the strongest interaction. This suggests that the residue is not directly responsible for the additional interaction energy. In order to identify the underlying molecular determinants, the region surrounding E395 was modeled based on the Kv1.2 crystal structure (29). The structures of both wildtype Kv1.2 and of the E395A mutant (E327A in Kv1.2) were compared after an energy minimization protocol which allowed residues in the surrounding of E395 to move freely while the rest of the molecule remained fixed. When glutamate 395 was replaced by alanine, a structural reorientation in the side chains of Y485 and R394 was observed (Fig. 5D). This adjustment reduced the distance between the hydroxyl group of Y485 and the guanidinium group of R394, implying a stronger interaction between these oppositely charged groups. The stronger bond may account for the voltage sensor immobilization seen experimentally with E395A.

In order to verify that the strong bond indeed results in voltage sensor immobilization, we neutralized both R394 and E395 simultaneously (R394A-E395A; RE-AA). In RE-AA, the slow OFF-gating components are preserved (Fig. 6A), and, unlike E395A, no voltage sensor immobilization was observed. The RE-AA double mutant resembles the W434F control in both gating phenotype and energetics (Fig. 6A & B). In contrast, the gating currents of the E395A-Y485A (EY-AA) double mutant lost the slow OFF-gating components (Fig. 6A) as was seen in the single mutation Y485A (Fig. 2), and exhibited QV relations similar to Y485A (Fig. 6B). Thus, in both double mutants EY-AA and ER-AA, the strong stabilization of the activated state developing in the absence of a charge at position 395 is eliminated, confirming that, in E395A, a strong bond develops between Y485 and R394. Stabilization of the activated state in the RE-AA double mutant is formed by a bond between Y485 and L398, 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. 4A & 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 elsewhere (namely, between R394 and Y485). Inspection of the Kv1.2 crystal structure indicates that V476 is located close to E395 of the adjacent subunit. We assume that V476 helps to properly position E395 in the context of an RELY intersubunit interaction. Replacement of V476 with alanine allowed E395 to swing away allowing the strong bond between R394 and Y485.

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 ascertain that the electromechanical coupling between 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 wildtype and mutant channels (E395A/D, Y485A) in response
to a series of depolarizations. Similar to the QV gating relations (Fig. 3), FV relations of E395A-A359C were negatively shifted compared to control, confirming 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’s QV relations. In Y485A-A359C, a shift to more positive potentials and a shallower slope of the GV curve compared to A359C was observed. QV and GV were, thus, further separated than in wildtype and the voltage dependence was less steep.

Nevertheless, since 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 Y485 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 Boltzmann 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 Experimental Procedures). This indicated that pore opening is governed 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 dependencies 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 (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 R394, E395, L398 and Y485, 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).

In order to confirm that the RELY interaction develops also under physiological conditions of ion permeation, we carried out fluorescence voltage-clamp measurements of the conducting mutants as well as their respective wildtype 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 wildtype 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 wildtype and T449Y was observed. Comparison of the fluorescence decay to W434F revealed, however, that wildtype fluorescence initially returned significantly faster than W434F fluorescence (Fig. 8C). The fast initial decay was
followed by a slower component, which is comparable to (or slower than) W434F-M356C. Accordingly, we found two time constants each for wildtype and W434F. In W434F, the time constants were slower in comparison to wildtype 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 to 40% in wildtype (Fig. 8B). The time constants found in the OFF-gating currents of W434F were consistent with the major faster component in the OFF-fluorescence decay. The slow component found in the OFF-gating current kinetics of W434F thus seems to be represented by the slower fluorescence component in wildtype and the faster one in W434F.

We next compared E395A, E395D and Y485A fluorescence data with wildtype in order to confirm that their effects on the gating currents persist in the conducting channel. The stabilization of the voltage sensors was evident when comparing the fluorescence decay of E395A in the conducting (A359C or M356C-T449Y) channels to their respective controls (Fig. 7B). The voltage sensor movement upon return from depolarizations > -60 mV was, like in the non-conducting mutant, significantly slower. No C-type inactivation was observed during the time course of the depolarizing pulse, indicating that the voltage sensors are still immobilized under physiological ion conduction and this immobilization is independent of C-type inactivation.

When comparing the fluorescence signals of Y485A and E395D with wildtype 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 wildtype 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+ channel blocker 4-aminopyridine (4-AP) on gating currents (W434F) and ionic currents (wildtype) 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 wildtype, ionic current was blocked by 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 R394 and Y485 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 wildtype. 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 Y485 represent late closed states in the activation pathway: a 5-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) GVs which follow a double Boltzmann characteristic. These effects have to be explained. We, therefore, fitted the gating current traces to a 5-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 (suppl. 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 (C$\rightarrow$A1) and -83.4 mV (A1$\rightarrow$A2) (Suppl. Fig. 1). The last transition (A3$\rightarrow$AS) 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. In E395A, we found two differences compared to W434F; first, the last transition from A3$\rightarrow$AS 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 $V_{1/2}$ of the C$\rightarrow$A1 transition was shifted to -38 mV. The $V_{1/2}$ of the second transition (A1$\rightarrow$A2) 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$\rightarrow$A1 transition was – although slightly accelerated – similar to W434F with a $V_{1/2}$ 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 wildtype. 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 Y485. While E395A stabilizes the final open state (AS) strongly, in Y485A, the rate constants suggest that the state is never entered, confirming 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 (R394, E395, L398) 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 E395, L398 and Y485 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, E395 and L398, fall within the region mapped by Soler-Llavia et al. (18) to disturb electromechanical coupling. Y485 is part of the YFYH 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 Y485 for the open state stabilization that we suggest here, in particular as the authors describe that the V½ of the GV was not shifted in this channel thus verifying that the electromechanical coupling is not interrupted by mutating Y485 (30). As for F481, it is located directly above Y485 such that it is likely that the bulky tryptophane 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. Due to the lack of open state stabilization, the last transition has a shallower voltage-dependence, leading to the double Boltzmann GVs of Y485A and E395D, although the QV remained the same.

The different effects of the Y485A and E395D mutations on QV and GV are explained by the different dependencies 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

\[ Q = l \left( 1 - \frac{I}{I + \frac{a}{\delta} \left( 1 + \frac{\gamma}{\delta} \right)} \right) \]  

(5)

whereas the conductance will be given by \( A_S \):

\[ G = A_S = \frac{l}{I + \frac{\delta}{\gamma} \left( 1 + \frac{\gamma}{\delta} \right)} \]  

(6)

As outlined in the Experimental Procedures section, subsequent transitions cannot shift the equilibrium (and thus the QV) to more positive potentials than the equilibrium of the charge carrying transition \( V_{1/2} = \beta \) 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 QV) to more negative potentials. The most positive \( V_{1/2} \) observed were -28 mV and -32 mV for L398I and R394A, respectively. A value close to these potentials should, thus, be smaller or equal to the “native” \( V_{1/2} \) 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 \( V_{1/2} \) of the GV 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 GV, in contrast, is controlled primarily by the last transition. According to equation (6), an earlier transition

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1 H486A of the YFYH motif did not have any effect.

2 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 refer to Experimental Procedures).
position E395D and Y485A mutants explain the opposing effects on GV and QV in the electromechanical coupling, we are thus able to just by changing the subsequent transitions of electromechanical coupling, we are thus able to explain the opposing effects on GV and QV in the E395D and Y485A mutants.

In this context, the fact that mutating position E395 leads to increasing shift of the V½ of the QVs following the electrostatic charge of the residue (E395D +5 mV, -E 0 mV, -A -16 mV, -R -30 mV) suggests that a more negative charge at this position stabilizes the activated state of the voltage sensor. Mutations at this position had a strong influence on the V½ of the QV while elimination of open state stabilization in E395D, L398V/I or Y485A/H/C only had small effects on the QV relations. 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 QV, 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 prior to the actual open state stabilization. Considering that the Y485F mutant did not express, which indicates an important role for Y485’s hydroxyl group, it is likely that the electrostatic interaction between E395, R394 and the polar hydroxyl group of Y485 is responsible for the dependence on the electrostatic charge of E395. Accordingly, in the EY-AA double mutant no significant shift of the QV was observed.

For the positions L398 and R394, 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 QV 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, asparagine and glutamate – all longer than alanine – have in common an –NH₂ or -OH group at the Cδ position suggesting at this position the interaction with the tyrosine occurs. One likely possibility would be a hydrogen bond with the hydroxyl group of Y485. This is supported by the fact that R394 forms a very strong interaction with Y485 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 L398. The V½ of the L398 mutants is shifted with respect to wildtype in the order L398I (+18 mV), -V (+7 mV), -L (0 mV), -N (-3 mV) and -W (-11 mV); of these mutations only L398I and -V eliminated the slow OFF-gating component. The single difference between isoleucine and leucine is a methyl group (Cδ2 in leucine) attached to the Cβ instead of Cγ. Asparagine, in contrast, has the identical basic structure as leucine but has polar groups attached to Cδ2, asparagine has no significant influence on the binding to the tyrosine. The group at position Cδ2, thus, appears to interact with Y485 in order to stabilize it in the open state. The position of L398 in the Kv1.2 crystal structure suggests formation of a CH-π-interaction (45-47) between the aromatic ring of Y485 and Cδ2. Neither isoleucine nor valine contain a Cδ2 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 tryptophane 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 E395 and R394 interact with the hydroxyl group of Y485. This interaction leads to a conformational change, during which Y485 enters a pocket formed by R394, E395 and L398 (and possibly V476; Fig. 1A) where Y485 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. While 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 L398-Y485 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 electromechanical 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), which 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.

Reference List
**Figure Legends**

**Figure 1.** Candidate interactions among residues on the S4-S5 linker and S6 of adjacent subunits in Shaker K⁺ channels.

(A) Side chains of the candidate amino acids (numbered as in Shaker) shown in stick (left) and spacefilling (middle, right) representation in the Kv1.2 crystal structure (29). The S4-S5 linker and S6 of one subunit (I) and the S6 C-terminus of the adjacent subunit (IV) are shown. Rightmost image shows the same region as the other structures except it is rotated by ~180°. (B) Alignment of sequences spanning the S4-S5 linker, N-terminal S5, and S6 of Drosophila (Shaker, Shab, Shaw, Shal), mammalian (Kv), and bacterial (KvAP, MLotiK) potassium channels. Using Shaker as a template, amino acids commonly conserved in the other channels are bolded, and residues involved in an S4-S5 linker/S6 interaction, shown in (A), are highlighted in yellow.

**Figure 2.** Gating current profiles in Shaker mutants.

Gating current traces representative of each mutation are shown following subtraction of capacitive currents using a P/4 protocol. Cut-open oocytes expressing W434F (control) or the various mutants were held at -90 mV and depolarizations were made in 10 mV increments up to +60 mV to +100 mV.

**Figure 3.** Voltage dependence of gating charge (Q) in Shaker mutants.

Boltzmann fits comparing normalized gating charge (Q) as a function of voltage in cut-open oocytes expressing W434F control (gray) of the various mutants as labeled in the graphs. For E395A, the maximum OFF-gating charge was normalized to the average, normalized OFF gating charge of W434F measured at the same depolarization.

**Figure 4.** Recovery of off-gating charge by reducing resting potential to -120 mV in E395A and V476A.

(A) Q_{OFF}/Q_{ON} ratio as a function of voltage in E395A (left; n=4) and V476A (right; n=3). Black squares, W434F, V_h = -90 mV, n=5; red filled circles, E395A/V476A V_h = -90 mV; red hollow circles, E395A/V476A V_h = -120 mV. Error bars show s.d. (B) At a resting potential of -120 mV the voltage sensors are released from their immobilized state and allowed fitting of the data into a single Boltzmann curve. OFF-gating charge and voltage relations between W434F (n=5) and E395A (left; n=4) and V476A (right; n=3), respectively. Black squares, W434F; red filled circles, E395A/V476A V_h = -90 mV; red empty circles, E395A/V476A V_h = -120 mV. Error bars show s.d. (C) Representative gating traces elicited in the E395A mutant at a resting potential of V_h = -120 mV (top). (bottom) comparison of OFF-gating between W434F (black) and W434F-E395A (red). (D) The fluorescence trace compares voltage sensor movement after various repolarizations as shown after depolarization to +20 mV. (bottom) Comparison of OFF-gating currents for different holding potentials (black trace, V_h = -90 mV; red trace, V_h = -120 mV).

**Figure 5.** Charge immobilization of the voltage sensor in the E395A-W434F Shaker mutant.

(A) (left) Voltage-clamp fluorometry results comparing W434F and E395A. Shown are representative fluorescent signals after depolarizations to +20, -20, and -60 mV (from V_h = -90 mV) for W434F (black traces) and E395A (red traces). (right) The time constants of the fluorescent decay curves during repolarization were plotted as a function of voltage (black A359C-W434F; red E395A-A359C-
W434F, $V_h = -90$ mV, $n=5$ or 6 for W434F and E395A respectively, error bars show s.d.). (B) (left) Representative gating currents of cut-open oocytes expressing W434F or E395A after two sequential saturating depolarizations at +20 mV. The interpulse interval ranged from 1 to 301 ms in 25 ms steps. Capacitive currents were subtracted with P/4 protocol. (right) Plot of the maximum amplitude of the ON-gating current after the second depolarizing pulse (P2) normalized to the ON-gating current following the first pulse (P1) as a function of the interpulse interval. Results were fit to an exponential curve with $\tau = 7$ ms (W434F, black squares) and $\tau = 70$ ms (E395A, red circles) ($n=5$, error bars show s.d.). (C) (left) Two pulse protocol varying the duration of the first depolarizing pulse at -60 mV from 20 ms to 200 ms in 20 ms intervals. Capacitive currents were subtracted with P/4 protocol. Shown are gating currents produced in cut-open oocytes expressing W434F or E395A. (right) Maximum amplitude after P2 normalized to that after P1 plotted as a function of the duration of P1. Average data were fit into an exponential decay function with one time constant $\tau_1$ values were 235.7 and 61.4 ms for W434F (black squares) and E395A (red circles), respectively ($n=4$, error bars show s.d.). (D) S4-S6 regions of wildtype and E327A mutant Kv1.2 (E395A in Shaker) were subjected to an energy minimization protocol. Molecular backbones of the minimized WT (green) and E327A (orange) mutant structures were superimposed. Residue numbering is according to Shaker.

Figure 6. Gating current profiles in Shaker double mutants.

(A) Gating current traces representative of R394A-E395A (RE-AA) and E395A-Y485A (EY-AA) double mutants are shown following subtraction of capacitive currents using a P/4 protocol. Oocytes expressing W434F (control) or a double mutant were held at a resting potential of -90 mV and depolarizing pulses in 10 mV increments were applied. (B) Boltzmann fits of ON- and OFF-gating currents in cut-open oocytes expressing RE-AA (red, $n=5$) and EY-AA (blue, $n=5$) versus W434F control (gray, $n=5$). Error bars show s.d.

Figure 7. Electromechanical uncoupling of the voltage sensor and pore in Y485A and E395D mutants.

(A) Plots of conductance ($\Delta G/G_{\text{max}}$) and fluorescence ($\Delta F/F_{\text{max}}$) as a function of voltage in E395A-A359C, Y485A-A359C, and E395D-A359C mutants. Black filled and hollow symbols represent G and F of the mutants (E395A, G: $n=8$, F: $n=4$; Y485A, G: $n=6$, F: $n=8$; E395D, G: $n=6$; F: $n=6$; error bars show s.d.). (B) Voltage-clamp fluorometry results comparing A359C with Y485A-A359C and E395D-A359C (right) and comparing M356C-T449Y with E395A-M356C-T449Y (left). Shown are representative fluorescent signals after depolarizations to +20 and -20 mV (from $V_h = -90$ mV) of mutant as shown (red traces) compared to their respective control (black traces).

Figure 8. Voltage sensor movement in the presence of permeating ions.

(A) Time constants of double exponential fits to fluorescence decay after depolarizing pulses for ShakerIR-M356C (black), -M356C-T449Y (red) and -M356C-W434F (blue). (B) Fraction of the slower time constant in fluorescence traces analyzed in (A). (C) Comparison of two fluorescence decays after a depolarizing pulse to +20 mV (Shaker-IR-M356C (WT) red; -M356C-W434F black).

Figure 9. Slow OFF gating components during voltage sensor deactivation are partially restored by 4-aminopyridine (4-AP) in the E395A mutant.

(A) Representative gating current traces in W434F control, E395A, and Y485A mutants before and after addition of 10 mM 4-AP (W434F, $n=4$; E395A, $n=3$; Y485A, $n=5$). (B) 5-state model used to fit the data (left; see Experimental Procedures) and reduced model combining all charge carrying steps into the first transition (right). (C) (left) GV and double Boltzmann fit of ShakerIR-Y485A. The dashed lines
visualize the two components of the double Boltzmann relation; (right) Modeling the effect of destabilization of the last transition on QV and GV. The first two transitions of the reduced model were left unaltered whereas the values from the fit (left; $V_{1/2} = 14.8$ mV, $dV = 38$ mV) were used for the last transition (red, Y485A, blue WT, solid line QV, dashed line GV; see Discussion for details).

**Table 1. Summary of gating current results in Shaker mutants.**

QV were fit into a single Boltzmann distribution as described in Methods. $V_{1/2}$ represents the voltage at which 50% of maximal charge has been moved. $dV$ represents the slope of the Boltzmann curve. $\Delta V_{1/2}$ represents the difference in $V_{1/2}$ between the respective mutant and W434F non-conducting control. N.D. indicates that the OFF-gating curves could not be fit into a single Boltzmann function. § signifies mutants in which gating currents were not detected.

**Table 2. Summary of ionic current and fluorescence results in Shaker mutants.**

GV and FV relations were fit into a single Boltzmann distribution as described in Methods. $V_{1/2}$ represents the voltage at which 50% of 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.
**Batulan et al. Figure 1**

A

![Diagram with labeled amino acids and positions](image)

B

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Batulan et al. Figure 2

W434F 1 µA
R394A 0.5 µA
R394Q 2 µA

E395A 2 µA
E395R 1 µA
E395D 0.5 µA

L398N 5 µA
L398W 1 µA
L398V 1 µA

V476A 1 µA
Y485A 1 µA
Y485H 1 µA
Batulan et al. Figure 9

A

10 mM 4-AP

Before

1 μA
10 ms

After

2 μA
10 ms

W434F

E395A

Y485A

B

C \overset{0.45Q}{\leftrightarrow} A_1 \overset{0.55Q}{\leftrightarrow} A_2 \overset{\beta}{\leftrightarrow} A_3 \overset{\gamma}{\leftrightarrow} A_S \overset{Q}{\alpha} C \overset{\delta}{\leftrightarrow} A_2 \overset{\gamma}{\leftrightarrow} A_3 \overset{\delta}{\leftrightarrow} A_S

C

$\frac{G}{G_{\text{max}}}$ vs. membrane potential (mV)

$\frac{Q}{Q_{\text{max}}}$ vs. membrane potential (mV)
<table>
<thead>
<tr>
<th>Shaker construct</th>
<th>$I_{gating}$</th>
<th>$Q_{ON}$</th>
<th>$Q_{OFF}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{1/2}$ (mV)</td>
<td>$dV$ (mV)</td>
<td>$\Delta V_{1/2}$ (mV)</td>
</tr>
<tr>
<td>W434F</td>
<td>-45.9±0.8</td>
<td>8.7±0.7</td>
<td>-53.2±0.7</td>
</tr>
<tr>
<td>R394A</td>
<td>-32.5±0.7</td>
<td>6.3±0.6</td>
<td>+13.4</td>
</tr>
<tr>
<td>R394Q</td>
<td>-47.9±1.3</td>
<td>8.4±1.1</td>
<td>-2.0</td>
</tr>
<tr>
<td>R394E</td>
<td>-46.0±1.0</td>
<td>7.7±0.9</td>
<td>-0.1</td>
</tr>
<tr>
<td>E395A</td>
<td>-62.2±0.7</td>
<td>7.1±0.6</td>
<td>-16.3</td>
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<tr>
<td>(Vh = -90 mV)</td>
<td>-62.3±0.5</td>
<td>7.8±0.4</td>
<td>-16.4</td>
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<tr>
<td>(Vh = -120 mV)</td>
<td>-40.8±0.7</td>
<td>11.6±0.6</td>
<td>+5.1</td>
</tr>
<tr>
<td>E395D</td>
<td>-76.2±0.7</td>
<td>9.1±0.6</td>
<td>-30.3</td>
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<tr>
<td>E395R</td>
<td>-28.0±1.9</td>
<td>11.0±2.1</td>
<td>+17.9</td>
</tr>
<tr>
<td>L398A</td>
<td>§</td>
<td>§</td>
<td>§</td>
</tr>
<tr>
<td>L398N</td>
<td>-48.8±0.4</td>
<td>9.7±0.4</td>
<td>-2.9</td>
</tr>
<tr>
<td>L398V</td>
<td>-38.9±1.0</td>
<td>12.1±0.8</td>
<td>+7.0</td>
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<tr>
<td>L398I</td>
<td>-59.4±1.9</td>
<td>24.4±1.4</td>
<td>-13.5</td>
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<tr>
<td>V476A</td>
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<td>9.1±1.4</td>
<td>-19.6</td>
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<tr>
<td>(Vh = -90 mV)</td>
<td>-60.9±0.7</td>
<td>7.6±0.6</td>
<td>-15.0</td>
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<tr>
<td>(Vh = -120 mV)</td>
<td>-48.9±0.6</td>
<td>10.8±0.5</td>
<td>-3.0</td>
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<tr>
<td>Y485A</td>
<td>§</td>
<td>§</td>
<td>§</td>
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<tr>
<td>Y485H</td>
<td>-53.8±0.4</td>
<td>10.4±0.4</td>
<td>-7.9</td>
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<tr>
<td>R394A-E395A</td>
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<td>R394E-E395R</td>
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<td>6.3±0.3</td>
<td>-18.3</td>
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<tr>
<td>E395A-Y485A</td>
<td>-51.9±0.6</td>
<td>12.7±0.6</td>
<td>-6.0</td>
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</tbody>
</table>
Table 2

<table>
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<tr>
<th>Shaker construct</th>
<th>$I_{ionic}$</th>
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<th>$\Delta F/F_{max}$</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$V_{1/2}$ (mV)</td>
<td>$dV$ (mV)</td>
<td>$\Delta V_{1/2}$ (mV)</td>
<td>$n$</td>
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<tr>
<td>A359C</td>
<td>-21.0±1.2</td>
<td>15.9±1.0</td>
<td>-39.6±2.7</td>
<td>20.9±2.3</td>
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<tr>
<td>E395A-A359C</td>
<td>-52.1±0.6</td>
<td>7.4±0.5</td>
<td>-58.4±1.3</td>
<td>11.1±0.1.1</td>
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<tr>
<td>E395D-A359C</td>
<td>-14.9±1.8</td>
<td>11.0±1.8</td>
<td>-34.8±0.3</td>
<td>12.9±0.3</td>
</tr>
<tr>
<td>Y485A-A359C</td>
<td>-17.3±1.8</td>
<td>11±1.4</td>
<td>-33.9±0.6</td>
<td>15.1±0.6</td>
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<tr>
<td>A359C-W434F</td>
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<td>-33.7±0.3</td>
<td>12.5±0.3</td>
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<tr>
<td>E395A-A359C-W434F</td>
<td>—</td>
<td>—</td>
<td>-56.2±0.5</td>
<td>12.3±0.4</td>
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</tbody>
</table>
An intersubunit interaction between S4-S5 linker and S6 is responsible for the slow off-gating component in shaker K+ channels
Zarah Batulan, Georges Anthony Haddad and Rikard Blunck

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