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A Multipoint Hydrogen-Bond Network Underlying KcsA C-Type Inactivation

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ABSTRACT In the prokaryotic potassium channel KcsA activation gating at the inner bundle gate is followed by C-type inactivation at the selectivity filter. Entry into the C-type inactivated state has been directly linked to the strength of the H-bond interaction between residues Glu-71 and Asp-80 behind the filter, and is allosterically triggered by the rearrangement of the inner bundle gate. Here, we show that H-bond pairing between residues Trp-67 and Asp-80, conserved in most K^+ channels, constitutes another critical interaction that determines the rate and extent of KcsA C-type inactivation. Disruption of the equivalent interaction in *Shaker* (Trp-434-Asp-447) and Kv1.2 (Trp-366-Asp-379) leads also to modulation of the inactivation process, suggesting that these residues also play an analogous role in the inactivation gating of Kv channels. The present results show that in KcsA C-type inactivation gating is governed by a multipoint hydrogen-bond network formed by the triad Trp-67-Glu71-Asp-80. This triad exerts a critical role in the dynamics and conformational stability of the selectivity filter and might serve as a general modulator of selectivity filter gating in other members of the K^+ channel family.

INTRODUCTION

The essential role that K^+ channels play in cell excitability is a consequence of the unique interplay between activation and inactivation gating. In K^+ channels, the pore-domain contains all the structural components required to carry out the functions of permeation, selectivity, and gating. Activation gating is typically associated with stimulus-dependent conformational changes at the inner bundle gate (1–3), whereas C-type inactivation is associated with structural rearrangements at the selectivity filter and the neighboring extracellular vestibule (4–9).

There is longstanding evidence showing that a variety of mutations at or near the selectivity filter directly affect the gating behavior of K^+ channels. In Kv2.1 a point mutation at the selectivity filter (D378E) affects single-channel behavior, presumably by destabilizing the open state (10). Moreover, mutations near the selectivity filter and the pore helix of *Shaker*, hERG, and inward rectifiers also have large effects on gating properties (11–14). Substitution of a conserved tryptophan residue to phenylalanine at the pore helix in *Shaker* (W434F) abolishes ionic currents without affecting the intracellular gate (15). It was later shown that this mutation results in a constitutively C-type inactivated channel (16). In *Shaker*, mutations at Tyr-445 (to either proline or alanine) and Asp-447 (to asparagine) also block ion conduction, highlighting the importance of these residues to the stability of the conductive form of the selectivity filter (17,18).

A series of crystal structures of KcsA trapped in various degrees of gate opening and ion occupancy have provided direct evidence to the type and extent of the structural changes associated with the C-type inactivated state (6). These changes, driven by either the local concentration of permeant ions (19) or via allosteric interactions during gating (6,20), are consistent with the idea that the selectivity filter and adjacent structures are intrinsically flexible (21,22). Indeed, recent NMR studies on KcsA reveal considerable conformational changes in the backbone angle of Tyr-78 between the closed (pH 7) and open-inactivated forms of the channel (23) as well as a reorientation around Val-76 (24). Similar changes have been observed as a result of the binding of the pore blocker Kaliotoxin (25).

The crystal structure of the noninactivating KcsA mutant E71A shows that the electron density of the Trp-67, equivalent to *Shaker* Trp-434, has dual rotameric occupancy. This has led to the suggestion that large structural excursions likely take place in this tryptophan side chain during gating events at the filter (4–9). Consistent with this suggestion, a structural comparison of the conductive and nonconductive conformations of the KcsA selectivity filter reveals that in addition to the collapse of the conductive pathway, the aromatic residues that surround the selectivity filter undergo slight rearrangements (Fig. S1 in the Supporting Material). Moreover, Marius et al. (26) suggested that anionic lipids could modulate the open probability of KcsA through its interaction with Trp-67. Put together, these pieces of evidence suggest that Trp-67 participates in critical intraprotein interactions and is energetically coupled to the surrounding lipids.

Here, we have investigated the mechanistic role of the highly conserved aromatic girdle (19,22) that surrounds

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the selectivity filter of most K^+ channels. In particular, we have focused our attention on the Trp-67-Asp-80 interaction between the selectivity filter and pore helix as a potential partner in the hydrogen-bond network behind the selectivity filter in KcsA. We find that aromatic substitutions at Trp-67 do not significantly affect the overall kinetics and extent of C-type inactivation, as long as the ability to form hydrogen bonds is preserved (W67Y). However, inability to establish H-bond interactions with Asp-80 (W67F) leads to a severe modulation of the inactivation process. These observations were tested on *Shaker* and Kv1.2, supporting the idea that similar interactions play a determinant role in defining the energetics of C-type inactivation throughout the K^+ channel superfamily.

METHODS

Mutagenesis and channel biochemistry

Mutations were made using the QuickChange site direct mutagenesis kit (Stratagene, La Jolla, CA). A pQE32 vector containing wild-type (WT) KcsA and mutants with the RGS-(6×His) epitope at the N-terminus was used for protein expression in *Escherichia coli* XL1-blue cells. KcsA purification and liposome reconstitution was carried out as described elsewhere (2,27). Briefly, membranes containing KcsA were homogenized and solubilized in 200 mM KCl + 50 mM Tris-base buffer containing dodecyl maltoside at room temperature, spin-down at 100,000 × *g* for 1 h, and purified with a Co^{2+}-based metal-chelate chromatography resin. The quality of the purified protein was checked by gel-exclusion chromatography in a Superdex 200 column. Purified channels were reconstituted in preformed asolectin liposomes at several lipid/protein ratios by the dilution method.

Liposome patch-clamp

Electrophysiological measurements on proteoliposomes were performed using patch-clamp techniques as described (28,29). Single-channel records were obtained from 1:5000–10,000 protein/lipid ratio and macroscopic currents were measured at a 1:100 (mass/mass). The liposome suspension was centrifuged for 1 h at 100,000 × *g* and the pellet (~10 mg of lipids) was resuspended in 60 μL of rehydration buffer. Proteoliposomes were dried overnight in a desiccation chamber under vacuum for ~12 h and subsequently rehydrated with 20 μL of buffer. Unless explicitly stated, patch-clamp measurements were done in symmetrical conditions: 200 mM KCl and 5 mM 4-morpholine propanesulfonic acid buffer, pH 4.0 at room temperature. Ionic currents were recorded with an Axopatch 200B, at 5 kHz (–3 dB) and sampled at 40 kHz. Macroscopic currents were recorded after a pH jump using gravity fed RCS-160 fast solution exchanger (Bio-Logic, Knoxville, TN) by. Ion selectivity was evaluated under biionic (K^+/Na^+) conditions, using 1.2 s ramp protocols between −200 and +200 mV. Single-channel analyses were done using pCLAMP 9 (Axon Instruments, Union City, CA).

Two-electrode voltage-clamp

The cRNA from rat Kv1.2 (rKv1.2) and *Shaker* Δ4–46 channel (ShIR) (30) were prepared with the mMESSAGE mMACHINE T7 kit (Ambion, Austin, TX) following linearization of plasmid cDNA. Defolliculated stage IV and V *Xenopus* oocytes were injected with 0.2 ng WT channels or 5 ng mutants and cultured in ND96 supplemented with 50 μg/mL gentamicin and 1% penicillin/streptomycin at 16°C. Whole-cell currents were measured 12 h postinjection using a Geneclamp 500 amplifier (Axon Instruments). Data were sampled at 5 kHz and recorded using Clampex software (Axon Instruments). Leak and capacitance were subtracted online using a P/4 protocol. Bath solution was (in mM) 96 NaCl, 4 KCl, 1 $MgCl_2$, 0.3 $CaCl_2$, 10 HEPES (pH 7.6).

Molecular dynamics

Molecular dynamics was performed as previously described (5). The simulation system was represented by an atomic model of KcsA (PDB ID: 1K4C) channel embedded in dipalmitoylphosphatidylcholine surrounded by an aqueous solution of 150 mM KCl. The microscopic system was composed of KcsA tetramer of 404 amino acids (6284 atoms), 112 dipalmitoylphosphatidylcholine molecules, 6384 water molecules, 3 K^+ ions in the pore (S0-S2-S4 positions in the selectivity filter). To make the entire system electrically neutral, 6 K^+ and 21 Cl^- were added in the bulk solution to mimic 150 mM KCl. All the calculations were performed using c29a2 or c32a2 of the biomolecular simulation program CHARMM (31). The simulation methodology has been described previously (32). W67F and W67Y mutants are generated in silico and carefully equilibrated before production run.

RESULTS

Functional role of the Trp-67-Asp-80 interaction in the KcsA selectivity filter

The interaction between Glu-71 and Asp-80 is one of the key determinants of C-type inactivation in KcsA (4,5). However, this particular pairing is only found in prokaryotic K^+ channels and some eukaryotic inward rectifiers (33) and is absent in a number of channels that exhibit robust C-type inactivation. Thus, we have examined additional interactions in the hydrogen-bond network behind the selectivity filter as potential contributors to C-type inactivation in Kv channels. In KcsA, we focused our attention on the interaction between residues Trp-67 and Asp-80, a partnership conserved in most K^+ channels (Fig. 1 *a*). These two residues interact through a hydrogen bond between the indole nitrogen in Trp-67 and the carboxyl group of Asp-80 (Fig. 1 *b*).

Ensemble macroscopic currents elicited by pH jump experiments under depolarizing conditions (+150 mV) reveal that disruption of the Trp-67-Asp-80 H-bond by a phenylalanine substitution (W67F, Fig. 2 *a*) leads to noninactivating macroscopic currents, functionally similar to those generated by the E71A mutation (Fig. 2 *c*). However, a tyrosine substitution (W67Y, Fig. 2 *b*) displays robust C-type inactivation, essentially identical to that in WT KcsA (Fig. 2 *c*). We reasoned that only tyrosine (with its hydroxyl moiety) is able to establish a hydrogen bond with Asp-80 (Fig. 2 *b*), therefore preserving the internal forces driving the filter to collapse. The macroscopic current behavior was fully recapitulated at the steady-state single-channel level (Fig. 2 *d*), where the long closed time periods characteristic of WT KcsA inactivation ($t_c > 10$ s) are only absent in the W67F and E71A mutants (with mean open times of ~100 ms). Trp-67 mutants were fully selective to K^+ against Na^+ under biionic conditions (Fig. S2), though the noninactivating mutants displayed a higher apparent

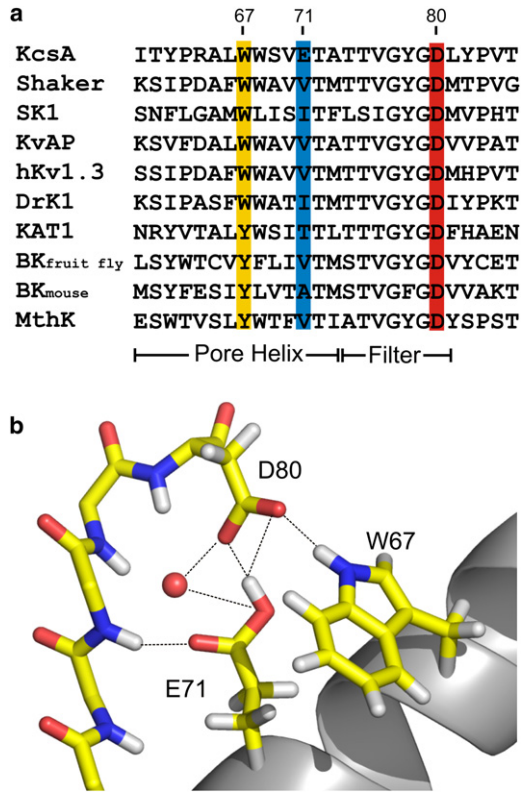

FIGURE 1 Nature of the hydrogen-bond network surrounding the K^+ channel selectivity filter. (*a*) Sequence alignment of KcsA and a series of Kv channels. We highlight the members of the Glu-71-Asp-80-Trp-67 interacting triad and show their conservation for a variety of Kv channels. (*b*) High-resolution crystal structure of the KcsA selectivity filter and adjacent structures (1K4C) (19). The Glu-71-Asp-80 and Trp-67-Asp-80 hydrogen-bond interactions between the pore helix and external vestibule are implicated as the driving force for C-type inactivation.

reversal potential (4). Previous attempts to substitute Trp-67 (4–9) and Asp-80 by alanine yielded channels with compromised hydrodynamic stability, according to size exclusion chromatography.

Unexpectedly, although at positive potentials W67F clearly inhibits entry to the inactivated states, normalized macroscopic currents of W67F at −150 mV show an enhanced rate of inactivation relative to WT KcsA (Fig. 3 *a*, *middle panel*). Ionic current density for W67F (Fig. 3 *a*, *right panel*) was much lower than WT at hyperpolarizing potentials, even when the amount of current was the same at depolarizing potentials, suggesting that channels at −150 mV might be either preinactivated (34,35) or inactivate faster than the activation process (36). Steady-state single-channel records of W67F at hyperpolarizing potentials are dominated by very long silent periods interrupted by a burst of activity, mirroring the behavior of WT and inactivating mutants (Fig. 3 *b*, *bottom*).

To better characterize the inactivation process in the W67F mutant, we measured its macroscopic current behavior under asymmetric conditions (200 mM K^+ intracellular/5 mM K^+ + 195 mM glycine extracellular; E_{rev} ~ −85 mV). Our results show that inactivation is relieved at extreme depolarizing potentials, but is present at less depolarizing and hyperpolarizing ones with an increasing rate of inactivation relative to WT KcsA (Fig. 3 *c* and Fig. S3). Furthermore, the enhancement of inactivation by the W67F substitution to less depolarizing and hyperpolarizing potentials resembles, to a lesser extent, the results of the deeply C-type inactivated mutant W434F in *Shaker*.

On the influence of hydrogen-bond interactions behind Kv channel selectivity filters

Although the Glu-71-Asp-80 interaction is absent in Kv channels (Fig. 1 *a*), most eukaryotic K^+ P-loops preserve the equivalent KcsA interaction between the Trp-67 and Asp-80 (i.e., Trp-434-Asp-447 in *Shaker* and Trp-366-Asp-379 in Kv1.2). This Trp-Asp hydrogen bond has been shown, using molecular dynamics simulations, to be among the strongest interactions at the selectivity filter of Kv channels and was suggested to play an equivalent role in gating, as it does in KcsA (37). Therefore, we evaluated the influence of the interaction between the eukaryotic equivalents of KcsA positions 67 and 80 in mutated *Shaker* and Kv1.2 channels by two-electrode voltage clamp.

In *Shaker*, substitution of Trp-434 for a phenylalanine abolishes ionic currents without affecting the behavior of the intracellular gate or the voltage-sensing domain (15). In contrast, placing a tyrosine at the same position (Fig. 4 *a*) allows for a robust ionic current, with a very fast C-type inactivation process (30 ms). A similar set of substitutions in Kv1.2 led to the same overall result. Fig. 4 *b* and Fig. S5 show that W366Y mutant in Kv1.2 enhances the inactivation process, however to a lesser extent than in *Shaker*. Furthermore, W366F, which in *Shaker* fully abrogates ion conduction, is able to support macroscopic currents in Kv1.2, although with a sharply accelerated C-type inactivation time constant (Fig. 4 *b*, inset). The present results confirm the importance of the interactions between the pore helix and the outer vestibule not only for KcsA but also for *Shaker* and Kv1.2 and suggest that the hydrogen-bond network behind the selectivity filter serves as a critical modulator for C-type inactivation throughout the K^+ channel family.

DISCUSSION

Although the structure of the selectivity filter is highly conserved among K^+ channels (19,38,39), their great diversity in kinetics and gating modes pose a challenge to generalize a common gating mechanism. A key structural component in most K^+ channel selectivity filters is the presence of a conserved ring of aromatic residues involved in intra- and inter-subunits contact, called the aromatic girdle (22). In KcsA and Kv1.2 crystal structures, this aromatic

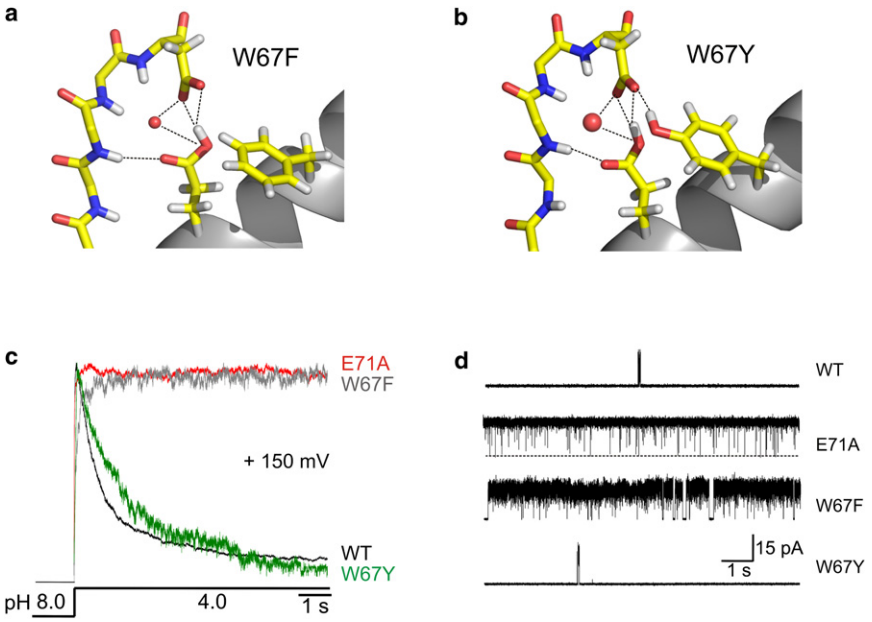

FIGURE 2 Influence of aromatic substitutions at Trp-67 in KcsA. Image of a single P-loop subunit with the putative hydrogen-bond network extracted from the last step of a 5 ns molecular dynamics simulations W67F (*a*) and W67Y (*b*). (*c*) Normalized macroscopic K^+ currents activated by pH jump (from 8 to 4) in WT-KcsA and mutants W67F, W67Y, and E71A. Traces were obtained at depolarizing potentials in symmetric 200 mM KCl. (*d*) Representative single-channel traces from WT-KcsA and mutants W67F, W67Y, and E71A obtained at pH 4 and +150 mV in symmetric 200 mM KCl.

ring is formed by the tyrosine from the signature sequence (TVGYGD), hydrogen bonded with a tryptophan side chain on the pore helix from the adjacent subunit. This network has been suggested to stabilize the structure of the selectivity filter (19,22,38), presumably by contributing to a putative rigidity necessary to sustain precise carbonyl-ion coordination in the filter. Additionally, a hydrogen-bond interaction between the tryptophan on the pore helix and

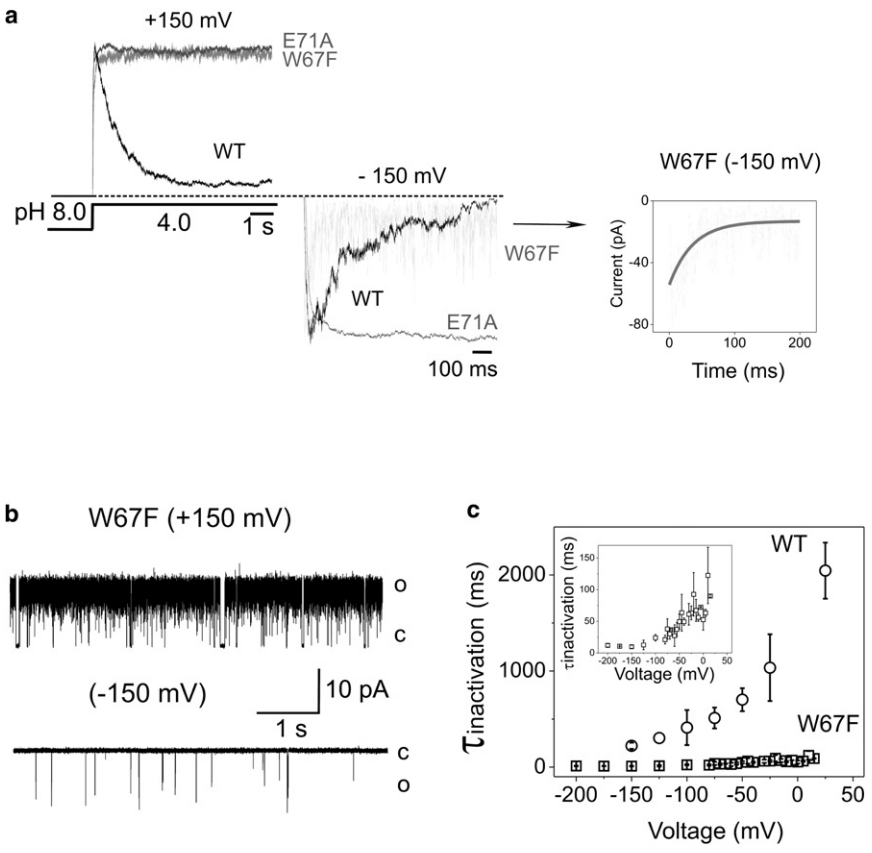

FIGURE 3 C-type inactivation in W67F mutant. (*a*) Normalized macroscopic currents in W67F mutant show a slower inactivation time constant at depolarizing potentials (+150 mV) and a faster time constant at hyperpolarizing potentials (−150 mV, 30.71 ± 17.38 ms), when compared to WT currents (−150 mV, 500 ± 220 ms; +150 mV, 1300 ± 789 ms, *left* and *middle panel*). Right panel shows a higher resolution detail of W67F macroscopic current at hyperpolarizing potentials (−150 mV). (*b*) Representative single channel traces from W67F obtained at pH 4 and ± 150 mV, under symmetric 200 mM KCl. The long closed time periods characteristic of inactivation are present at hyperpolarizing potentials (*bottom*, −150 mV) but absent at depolarizing ones (*top*, +150 mV). (*c*) Comparison of the time constant of inactivation shows that W67F (*square*) inactivates much faster at less depolarizing and hyperpolarizing potential in asymmetric conditions than WT KcsA (*circle*). Data shown are means ± SD $n > 10$.

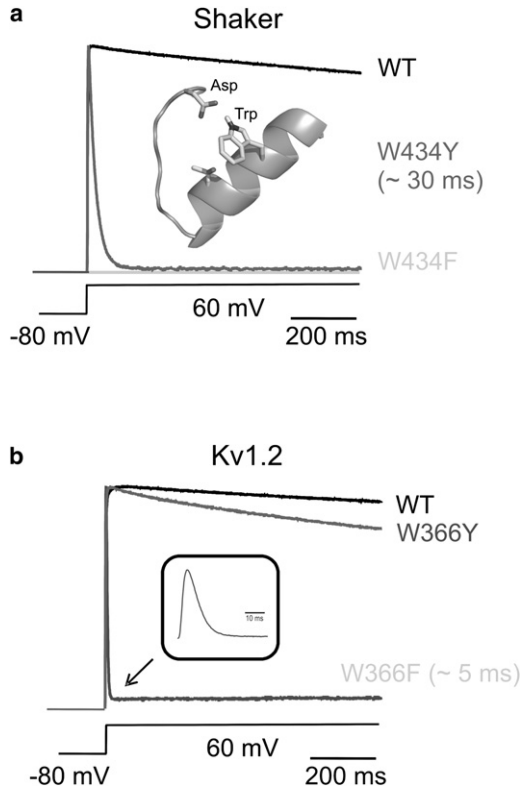

FIGURE 4 Hydrogen-bond interactions at the selectivity filter serve as the basis for C-type inactivation in eukaryotic Kv channels. (*a*) Normalized macroscopic currents of WT *Shaker*, W434F, and W434Y mutants are shown. W434Y substitution shows robust ionic current and faster time constant of inactivation than WT *Shaker*. Inset shows the single P-loop subunit of Kv1.2 (19,22,38). (*b*) Normalized macroscopic currents of WT Kv1.2, W366F, and W366Y mutants are shown. W366Y mutation in Kv1.2 enhance the inactivation process, however to a lesser extent than in *Shaker*. However, in contrast to *Shaker*, W366F Kv1.2 shows robust macroscopic current with a fast time constant of inactivation. Inset shows a higher resolution detail of W366F macroscopic current. Currents were elicited by a depolarizing voltage step from a holding potential of −80 mV to +60 mV.

the aspartic acid of the signature sequence in the extracellular loop (Fig. 1, *a* and *b*) is not only highly conserved but was estimated to be the strongest interaction at the selectivity filter (33,37). However, recent structural evidence shows that the selectivity filter and adjacent elements appear to be far more dynamic than initially postulated and this possible flexibility might be a critical requirement during gating behavior at the selectivity filter (4–6).

In KcsA, the attractive interaction between Glu-71 in the pore helix and Asp-80, at the top of the selectivity filter, has been shown to be one of the key forces driving the filter toward its nonconductive conformation. Disrupting this interaction relieves C-type inactivation, thus increasing the steady-state open probability (4) in a highly cooperative way (40). In other K^+ channels, the equivalent to KcsA Trp-67 is highly conserved, being populated by tryptophan or tyrosine. This suggests that amino acids with the ability to establish hydrogen bond might be favored at this position. Aromatic residues at the filter have been shown to undergo considerable conformational changes, presumably involved in KcsA gating (Fig. S1) (4,23,25,41,42). In this work, we have evaluated the role of conserved aromatic residues in the pore helix of KcsA (Trp-67), *Shaker* (Trp-434), and Kv1.2 (Trp-366), as part of a multipoint H-bond network that modulates C-type inactivation in K^+ channels.

How does W67F modulate KcsA selectivity filter? We suggest that at depolarizing potentials, the carboxylate group of Glu-71 orients toward the intracellular end of the channel (Fig. 5), weakening the Glu-71-Asp-80 interaction (critical for inactivation) and thereby increasing the flexibility of Asp-80. This flexibility would be restricted by a second interaction between Trp-67 and Asp-80 in the WT. However, in the absence of this hydrogen bond (W67F), Asp-80 might be able to fluctuate more extensively, reducing the effectiveness of the Glu-71-Asp-80 interaction, and thus decreasing the extent of inactivation at depolarizing potentials (Fig. 3 *a*, *left panel*). In contrast, hyperpolarizing voltages should favor the Glu-71-Asp-80 interaction, increasing the extent of inactivation as shown in the middle panel of Fig. 3 *a*. The lack of hydrogen bond between Phe-67 and Asp-80 might allow for a stronger interaction between Glu-71 and Asp-80 at negative potentials, increasing the rate of inactivation in the W67F substitution (Fig. 3 *a*, *b*, and *c*).

We have shown that the strength of the interaction between Glu-71 and Asp-80 directly correlates with the rate of entry into the inactivated state (5). The fact that W67F proceeds with a faster time constant of inactivation when compared to WT KcsA (to less depolarizing and

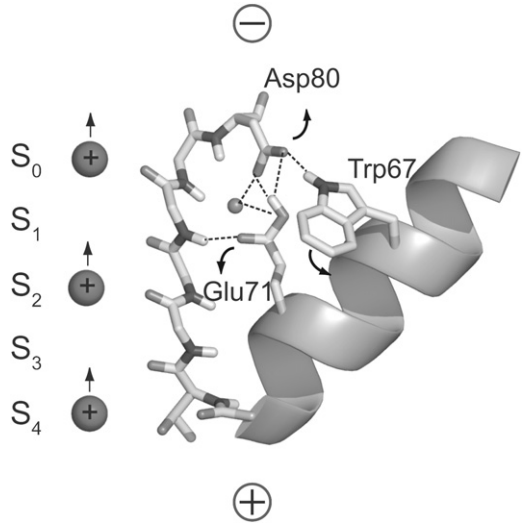

FIGURE 5 Mechanistic interpretation of the role of a multipoint hydrogen-bond network at the KcsA selectivity filter (1K4C) (19). At positive potentials, reorientation of Glu-71 away from Asp-80 weakens the Trp-67-Glu-71-Asp-80 interaction triad. Negative potentials reorient Glu-71 toward Asp-80, strengthening the interaction triad.

hyperpolarizing potentials) resembles, to a lesser extent, the results of the deeply C-type inactivated mutant W434F in *Shaker* (Fig. 3 *c*). This result would imply that the absence of a Phe-67-Asp-80 interaction potentiates the Glu-71-Asp-80 interaction, accelerating inactivation. Together, these results are consistent with the idea that KcsA inactivation is also modulated by the Trp-67-Asp-80 interaction, forming a multipoint hydrogen-bond network anchored by Asp-80. This mechanism is likely to be responsible for C-type inactivation in Kv channels, even if the composition and strength of the hydrogen-bond interaction network between the selectivity filter and the pore helix vary from channel to channel. Indeed, mutations to Phe and Tyr in W434 in *Shaker* and W366 in Kv1.2 play a critical role in modulating entry into the inactivated state (Fig. 4). Thus, small differences in this network of interactions might account for the large variability in the degree and kinetics of C-type inactivation throughout the K^+ channel family.

SUPPORTING MATERIAL

Five figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495S0006-3495(11)00324-9.

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The authors declare they have no competing financial interests.

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