Pore Helices Play a Dynamic Role as Integrators of Domain Motion during Kv11.1 Channel Inactivation Gating*

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Background: Potassium channel pore helices are structurally important, but their contribution to the dynamics of inactivation gating is unclear.

Results: Kinetic analysis reveals early and late pore helix motions during inactivation gating of Kv11.1 channels.

Conclusion: Pore helices act as bidirectional integrators of domain motions during inactivation gating.

Significance: Mechanistic insights into inactivation are crucial to understanding how Kv11.1 channels regulate cardiac electrical activity.

Proteins that form ion-selective pores in the membrane of cells are integral to many rapid signaling processes, including regulating the rhythm of the heartbeat. In potassium channels, the selectivity filter is critical for both endowing an exquisite selectivity for potassium ions, as well as for controlling the flow of ions through the pore. Subtle rearrangements in the complex hydrogen-bond network that link the selectivity filter to the surrounding pore helices differentiate conducting (open) from nonconducting (inactivated) conformations of the channel. Recent studies suggest that beyond the selectivity filter, inactivation involves widespread rearrangements of the channel protein. Here, we use rate equilibrium free energy relationship analysis to probe the structural changes that occur during selectivity filter gating in Kv11.1 channels, at near atomic resolution. We show that the pore helix plays a crucial dynamic role as a bidirectional interface during selectivity filter gating. We also define the molecular bases of the energetic coupling between the pore helix and outer helix of the pore domain that occurs early in the transition from open to inactivated states, as well as the coupling between the pore helix and inner helix late in the transition. Our data demonstrate that the pore helices are more than just static structural elements supporting the integrity of the selectivity filter; instead they play a crucial dynamic role during selectivity filter gating.

Potassium-selective ion channels are critical signaling proteins in almost all cell types (1). Conduction of potassium ions through the central pore of tetrameric potassium channels can be switched on and off by gates at either end of the conduction pathway (1). The selectivity filter, located at the extracellular end of the conduction axis (2), is critical not only for the exquisite selectivity for potassium ions (3, 4), but also for gating (5–9). Gating at the selectivity filter is commonly referred to as C-type inactivation. Although a conformational rearrangement of the selectivity filter (10) underlies the final step of inactivation gating, there is increasing evidence, from a variety of potassium channels (11–13), that more widespread rearrangements of the channel protein precede this final nonconducting conformation.

Crystal structures of potassium channel proteins indicate that each loop of the selectivity filter, one from each of the four subunits, forms a complex network of intra- and intersubunit contacts with a short helical segment termed the pore helix (2, 9, 10, 14, 15). This network appears to be crucial for maintaining ion selectivity and C-type inactivation gating (2, 5, 10, 14, 15). Although these static structures have been enormously valuable, we still do not understand the dynamic nature of the conformational changes that mediate the interconversion between the stable open and inactivated states of potassium channels. Rate equilibrium free energy relationship (REFER) analysis, otherwise termed φ-value analysis, is a powerful protein engineering technique (16–18) that enables an interrogation of the ensemble of transient intermediate states that proteins must traverse as they move from one stable end state to the next, and thereby provides information about the underlying conformational changes that occur during gating transitions. Auerbach and colleagues have demonstrated experimentally (19–21) and theoretically (22, 23) that REFER analysis can be used to infer the relative timing of residue and/or domain motions during gating transitions. Specifically, within the range of φ-values from 0 to 1, residues with higher values are involved in earlier transitions than those with lower values (13, 20–22).

Kv11.1 channels pass the rapid delayed rectifier current, \( I_{Kr} \), in cardiac myocytes and are one of the best studied, and perhaps most clinically relevant, examples of channels that undergo C-type inactivation (24). Inherited mutations that enhance (25) or reduce (26, 27) inactivation result in delayed or accelerated repolarization of the heart, respectively, and consequently

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† This article contains supplemental Table 1.

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2 The abbreviations used are: REFER, rate equilibrium free energy relationship; Kv, voltage gated potassium channel.
greatly increase the risk of sudden cardiac arrest (26–28). Kv11.1 channels are also of great interest in terms of understanding selectivity filter gating as the open-to-inactivated state transition can be easily isolated from the much slower closed-to-open state gating transition (29, 30), making it amenable to study by REFER analysis (13). Previously, we used REFER analysis to show that Kv11.1 channel inactivation is initiated by a loss of K⁺ ions from the selectivity filter followed by a sequence of consecutive conformational changes involving energetically connected but physically separate domains of the channel, analogous to the opening and closing of a Japanese puzzle box (13). A crucial missing piece of the puzzle, however, is how the motion of these domains during inactivation gating is energetically coupled to the selectivity filter both at the beginning and at the end of the inactivation process. Although the pore helix is an obvious candidate for this role, previous mutations within this domain often resulted in nonfunctional channels or channels with altered ion selectivity that are not suitable for REFER analysis (13). Here, we have combined REFER and thermodynamic mutant cycle analyses, with a much more extensive scanning mutagenesis of the Kv11.1 pore helix, to show that the pore helix plays a crucial dual role in coordinating the allosteric rearrangements that underlie both early and late steps in the C-type inactivation gating transition.

EXPERIMENTAL PROCEDURES

Molecular Biology—Kv11.1 cDNA (a gift from G. Robertson, University of Wisconsin, Madison, WI) was subcloned into a pBluescript vector containing the 5’-untranslated region (UTR) and 3’-UTR of the Xenopus laevis β-globin gene (a gift from R. Vandenberg, University of Sydney). Mutagenesis of Kv11.1 cDNA was performed using the QuikChange method (Agilent Technologies, Santa Clara, CA) and confirmed by DNA sequencing. Linearization of the plasmid was performed using BamHI-HF (New England Biolabs, Ipswich, MA), and cRNA was in vitro transcribed using the mMESSAGE mMACHINE kit (Ambion).

Electrophysiology—Female X. laevis frogs were purchased from Nasco (Fort Atkinson, WI). All experiments were approved by the Garvan/St Vincent’s Animal Ethics Committee. Following anaesthetization in 0.17% w/v tricaine, the ovarian lobes were removed through a small abdominal incision. The follicular cell layer was removed by 2–h digestion with 1 mg/ml collagenase A (Roche Applied Science) in Ca²⁺-free ND96 solution containing (in mM): 96 NaCl, 2 KCl, 1.0 MgCl₂, and 5 Heps (pH adjusted to 7.5 with NaOH). After rinsing with ND96 (as above, plus 1.8 mM CaCl₂), stage V and VI oocytes were isolated and stored at 18 °C in tissue culture dishes containing ND96 supplemented with 2.5 mM pyruvic acid sodium salt, 0.5 mM theophylline, and 10 μM/ml gentamicin. X. laevis oocytes were injected with cRNA and incubated at 18 °C for 12–48 h prior to electrophysiological recordings. Two-electrode, voltage clamp experiments were performed at room temperature (20–22 °C) using a GeneClamp 500B amplifier (Molecular Devices Corp., Sunnyvale, CA). Glass microelectrodes were filled with 3 M KCl and had tip resistances of 0.3–1.0 mV. Oocytes were perfused with ND96 solution (see above). Data analysis was performed using pClamp software (Version 10, Molecular Devices), Microsoft Excel software (Microsoft Corp.), and Prism 6 (GraphPad Software Inc., La Jolla, CA). All data are shown as mean ± S.E.

REFER Analysis—Rates for the onset of inactivation, the forward transition in the pathway, were measured using the triple pulse protocol shown in Fig. 1A. From a holding potential of −90 mV, cells were depolarized to +40 mV for 500 ms so that channels became inactivated. For some mutant channels, a more depolarized potential (+80 mV) was required to produce full inactivation. A subsequent voltage step to −90 or −110 mV for 10 ms enabled channels to recover from inactivation into the open state. Finally, the potential was stepped to incremental voltages between −80 mV and +80 mV, and rates of inactivation at each voltage were obtained by fitting a single exponential to the respective decaying current trace (see Fig. 1A, black lines). Rates of recovery from inactivation, the backward transition in the pathway, were measured using the two-step protocol shown in Fig. 1B. Channels were opened and activated by an initial 1-s depolarizing step to +40 mV (or +80 mV) from a holding potential of −90 mV, before channels were allowed to recover from inactivation by stepping to a range of negative potentials. In the resulting “hooked” tail current, the initial rapid current increase represents the recovery from inactivation (see Fig. 1B, black lines), whereas the much slower decay in current occurs due to channel deactivation. Rates for recovery from inactivation were obtained from the fastest time constant of a double exponential function fitted to the hooked tail currents.

At each particular voltage (V), the observed rate constant (k_{obs,V}) is equal to the sum of the forward (k_{inact,V}) and backward (k_{rec,V}) rates. When plotted against voltage (see Fig. 1C), the logarithm of k_{obs} exhibits a typical chevron phenotype, which is indicative of a reaction dominated by a single transition state (31). The linear portions of the two arms of the chevron plot were extrapolated (see Fig. 1C, dotted lines) to derive unilateral forward (k_{inact,0}) and backward (k_{rec,0}) rates at 0 mV. The equilibrium rate constant (K_{eq}) at 0 mV was then calculated as

$$K_{eq,0} = \frac{k_{inact,0}}{k_{rec,0}}$$

(Eq. 1)

Following mutation of a residue of interest, a φ-value was calculated from the ratio of the change in energetics of the transition state as compared with WT, which is proportional to the change in the logarithm of the unidirectional forward rate constant (k_{inact,0}), relative to the change in energetics between the stable ground states, which is proportional to the change in the logarithm of K_{eq,0} (13, 16, 22).

$$\phi = \frac{\log (k_{inact,0,mut}) - \log (k_{inact,0,WT})}{\log (K_{eq,0,mut}) - \log (K_{eq,0,WT})} = \frac{\Delta \log (k_{inact,0})}{\Delta \log (K_{eq,0})}$$

(Eq. 2)

For each point mutation, the φ-value was calculated using Equation 2. Informative φ-values are those between 0 and 1, where high φ-values indicate that the mutation causes a perturbation at an early stage in the transition pathway, whereas a low φ-value indicates a perturbation at a late stage in the pathway.
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Not all mutations produce $\phi$-values between 0 and 1. For instance, mutations that perturb the structure of the stable open and inactivated end states, or mutations that alter the transition pathway between the stable end states, tend not to produce $\phi$-values between 0 and 1 and do not provide information about the wild-type protein.

At a given residue, it is possible for some amino acid substitutions to cause perturbations that are uninformative, as outlined above, whereas other substitutions are informative. For this reason, it is more accurate to examine families of mutations at a specific residue and calculate an overall $\phi$-value from the slope of a linear regression between $\log(K_{\text{eq,0}})$ versus $\log(K_{\text{eq,0}})$ (term a REFER plot). Mutations that do not fall within the linear relationship are unable to provide information about the wild-type protein and are therefore removed from the REFER analysis. This methodology provides a more accurate estimation of the relative timing of the motion of a given residue or domain during a gating transition.

Double Mutant Cycle Analysis—To test for an energetic coupling between a pore helix residue and a residue in one of the neighboring domains during the inactivation gating transition of Kv11.1 channels, we combined the technique of REFER analysis with double mutant cycle analysis. For each pore helix residue of interest, the largest perturbing mutation, measured as $\Delta \log(K_{\text{eq,0}})$, was introduced by one or more of the mutations. As was the case in the recent discovery of a clinically occurring pore helix mutation, T618I, that results in a marked alteration of inactivation gating (27) prompted us to re-examine the role of the pore helix in Kv11.1 inactivation, starting with an analysis of the T618I channel. Rates of onset and recovery from inactivation were obtained from families of current traces for WT and T618I mutant channels, as shown in Fig. 1, A and B (see “Experimental Procedures”). When plotted against voltage, the logarithm of the observed rate constants for inactivation in WT and T618I channels exhibits typical chevron phenotypes indicative of a reaction dominated by a single transition state (Fig. 1C) (31). From the chevron plots, it is clear that the T618I point mutation predominantly perturbs the unidirectional forward rate constant, $k_{\text{inact}}$, whereas having a minimal impact on the reverse rate constant, $k_{\text{rec}}$. Derived unidirectional forward ($k_{\text{inact}}$) and backward ($k_{\text{rec}}$) rate constants measured at a voltage of 0 mV were used to calculate the equilibrium constant for inactivation at 0 mV ($K_{\text{eq,0}}$) (see “Experimental Procedures,” Equation 1). In turn, values for $K_{\text{eq,0}}$ and $k_{\text{inact,0}}$ for WT and T618I mutant channels were used to calculate a $\phi$-value (see “Experimental Procedures,” Equation 2). Because the T618I mutation predominantly perturbs $k_{\text{inact,0}}$, this results in a high $\phi$-value (0.88 ± 0.02, $n = 15$) and implies that mutation T618I perturbs the inactivation transition at a very early stage in the gating reaction.

To optimize our chances of finding more mutant channels that were amenable to REFER analysis, we performed three separate mutagenesis scans of the pore helix, the boundaries of which were identified from the alignment shown in Fig. 2A. Residues Tyr-611 to Leu-622 were each mutated to alanine (Fig. 2B), isoleucine (Fig. 2C), or valine (Fig. 2D). Many (21/34) of the mutant channels were either nonexpressing or nonfunctional, exhibited altered selectivity for potassium ions, or abolished C-type inactivation over the voltage range studied (see supplemental Table 1), further emphasizing the importance of this region in channel function. Highlighted in black in Fig. 2, B–D, are mutant channels that exhibit a change in the equilibrium constant ($\Delta \log(K_{\text{eq,0}})$) of more than ±0.5 log units relative to WT, which has been shown to be a suitable cut-off criterion for the determination of an accurate $\phi$-value (13, 19). Sufficient perturbations to the inactivation transition were observed for the $\phi$-value of the double mutant to test for a native or non-native interaction. For example, the $\phi$-value derived from the double mutant T618I,I560A was >1, indicating an alteration to the transition pathway. In contrast, the $\phi$-value of the double mutant T618I,I567A was within the normal range (0.89 ± 0.03, $n = 7$), and similar to T618I alone (0.85 ± 0.02, $n = 15$), indicating a perturbation to the native transition pathway that suggests a native interaction between Thr-618 and Ile-567 during inactivation gating.

Homology Structure Generation—The sequence alignment in Fig. 4A was used to generate the homology model of Kv11.1 channels using the program Swiss PdbViewer (32) and optimized using SWISS-MODEL Workspace (33, 34).

RESULTS

Numerous mutations in the pore helix region of Kv11.1 channels result in nonfunctional channels (13, 35). However, the recent discovery of a clinically occurring pore helix mutation, T618I, that results in a marked alteration of inactivation gating (27) prompted us to re-examine the role of the pore helix in Kv11.1 inactivation, starting with an analysis of the T618I channel. Rates of onset and recovery from inactivation were obtained from families of current traces for WT and T618I mutant channels, as shown in Fig. 1, A and B (see “Experimental Procedures”). When plotted against voltage, the logarithm of the observed rate constants for inactivation in WT and T618I channels exhibits typical chevron phenotypes indicative of a reaction dominated by a single transition state (Fig. 1C) (31). From the chevron plots, it is clear that the T618I point mutation predominantly perturbs the unidirectional forward rate constant, $k_{\text{inact}}$, whereas having a minimal impact on the reverse rate constant, $k_{\text{rec}}$. Derived unidirectional forward ($k_{\text{inact}}$) and backward ($k_{\text{rec}}$) rate constants measured at a voltage of 0 mV were used to calculate the equilibrium constant for inactivation at 0 mV ($K_{\text{eq,0}}$) (see “Experimental Procedures,” Equation 1). In turn, values for $K_{\text{eq,0}}$ and $k_{\text{inact,0}}$ for WT and T618I mutant channels were used to calculate a $\phi$-value (see “Experimental Procedures,” Equation 2). Because the T618I mutation predominantly perturbs $k_{\text{inact,0}}$, this results in a high $\phi$-value (0.88 ± 0.02, $n = 15$) and implies that mutation T618I perturbs the inactivation transition at a very early stage in the gating reaction.

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five mutant channels: T618A/V/I, F619V, and L622V, in addition to the previously identified S621A (13).

ϕ-values derived from individual mutant channels provide temporal information regarding the effect of a specific mutation in perturbing the transition pathway, but not necessarily about the role of the native amino acid residue per se. A single mutation may affect two or more different processes that fortuitously cancel each other out to give a ϕ-value between 0 and 1. Such a fortuitous combination will not occur with every mutation at that single residue. Therefore, a better estimation of ϕ-values can be derived from REFER plots of log(K_{inact,o}) versus log(K_{eq,o}) for a family of mutations at a single residue or region of the channel (20) (see “Experimental Procedures”). We therefore performed more extensive mutagenesis of the three most informative residues identified from our scans (Fig. 3A): panel i, Thr-618; panel ii, Phe-619; and panel iii, Leu-622.

Mutation of residue Thr-618 (to Ala, Cys, Ile, Leu, Met, Asn, Gln, Ser, or Val) produced two mutant channels, T618V and T618I, with Δlog(K_{eq,o}) more than ± 0.5 log units (Fig. 3A, panel i). The ϕ-value derived from the REFER plot for all the Thr-618 mutants was 0.85 ± 0.05 (Fig. 3B, panel i). Mutant channel T618L (Fig. 3B, panel i, circled in gray) did not fit within the linear relationship and so was excluded from the analysis.

Due to strong hyperpolarizing shifts in the voltage dependence of inactivation, we did not observe currents for T618A or T618S channels under normal recording conditions, i.e. with an extracellular K⁺ concentration ([K⁺]o) of 2 mM. However, C-type inactivation gating in Kv11.1 channels is sensitive to changes in [K⁺]o, such that an increase in [K⁺]o causes a depolarizing shift in the voltage dependence of inactivation (30, 36). T618A and T618S mutant channels were therefore recorded in 20 mM [K⁺]o, indicated by gray bar, and compared with WT and T618I channels under the same conditions (Fig. 3B, panel i). Although we were unable to accurately derive values for T618S due to an ultra-rapid onset of inactivation, the ϕ-value derived from the REFER plot of the remaining Thr-618 mutants in 20 mM [K⁺]o was 0.84 ± 0.08, which is in very good agreement with that observed in 2 mM [K⁺]o, 0.85 (Fig. 3B, panel i).

Further mutagenesis of residue Phe-619 (to Ala, Cys, Gly, His, Ile, Leu, Asn, Gln, Ser, Thr, Val, Trp, or Tyr) resulted in three channels with Δlog(K_{eq,o}) more than ± 0.5 log units (F619T, F619Y, and F619V; Fig. 3A, panel ii). The ϕ-value derived from the slope of the REFER plot for Phe-619 was 0.14 ± 0.23 (Fig. 3B, panel ii). This indicates that residue Phe-619 undergoes a conformational change late in the reaction pathway between the open and inactivated states. The low ϕ-value derived for Phe-619 mutations is similar to that observed previously for S621A (ϕ-value = 0.26) (13). Further

The SEARCH and SEEK on Kv11.1 and KvαP for Kv11.1, F619V, and L622V, in addition to the previously identified S621A (13).
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FIGURE 3. Mutations at residues Phe-619 as compared with Thr-618 or Leu-622 affect different steps during inactivation gating of Kv11.1 channels. A, shifts (relative to WT) in the $\Delta \log(K_{eq,0})$ following mutation of residues Thr-618 (panel i), Phe-619 (panel ii), or Leu-622 (panel iii). Mutant channels are represented by single letter amino acid code. Data are presented as mean ± S.E. for $n = 6$–13 oocytes (see supplemental Table 1). Dashed lines indicate $\Delta \log(K_{eq,0})$ of ±0.5 log units. Black bars indicate mutant channels from which individual $\phi$-values were obtained. Gray bars indicate mutants recorded in 20 mM $K^+$, indicating an energetic coupling. However, the $\phi$-value derived from the double mutant lies outside of the normal range (0–1), suggesting that the double mutant had somehow altered the transition pathway rather than just perturbing the native (WT) pathway (see supplemental Table 1 and “Experimental Procedures”). Combining T618I and I560A produced channels with a $\Delta \log(K_{eq,0})$ that was also clearly not additive to that of the individual mutants alone (Fig. 4D). In this case the $\phi$-value derived from the double mutant T618I,I567A was within the normal range (0.89 ± 0.03, $n = 7$) and was similar to T618I alone (0.88 ± 0.02, $n = 15$) indicating a native interaction between Thr-618 and Ile-567 during inactivation gating. The remaining S5 mutant, L564A, was only partially additive when combined with T618I (Fig. 4D). By contrast, combining T618I with previously identified perturbing mutations within the S6 (V644A) or the S5-P linker (D591K) (13, 20–22), two helices that we predict would not energetically couple with Thr-618, produced mutant channels for which the $\Delta \log(K_{eq,0})$ values were completely additive when compared with the individual mutants alone (Fig. 4D). These data provide strong evidence for an energetic coupling between Thr-618 of the pore helix and the S5 helix and indicate that this interaction forms an important early step in the inactivation transition pathway.

mutagenesis of Ser-621 produced either no observable currents (Thr, Val, Asp) or channels with altered $K^+$ selectivity (Cys, Gly) (13), so we were not able to confirm the $\phi$-value for Ser-621.

Mutagenesis of residue Leu-622 (Val, Cys, Phe, Met, Asn, Gin, Ser, or Tyr) did not produce any channels, other than L622V, with a sufficient change in $\Delta \log(K_{eq,0})$ to provide an accurate individual $\phi$-value. A REFER plot of the functional mutants produced a $\phi$-value of 0.90 ± 0.22 (Fig. 3B, panel iii), which is similar to that obtained for Thr-618. Therefore, in contrast to the low $\phi$-values for Phe-619 and Ser-621, both Thr-618 and Leu-622 have high $\phi$-values, indicating that Thr-618 and Leu-622 undergo conformational rearrangements very early in the transition between the open and inactivated states of Kv11.1.

In our previous study, we found that $\phi$-values for inactivation gating in Kv11.1 channels ranged from 0 to 1 (13). The highest $\phi$-value (~1) was observed in response to increasing the concentration of external potassium ions, with diminishing $\phi$-values then obtained from mutational analysis of the S5 helix (~0.75), S5P linker (~0.6), S4 helix (~0.55), S4S5 linker (~0.45), and S6 helix (0.3). In the present study, the $\phi$-value derived from families of mutations at residues Thr-618 or Leu-622 (~0.85) is intermediate between those for the initial loss of $K^+$ ions from the selectivity filter ($\phi$ ~1) and of the S5 helices ($\phi$ = 0.75), suggesting an energetic coupling between these domains. When viewed on a Kv11.1 channel homology model, based on the crystal structure of a Kv1.2/2.1 channel chimera (14) (Fig. 4A), the side chains of Thr-618 and Leu-622 face away from the pore helix and directly into the S5 helix on the same subunit (Fig. 4B), supporting the notion that these two domains are coupled.

To experimentally test for an energetic coupling between the pore helix and nearby parts of the Kv11.1 channel protein, we combined the largest perturbing mutation at each pore helix residue of interest (T618I, F619V, L622V, or S621A) with perturbing mutations of residues within the S5 helix (I560A, L564A, or I567A), the S5P linker (D591K), or the S6 helix (V644A). The schematic in Fig. 4C demonstrates the principle of double mutant cycle analysis when combined with REFER analysis, using T618I and I560A as an example. If an energetic coupling exists between Thr-618 and Ile-560, we would expect that the perturbations brought about by the individual mutations, measured by $\Delta \log(K_{eq,0})$, would not be additive when combined in the double mutant (i.e. $\Delta G_{xy}$ more than ±0.5 log units from the sum of $\Delta G_x + \Delta G_y$ see “Experimental Procedures”). Conversely, additive effects in the double mutant would indicate a lack of energetic coupling (i.e. $\Delta G_{xy} \neq \Delta G_x + \Delta G_y$). The double mutant T618I,I560A exhibits a $G_{xy}$ that is more than ±0.5 log units from $\Delta G_x + \Delta G_y$ (Fig. 4, C and D), indicating an energetic coupling. However, the $\phi$-value derived from the double mutant lies outside of the normal range (0–1), suggesting that the double mutant had somehow altered the transition pathway rather than just perturbing the native (WT) pathway (see supplemental Table 1 and “Experimental Procedures”). Combining T618I with another S5 helix residue mutation, I567A, produced channels with a $\Delta \log(K_{eq,0})$ that was also clearly not additive to that of the individual mutants alone (Fig. 4D). In this case the $\phi$-value derived from the double mutant T618I,I567A was within the normal range (0.89 ± 0.03, $n = 7$) and was similar to T618I alone (0.88 ± 0.02, $n = 15$) indicating a native interaction between Thr-618 and Ile-567 during inactivation gating. The remaining S5 mutant, L564A, was only partially additive when combined with T618I (Fig. 4D). By contrast, combining T618I with previously identified perturbing mutations within the S6 (V644A) or the S5-P linker (D591K) (13, 20–22), two helices that we predict would not energetically couple with Thr-618, produced mutant channels for which the $\Delta \log(K_{eq,0})$ values were completely additive when compared with the individual mutants alone (Fig. 4D). These data provide strong evidence for an energetic coupling between Thr-618 of the pore helix and the S5 helix and indicate that this interaction forms an important early step in the inactivation transition pathway.

Next, we tested for energetic interactions with the other pore helix residue, Leu-622, which exhibited a high $\phi$-value (0.90), using the mutation L622V (Fig. 4E and supplemental Table 1). Combining L622V with I560A, L564A (both S5 helix), or D591K (S5P linker) produced completely additive effects, whereas combinations with I567A on the S5 helix or V644A on the S6 helix were nonadditive (Fig. 4E). This supports the notion that the residues that undergo a conformational rearrangement early in the inactivation gating transition, that is...
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Thr-618 and Leu-622, are energetically coupled to the S5 helix. Given the nonadditive perturbations by L622V and V644A, we cannot rule out the possibility that Leu-622 may also be energetically coupled to the S6 helix, but this is not reflected in its overall \( \phi \)-value.

In contrast to the high \( \phi \)-values derived from families of mutations at Thr-618 or Leu-622, mutation of either Phe-619 or Ser-621 produced low \( \phi \)-values (<0.3), which indicates a second distinct role for the pore helices at a much later stage in the transition pathway. Based on our previous work, this would follow the motion of the S6 helix (\( \phi \sim 0.3 \), (13)). Although our Kv11.1 homology model (Fig. 5A) does not support a direct intrasubunit interaction between Phe-619 and the S6 helix, it is consistent with an intersubunit interaction between these two helices. Conversely, the side chain of Ser-621 faces directly toward the S6 helix on the same subunit, suggesting an intrasubunit interaction (Fig. 5A).

Consistent with our homology model, combining F619V with the S5 mutants I560A or L564A, or with the SSP linker mutation D591K, produced entirely additive perturbations, indicating no direct energetic coupling (supplemental Table 1). Although we did not observe additive perturbations when F619V was combined with the S5 residue I567A, the \( \phi \)-value for this double mutant was outside of the meaningful range of between 0 and 1, suggesting a switch to a non-native transition pathway. Further examination of our homology model suggests that the side chain of residue Phe-619 is in close proximity to the side chains of three residues (Ile-642, Met-645, and Leu-647) on the neighboring S6 helix. We mutated all three of these S6 helix residues to Asn, Ser, or Val (Fig. 5B and supplemental Table 1). Mutation of Met-645 produced channels that either did not express (Val) or had altered selectivity for potassium ions (Asn, Ser) and therefore could not be used for REFER analysis. Although mutation of Leu-646 to serine produced channels with \( \Delta \log(K_{eq,0}) \) more than \( \pm 0.5 \) log units, it also exhibited a \( \phi \)-value of <0, indicative of a non-native interaction, and was therefore unsuitable for further study. Of the Ile-642 mutations, I642S provided the largest perturbation to \( \Delta \log(K_{eq,0}) \) and was initially chosen for double mutant cycle analysis. However, although F619V and I642S produce very similar effects on both the forward (\( k_{inact,0} \)) and the backward (\( k_{rec,0} \)) rate constants, as well as similar \( \phi \)-values, the double mutant, F619V,I642S, exhibited a very different phenotype that included an altered selectivity for potassium ions and reverse helix and Ile-560 on the S5 helix. Perturbations to inactivation gating, measured by \( \Delta \log(K_{eq,0}) \) relative to WT, caused by the individual mutations, T618I (\( x \)) and I560A (\( y \)), are compared with those of the double mutant T618I+I560A (\( xy \)). An energetic coupling is indicated when \( \Delta G_{xy} \) is more than \( \pm 0.5 \) log units from \( \Delta G_{x} + \Delta G_{y} \) (see “Experimental Procedures”). Measured values of \( \Delta \log(K_{eq,0}) \) values are indicated in parentheses for \( x, y, xy, D \) and E, double mutant cycle analysis to test for energetic coupling of pore helix residues, Thr-618 (D) or Leu-622 (E), with residues on the S5 helix (Ile-560, Leu-564, or Ile-567), S6 helix (Val-644), or SSP linker (Asp-591). Measured values of \( \Delta \log(K_{eq,0}) \) are shown with solid black bars, whereas predicted additive values (\( \Delta G_{x} + \Delta G_{y} \)) are indicated by open white or green bars. Data are presented as mean ± S.E. for \( n = 6–15 \) oocytes (see supplemental Table 1). Bars in green represent mutant pairs that have a significant interaction and a \( \phi \)-value in the informative range (0–1). Although T618I+I560A exhibits a \( \Delta G_{xy} \) that is more than \( \pm 0.5 \) log units from \( \Delta G_{x} + \Delta G_{y} \) suggesting an energetic coupling, the \( \phi \)-value lies outside of the normal range (0–1), indicating a deviation from the native transition pathway (see “Experimental Procedures”).

FIGURE 4. Double mutant cycle analysis of pore helix residues Thr-618 and Leu-622. A, sequence alignment of the S5 to S6 helices of Kv1.2/2.1 and Kv11.1 channels. Due to the much longer SSP linker in Kv11.1 channels as compared with Kv1.2/2.1 channels, this region is excluded from the alignment, denoted by the break. The transmembrane regions of Kv1.2/2.1 are highlighted in gray according to the DSSP secondary structure of 2R9R.pdb from the Protein Data Bank. B, extracellular view of a homology model of the Kv11.1 channel created using the Kv1.2/2.1 chimera crystal structure (14) as a template, according to the alignment shown in A. The boxed region shows the entire four-subunit homology model, whereas the larger image is an amplified view of the boxed region in the inset. The image shows the pore helix, selectivity filter, upper S5 and S6 helices of one subunit (subunit 1 colored green), as well as the upper S6 domain of the neighboring subunit (subunit 2 colored blue). Amino acid side chains of key residues Thr-618 and Leu-622 on the S5 helix of subunit 1 are labeled. C, schematic of double mutant cycle analysis to test for energetic coupling between residues Thr-618 on the pore helix and Ile-560 on the S5 helix. Perturbations to inactivation gating, measured by \( \Delta \log(K_{eq,0}) \) relative to WT, caused by the individual mutations, T618I (\( x \)) and I560A (\( y \)), are compared with those of the double mutant T618I+I560A (\( xy \)). An energetic coupling is indicated when \( \Delta G_{xy} \) is more than \( \pm 0.5 \) log units from \( \Delta G_{x} + \Delta G_{y} \) (see “Experimental Procedures”). Measured values of \( \Delta \log(K_{eq,0}) \) values are indicated in parentheses for \( x, y, xy, D \) and E, double mutant cycle analysis to test for energetic coupling of pore helix residues, Thr-618 (D) or Leu-622 (E), with residues on the S5 helix (Ile-560, Leu-564, or Ile-567), S6 helix (Val-644), or SSP linker (Asp-591). Measured values of \( \Delta \log(K_{eq,0}) \) are shown with solid black bars, whereas predicted additive values (\( \Delta G_{x} + \Delta G_{y} \)) are indicated by open white or green bars. Data are presented as mean ± S.E. for \( n = 6–15 \) oocytes (see supplemental Table 1). Bars in green represent mutant pairs that have a significant interaction and a \( \phi \)-value in the informative range (0–1). Although T618I+I560A exhibits a \( \Delta G_{xy} \) that is more than \( \pm 0.5 \) log units from \( \Delta G_{x} + \Delta G_{y} \) suggesting an energetic coupling, the \( \phi \)-value lies outside of the normal range (0–1), indicating a deviation from the native transition pathway (see “Experimental Procedures”).
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**FIGURE 5. Double mutant cycle analysis indicates that the pore helix residues Phe-619 and Ser-621 are energetically coupled to the S6 domain during inactivation gating of Kv11.1 channels.** A, amplified view of the homology model shown in the inset demonstrates that Phe-619 in the pore helix of subunit 1 (colored green) faces residue Ile-642 (also Met-645 and Leu-646, side chains not shown) in the S6 helix of the neighboring subunit 2 (colored blue), whereas Ser-621 in subunit 1 faces toward Val-644 in the S6 helix of the same subunit. B, shifts (relative to WT) in log($K_{eq}$) following point mutations (to Asn, Ser, or Val) of S6 helix residues Ile-642, Met-645 or Leu-646. Dashed line indicates Δlog($K_{eq}$) of ±0.3 log units, which has been previously shown to be a minimum requirement to derive an accurate $\phi$-value (13). Black bars indicate mutant channels that met this requirement. * denotes channels that failed to express, and # denotes channels with altered selectivity for $K^+$ ions. Both L646N and L646S exhibit $\phi$-values <0. C and D, double mutant cycle analysis to test whether Phe-619 (C) or Ser-621 (D) are energetically coupled to the S6 helix residues Ile-642 and Val-644 in the inactivation gating. Solid black bars show measured values of Δlog($K_{eq}$) (relative to WT), whereas open white or blue bars indicate the predicted additive values ($\Delta G_i + \Delta G_j$). All data are presented as mean ± S.E. for $n=$5–12 oocytes (see supplementary Table 1). Bars in blue represent mutant pairs that have a significant interaction and a $\phi$-value in the informative range (0–1). Although S621A + I642V exhibits a $\Delta G_i$ that is more than ±0.5 log units from $\Delta G_i + \Delta G_j$, suggesting an energetic coupling, the $\phi$-value lies outside of the normal range (0–1), indicating a deviation from the native transition pathway (see “Experimental Procedures”).

To further investigate our homology model, we tested for energetic coupling between the pore helix residue Ser-621 and the S6 helix residues Val-644 or Ile-642. Data in Fig. 5D show that perturbations caused by S621A and V644A are clearly not additive when combined in the double mutant. All other double mutant combinations with S621A, including I642V, produced channels with $\phi$-values outside of the meaningful range (Fig. 5D and supplemental Table 1). Our data are consistent with a direct intrasubunit coupling between Ser-621 on the pore helix and Val-644 in the S6 helix. Given that the $\phi$-values for Phe-619 and Ser-621 are small (<0.3), we suggest that the energetic coupling between the pore helix and S6 helix is important at a late stage of the inactivation transition pathway.

**DISCUSSION**

Inactivation gating involves the interconversion between two stable selectivity filter conformations, one conducting and the other nonconducting (37). Increasing evidence from studies of KirBac (11) and KcsA (12) channels suggests that more global dynamic conformational rearrangements within the channel protein are likely to accompany the interconversion between the open and inactivated conformations. Previously, we showed that the transition between the open and the inactivated stable end states of Kv11.1 channels involves complex multidomain motions, analogous to the opening and closing of a Japanese puzzle box (13). One domain conspicuously absent from this scheme was the pore helix, which has been shown to be critical for inactivation gating in a number of potassium channels (10, 35, 38–43). Here, extensive mutagenesis of the pore helix of Kv11.1 channels reveals an important dual role in energetically coupling the selectivity filter to the remainder of the channel at both early (involving residues Thr-618 and Leu-622) and late (involving residues Phe-619 and Ser-621) stages of the inactivation transition pathway (Fig. 6). Thus, in addition to maintaining the structural integrity of the Kv11.1 channel selectivity filter, the pore helix also acts as a bidirectional interface for coupling of the selectivity filter to the rest of the channel during inactivation gating.

The $\phi$-values derived from families of mutations at Thr-618 or Leu-622 (~0.85) lie between those representing the initial loss of potassium ions from the selectivity filter ($\phi$~1) and motion of the S5 helices ($\phi$=0.75). This suggests that Thr-618 and Leu-622, which lie on one face of the pore helix (Fig. 4B), energetically couple the selectivity filter to the S5 helix such that loss of potassium ions is transmitted to S5 motion and the subsequent widespread conformational changes that precede inactivation (13). In an alternative model, loss of potassium ions from the selectivity filter could represent a necessary, but dis-
Inactivated

FIGURE 6. Model of the pore helix as an orchestrator of multidomain motions during the transition of Kv11.1 channels from the open to the inactivated state. The channel schematic shows the transmembrane segments of two opposing subunits (labeled in the right-hand subunit). In the left-hand subunit, the helices are numbered and color-coded according to the temporal sequence of events that occur during inactivation gating, with red (1) representing the first step and purple (9) representing the final step in the inactivation transition. The pore helix (residues Thr-618 and Leu-622) couples an initial loss of potassium ions from the selectivity filter with motion of the S5 domain (numbered 2). Sequential motions of the S5, S5P, S4, S4-S5 linker, and S6 domains of the channel are then transmitted back through the pore helix (Phe-619 and Ser-621, numbered 8) to induce a putative final conformational rearrangement of the selectivity filter that is thought to underlie the stable inactivated state. The pore helix therefore acts as a bidirectional integrator of motions between the selectivity filter and the rest of the channel during inactivation gating.

The exact nature of the motion of the S6 helices is unclear. It occurs in Kv11.1 channels (Gly-648 to Tyr-667) undergoes a rotation at some point during the transition between open and inactivated states and that this rotation enhances sensitivity to block by some drugs (48, 49). Whether a similar rotation or a distinct conformational change occurs in the upper (extracellular) half of the S6 helix (Phe-640 to Ile-647) remains to be determined.

There are likely to be additional interactions between the pore helix and S6 helix beyond those involving Phe-619 and Ser-621 identified here. For instance, in KcsA channels, the cooperativity between activation and C-type inactivation gating is thought to involve an intrasubunit connection between Thr-74/Thr-75 (equivalent to Thr-623/Ser-624 in Kv11.1) at the base of the pore helix and Phe-103/Met-96 (equivalent to Tyr-652 and Met-645 in Kv11.1) in the S6 helix (50). A previous study was unable to derive accurate $\phi$-values from mutations at Thr-623/Ser-624 or Tyr-652 (13), so we cannot confirm or refute whether a similar interaction to that observed in KcsA occurs in Kv11.1 channels.
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It is important to note that our study is limited to those residues for which we can gain an accurate $\Phi$-value. However, it is likely that residues for which we cannot derive a $\Phi$-value will also play key roles in Kv11.1 inactivation gating. For instance, mutation of residues Tyr-616 or Phe-617 produced either non-expressing channels (Ala/Val mutants), attributed to failed trafficking to the membrane (35), or currents with altered selectivity and greatly reduced inactivation (Ile mutants). Both residues have conserved aromatic side chains across the potassium channel family (Fig. 2A), and the equivalent residues in KcsA (Trp-67 and Trp-68) and in Shaker (Trp-434 and Trp-435) have important roles in inactivation gating (10, 41, 42), possibly through a direct interaction with the side chains of residues within the selectivity filter (51).

The pore helix clearly plays a critical role in maintaining the structural integrity of the selectivity filter of potassium channels (2, 4, 14). To this role we can now add that it undergoes multiple conformational changes during the transition between conducting and nonconducting states of the filter. Further, we demonstrate that the pore helix interfaces with distinct domains during different stages (early and late) of the inactivation gating transition (Fig. 6). That the pore helix acts to integrate multiple allosteric inputs that influence the state of the selectivity filter is entirely consistent with our previous suggestion that inactivation gating is analogous to the opening and closing of a Japanese puzzle box (13). A dynamic role for the pore helix is also consistent with recent molecular dynamics studies indicating that ion selectivity is not dependent on static selectivity filter structures (52). Although our work has focused on the Kv11.1 channel, there is no doubt that the pore helix plays a critical role in inactivation gating in all channels. The high degree of conservation within the pore helices of potassium channels across the entire family (53) is indicative of a high degree of structural similarity, and we also suggest that it will have conserved functional similarity in terms of selectivity filter gating. In this context, the subtle side chain variations could account for different levels of conformational flexibility in the pore helix and hence the diversity of kinetics of selectivity filter gating among potassium channels.

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