Mode shift of the voltage sensors in Shaker K^+ channels is caused by energetic coupling to the pore domain

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The voltage sensors of voltage-gated ion channels undergo a conformational change upon depolarization of the membrane that leads to pore opening. This conformational change can be measured as gating currents and is thought to be transferred to the pore domain via an annealing of the covalent link between voltage sensor and pore (S4-S5 linker) and the C terminus of the pore domain (S6). Upon prolonged depolarizations, the voltage dependence of the charge movement shifts to more hyperpolarized potentials. This mode shift had been linked to C-type inactivation but has recently been suggested to be caused by a relaxation of the voltage sensor itself. In this study, we identified two ShakerIR mutations in the S4-S5 linker (I384N) and S6 (F484G) that, when mutated, completely uncouple voltage sensor movement from pore opening. Using these mutants, we show that the pore transfers energy onto the voltage sensor and that uncoupling the pore from the voltage sensor leads the voltage sensors to be activated at more negative potentials. This uncoupling also eliminates the mode shift occurring during prolonged depolarizations, indicating that the pore influences entry into the mode shift. Using voltage-clamp fluorometry, we identified that the slow conformational change of the S4 previously correlated with the mode shift disappears when uncoupling the pore. The effects can be explained by a mechanical load that is imposed upon the voltage sensors by the pore domain and allosterically modulates its conformation. Mode shift is caused by the stabilization of the open state but leads to a conformational change in the voltage sensor.

The Journal of General Physiology

INTRODUCTION

In the gating mechanism of the voltage-gated ion channels, a depolarization of the membrane prompts voltage sensors (transmembrane helices S1-S4) to undergo conformational changes, which then trigger the central ion conducting pore (transmembrane helices S5-S6) to open. The conformational changes of the voltage sensor are manifested as gating currents, small transient currents caused by the movement of the positively charged arginines of the fourth transmembrane domain S4 through the electric field. After prolonged depolarization more positive than −60 mV, the voltage sensors require stronger hyperpolarizations to return to their resting position than the voltage required to move the voltage sensors to the activated state. As a consequence, the voltage dependence of the gating charges Q that are moved upon polarization to varying voltages V (QV) shifts to more negative potentials compared with the QV obtained when holding the resting potential at −90 mV. This behavior is referred to as mode shift.

The occurrence of the mode shift in voltage-dependent ion channels is well documented. It was first observed in QVs from squid axons (Bezanilla et al., 1982a) and was later also described for potassium channels (Fedida et al., 1996; Olcese et al., 1997, 2001; Piper et al., 2003), sodium channels (Bezanilla et al., 1982a; Kuzmenkin et al., 2004), hyperpolarization-activated cyclic nucleotide–gated (HCN) channels (Männikkö et al., 2005; Bruening-Wright and Larsson, 2007), and calcium channels (Brum and Rios, 1987; Shirokov et al., 1992).

In HCN channels, the mode shift is more distinct (Männikkö et al., 2005; Bruening-Wright and Larsson, 2007; Bruening-Wright et al., 2007) and is required for a stable rhythmic firing of pacemaker cells. However, the physiological role of the mode shift in the voltage-gated K^+ channel Shaker from *Drosophila melanogaster* and its human homologues, the Kv1 channels, is not yet fully understood. It is likely that the mode shift ensures rapid repolarization to the resting potential, as the Kv1 channels are responsible for the repolarization of the membrane after an exciting potential and protect against too fast reactivation (Geiger and Jonas, 2000; Sutachan et al., 2005). The channels start to open around −50 mV after the onset of an action potential, and without an additional mechanism, the forward and backward movement of gating charges should occur at the identical potentials. However, the mode shift of the QV makes certain that the Kv channels do not close or recover from inactivation before the resting potential of −70 mV

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Abbreviations used in this paper: Ci-VSP, *C. intestinalis* voltage sensor–containing phosphatase; HCN, hyperpolarization-activated cyclic nucleotide gated; HERG, human ether-a-go-go–related gene.

The Rockefeller University Press $30.00
J. Gen. Physiol. Vol. 137 No. 5 455–472
www.jgp.org/cgi/doi/10.1085/jgp.201010573

has been reached again, thus preventing too early closure and too rapid firing, respectively. At the resting potential, the channels are thus closed under steady-state conditions but are open (or inactivated) after a prolonged excitation to readjust the resting potential.

The mode shift has been correlated with slow C-type inactivation. In contrast to N-type inactivation, which is caused by the N-terminal ball peptide blocking the pore from the cytosolic entry (Bezanilla and Armstrong, 1977; Hoshi et al., 1990; Zagotta et al., 1990), in C-type inactivation, the selectivity filter itself enters a nonconductive conformation (Ehrenstein and Gilbert, 1966; Choi et al., 1991; Hoshi et al., 1991; Yellen et al., 1994; Blunck et al., 2006; Cordero-Morales et al., 2006; Cuello et al., 2010a,b). The time course of developing the mode shift has been shown to coincide with the time course of entering the inactivated state (Olcese et al., 1997). It has also been observed in the ShakerIR-T449V-I470C mutant (Holmgren et al., 1997), which is thought to enter into a conducting state with a conformation similar to that of C-type inactivation. However, a noninactivating mutant of the HERG (human ether-a-go-go–related gene) channel also shifts the QV (Piper et al., 2003), as well as the noninactivating HCN channels (Männikkö et al., 2005; Bruening-Wright and Larsson, 2007; Bruening-Wright et al., 2007), indicating that, although C-type inactivation is correlated with the mode shift, inactivation is not a prerequisite for the shift.

A recent discovery of the mode shift of the QV even in voltage-dependent phosphatases of *Ciona intestinalis* (Ci-VSP [*C. intestinalis* voltage sensor–containing phosphatase]; Murata et al., 2005) has led to the suggestion that the shift is an intrinsic property of the voltage sensor caused by a relaxation of the S4 from a 3_{10} to an α-helical conformation (Villalba-Galea et al., 2008). Ci-VSP consists of the voltage sensor domain (S1-S4) linked to a phosphatase whose activity is governed by the membrane potential. Thus, it does not contain a pore domain, and no C-type inactivation may be defined for this protein. Therefore, an influence of the pore domain is unlikely, unless it is replaced by the enzymatic domain. In addition, the shift is still observed in the isolated voltage sensor of Ci-VSP after truncation of the enzymatic domain (Villalba-Galea et al., 2008). Findings by Bruening-Wright and Larsson (2007) support the idea that the mode shift occurs in the S4. They identified a slow conformational change in the S4 of HCN channels that coincides with the shift from one mode to the other. A similar conformational change has been found in the fluorescence signal of Ci-VSP (Villalba-Galea et al., 2008).

To resolve the apparent contradiction between the dependence of the mode shift on the state of the pore and its occurrence in an isolated voltage sensor, we investigated the influence of the coupling between pore domain and voltage sensor (electromechanical coupling) on the development of the mode shift. The molecular determinants underlying the electromechanical coupling between the voltage sensor movement and opening of the pore in voltage-gated ion channels has been narrowed down to an interaction between the S4-S5 linker, the only covalent link between voltage sensor and pore, and the C-terminal end of the S6 either of the same (Chen et al., 2001; Lu et al., 2001, 2002; Tristani-Firouzi et al., 2002; Decher et al., 2004; Long et al., 2005b; Labro et al., 2008; Muroi et al., 2010) or the neighboring subunit (Batulan et al., 2010). The annealing between the S4-S5 linker and the S6 is one of several close contacts in the tertiary and quartiary structure observed in the crystal structure of the human homologue to Shaker Kv1.2 (Long et al., 2005a). In this study, we used mutations that fully uncouple the voltage sensor movement from pore opening to investigate to what extent the pore domain influences the conformation of the voltage sensor.

MATERIALS AND METHODS

Molecular biology and channel expression

Experiments for this study were obtained using the ShakerIR-H4 channel, in which N-type inactivation was removed by deletion (Δ6–46; Hoshi et al., 1990). We also used the nonconducting Shaker-H4-Δ(6–46)-W434F (Perozo et al., 1993) for gating current measurements. For simultaneous fluorescence measurements, the mutation A359C was introduced. Point mutations were generated using site-directed mutagenesis (QuikChange; Agilent Technologies), and sequences were verified in a sequencing facility. Oocytes from *Xenopus laevis* were surgically obtained. Follicular membrane was removed according to a standard collagenase treatment. cRNA was in vitro transcribed (mMachine T7; Invitrogen), and 46 nl was injected into each oocyte with concentrations of 0.1–1 μg/μl. After injection, oocytes were incubated in Barth solution at 18°C for 1–3 d before electrophysiological recordings.

Electrophysiology and voltage-clamp fluorometry

Measurements were performed with the cut-open voltage-clamp fluorometry technique for spatial voltage homogeneity and fast temporal resolution (Taglialatela et al., 1992; Cha and Bezanilla, 1997; Batulan et al., 2010). Macroscopic currents were recorded and registered using GPatch acquisition software (Department of Anesthesiology, University of California, Los Angeles, Los Angeles, CA). Oocytes were placed in a three-part chamber, top, middle, and bottom, containing an external solution (115 mM NMDG, 10 mM Hepes, and 2 mM $Ca(OH)_2$) adjusted to pH 7.1 using MES (methanesulfonic acid), and then permeabilized by exchanging external solution in the bottom chamber with 0.2% saponin so the current could be injected directly into the oocyte. Saponin was replaced by an internal solution (10 mM Hepes, 2 mM EDTA, and 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 performed at room temperature.

Capacitive current was subtracted from the current traces with the P/4 protocol. For gating currents, oocytes were held at −90 mV, −120 mV (I384C), or −140 mV (I384A) with pulse potential starting from −120 mV ranging to 50 mV and 180 mV in 10-mV increments. Gating and ionic current were analyzed with Analysis software (Department of Anesthesiology, University of California, Los Angeles).

The total gating charge was calculated by integrating the gating current as a function of time. The relation between the gating charge (Q) as a function of voltage (V) was fit, using commercial software, to a single Boltzmann equation: $Q/Q_{max} = (1 + \exp(-(V - V_{1/2})/dV))^{-1}$, with dV being the slope factor and $V_{1/2}$ the potential with $Q/Q_{max} = 0.5$. Correspondingly, the conductance (G) as a function of voltage (V) was fit to $G/G_{max} = (1 + \exp(-(V - V_{1/2})/dV))^{-1}$, with dV being the slope factor and $V_{1/2}$ the potential with $G/G_{max} = 0.5$. Significance of shifts between QV or GV relations was determined by one-way ANOVA.

RESULTS

Shaker mutations I384N and F484G uncouple the voltage sensor movement from pore opening

Our first goal was to identify mutations that fully uncouple the pore movement from the voltage sensors in the coupling region of the Shaker K^+ channel but not inside the voltage sensor or charge transfer center. Mutations in the lower S4 (Smith-Maxwell et al., 1998; Ledwell and Aldrich, 1999; Silverman et al., 2003; Tao et al., 2010) tend to change the intrinsic transitions and properties of the voltage sensor itself and thus cannot be used to conclusively determine the effect of the coupling between the voltage sensor and the pore. The energy generated by the voltage sensor has been suggested to be transferred to the pore domain by a link between the S4-S5 linker and the C terminus of the S6 beyond the PVP kink. We thus looked in the crystal structure of the human homologue of Shaker, Kv1.2, for possible candidates in that region to fully uncouple voltage sensor and pore within one subunit. We found, as the most likely interaction partners, the isoleucine I384 on the N-terminal S4-S5 linker and the two phenylalanines F481 and F484 on the S6 (Fig. 1 A). The corresponding residue in Kv1.5, I422, forms together with F519 and F522 a hydrophobic contact to promote voltage sensor–pore coupling (Labro et al., 2008). In Shaker, according to the interacting regions found in the crystal structure of Kv1.2, I384 seems to fit snuggly into a hydrophobic pocket formed by the two phenylalanines F481 and F484. All three residues I384, F481, and F484 are close to the regions identified by Lu et al. (2001, 2002) as important for electromechanical coupling in Kv channels. F481 and F484 are located within the region N480-Y485, whereas I384 is positioned one residue before the interacting region L385-S392 on the S4-S5 linker.

To achieve uncoupling, the isoleucine I384 and the two phenylalanines F481 and F484 were then mutated in the ShakerIR-W434F background to an asparagine (I384N) and glycines (F481G and F484G), respectively. Compared with isoleucines, asparagines have a similar size but are more hydrophilic and thus likely disturb the suspected hydrophobic interaction. In contrast, the phenylalanines were replaced with smaller-sized glycines to remove their aromatic ring that reach, like a lever, deep into the contact area. Out of the three mutants I384N-W434F, F481G-W434F, and F484G-W434F, we were able to obtain gating currents only from the two mutants, ShakerIR-W434F-I384N and ShakerIR-W434F-F484G (Fig. 1 B).

The analysis of the gating currents revealed that the QV, the relation between integrated gating charge and depolarization potential, of both mutants was shifted to more negative potentials ($V_{1/2} = -68.5 \pm 0.6$ mV and -67.7 ± 0.6 mV for I384N and F484G, respectively; Fig. 1 C and Table I), indicating that the voltage sensor requires less energy to transit to the activated state or, depending on the perspective, more energy to return to the resting state. When we determined the voltage dependence of the pore opening in the corresponding conducting ShakerIR-A359C mutants, we found that the conductance voltage relations (GV) of I384N and F484G were, in contrast to the QVs, far shifted to positive potentials (Fig. 1 D). For pulses up to 200 mV, the conductance did not even saturate. We thus could not define a $V_{1/2}$ for these mutants.

The amplitude of the ionic currents was significantly lower than generally observed in other mutants. This might of course simply be the result of lower expression efficiency but could also result from very low open probability, and indeed, for I384N, we were able to measure gating and ionic currents simultaneously, confirming a low open probability (Fig. 2 A). From their respective amplitudes, we calculated the open probability. Under the assumption that in this mutant, as in wild-type Shaker, during the activation of each channel 13 e are moved through the electric field, we can determine the number of channels present in the clamped membrane from the total gating charge measured for a saturating depolarizing pulse. Assuming a conductance of 11 pS (Lopez et al., 1994), we can also determine how many channels are open at 195 mV. From both results, we deduced that only ~0.8% of the channels present were open at 195 mV.

The drastic separation between QV and GV indicates that in the mutants I384N and F484G, the voltage sensor movement is no longer energetically coupled to pore opening because the range in which the voltage sensor movement occurred (less than −10 mV) hardly overlaps with the range in which the pore opens. The latter only begins at −20 mV to 0 mV for I384N and F484G, respectively, and does not saturate at potentials more negative than 200 mV. As the conductance still increases significantly although the charge movement is already saturated, it follows that voltage sensor activation does not lead directly to pore opening anymore. If the energetic coupling would have remained the same and only one transition would have been changed, both QV and GV would have been shifted in the same direction.

To confirm that the mutants energetically uncouple the voltage sensor from the pore also in the conducting mutant, we compared the fluorescence voltage relationships (FV) of the conducting ShakerIR-A359C-I384N and ShakerIR-A359C-F484G mutants to their GVs

obtained simultaneously. We first labeled the cysteine in the S3-S4 linker at position A359C with tetramethylrhodamine maleimide such that fluorescence intensity reports the state of the voltage sensor (Mannuzzu et al., 1996; Cha and Bezanilla, 1997; Batulan et al., 2010). Simultaneous monitoring of the gating (FV) and conductance (GV) of ShakerIR-A359C, ShakerIR-A359C-I384N, and ShakerIR-A359C-F484G revealed that in the

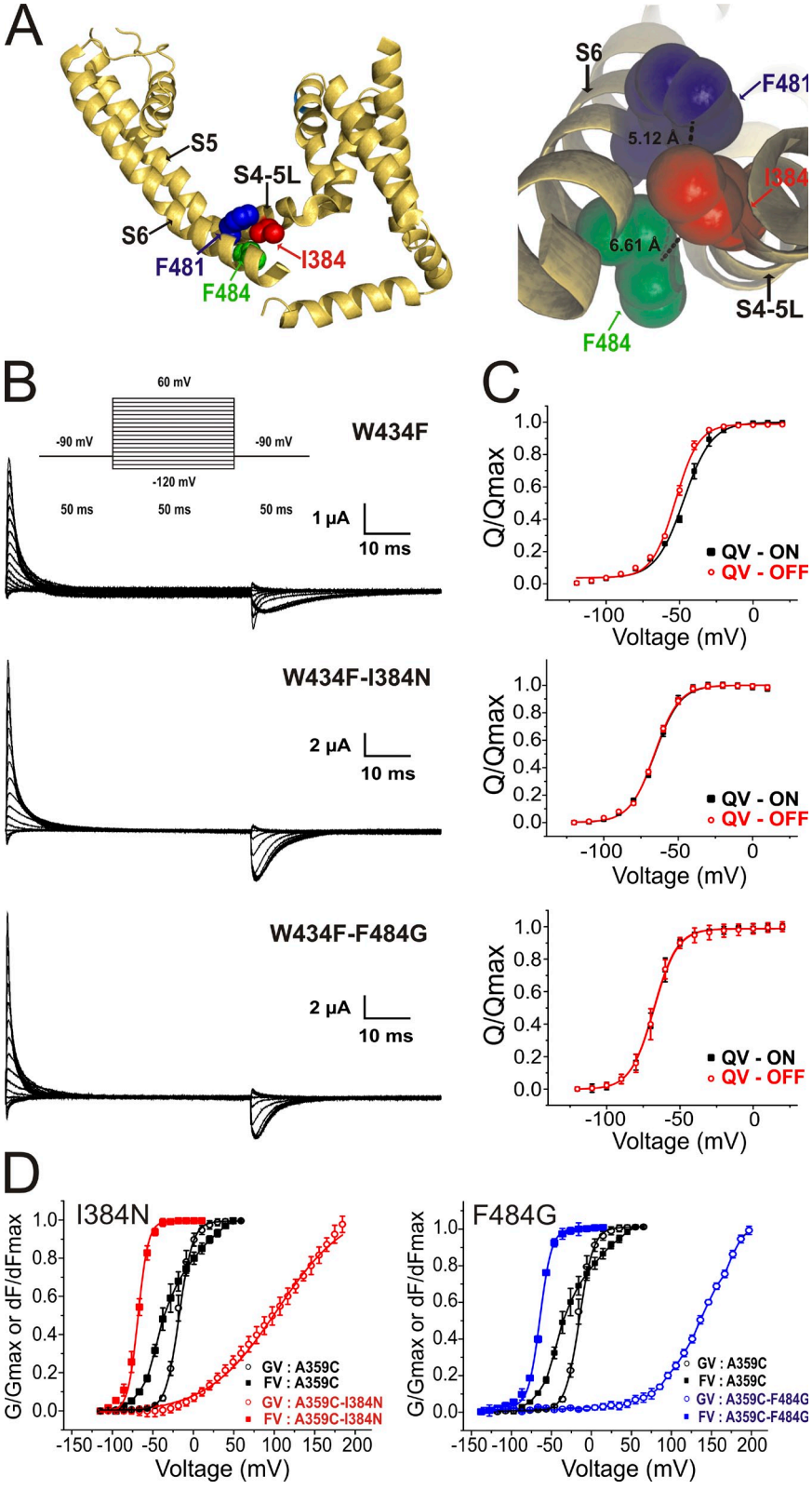


Figure 1. Uncoupling mutants I384N and F484G. (A) Location of the uncoupled mutants in the Kv1.2 crystal structure (Long et al., 2005a). Shown is a single subunit. I384 is located on the S4-S5 linker, and F481 and F484 are located on the C-terminal S6, which anneals to the S4-S5 linker. (right) Magnification of the interaction site. (B) Gating currents of ShakerIR-W434F, ShakerIR-W434F-I384N, and ShakerIR-W434F-F484G in response to a series of depolarizing pulses from a holding potential of V_{hold} = −90 mV to a potential of −120 to 60 mV in steps of 10 mV for 50 ms. (C) QV_{ON} and QV_{OFF} of ShakerIR-W434F, ShakerIR-W434F-I384N, and ShakerIR-W434F-F484G. (D) Fluorescence voltage (FV) and conductance voltage (GV) relations for ShakerIR-A359C, ShakerIR-A359C-I384N, and ShakerIR-A359C-F484G elicited from a series of depolarizing pulses from a holding potential of V_{hold} = −90 mV to a potential of −120 to 60 mV/200 mV in steps of 10 mV for a duration of 50 ms. QV and GV are far separated in the uncoupled mutants I384N and F484G. (C and D) Data are shown as mean ± SD.

uncoupled mutants, the FV, like the QVs obtained earlier from the nonconducting mutants, shifted to more negative potentials (Fig. 1 D), whereas the GV of ShakerIR-A359C-I384N and ShakerIR-A359C-F484G, as in the conducting nonlabeled mutants, shifted to the more positive potentials so that also here no overlap between QV (FV) and GV is observed. These results confirm in simultaneous measurements of charge movement and pore opening that both processes occur energetically uncoupled.

Uncoupled mutants show a slow off-gating component

The gating currents obtained from uncoupled mutants were faster than those obtained from ShakerIR-W434F, which is perhaps an expected result from uncoupled mutants that extricate the pore from the workings of the voltage sensors and thus energetically free the voltage sensors (Figs. 1 B and 2 B). However, what was unexpected was that the off-gating currents showed a slow onset and decay (Fig. 1 B), seemingly contrary to the current understanding that the slow component observed in W434F is caused by an open state stabilization (Batulan et al., 2010). The onset disappears from the gating currents obtained from depolarizing pulses to voltages where the pore does not open (Perozo et al., 1993; McCormack et al., 1994; Chen et al., 1997). However, in our uncoupled mutants, a slow onset before the exponential decay was observed in spite of the pore being closed. The shape of the off-gating currents did not change even upon depolarizations up to potentials of 180 mV, at which the channels opened partly. In this range, also no further charge movement was observed.

A slow onset in the gating currents can be observed only if the voltage sensors are stabilized in the activated state, and leaving the stabilized state should be the rate-limiting step upon return to the resting state. However, whereas in ShakerIR-W434F, the slow component only develops if the pore is open during open state stabilization (Perozo et al., 1993; McCormack et al., 1994; Batulan et al., 2010), here, a slow component develops that is not seen in the closed ShakerIR-W434F, the activated-not-open ILT mutant (Ledwell and Aldrich, 1999), or the open state stabilization–deficient mutant ShakerIR-W434F-Y485A. The stabilization of the voltage sensor in the uncoupled mutants thus has a different origin than the open state stabilization observed in ShakerIR-W434F.

Uncoupling removes the shift between on and off-gating

One feature in the QVs of I384N and F484G that caught our attention was the missing shift between the QV_{ON} and QV_{OFF}, which are obtained at the beginning and the end of the pulse, respectively (Fig. 1 C). In theory, all gating charges that become activated during a depolarizing pulse should return to their resting position when resetting the channel to the initial state. Thus, plotting the gating charge that moved during the onset of a depolarizing pulse (Q_{ON}) and the one that moved during the return to resting potential (Q_{OFF}) as a function of the pulse potential (V) should lead to identical curves. However, in practice, the charges return too slowly to resolve their gating current over the current noise, and consequently, Q_{OFF} is smaller than Q_{ON} so that the QV_{OFF} shifts slightly left of the QV_{ON} after normalization (Fig. 1 C). This shift between the QV_{ON} and QV_{OFF} was notably absent in the QVs of the uncoupled mutants I384N and F484G.

The shift between the QV_{ON} and QV_{OFF} observed in W434F is likely also linked to the mode shift, the shift of the QV which occurs during prolonged depolarizations (Bezanilla et al., 1982b; Fedida et al., 1996; Olcese et al., 1997). The reason is that the Q_{OFF} is obtained, in contrast to the Q_{ON}, after a depolarization of 50–100 ms, meaning that the channels will, at least partly, have entered the shifted mode. Because the

TABLE I

Summary of results of Boltzmann fits to the QVs and GVs of the mutant channels

Mutation	$QV_{90/ON}$ (W434F)		QV_{OFF} (W434F)		QV_0 (W434F)		W434F (*n*)	GV (A359C)		FV_{90} (A359C)		FV_0 (A359C)		A359C (*n*)
	$V_{1/2}$	dV	$V_{1/2}$	dV	$V_{1/2}$	dV		$V_{1/2}$	dV	$V_{1/2}$	dV	$V_{1/2}$	dV	
	mV	*mV*	*mV*	*mV*	*mV*	*mV*		*mV*	*mV*	*mV*	*mV*	*mV*	*mV*	
ShakerIR	−49.2 ± 0.7	8.5 ± 0.4	−53.0 ± 0.5	7.6 ± 0.3	−74.4 ± 0.5	9.0 ± 0.1	22	−22.5 ± 0.2	6.3 ± 0.4	−47.1 ± 0.5	14.6 ± 0.6	−115.3 ± 9.2	14.9 ± 0.4	11
I384N	−68.5 ± 0.6	8.0 ± 0.3	−69.8 ± 0.3	8.3 ± 0.3	−69.2 ± 0.2	7.9 ± 0.2	10			−71.6 ± 1.1	10.7 ± 0.8	−72.5.4 ± 0.8	13.4 ± 0.1	8
F484G	−67.7 ± 0.6	8.0 ± 0.2	−68.0 ± 0.4	8.0 ± 0.3	−71.6 ± 0.3	7.1 ± 0.2	6			−70.3 ± 0.7	10.6 ± 0.2	−70.9 ± 1.1	14.3 ± 0.6	5
Y485A	−48.5 ± 0.4	10.9 ± 0.3	−49.1 ± 0.2	11.6 ± 0.2	−55.1 ± 0.8	13.4 ± 0.7	5							
I384A*	−34.6 ± 0.1	13.2 ± 0.9	−36.6 ± 0.1	15.2 ± 1.0	−106.5 ± 0.4	9.0 ± 0.3	4	−32.6 ± 0.2	6.6 ± 0.2					6
I384A	−37.8 ± 0.4	12.9 ± 0.4			−106.5 ± 0.4	9.0 ± 0.3	5	−34.7 ± 0.4	6.6 ± 0.2	−39.2 ± 1.2	11.3 ± 1.4	−145 ± 5.4	11.3 ± 1.2	6
F484A	−58.5 ± 0.5	8.5 ± 0.3	−61.0 ± 0.6	11.7 ± 0.5	−63.8 ± 0.2	9.4 ± 0.4	5	−29.2 ± 0.6	8.3 ± 0.6					5
I384C*	−48.9 ± 0.5	11.5 ± 0.9	−53.0 ± 0.4	13.6 ± 0.7	−114.3 ± 0.8	9.3 ± 0.3	4	−37.9 ± 0.6	7.5 ± 0.4					4

$V_{1/2}$, ± SD, and dV (see Materials and methods) of single Boltzmann fits of QV_{90}, QV_{OFF}, QV_0, GV, FV_{90}, and FV_0 are given for ShakerIR wild type and the mutants I384N, F484G, Y485A, I384A, F484A, and I384C. The secondary mutation (W434F or A359C) is given on top of each column. The GV and QV_{90} values (QV_{ON} and QV_{OFF}) of I384A* and I384C* are obtained from holding potentials of V_{hold} = −140 mV and −120 mV, respectively, because of the strongly shifted QV_{OFF}. QV_{90} and QV_{140} of I384A are not significantly displaced with respect to one another. The *n* given in the table indicates the number of fully independent measurements (different batch of oocytes). All blank cells indicate that the data have not been determined.

QV_{ON}–QV_{OFF} shift is related to the mode shift, its absence in the uncoupled mutants suggests that the mode shift will also be influenced by uncoupling. The more robust effect of the mode shift motivated us to verify whether it is affected in the two uncoupled mutants.

Pulsing to more hyperpolarized potentials from a holding potential of V_{hold} = 0 mV, we determined the QV_0 of ShakerIR-W434F and confirmed that it had shifted to more negative potentials with respect to the QV_{90} obtained at V_{hold} = −90 mV (−73.4 ± 0.4 mV at V_{hold} = 0 mV vs. −49.2 ± 0.7 mV at V_{hold} = −90 mV; Fig. 2 C). It suggests that after the pore has opened, the return path is energetically altered and more energy is required to deactivate the voltage sensors. This behavior has been modeled with a parallel return path previously (Bezanilla et al., 1982a, 1994; Olcese et al., 1997; Villalba-Galea et al., 2008).

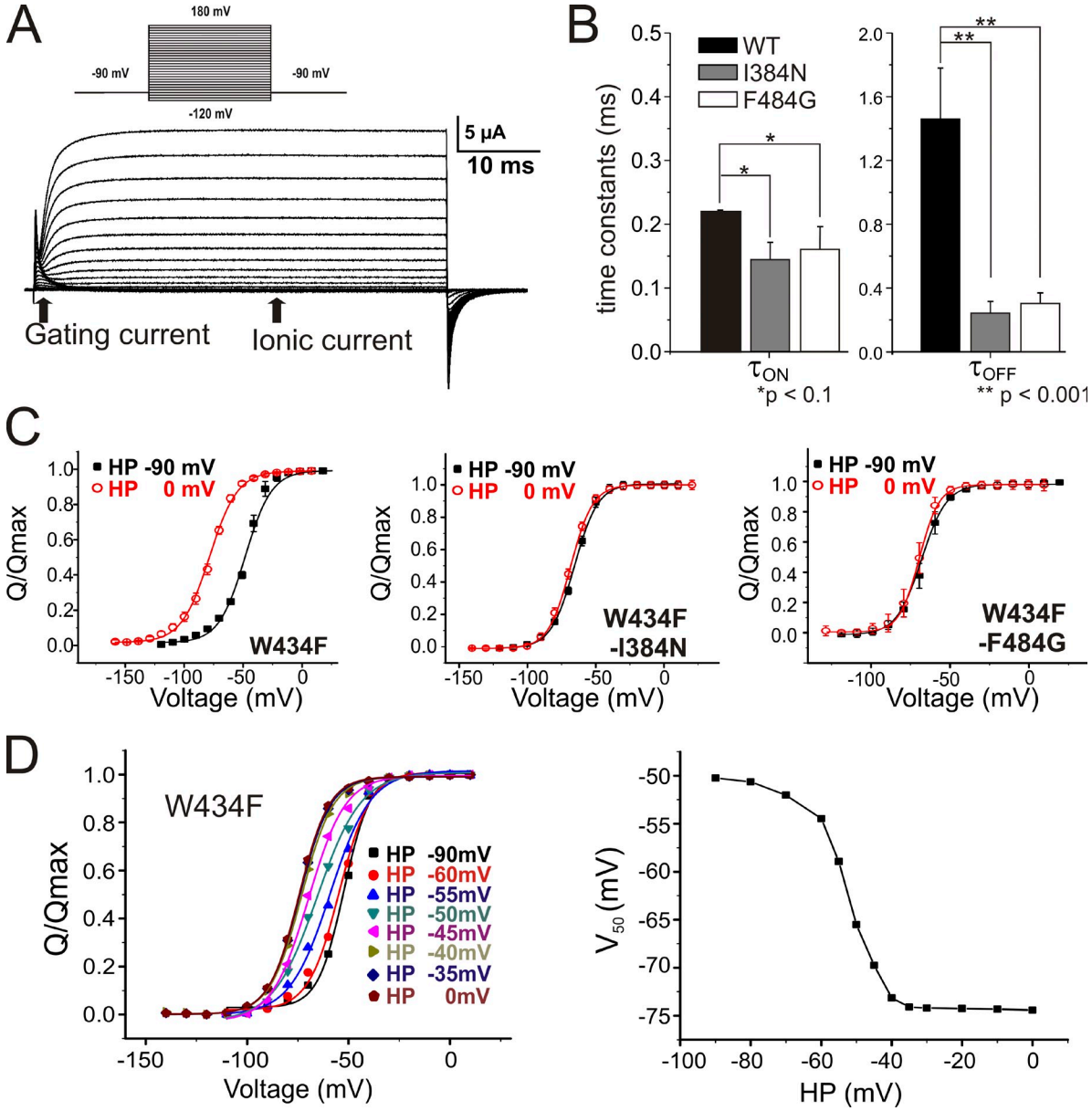


Figure 2. Mode shift is removed in uncoupled mutants I384N and F484G. (A) Gating and ionic currents in response to a series of depolarizing pulses from a holding potential of V_{hold} = −90 mV to a potential of −120 to 180 mV in steps of 10 mV elicited from ShakerIR-I384N. Both gating and ionic currents are detectable. (B) Time constants from exponential fits of the gating currents (saturating depolarizations) obtained from W434F (n = 7), W434F-I384N (n = 6), and W434F-F484G (n = 4). (C) QV curves were elicited for holding potentials at −90 (QV_{90}) and at 0 mV (QV_0) of ShakerIR-W434F, ShakerIR-W434F-I384N, and ShakerIR-W434F-F484G in response to a series of depolarizing pulses from −160 to 60 mV in steps of 10 mV. No shift of the QVs is observed in the uncoupled mutants ($P > 0.3$) except for W434F ($P < 0.0001$). (D) QV relations of ShakerIR-W434F in response to a series of pulses from a given holding potential (HP) to variable voltages. (right) $V_{1/2}$ of single Boltzmann fits as a function of the holding potential of the data on left. The shift occurs at voltages between −60 and −40 mV. (B–D) Data are shown as mean ± SD.

Strikingly, when we repeated the aforementioned experiments obtained from ShakerIR-W434F with the ShakerIR-W434F-I384N and ShakerIR-W434F-F484G mutants, we found that the QV_0 remained largely unchanged ($V_{1/2}$ = −69.2 ± 0.2 mV and −71.6 ± 0.3 mV for I384N and F484G, respectively) and almost superposed with the left-shifted QV_{90} ($V_{1/2}$ = −68.5 ± 0.4 mV and −67.7 ± 0.6 mV for I384N and F484G, respectively). Uncoupling thus removed the mode shift (Fig. 2 C and Table I), and only under the influence of the pore domain do both shifts of QV_{OFF} and QV_0 with respect to QV_{90} (QV_{ON}) occur.

These effects may be interpreted by regarding the energy levels. A shift in the QV is associated with the difference in the free energy ΔG between the initial and final state. Relative to the ShakerIR-W434F QV_{90} obtained for depolarizations from −90 mV, we found two different shifts to more negative potentials, the shift of the QV_{90} of the uncoupled mutants as well as the mode shift, the shift of the QV_0 of ShakerIR-W434F. As the voltage sensors are in their resting position at negative membrane potentials, a negative shift of the QV translates into less energy required to activate the voltage sensors and vice versa. Thus, the negative shift of the QV_{90} in the uncoupled mutants indicates that activation of the sensors in the absence of pore opening requires less free energy. However, during longer depolarizations to 0 mV, the QV of both coupled and uncoupled mutants superpose (Fig. 2 C) because, although the QVs and thus the energy levels of the uncoupled mutants do not change, the wild-type channel's QV_0 is shifted to more negative potentials, indicating that more work is required to return the voltage sensors to their resting state (and less to activate them). Because only the wild-type channel and not the uncoupled mutants is affected by the prolonged depolarization and, additionally, both shifts, the shift in the QV_{90} caused by uncoupling and the mode shift of the wild-type channel, are of equal value, the question arises whether both shifts and their conjugated differences in free energy (ΔΔG) have the same underlying mechanism.

Let us consider the effect of uncoupling first. Under the assumption that the uncoupling mutations did not affect either the intrinsic voltage sensor movement or the properties of pore opening, the energy liberated by uncoupling the voltage sensor from the pore in I384N and F484G is, in the wild type, required to overcome the mechanical load to open the pore. According to the actio = reactio principle, then, the pore pushes or applies force against the sensor, thus preventing it from activating at more negative potentials. If, as mentioned in the previous paragraph, both shifts and their conjugated ΔΔG's have the same underlying mechanism, this would suggest that the mechanical load is taken off the voltage sensors during prolonged depolarizations to 0 mV in a process of open pore stabilization, which would leave the voltage sensors in the same energetic state as the uncoupled mutants.

Shift is mediated during stabilization of the pore in the open state

To test whether our aforementioned hypothesis is true that the changes in ΔG upon both uncoupling and prolonged depolarizations are caused by the release of the mechanical load that the pore puts on the voltage sensors, we verified whether blocking the open pore stabilization will suppress the mode shift, shift in the QV_0 with respect to QV_{90}, while leaving unchanged the QV_{90} of the wild-type channel. The most likely candidate for the stabilizing interaction is the open state stabilization that gives rise to the slow component in ShakerIR-W434F off-gating currents (Batulan et al., 2010). An indication that open state stabilization influences the mode shift becomes evident when plotting the $V_{1/2}$ of the QV as a function of V_{hold}. The shift of the $V_{1/2}$ occurs in the voltage range between −70 and −40 mV (Fig. 2 D). In this range, the slow component in the off-gating current of ShakerIR-W434F also develops. Thus, open state stabilization and development of the mode shift correlate with one another.

To prove conclusively that the open state stabilization causes the mode shift, we used the mutation Y485A, which prevents open state stabilization but leaves intact the coupling between the voltage sensor movement and pore opening (Batulan et al., 2010). We obtained the two following results. First, similar to the QVs in the uncoupled mutants, the QVs of Y485A obtained at 60 (where the pores are open) and 0 mV were only slightly shifted compared with its QV_{90} (Fig. 3 A), suggesting that Y485A, like the uncoupled mutants, removes the mode shift. Second, the $V_{1/2}$ of Y485A remained at −50 mV (Table I), like the QV_{90} of W434F, and not around −70 mV as the uncoupled mutants. So, in spite of the fact that the mode shift is also removed in Y485A, this mutant remains in a different mode than the two uncoupled mutants I384N and F484G. This is in accordance with our hypothesis. It supports the idea that the mode shift is caused by the release of the mechanical load and that the responsible stabilization of the open pore is the stabilization also giving rise to the slow component of the gating currents.

Strong coupling increases mode shift

The aforementioned results suggest that the pore is applying a load onto the voltage sensor during activation and pore opening. Once the pore is stabilized, this load is lifted. They also suggest that the stabilized pore has energetically only little influence on the deactivation of the voltage sensor as the QV is identical to the uncoupled mutants. This would suggest that closing of the pore is not required before voltage sensor deactivation. To further investigate this, we looked for a mutation that creates a tighter coupling between voltage sensor

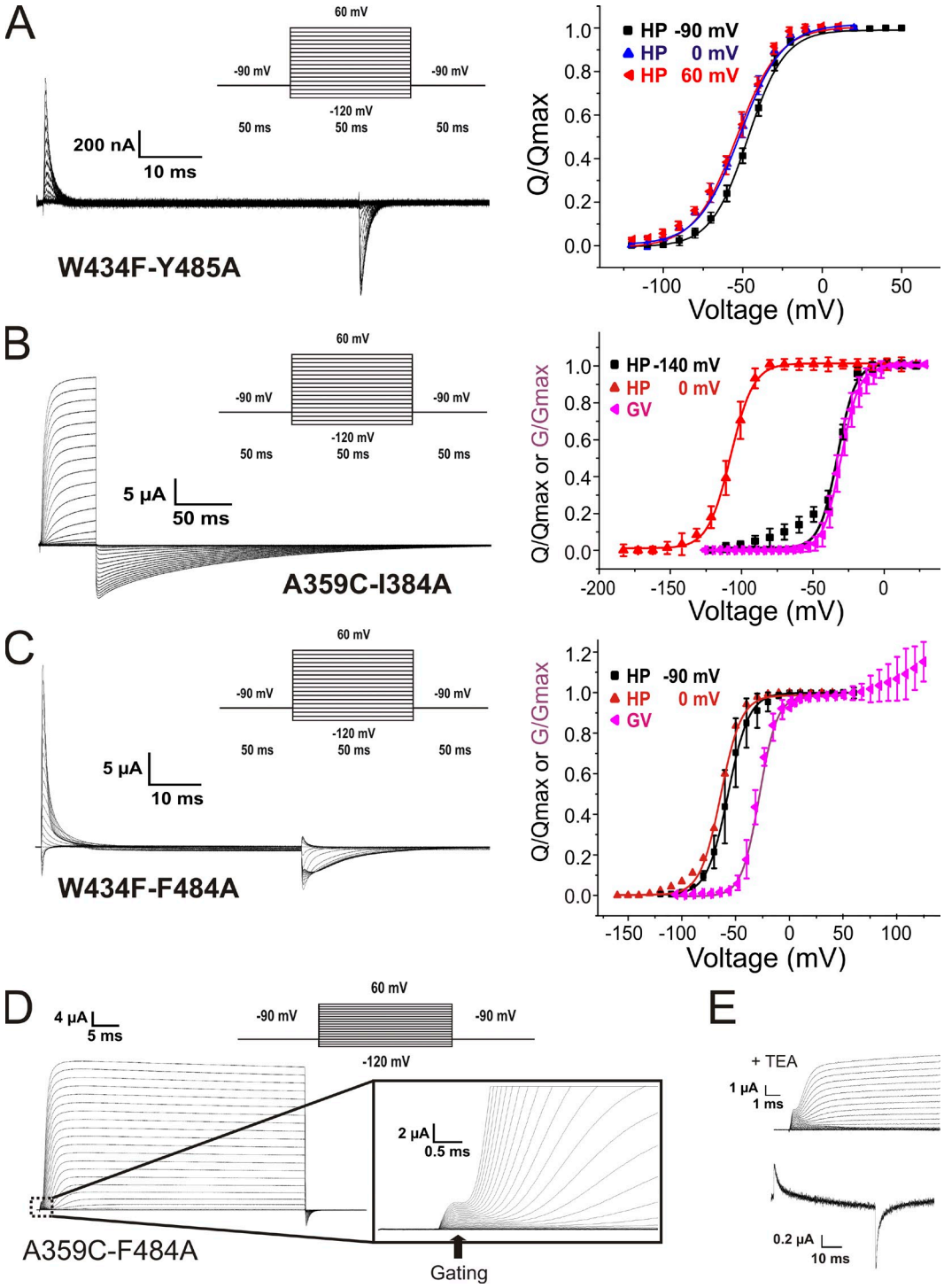


Figure 3. Open state stabilization, strong and weak coupling. (A) Gating currents of ShakerIR-W434F-Y485A in response to a series of depolarizing pulses (50 ms) from a holding potential (HP) of −90 mV to voltages between −120 and 60 mV in steps of 10 mV. (right) Corresponding QV relations for holding at 60, 0, and −90 mV are shown. Only a very modest mode shift is observed ($P > 0.2$). (B) Ionic currents of ShakerIR-A359C-I384A in response to a series of depolarizing pulses (50 ms) from a holding potential of −90 mV to voltages between −120 and 60 mV in steps of 10 mV. (right) Corresponding QV relations for holding at 0 and −140 mV and the GV (V_{hold} = −140 mV) are shown. The QVs are significantly shifted ($P < 0.0001$). (C) Gating currents of ShakerIR-W434F-F484A in response to a series of depolarizing pulses (50 ms) from a holding potential of −90 mV to voltages between −120 and 60 mV in steps of 10 mV. (right) Corresponding QV relations for holding at 0 and −90 mV are shown. They are not significantly shifted to one another ($P > 0.7$). (A–C) Data are shown as mean ± SD. (D) Gating and ionic currents of ShakerIR-F484A in response to depolarizing pulses from −90 mV to voltages between −120 and 60 mV in steps of 10 mV. (E) Effect of 100 mM TEA (top) and change in reversal potential to −30 mV in the presence of TEA (bottom; V = −70 mV). Only ionic current is affected, whereas gating currents remain the same.

movement and pore opening. We chose the mutation I384A because alanine is, although smaller than isoleucine, still hydrophobic. We determined the QVs at −90 mV and, because of the shift of the $QV_{OFF,}$ also at −140 (QV_{90}) and 0 mV (QV_0) as well as the conductance voltage relationship GV at −90 and −140 mV (Fig. 3 B). The QV_{90} shifted to more positive potentials with respect to W434F (−34.6 mV; Table I), indicating that even more energy than in W434F was required to transit into the activated state. At the same time, the GV was shifted negatively (−32.6 mV), but to the same potential as the QV_{90}. The effect on QV and GV was inverse to the one caused by uncoupling. Pore opening followed the voltage sensor movement directly; the voltage sensor movement was thus very tightly coupled to pore opening. When we then held the resting potential at 0 mV, the QV_0 was strongly shifted to more negative potentials (−107 mV). The strong coupling between pore opening and the voltage sensor therefore keeps the voltage sensor in the activated state, and only strong hyperpolarizations can bring the sensors back to their resting state. At the same time, ionic currents deactivated very slowly (Fig. 3 B). This suggests that in I384A, not only can the voltage sensor open the pore, but also the pore can keep the voltage sensor from returning to the resting state.

Weak coupling leads to low open probability

We also mutated the phenylalanine 484 in the S6 to alanine, as alanine, although still small, is more hydrophobic than glycine. In contrast to I384A, mutating F484A led to charge movement at more negative potentials and only to a slight shift between holding potentials at 0 and −90 mV (−58.5 ± 0.5 mV vs. −63.8 ± 0.2 mV; Fig. 3 C and Table I). These are almost the characteristics observed with the uncoupled mutants. However, the GV of F484A followed a Boltzmann distribution with $V_{1/2}$ = −29 mV, close to the wild-type channel. On first sight, these results would seem to contradict our findings that the shift is related to pore stabilization if it were not for a second phase in the GV (Fig. 3 C, right) and a transient component in the onset of the current traces (Fig. 3 D). At potentials more positive than 60 mV, the GV increased again in a second phase without reaching saturation in the voltage range tested (170 mV). This second phase is thus similar to the GVs observed of the uncoupled mutants. The transient component in the ionic currents turned out to be gating currents as they remained unaffected when we shifted the reversal potential of the ionic current to −30 mV or blocked the channel with TEA or 4-aminopyridine (Fig. 3 E). Both features indicate that the channel was not fully open at the first plateau of the GV around 25 mV. The amplitude of the gating currents at the first plateau was approximately 1/15 of the ionic currents, which allows us to calculate the open probability in this region. Similar to I384N, we calculated the number of channels present in the clamped membrane based on the total gating charge and the number of open channels from the conductance. We found that the second number is ∼50 times smaller than the number calculated from the gating charges; correspondingly, in the second phase even at 170 mV, it was still 34-fold lower. It is unlikely that we changed the gating charge per channel (13 *e*) because we did not directly mutate the voltage sensor. If we assume that we also did not significantly alter the open channel conductance (11 pS), we must have altered the number of channels that were opened by the activation of the voltage sensors ($P_{o,MAX} \cong 0.035 \pm 0.003$).

The alternative is also consistent with our results; if indeed the single channel conductance was not constant but reduced 50-fold while neither pore residues nor electrostatic residues at the cytosolic entry to the ion conducting path were altered, the ions must have been hindered from entering the water-filled cavity. This would suggest that the helical bundle crossing at the cytosolic entry to the pore did not open to its full extent, but instead only a leak current (∼0.2 pS) was permitted. Thus, in both cases, we effectively uncoupled the voltage sensor from pore opening with only residual pore opening occurring, and consequently, the QVs were similar to the uncoupled mutants.

W434F mutation but not C-type inactivation influences the mode shift mechanism

The lack of conductance in the mutant ShakerIR-W434F has been proposed to be caused by continuous C-type inactivation or by entering C-type inactivation very rapidly upon depolarizations (Yang et al., 1997). In light of the works that have linked C-type inactivation and mode shift (Olcese et al., 1997, 2001), the concern may thus be raised that the inactivated W434F channel mutation might have influenced the development of the mode shift. Therefore, we verified whether the same shift of the voltage sensor movement occurred in the conducting mutant during normal ion permeation by simultaneously following voltage sensor movement and pore opening with voltage-clamp fluorometry in the ShakerIR-A359C mutants.

When holding the resting potential at 0 mV, the FV_0 relationship ($V_{1/2}$ = −115 mV; Table I) of the ShakerIR-A359C mutant shifted compared with the FV_{90} ($V_{1/2}$ = −47 mV; Fig. 4 A). Although the FV_{90} superposed with the QV_{90} of ShakerIR-W434F, the FV_0 was, compared with the QV_0 of ShakerIR-W434F, shifted to more negative potentials by $\Delta V_{1/2}$ = −41 mV. However, when we repeated the same experiments with the uncoupled mutants I384N and F484G, the FV_0, just as the QVs in the W434F mutants, did not significantly shift with respect to the FV_{90} ($V_{1/2}$ = −71 mV and −70 mV for I384N and F484G, respectively; Fig. 4 A and Table I), and both superposed with the QVs of the respective ShakerIR-W434F mutants. All FVs of the respective ShakerIR-W434F

mutants, including the FV_0 of ShakerIR-A359C-W434F, superposed with the QVs obtained earlier in the W434F background (Fig. 4 B), confirming that the fluorescence intensity reports voltage sensor movement.

The shift of the FV_0 of ShakerIR-A359C beyond −70 mV indicated that not only, like in the W434F background, are the voltage sensors relieved from their mechanical load, but also additional energy is necessary to return the voltage sensors to their resting position. We were able to rule out the possibility that this energy corresponds to the energy required by the strongly coupled mutant I384A to return the voltage sensors; when we tested the ShakerIR-A359C-I384A mutant, its FV_0 was also shifted by the same amount to more negative potentials to $V_{1/2}$ = −145 mV ($\Delta V_{1/2}$ = −39 mV), whereas the FV_{90} remained at $V_{1/2}$ = −39 mV as the QV_{90} in the W434F background (Table I).

The additional stabilization of the open state seems to occur in the pore domain. For one, the W434F mutation is located near the selectivity filter of the pore so that a direct influence on the voltage sensor is unlikely. For another, uncoupling (I384N and F484G) prevents the

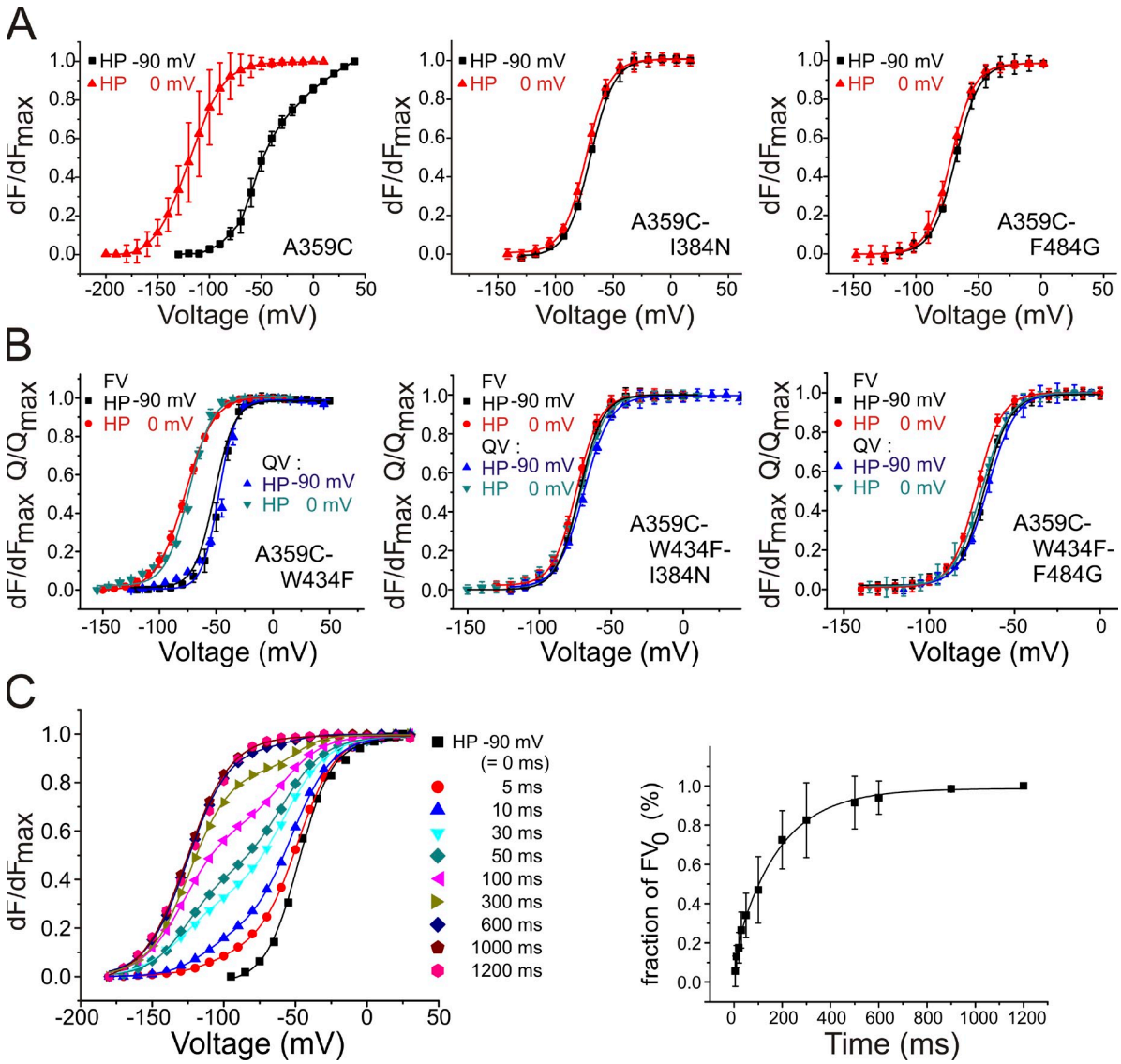


Figure 4. Mode shift in Shaker K^+ channels. (A) FV curves for the three mutants ShakerIR-A359C, ShakerIR-A359C-I384N, and ShakerIR-A359C-F484G for holding potential (HP) at −90 and 0 mV in response to a series of depolarizing pulses to a potential of −120 to 60 mV and 40 to −160 mV, respectively. Reversal potential was adjusted to 0 mV. The FVs of A359C were significantly shifted ($P < 0.0001$), and the uncoupled mutants were not ($P \geq 0.3$). (B) Comparison of FV and QV curves of the three mutants ShakerIR-A359C-W434F, ShakerIR-A359C-W434F-I384N, and ShakerIR-A359C-W434F-F484G for holding potentials at −90 and 0 mV in response to voltage pulses of 50-ms duration to voltages ranging between −160 and 50 mV in steps of 10 mV. (C) FV of ShakerIR-A359C from a holding potential of −90 mV to a prepulse of 0 mV for variable duration (0–1,200 ms) followed by a series of pulses of 50 to −160 mV in steps of 10 mV. The FV curves were fitted to a sum of two Boltzmann curves. (right) The fraction of the FV_0 Boltzmann as a function of the duration of the depolarizing prepulse is shown. (A–C) Data shown are mean ± SD.

additional energy observed in ShakerIR to develop or to be transferred to the voltage sensor as we do not observe the mode shift in the uncoupled mutants. Thus, in both ShakerIR and ShakerIR-W434F, the mode shift appears to be caused by stabilization in the pore domain and is prevented by energetic uncoupling of the voltage sensor.

Presence of the mode shift not only in the W434F background but also in ShakerIR-A359C indicated that the mode shift is not caused by C-type inactivation in the W434F mutant. Nevertheless, we did observe a difference in the mode shift between ShakerIR and the ShakerIR-W434F mutant. This difference does not seem to be linked to C-type inactivation. If we plot the FV_0 after a depolarizing prepulse to 0 mV for variable durations between 0 and 1,200 ms (Fig. 4 C, left), we find that the resulting distribution is a superposition of two Boltzmann curves representing the FV_{90} and the mode-shifted FV_0. We can plot the fraction of channels in both modes as a function of the duration of depolarization and thus determine the time constants for entering the mode shift (τ_{slow} = 181 ± 14 ms and τ_{fast} = 10 ± 4 ms; Fig. 4 C, right). This time constant is faster than the time constant for entering C-type inactivation.

Coupling leads to a slow conformational change of the S4

Uncoupling in I384N and F484G not only inhibited the mode shift in both the ShakerIR and ShakerIR-W434F background but also removed a slow conformational change of the S4 in the voltage sensor during prolonged depolarizations. This slow conformational change manifested itself in a slow increase in the fluorescence change in ShakerIR-A359C in response to a depolarizing pulse (Fig. 5, A and C). The time constant of the slow conformational change was in the range of 100–200 ms (τ_3 = 146 ± 29 ms at 0 mV) and thus much slower than voltage sensor activation or pore opening (Fig. 5 B). When we compare the slow time constant in the fluorescence with the time constant for entering the shifted mode (Fig. 4 C, right), we find that both correlate well. In the uncoupled mutants, in contrast, this slow conformational change was not observed (Fig. 5 C). Instead, the fluorescence saturated after ∼25 ms for saturating pulses (Fig. 5 A). The slowest time constant observed was around 20 ms (Fig. 5 B).

In the W434F background, the slow component was also absent in the ShakerIR-A359C-W434F mutant. The reason was that the W434F mutant enters the mode-shifted state much faster. When depolarizing to 0 mV for variable duration (0–50 ms; Fig. 5 D, left) before determining the QV_0, we find a time constant for entering the mode-shifted state of 4–9 ms (Fig. 5 D, right), which is faster than the slowest time constant found in the fluorescence time course of ShakerIR-A359C-W434F. We will therefore not be able to observe the conformational change in the fluorescence signal. The different time courses of ShakerIR and ShakerIR-W434F indicate that the additional stabilization of the wild-type channel occurs on a much slower time scale than the mode-shift in the W434F background.

The slow conformational change observed in the fluorescence signal of ShakerIR but not in ShakerIR-I384N and ShakerIR-F484G not only confirms the idea that uncoupling blocks the mode shift, which seems mediated by open state stabilization in the pore, it also confirms that the mode shift is related to conformational changes in the S4 of the voltage sensor. Slow conformational changes of the S4 helix were observed during prolonged depolarizations using voltage-clamp fluorometry both in HCN channels (Bruening-Wright and Larsson, 2007) and in the Ci-VSP (Villalba-Galea et al., 2008). The time constants found in those measurements correlated well with the observed value for ShakerIR-A359C. So in spite of the voltage sensors being the driving force for the pore opening in response to depolarizations, the state of the pore (closed, open, and open stabilized) also induces conformational changes back in the S4, which give rise to the mode shift observed in wild-type ShakerIR.

DISCUSSION

By fully uncoupling voltage sensor movement and pore opening in the mutants I384N and F484G, we aimed to define the mutual influence between the voltage sensors and pore domain. Our data suggest that the mode shift originates from the mechanical load that the pore imposes upon the voltage sensors but also that it is the S4 that changes its conformation in the process.

Energetic uncoupling of the voltage sensors and pore domain

Our uncoupled mutants I384N and F484G are located in the region of the S4-S5 linker and the C-terminal S6 where an interaction forms between the S4-S5 linker and the S6 of the same subunit that mediates electromechanical coupling. The two residues, together with F481, seem to tightly interact according to the crystal structure of Kv1.2 (Lu et al., 2001, 2002; Long et al., 2005a). Although F484 is positioned in the motif N480-Y485 on the S6 (Lu et al., 2002), I384 precedes the putative interacting motif L385-S392 on the S4-S5 linker (Lu et al., 2002).

Uncoupling voltage sensors and the pore domain from one another led to a separation of the voltage sensor movement (QV) and pore opening (GV), with overlap between both processes only over a small voltage range. The negative shift of the QV_{90} in our uncoupled mutants indicated that the voltage sensors required less energy to transit to the activated state. I384N and F484G are not the first mutants found to influence both gating and conductance differently. Previously, other mutants have been described (Seoh et al., 1996; Schoppa and Sigworth, 1998; Soler-Llavina et al., 2006; Batulan et al., 2010;

Muroi et al., 2010) that also disturb the energetic coupling between voltage sensor and pore. However, in our mutants I384N and F484G, the uncoupling was much more pronounced; it was almost complete. In I384N, even at potentials of 190 mV, only 0.8% of the channels opened even though all voltage sensors were in the activated position in both the nonconducting as well as wild-type ShakerIR. However, the fact that the channels still opened to a small extent indicates that a voltage-dependent step prevailed.

Based on the fact that the mutations were neither in the voltage sensor nor in the pore region, we can assume that the intrinsic properties of both domains were not altered. This suggests that the energy gained by uncoupling

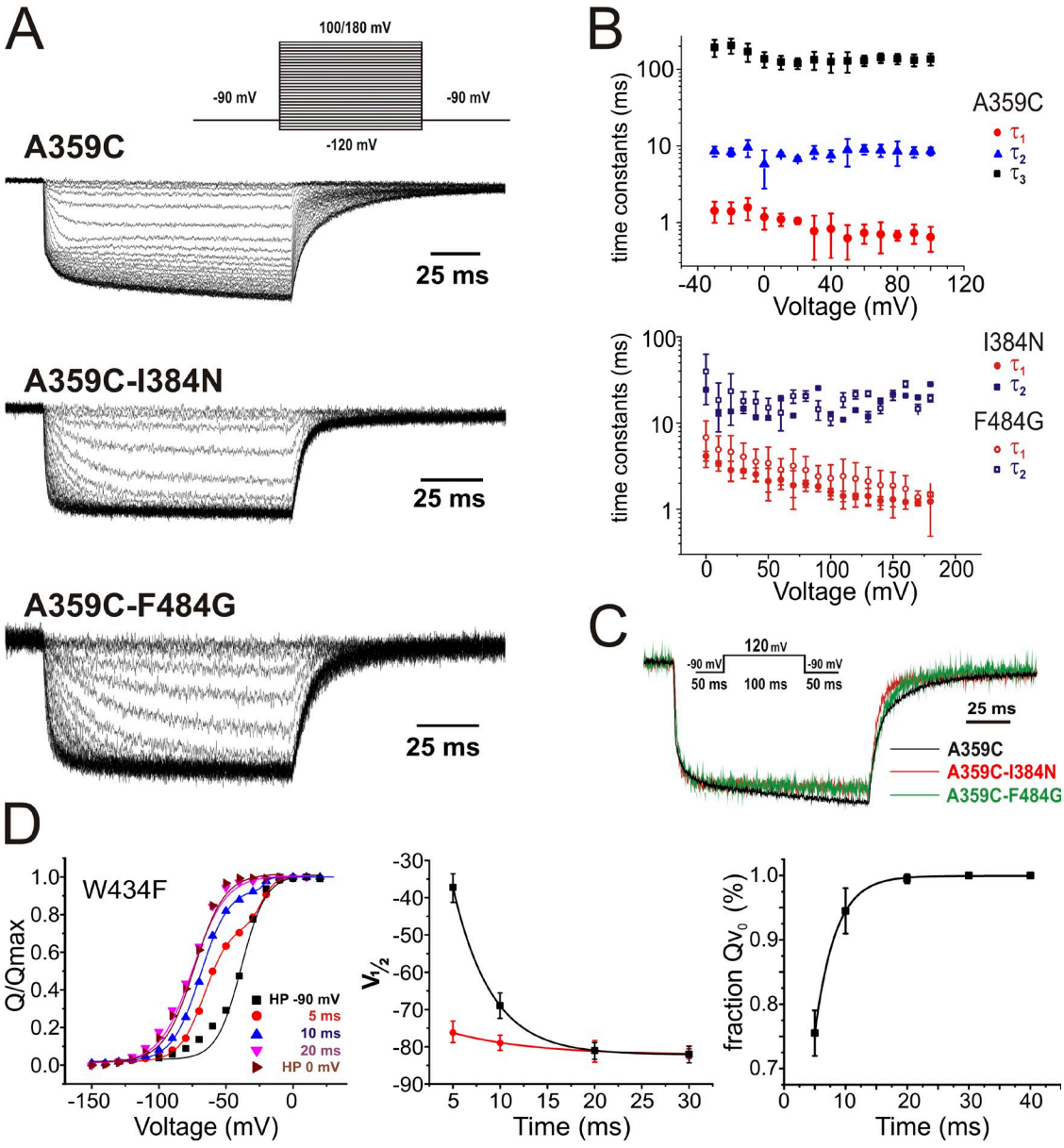


Figure 5. Fluorescence signal indicating conformational changes of S4. (A) Fluorescence response to depolarizing pulses from −90 mV to voltages between −120 and 100 mV and 180 mV for ShakerIR-A359C and ShakerIR-A359C-I384N/F484G, respectively, for a duration of 100 ms. Channels are fluorescently labeled with tetramethyl-rhodamine maleimide at position A359C on top of the S4. (B) Time constants of exponential fits of the onset of the fluorescence signal obtained in A. (C) Comparison of fluorescence signals for saturating pulses of ShakerIR-A359C ($V_{1/2}$ = −37.5 mV), ShakerIR-A359C-I384N ($V_{1/2}$ = −55.4 mV), and ShakerIR-A359C-F484G ($V_{1/2}$ = −56.8 mV). (D) QV of ShakerIR-W434F from a holding potential (HP) of −90 mV to a prepulse of 0 mV for variable duration (0–50 ms) followed by a series of pulses of 30 to −160 mV in steps of 10 mV. The QV curves were fitted to a sum of two Boltzmann curves. (middle) The shift of the $V_{1/2}$ of the two Boltzmann distributions as a function of the duration of the depolarizing prepulse is shown. The shifts were fitted to single exponentials with time constants of τ = 4.0 and 8.8 ms for more positive and more negative ones, respectively. (right) The fraction of the QV_0 Boltzmann as a function of the duration of the depolarizing prepulse is shown. (B and D) Data shown are mean ± SD.

the sensors is used for pore opening in the coupled (wild type) channel. The pore thus places a mechanical load onto the voltage sensors. This additional load implies that the pore is closed in its relaxed position and only opens with the help of the voltage sensors.

An alternative explanation for the opposing shift of QV and GV would be a strongly stabilized activated-not-open state preceding the open state in a sequential model. However, the mathematical description of such a model would be identical to uncoupling. Therefore, to decide whether uncoupling or stabilization of an intermediate state is more likely, the molecular determinants of the interaction have to be considered. A likely mechanism that led to uncoupling is the disturbance of the hydrophobic interaction between the S6 and the S4-S5 linker. The strength of coupling seems determined by a hydrophobic cleft formed by the C-terminal S6, into which the residues I384 and T388 of the S4-S5 linker insert (Labro et al., 2008). Introducing a hydrophilic residue in this cleft by replacing an isoleucine with an asparagine disturbed the coupling energy. Conversely, when we added an alanine in the same residue (I384A), the smaller hydrophobic residue led to an even stronger bond. However, given that introducing a cysteine at position I384(C; Table I) gave a similar phenotype as the alanine (I384A), the effect might be more complex than merely a hydrophobic interaction. On the opposite S6, the aromatic rings of the phenylalanines seem essential for the formation of the hydrophobic cleft. Replacing the phenylalanine with a small residue, either glycines or alanine (F484G/A), led to uncoupling. A similar nonpolar interaction linking a phenylalanine and an isoleucine on two adjacent α helices near the selectivity filter in KcsA has recently been described to be involved in the coupling mechanism between activation and C-type inactivation (Cuello et al., 2010a).

The aforementioned alternative explanation, in which the activated voltage sensor is stabilized by the mutation I384N in the activated-not-open state, would imply that introducing an asparagine at this position leads to a strong link between the S4-S5 linker and its environment. Although this might still be conceivable, it is more difficult to imagine that the same effect occurs by the mutation F484G on the S6. It would also be difficult to explain why the stronger coupling (I384A), which shifts the QV_0 to even more negative potentials, leads to a negative shift of the GV and not to a separation between QV and GV.

Also, a destabilization of the resting state of the voltage sensor is not a possible explanation because then the GV would be shifted to more negative potentials. The exception would be that a single point mutation in the S4-S5 linker or S6 produced both the destabilization of the S4 and uncoupling of the pore from the voltage sensor. How likely does a single point mutation in the S4-S5 linker alter the intrinsic properties of the voltage sensor or the pore itself? According to the crystal structure, both residues I384 and F484 point toward the interaction area between the S4-S5 linker and the C-terminal S6 (Fig. 1 A). A direct influence of both residues on the structure of voltage sensor or pore is therefore unlikely. The possibility remains that uncoupling leaves the pore and/or voltage sensor domain free to relax and assume an alternative conformation. However, such an allosteric change in conformation would be a structural consequence from the uncoupling, which is the mechanism that we suggest in this study (see Mode shift is accompanied by movement of the voltage sensors). The voltage sensor, for instance, would assume its intrinsic conformation in the absence of the mechanical load from the pore.

All things considered, uncoupling caused by low interaction between the S4-S5 linker and S6 seems to best explain the effects evoked by the mutations I384N and F484G, and we can assume that our uncoupling mutations I384N and F484G leave the intrinsic properties of the voltage sensors and the pore unaltered and merely influence the coupling between both domains. Under these assumptions, the difference in free energy that is needed to activate the voltage sensors will give a measure of the energy required in the wild-type channel to open the pore. Accordingly, we can approximate the energy required to open the pore from the shift of the QV in I384N and F484G. Because the slope of the QV does not alter during its shift, we can directly calculate the difference in free energy $\Delta\Delta G$ from the $\Delta V_{1/2} \cong -22 \pm 3$ mV and find that each voltage sensor has to procure 1.6 kcal/mol. As four voltage sensors are necessary to open the pore, pore opening requires 6.4 kcal/mol. However, this is just an approximation as other small structural changes in voltage sensor or pore domain might have altered the kinetics too.

The mode shift is caused by a change in the mechanical load on the voltage sensor

Coming back to the mechanical load produced by the pore onto the voltage sensors, the energy that acts on the voltage sensor during opening also seems linked to the mode shift of the voltage sensors observed in voltage-dependent ion channels (Bezanilla et al., 1982a; Brum and Rios, 1987; Shirokov et al., 1992; Fedida et al., 1996; Olcese et al., 1997, 2001; Piper et al., 2003; Kuzmenkin et al., 2004; Männikkö et al., 2005; Bruening-Wright and Larsson, 2007; Bruening-Wright et al., 2007). During the mode shift at longer depolarizations, the voltage sensors shift their voltage dependence and, consequently, return to their resting position at more negative membrane potentials. Uncoupling of the voltage sensor from the pore domain in I384N and F484G prevented the QV to be shifted when holding the resting potential at −90 mV (QV_{90}) with respect to holding it at 0 mV (QV_0). This suggests an interpretation based on the free energy exchanged between the systems for the QVs.

The voltage sensor is the generator that has to carry a certain mechanical load, the opening of the pore, which has different consequences dependent on the mutant. First, if no mechanical load exists (I384N, F484G, and F484A), the voltage sensor follows the QV intrinsic to the voltage sensors (approximately −70 mV) at all holding potentials. Second, as soon as the pore is coupled to the voltage sensor (wild type), the voltage sensor has to provide the energy for pore opening and enters the normal activated state, following the more positive QV_{90} of V_{hold} = −90 mV. Under prolonged depolarizations, the pore is stabilized in the open state and therefore relieves the voltage sensor from the mechanical load. Consequently, the voltage sensors relax and deactivate, following the more negative QV_0. Third, if the pore is not stabilized in the open state (Y485A), the mechanical load onto the voltage sensor remains, and the QV follows at all holding potentials the QV_{90}. Thus, open pore stabilization is responsible in the wild-type channel for lifting the mechanical load and thereby shifting the voltage dependence of the voltage sensor.

The aforementioned hypothesis assumes that uncoupling is the underlying reason for the negative shift of the QV. This is a reasonable assumption, as we already discussed (see Energetic uncoupling of the voltage sensors and pore domain), because otherwise two point mutations at different positions in the protein would lead to both uncoupling and a stabilization of the activated state of the voltage sensor. Although we cannot completely exclude the latter possibility, the underlying mechanism would remain the same even if it were the case; the fact that in Y485A no mode shift occurs in spite of the channels being open indicates that still open pore stabilization is responsible for the mode shift.

In the absence of the mutation W434F behind the selectivity filter, the voltage sensors require more energy to return to their resting state than the uncoupled mutants, indicating that here, not only the load of pore opening is relieved from the voltage sensors, but in addition the pore appears to have formed an interaction that further stabilizes it in the open state beyond the open state stabilization found in W434F (Fig. 6 A). This additional energy has to be overcome for the voltage sensors to return to their resting position, leading to the additional shift of the QV_0 to more negative potentials.

Our results indicate that the pore intrinsically prefers to be closed when the channel is in the resting position (i.e., before open state stabilization). In the uncoupled mutants, the pore is closed although the voltage sensors are fully activated, indicating that the pore intrinsically remains in the closed position unless stabilized by open pore stabilization. Only after prolonged depolarization, open state stabilization occurs and the pore autonomously remains open. One may say that the pore is a bistable system. This is even more so the case in ShakerIR than in the ShakerIR-W434F mutant because of its stronger stabilization of the open state. Provided that in ShakerIR the pore has formed an additional stabilizing interaction, whereas in the ShakerIR-W434F mutant the voltage sensor is moving freely, as we suggested (see Uncoupling removes the shift between on and off-gating), we can estimate the strength of the additional interaction from the difference of the two ΔΔGs obtained from the two mode shifts. The shift of the ShakerIR channel results in ΔΔG = 4.0 kcal/mol, leading to a difference with the Shaker-W434F mutant of 2.4 kcal/mol for the stabilization of the open pore (Fig. 6 A).

In contrast to our suggestion of a bistable system, it has been proposed previously that the pore's default state is the open state and that the channel is under constant strain in the resting position (i.e., at −70 mV; Bao et al., 1999; Upadhyay et al., 2009). This is based on the fact that at 0 mV, the pore is mainly in the open state and charge neutralization of the positive charges in the S4 leads to a shift to more negative potentials of the GV. However, this is misleading because in both cases the entire system of voltage sensor and pore were considered. The voltage sensor's neutral position is in the activated state (Gagnon and Bezanilla, 2009). Neutralizing gating charges will diminish the force into the resting state and thus stabilize the voltage sensor in its activated position and finally lead to pore opening. Therefore, the state of the pore in the wild-type channel at 0 mV does not provide evidence on the intrinsic state of the pore domain.

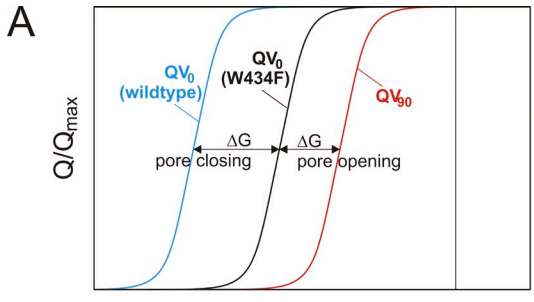
Our hypothesis that the pore is intrinsically closed before open pore stabilization is supported by the fact that in the ILT mutant, the GV lies in the positive voltage range (Ledwell and Aldrich, 1999; Gagnon and Bezanilla, 2010). However, in the ILT mutant, the final voltage sensor movement occurs during pore opening (Smith-Maxwell et al., 1998; Ledwell and Aldrich, 1999; Pathak et al., 2005), which again might influence the pore movement. The uncoupled mutants are different than the ILT mutants insofar as we do not see any charge movement with the final pore opening. However, as only 1% of all channels open, an eventual charge movement might not be detectable. Nevertheless, the uncoupled mutants differ also from the ILT mutants as the gating does not behave like activated-not-open channels (fast off-gating currents) but show a slow off-gating component, indicating that the activated voltage sensor is more stable. This supports our notion that the energy imposed by the pore is absent in the uncoupled mutants.

Weak and strong coupling modulate voltage sensor behavior

In the I384A channel, the energetic coupling to the pore seems to influence both directions activation and deactivation. Here, in contrast to the uncoupled mutants, QV_{90} and GV are not separated from one another, but instead, the QV is shifted to more positive and the GV to more negative potentials to such an extent that

QV and GV superpose. This indicates that voltage sensor movement and pore opening occur simultaneously. The union of QV and GV was the exact opposite of what was observed in the uncoupled mutants, indicating that here the coupling is stronger; in fact, voltage sensor and pore are so strongly coupled that no elasticity remains in the system. The voltage sensors can move only in conjunction with pore opening. This is different in the wild-type Shaker channel, where the voltage sensors move before pore opening. In I384A, the strong coupling means that each voltage sensor has to additionally overcome (part of) the energy barrier for pore opening. However, as more energy is transferred between voltage sensor and pore, the pore opens already at lower potentials.

Because of the bistable nature of the pore, also during closing/deactivation, the voltage sensors are held back by the pore. Before the pore can close or the voltage sensors can return to their resting position, open pore stabilization has to be broken again. For this reason, the mode shift of I384A is even stronger than that of wild type. However, in ShakerIR-W434F the voltage sensors also leave the activated state slower than without open state stabilization as indicated by the slow component in its off-gating currents. This indicates that also in the wild-type channel the voltage sensor does not move fully independently, whereas in I384A the voltage sensor is tightly coupled to pore movement.



B

Shaker	GLQILGRTLKASMRELGLL	VIVSNFNYFYHRET
KV1.1	------Q----------	-------------
Kv1.2	------Q----------	-------------
Kv1.3	------Q----------	-------------
Kv1.4	------H--R-------	-------------
Kv1.5	------K--Q-------	-------------
Kv1.6	------K--Q-------	-------------
Kv1.7	------Q--R-------	------S-------
Kv1.8	------Q----------	-------------
Kv2.1	---S--F--RR-YN-----	I--N--SE--KEQK
Kv3.1	--RV--H--R--TN-FL--	---N--GMY-SLAM
Kv4.1	--R---Y---SCAS---F-	------SRI--QNQ
Kv5.1	---T-TYA--R-FK-----	P-IN--VRY-NKQR
Kv6.1	---T--L-ARRCT--F---	S-FHT-SRS-LELK

Figure 6. Alignment of Kv channels and energetics of mode shift. (A) Mode shift of the charge movement in ShakerIR and ShakerIR-W434F and relationship with the energies of pore opening and closing. (B) Alignment of the S4-S5 linker and C-terminal S6 region of human Kv channels. I384, F481, and F484 are highlighted.

In contrast, the mutant F484A is only weakly coupled. The activation of the voltage sensors leads to the first phase of the GV, which saturates at 25 mV, suggesting that at this potential the equilibrium and maximal open probability linked to voltage sensor movement have been reached. However, the ratio between gating and ionic current indicates that only 2% of all channels opened in equilibrium or that the pore only opened to a small extent. Thus, voltage sensor movement and pore opening are energetically only weakly coupled. Because of this weak coupling and the resulting low open probability or small opening, the energetic state of the pore does not influence as much the voltage sensor movement. Consequently, the QVs of the F484A mutant correspond to the uncoupled mutants' ones, and the second phase of the GV does not saturate as in the uncoupled mutants, whereas the first phase of the GV of F484A was similar to the GV of wild-type ShakerIR as residual coupling prevailed.

Mode shift is accompanied by movement of the voltage sensors

The mode shift has been shown to be linked to C-type inactivation (Olcese et al., 1997), but our results suggest that, although C-type inactivation is linked to the mode shift, it is not its primary cause. First, the shift of the QV also occurs in the conducting ShakerIR and is thus not specific to the continuously C-type inactivated W434F mutant. Second, in I384A, those channels whose sensors are held back in the activated state after stabilization of the open state are not C-type inactivated because of the observed slow decay of the ionic current caused by the slow deactivation of the voltage sensors. However, we showed in the W434F background that the mechanical load is modulated during open state stabilization and thereby likely also during C-type inactivation. Cuello et al. (2010a) recently demonstrated the link between the region of electromechanical coupling (S4-S5 linker and S6), where the open state stabilization occurs (Batulan et al., 2010), and the selectivity filter, where C-type inactivation occurs. In the ShakerIR-I470C mutant (S6), although this link is disturbed (Holmgren et al., 1997; Cuello et al., 2010a), the same mode shift is observed (Olcese et al., 2001). Thus, it seems that the conformational change occurring in the coupling region during open state stabilization is decisive for inducing the mode shift but that C-type inactivation is linked to this process.

In contrast, Villalba-Galea et al. (2008) demonstrated that a mode shift occurs also for the isolated voltage sensor of Ci-VSP. The state entered when holding at depolarizing potentials is called the relaxed state and is thought to be caused by a transition from a 3_{10} to an α helix in the S4 of the voltage sensor. In the absence of the pore, only a conformational change of the voltage sensor itself is possible as an underlying mechanism.

With our proposed mechanism, we can reconcile why the mode shift on one hand is linked to C-type inactivation (Olcese et al., 1997, 2001) and open state stabilization but on the other hand also occurs in an isolated voltage sensor (Villalba-Galea et al., 2008). In ShakerIR-A359C, we observed a slow conformational change of the S4 during long depolarizations. Such a slow conformational change of the S4 has been linked to the relaxation of the voltage sensor in HCN channels and Ci-VSP (Bruening-Wright and Larsson, 2007; Villalba-Galea et al., 2008). It is therefore likely that also in ShakerIR both processes, the conformational change internal to the S4 and the mode shift induced by the mechanical load, share a common mechanism. In all three cases, the time course correlated with the time course for entering the mode shift. In addition, the slow fluorescence change was not observed in the uncoupled mutants ShakerIR-I384N or ShakerIR-F484G, indicating that uncoupling the pore from the voltage sensor prevented, or significantly slowed down, both the mode shift and the slow conformational change of the S4 in the voltage sensor. In the W434F background, the mode shift occurs within a few milliseconds such that the slow conformational change is not observed. It is also possible that the conformational change only arises in ShakerIR during the formation of the additional interaction, which led to a further shift of the FV_0 in ShakerIR, and does not happen in the ShakerIR-W434F mutants.

The possibility that the slow fluorescence change in the S4 is caused by additional voltage sensors entering the activated state is unlikely for two reasons; for one, 0 mV falls within the saturating region of the QV; for another, no steady increase in the ionic current was observed. Thus, our findings indicate that the pore allosterically alters the structure of the voltage sensor by transferring a mechanical load through annealing between the S6 and the S4-S5 linker.

Relation to other Kv channels

We showed in the ShakerIR channel that the coupling between pore and voltage sensor is necessary for the mode shift to occur. We used the Shaker channel to investigate the effects of uncoupling, as most previous data are available from this channel. However, our results are in all likelihood applicable to other voltage-gated ion channels. Comparing different human analogues of Shaker, the Kv1 channels, one realizes that the region of electromechanical coupling is highly conserved (Fig. 6 B). Only the positions R387 and K390 are replaced by Q, K, and H and Q and R, respectively. Among other Kv channel families, the region shows more variation. Nevertheless, the link between the S4-S5 linker and the C-terminal S6 has been confirmed for HCN (Decher et al., 2004) and HERG channels (Tristani-Firouzi et al., 2002), and it has been recently suggested for the voltage-gated sodium channels Na_V as well (Muroi et al., 2010). Although the mechanism of interaction between the S4-S5 linker and the S6 is conserved, the molecular determinants involved are different. For both HCN and HERG, electrostatic interactions play a dominant role (Tristani-Firouzi et al., 2002; Decher et al., 2004). For all these channels, HCN, HERG, and Na_V, the mode shift has also been described (Bezanilla et al., 1982a; Piper et al., 2003; Männikkö et al., 2005; Bruening-Wright and Larsson, 2007; Bruening-Wright et al., 2007) so that it is likely that a similar mechanism as described in this study exists in these channels.

The mode shift in HCN channels is more pronounced than in the K_V channels and is thought to be important for preventing arrhythmias (Männikkö et al., 2005; Bruening-Wright and Larsson, 2007; Bruening-Wright et al., 2007). In sodium channels, the shift might play a similar role as in the K_V channels, but also fast inactivation is controlled by depolarization and might therefore be subject to the mode shift. The mutual energetic influence between voltage sensor and pore domain thus seems to apply universally to a wide range of voltage-gated ion channels and to govern key functions such as recovery after excitation and stable firing rates (Männikkö et al., 2005; Bruening-Wright and Larsson, 2007; Bruening-Wright et al., 2007).

We would like to thank Mireille Marsolais for technical support.

This work was financially supported by the Canadian Institutes of Health Research (MOP-102689), the Canada Research Chairs (950-202965), and the Canada Foundation for Innovation. Groupe d'étude des protéines membranaires is a research group funded by the Fonds de la recherche en santé du Québec.

Submitted: 8 November 2010
Accepted: 5 April 2011

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