A specialized molecular motion opens the Hv1 voltage-gated proton channel

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The Hv1 proton channel is unique among voltage-gated channels for containing the pore and gate within its voltage-sensing domain. Pore opening has been proposed to include assembly of the selectivity filter between an arginine (R3) of segment S4 and an aspartate (D1) of segment S1. We determined whether gating involves motion of S1, using Ciona intestinalis Hv1. We found that channel opening is concomitant with solution access to the pore-lining face of S1, from the cytoplasm to deep inside the pore. Voltage- and patch-clamp fluorometry showed that this involves a motion of S1 relative to its surroundings. S1 motion and the S4 motion that precedes it are each influenced by residues on the other helix, thus suggesting a dynamic interaction between S1 and S4. Our findings suggest that the S1 of Hv1 has specialized to function as part of the channel’s gate.

Voltage-gated proton channels are expressed in a variety of cell types and tissues. They were first described in snail neurons 1 and were subsequently found in many different species across phyla, including cocolithophores, amoeboidozoa, echinoderms, tunicates and vertebrates 2. They have important physiological roles, for example, acid extrusion in lung epithelial cells 3 and sperm 4,5, and regulation of pH homeostasis in phytoplankton 6. In immune cells, voltage-gated proton channels are involved in charge compensation during production of reactive oxygen species by the NADPH oxidase complex 6–11. In microglia, voltage-gated proton channels increase brain damage after ischemic stroke by assisting in the production of reactive oxygen species 12. The voltage-gated proton channel Hv1 is a peculiar member of the superfamily of voltage-gated cation channels. ‘Classical’ voltage-gated cation channels are tetrameric, and each subunit contains a voltage-sensing domain (VSD), with transmembrane segments S1–S4, and a pore domain (PD), which contributes transmembrane proton channels are involved in charge compensation during production of reactive oxygen species by the NADPH oxidase complex 6–11. In microglia, voltage-gated proton channels increase brain damage after ischemic stroke by assisting in the production of reactive oxygen species 12. The voltage-gated proton channel Hv1 is a peculiar member of the superfamily of voltage-gated cation channels. ‘Classical’ voltage-gated cation channels are tetrameric, and each subunit contains a voltage-sensing domain (VSD), with transmembrane segments S1–S4, and a pore domain (PD), which contributes transmembrane segments S5 and S6 and an intervening P region to the central pore 13. However, after the cloning of Hv1 (refs. 14,15), it became clear that Hv1 has a markedly different architecture: it contains only the VSD and lacks the traditional PD. Hv1 is a dimer 16–18 with a separate pore in each subunit, and interaction between subunits results in cooperative gating 19,20. The cytosolic C-terminal region contains a coiled coil 21,22 that is necessary for dimerization but not for permeation, and the N-terminal region is also dispensable for proton conduction 16,18. Moreover, purified Hv1 can be functionally reconstituted in artificial bilayers 17,23, thus indicating that the VSD contains the channel’s voltage sensor, gate and pore. In Hv1, like its tetrameric cousins, the S4 segment moves outward upon membrane depolarization 19. S4’s third voltage-sensing arginine, R3, which enters the membrane at positive voltage 19, is important for proton selectivity 24. R3 appears to interact with D1 (D112 in human Hv1 (ref. 24)), an aspartate in the middle of S1 that is unique to Hv1 and is critical for proton selectivity 22,24 and selectivity against anions 25. These findings led to the hypothesis that S4’s outward motion places R3 into register with D1 to form the selectivity filter during channel opening. However, a channel with a truncated S4 that is missing R3 still supports a small voltage-gated proton current 26.

We set out to investigate the role of S1 in gating. We find that a voltage-dependent conformational change that is associated with channel opening increases the access of methanethiosulfonate (MTS) reagents from the internal solution to the face of S1 that contains D1 and therefore faces the pore, until deep into the membrane. Voltage- and patch-clamp fluorometry confirm that S4 moves in relation to its surroundings, with the timing and voltage dependence of the opening transition. This stands in contrast to S4, whose rearrangement precedes opening, as expected for voltage sensing. Our findings indicate that two distinct but interdependent rearrangements involving S1 and S4 take place during the gating process, and they suggest that channel opening involves a rearrangement of S1 that opens access for bulk water deep toward the selectivity filter.

RESULTS
S1 accessibility suggests voltage-dependent motion around S1
We tested for voltage-dependent changes in solvent accessibility for S1 cysteine mutants by measuring their rate of modification by membrane-impermeable MTS reagents. The substituted-cysteine accessibility method (SCAM), which assumes that the modification rate by MTS compounds is directly proportional to the solvent accessibility of the introduced cysteine, was previously used to demonstrate that S4 translocates through the membrane during the gating of voltage-dependent ion channels, including Hv1 (refs. 19,27–29). In total, we made 29 S1

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cysteine mutants of *C. intestinalis* Hv1 (CiHv1) (Fig. 1a). We tested external accessibility to 2-(trimethylammonium)ethylmethanethiosulfonate bromide (MTSET) of residues C terminal to D1 in two-electrode voltage clamp (TEVC) recordings and internal accessibility of residues N terminal to D1 in excised inside-out patch-clamp recordings.

For residues located at the extracellular end of S1, we used two protocols that kept the channel at depolarized potential either 60% or 10% of the time (Online Methods), thereby changing the relative time spent in the open and closed state. Wild-type (WT) current ran down by about 10% and 25% when depolarized 10% and 60% of the time, respectively (Supplementary Fig. 1a), and showed no effect of exposure to either extracellular sodium [2-sulfonatoethyl] methanethiosulfonate (MTSes) (Supplementary Fig. 1b) or MTSET (Supplementary Fig. 1c). At the external end of S1, only mutants K173C and D171C were affected. External MTSET irreversibly accelerated closure of K173C, but the rate of modification was not significantly state dependent (Fig. 1b–f). The rate of MTSET-induced inhibition of D171C was 2.2-fold faster with MTSET (Supplementary Fig. 1d) and 5.5-fold faster with MTSES (Fig. 1g–k) when depolarized 60% versus 10% of the time. The differential effects of MTSES and MTSET on D171C may be explained by local differences in surface electrostatics or by local changes in pH during gating that favor MTSET over MTSET at depolarized potentials. That the two MTS compounds of opposite charge show a voltage dependence of action nevertheless suggests a slight increase in accessibility around position 171 at depolarized potentials.

We next turned to residues located at the intracellular side of S1 (146–159). In inside-out patches, MTSET had no effect on WT channels (Supplementary Fig. 1e), but we detected modification of five cysteine mutants (Fig. 1a). One mutant (H150C) was too inconsistent in behavior to allow qualitative assessment of state dependence (Supplementary Fig. 1f). MTSET accelerated opening of V151C, I154C and V157C (Fig. 2a–e and Supplementary Fig. 1g,h) and inhibited I153C (by approximately 70%; Fig. 2f–h). All four positions showed a very sharp state dependency, with no or very little modification in the resting state at −80 mV and rapid modification in the open state at +80 mV (Fig. 2 and Supplementary Fig. 1).

In summary, the results suggest that the largest conformational changes around S1 take place at its internal side. Mapping of the internal positions onto the crystal structure of an mHv1-CiVSP-GCN4 chimera (PDB 3WKV, mHv1cc) reveals that all but position 151 lie on the same face of the helix as D1 in the middle of S1 (Supplementary Fig. 2), thus suggesting a large structural rearrangement around the VSD internal vestibule. The structural rearrangements around S1 differ from those in the region of S4, which undergoes substantial and converse exposure changes at internal and external ends.

### Environment changes of S1 correlate with channel opening

To identify the functional transition associated with the voltage-dependent change in S1 accessibility, we took advantage of the sharp state dependence of the I153C modification by MTSET and measured the modification rate at a range of voltages (Online Methods). The voltage dependence of the I153C modification rate by MTSET (accessibility-voltage relationship, A–V) was very close to the conductance-voltage relationship (G–V) of the channel before MTS modification.
Figure 2. Strong voltage dependence of accessibility at the internal end of S1. (a) Schematic for experiments in b–e showing modification of V151C by internal MTSET only at positive voltage (n = 4 patches). (b) V151C currents before (control) and during application, first at −80 mV, then during repeated steps to +80 mV (chronological color code from violet to red) and after washout (at −80 mV) of 200 µM MTSET. Inset, magnification of the current traces at stimulus onset. (c) MTSET-induced steady-state current increase at arrowhead in b, at −80 or +80 mV. Dashed line, absence of effect. (d) Outward-current change at −80 or +80 mV, with exponential (+80 mV) or linear (−80 mV) fit. (e) Rate constants of modification at −80 mV (k_{MTSET} = 1.6 ± 0.4 M^{-1} s^{-1}) and +80 mV (k_{MTSET} = 558 ± 60 M^{-1} s^{-1}). (f) Schematic for experiments in g–j showing modification of I153C by internal MTSET only at positive voltage. (g) Currents before (control) and during application, first at −80 mV, then at +80 mV and after washout (at −80 mV) of 1 mM MTSET. (h) MTSET-induced steady-state current inhibition at −80 mV (n = 5 patches) or +80 mV (n = 6 patches), measured at arrowhead in g. Dashed line, absence of effect. (i) Current in response to an +80-mV voltage step in the presence of MTSET, with single exponential fit (gray trace). (j) Rate constant of modification at +80 mV (k_{MTSET} = 920 ± 170 M^{-1} s^{-1}). ***P < 0.001 by two-tailed Student’s t-test; error bars, s.e.m.

Figure 3. Environmental changes around S1 are concomitant with gating. (a) Internal end: comparison of the voltage dependence of the normalized modification rate of I153C by MTSET (accessibility, green curve) with the voltage dependence of I153C conductance before MTS modification (G, black curve). For A–V curve (n = 3 patches for −20 and −10 mV data points; n = 2 patches for other data points), V1/2 = −27 ± 2 mV; kT/ζ0 = 14 ± 3 mV. For G–V curve (n = 6 patches), V1/2 = −30 ± 4 mV; kT/ζ0 = 7.61 ± 0.25 mV. (b–e) VCF ΔF5 at the outer end of S1, tracking opening and closing kinetics. (b) Cartoon and sequence alignment of CiHv1, hHv1 and mHv1 with TAMRA-MTS attached to I175C. (c) VCF current and fluorescence traces (as indicated in cartoon inset) of 175C* for steps from −80 mV to gray, −100 mV; blue, −40 mV; green, +20 mV; yellow, +60 mV; and red, +100 mV. (d) Left, superposition of normalized current (black) and fluorescence (red) for a voltage step to +100 mV (activation). Right, average fast and slow activation time constants (τon) of current and fluorescence (double exponential fit; n = 14 oocytes; P = 0.39 for fast τon and 0.12 for slow τon by two-tailed paired t tests). (e) Left, superposition of normalized current (black), fluorescence (red) and inverted fluorescence (pink) during repolarization from +100 to −80 mV (deactivation). Right, average deactivation time constants (τoff) of current and fluorescence (single exponential fit; n = 8 oocytes; P = 0.08 by two-tailed paired t test). Error bars, s.e.m., except in a, in which bars represent range. NS, not significant.
suggest that changes in S1 environment are associated with opening and closing of the channel gate.

When Hv1 opens, proton flux can modify the local pH and thereby alter gating properties and current kinetics. Even in the presence of strong pH buffer (ref. 19 and Online Methods), the pH is never perfectly controlled in oocytes. We therefore turned to patch-clamp fluorometry (PCF), which provides a tight control of intra- and extracellular pH, using symmetrical 100 mM HEPES solutions perfectly controlled in oocytes. We therefore turned to patch-clamp fluorometry (PCF), which provides a tight control of intra- and extracellular pH, using symmetrical 100 mM HEPES solutions.

Supplementary Fig. 4b,c), thus indicating that the ΔF at 175C* is caused by conformational changes of the channel, not by proton flux.

To confirm that the conformational changes tracked at the external end of S1 represent the opening and closing transitions, we perturbed channel gating by mutating aspartate D1 (D160) to glutamate and then analyzed the effect on the ΔF at 175C*. In hv1, mutation of D1 to glutamate does not disrupt proton selectivity25 but slows channel closure24. Similarly, we found that the D1E mutation in the 175C* background slowed deactivation by a factor of 4 while barely affecting the activation time constants (Supplementary Fig. 4d–f). The fluorescence kinetics changed by the same amount, and a good match between current and fluorescence kinetics in D1E 175C* remained (Supplementary Fig. 4e,f). Thus, a structural rearrangement around S1 accompanies the opening and closing steps.

S1 undergoes voltage-dependent motion
Do the changes in fluorescence and solution accessibility reflect a rearrangement of S1 itself, or are they indirect consequences of motion of another part of the protein, such as S4? Although S4 undergoes a major transmembrane motion that displaces gating charge19,34, it has also been proposed to undergo a conformational change during opening35, thus raising the possibility that environmental changes monitored at S1 may indirectly report S4 motion. If S1 itself underwent a rearrangement during the gating step, ΔFs tracking the gating step should be observed at several attachment sites pointing to different directions on S1. Position 173C labeled with Alexa488-maleimide (173C-Alexa) yielded sufficient ΔFs to permit detailed analysis. 173C-Alexa ΔFs contained two main components: a very fast component (Ffast) occurring during the depolarization- and repolarization-induced capacitive transients, and a slower fluorescence component (Fslow) (Supplementary Fig. 5a–c). Although Ffast was faster than channel

Figure 4 PCF fluorescence changes at S1 track gating. (a) PCF current and fluorescence traces of 175C* for steps from −80 mV to gray, −100 mV; blue, −40 mV; green, 0 mV; yellow, +40 mV; and red, +80 mV. Left inset, EMCCD fluorescence image of an inside-out giant patch from an oocyte expressing 175C labeled with TAMRA-MTS. Bright curved line circled in green is the voltage-clamped labeled membrane in the pipette. Right inset, cartoon of fluorescent inside-out patch with internal and external pH indicated. (b) F-V (red) and G-V (black) curves (measured at arrowheads in a). For G-V, V1/2 = 24 ± 6 mV and kTz20 = 13.5 ± 0.9 mV; for F-V, V1/2 = 19 ± 6 mV and kTz20 = 17 ± 1 mV (n = 4 patches). (c) Superposition of the normalized current (black) and fluorescence (activation, yellow) or inverted fluorescence (deactivation, orange) for a voltage step to +40 mV (activation) and back to −80 mV (deactivation). (d) Top, average fast (closed circles) and slow (open circles) time constants of current (black) and fluorescence (red) in response to different voltage steps (activation, double exponential fits). Bottom, average current and fluorescence time constants after repolarization to −80 mV (deactivation, single exponential fits). n = 6 patches. Error bars, s.e.m.; a.u., arbitrary units.
opening and closure and occurred at potentials hyperpolarized to channel opening (Supplementary Fig. 5c,d). $F_{\text{slow}}$ had kinetics and voltage dependence that matched channel gating (Supplementary Fig. 5d–i). According to the mHv1cc crystal structure, K173 points away from the VSD, and I175 is predicted to point toward the interior of the VSD31. Thus, at least two positions of the S1 segment pointing in different directions (and thus pointing toward different environments), 173 and 175, can track the gating transition. This supports the hypothesis that the $\Delta F$s at these positions reflect an S1 motion rather than changes in the environments to which the fluorophores point.

To examine S1 rearrangement in a more direct fashion, we considered an intriguing implication of a putative S1 motion. In the dimer, the outer end of S1 is close enough to the other subunit’s S1 to form a disulfide bond17. Therefore, an S1 motion would change the S1-S1 distance or orientation. Tryptophans quench the fluorescence of organic fluorophores in a distance- or orientation-dependent manner through electron transfer from the tryptophan side chain to the excited state of the fluorophore36–38. As a consequence, tryptophan-induced amplitude changes of dye fluorescence can be used to study structural rearrangements in proteins, as was previously shown in other ion channels39,40. We constructed tandem dimers with only one subunit containing a cysteine at position 174, at the external end of S1, and labeled it with TAMRA-MTS (174C*). In tandem dimers in which the 174C* subunit faced a WT subunit (WT-td-174C*), we detected no $\Delta F$ during a voltage step to +100 mV (Fig. 5a). In contrast, when the 174C* subunit faced a subunit with a tryptophan substitution at position V174 in S1 (V174W-td-174C*), we observed a clear $\Delta F$ in response to a +100-mV step voltage ($\Delta F/F = -1.3 \pm 0.3\%$; Fig. 5a,b). Because WT-td-174C* channels are fluoroscently silent, the $\Delta F$ of V174W-td-V174C* reflects quenching of TAMRA by the tryptophan at depolarized potentials. We thus find that the external ends of the two S1 segments in the dimer move in relation to each other after membrane depolarization, a result indicating that S1 does indeed undergo a structural rearrangement.

S1 and S4 undergo distinct motions and influence each other

Next, we tracked S4 motion with TAMRA-MTS at position 249 by using PCF (Fig. 6a). Hyperpolarization increased fluorescence, whereas depolarization decreased fluorescence (Fig. 6b). The F-V curve was negatively shifted as compared to the G-V curve (Fig. 6c). In addition, the $\Delta F$ took place during the fast current component (fluorescence $\tau_{\text{off}} = 12 \pm 6$ ms versus current fast $\tau_{\text{off}} = 21 \pm 9$ ms at +40 mV; Fig. 6d) and thus was faster overall than channel opening. We also observed a similar $\Delta F$ preceding gating in a kinetic and voltage-dependent manner at the nearby position 242C (Supplementary Fig. 6 and refs. 19,35), this being consistent with $\Delta F$s at 242C and 249C tracking the voltage-sensing rearrangement of S4. This is in contrast to the structural changes in the S1 region, which track opening in both a kinetic and a voltage-dependent manner.

Mutants of selectivity-filter residues D1 and R3 further distinguished S1 and S4 motions. In VCF, double-mutant neutralization with D1N R3S slowed $\Delta F$ deactivation kinetics only at 249C* but not at 175C* (Fig. 7a–d). In addition, D1N 175C* showed a rightward shift of both $\Delta F$-V relationships compared to 249C*, preserving a close match between the F-V and G-V in the mutant (a further confirmation that 175C* fluorescence tracks gating; Supplementary Fig. 7a). In contrast, the discrepancy between the voltage-dependent channel opening and S4 motion increased for D1N 249C* as compared to 249C* (Supplementary Fig. 7b). These results further support the interpretation that the $\Delta F$ observed at 175C* reflects not a motion of S4 but a distinct conformational change of S1 that is associated with gating.

Although S1 and S4 seem to move together during deactivation in the WT background (S1 and S4 $\Delta F$s both have off rates similar to the tail current), their kinetics differ in the D1N R3S background (Fig. 7c,d and Supplementary Fig. 7c,d). By neutralizing the selectivity-filter residues, we were thus able to decouple S1 and S4 motions during repolarization. We furthermore found that mutating R3 to serine induced a slight acceleration of deactivation $\Delta F_{\text{off}}$ for the S1 labeling site (175C*) (by about two-fold; Supplementary Fig. 7e,f) and shifted the F-V by around +20 mV to the right (Supplementary Fig. 7a). In the D1N 175C* background, however, the R3S mutation accelerated $\Delta F_{\text{off}}$ by 50-fold (comparison of $\tau_{\text{off}}$ of D1N 175C* and...
D1N R3S 175C* in Fig. 7 and Supplementary Fig. 7f), thus suggesting an interaction between D1 and R3 in the active state. Neutralization of D1 slowed the fast component of the ΔFoff of the S4 labeling site (249C*) by approximately eight-fold, introduced a brightening component to the dimming ΔFin (Supplementary Fig. 7g,h) and increased the steepness of the voltage dependence of the 249C* fluorescence signal (Supplementary Fig. 7b). Because, as shown above, S4 deactivation motion seems to be limited by S1 motion in the WT (Supplementary Fig. 7a), slowing of 249C* ΔFoff by the D1N mutation may be indirectly due to its slowing of S1 motion (by approximately 60-fold in the 175C* background; Supplementary Fig. 7e), thus reinforcing the idea of an interaction between S1 and S4 in the active state. These results suggest that interaction between S1 and S4 via their selectivity-filter residues D1 and R3 mutually influences the motions of these domains.

DISCUSSION

The Hv1 proton channel is unique because its VSD contains the channel's voltage sensor, gate and pore. A depolarization-driven outward motion of S4 has been proposed to trigger a subsequent rearrangement that opens the channel19,20,24,35,41. Using cysteine-accessibility analysis and fluorometry, we show that channel opening also involves a rearrangement of S1, whose unique D1 lines the selectivity filter in the pore14,35.

Several lines of evidence suggest that the state-dependent change in environment around S1 is caused by a structural rearrangement in S1 itself: (i) at position 1153C of S1, the A-V relationship closely tracked the G-V relationship, thus indicating that the rearrangement that changes the environment around S1 is associated with the opening transition; (ii) concordantly, fluorometry showed that the voltage dependence and kinetics of the structural rearrangement around the outer end of S1 occur during the opening and closing of the channel; (iii) in contrast, the rearrangement around the outer end of S4 preceded channel opening kinetically and occurred at more negative voltage; (iv) the association between S1 fluorescence change and channel opening was maintained when gating was perturbed by mutations, whereas the S4 fluorescence change was further dissociated from the open-closed transition; and (v) fluorescence quenching between a fluorophore attached to S1 in one subunit and a tryptophan introduced into the S1 of the other subunit revealed a voltage-dependent change in quenching that indicates a relative motion of the two S1 segments in the dimer. Together, accessibility and fluorometry results suggest that opening and closing of the gate of Hv1 involves a structural rearrangement of S1.

The four positions near the internal end of S1, which become more accessible to the internal solution at positive voltage, span the internal half of S1 and reach from the inside up to one helical turn before D1 (Supplementary Fig. 2), thus suggesting that S1 motion opens a deep internal crevice when the channel opens. Three of these residues, 153, 154 and 157, are located on the same face of the helix as selectivity residue D1 (ref. 31 and Supplementary Fig. 2) and therefore, like D1, are predicted to face the lumen of the open pore. Thus, channel opening appears to be accompanied by a rearrangement that opens solution access to the selectivity filter (Fig. 7e and Supplementary Fig. 8a). This could account for the selective open-state entry of bulky guanidinium blockers from the internal solution into the pore41,42.

The mHv1cc crystal structure was proposed to capture a closed state, on the basis of the presence of Zn2+ at the external site whose occupancy favors closure31. Although an internal vestibule is already present in the structure, it is too narrow to allow MTSET to reach cysteine at position 157, located in the lower hydrophobic plug, along with F198 and F230 (refs. 31,43,44). Although the pathway to positions 154 and 153 is wide enough to accommodate water in the crystal structure (Supplementary Fig. 8b,c), these residues are surrounded by I206, I262 and H150, which could hinder MTSET access (Supplementary Fig. 8c,d). This may also be the reason why docking of guanidinium blockers is hindered in the closed state42. Finally, position 151 points away from the VSD but is likely to either take part in the dimer interface or be buried in the membrane in membrane-embedded dimeric channels, thus being inaccessible to water-soluble MTS compounds. We therefore propose that after the voltage-sensing outward motion of S4 the channel opens via a motion that includes a rearrangement of S1 with respect to its surroundings, which widens the internal vestibule and thereby allows for solution access from the cytoplasm to the selectivity filter to enable proton flux (Fig. 7e and Supplementary Fig. 8a).

Depolarization induced a drastic increase in accessibility in the internal side of S1 but only mildly affected the accessibility at one site at the external side of S1. Still, this rearrangement at the external end of S1 was sufficient to generate an inter-S1 change in the
dimer that we detected as a tryptophan–quenching change. Thus, S1 appears to undergo a motion relative to its surroundings that pivots near its external end so that a larger rearrangement occurs at its internal end (Fig. 7e and Supplementary Fig. 8a). This is in contrast to the voltage-sensing motion of S4, whose outward transmembrane displacement by depolarization induces drastic and opposite changes of exposure at its internal and external ends19, as seen in Kv and Nav channels27,28,45.46. The mechanism that drives S1 motion still needs to be determined. S1 contains three negatively charged residues (D1, E167 and D171) that could move inward at positive voltage and therefore could carry some gating charge45. However, the gating charge of Hv1 appears to be mostly contained in the arginines of S4 (ref. 34), thus suggesting that S1 motion is a consequence of the voltage-dependent rearrangement of S4.

The gating rearrangement at the external end of S1 might contribute to intersubunit cooperativity19,20. S1 motion during the opening of one of the subunits would be expected to influence S1 motion and therefore the opening of the other subunit, as suggested recently by the influence of a mutation at the outer end of S1 on a cooperative rearrangement tracked by S4 (ref. 35).

Although in classical tetrameric voltage-gated channels S1 also appears to interact with S4 (refs. 47–50), the function and interactions of S1 in the dimeric Hv1 channel have diverged from those of classical channels. The S1 of Kv channels interacts with S5 and the pore helix51,52, which are absent in Hv1. In tetrameric channels, there is no contact between VSDs, whereas the S1 of Hv1 lies at the dimer interface between the VSDs17. Nevertheless, earlier voltage-clamp fluorometry on the Shaker Kv channel revealed a fast rearrangement in S1, although it was not resolved whether this corresponds to the voltage-sensing or opening steps53. The monomeric voltage-sensing phosphatase (VSP) also undergoes voltage-dependent conformational changes in S1 (ref. 54). However, in VSP, S1 and S4 fluorescence signals have similar properties, consistently with their participation in a common conformational step and in contrast to what we find here for Hv1. Thus, Hv1 seems to have a unique and specialized gating motion of S1 that allows access to the deep pore of the channel and enables proton flux through its VSD.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

L.M. and T.K.B. performed the experiments and analyzed data. L.M., T.K.B. and E.Y.I. designed the experiments and wrote the manuscript.

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ONLINE METHODS

Mutagenesis and expression in *Xenopus* oocytes. Female *Xenopus laevis* animals were housed and surged according to the guidelines of University of California, Berkeley's Animal Care and Use Committee (ACUC) (animal use protocol R187-0814). In some cases, *X. laevis* oocytes were purchased (Ecocyte). CiHV1 mutants were constructed via site-directed mutagenesis in the pSD6TF vector by QuikChange mutagenesis (Stratagene) and verified by sequencing. DNA was linearized with SfiI and transcribed to RNA with the SP6 mMessage mMachine Kit (Ambion). *X. laevis* oocytes were injected with 50 nL of RNA at a concentration of 0.5–2 µg/µL and incubated at 18 °C for 1–5 d in ND96 containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 5 mM pyruvate, and 100 mg/L gentamycin. pH 7.6. CiHV1 tandem dimers contained a 17–amino acid linker between the two Hv1 monomers with the sequence GGSSGGSGGSGGSGGSGG and were generated according to ref. 18.

Cysteine accessibility measurements in TEVC and patch-clamp recordings. Cysteine accessibility experiments to methanethiosulfonate (MTS) reagents were performed in TEVC and excised inside-out patch clamp. TEVC recordings were adapted from different protocols19,20. To minimize pH changes due to proton efflux, high-buffer solutions (100 mM HEPES) were used both intra- and extracellularly. On the day of the experiment, oocytes were injected with 50 nL of a solution of 1 M 2-[2-(hydroxyethyl)propyrazin-1-yl]ethanesulfonic acid (HEPES) at pH 7.0 (pH adjusted to 7.0 with KOH) and left 1–2 h at 18 °C to recover in the extracellular recording solution. This results in approximately 100 mM HEPES in the cytosol19. The extracellular recording solution contained 46 mM NaCl, 1 mM KCl, 1 mM MgCl2, 1 mM CaCl2, and 100 mM HEPES, adjusted to pH 7.5 with NaOH. Patch-clamp recordings were performed with symmetric pipette (extracellular) and bath (intracellular) solutions void of methanethiosulfonate, containing 100 mM HEPES, 30 mM methanesulfonic acid (MS), 5 mM N,N,N′,N′-tetraacetic acid (EGTA), adjusted to pH 7.0 with TEA hydroxide (>25 mM).

[2-(trimethylammonium)ethyl]methanethiosulfonate bromide (MTSET) and sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES) were purchased from Toronto Research Chemicals and stored as powder at −20 °C. On the day of the experiment, MTS powder was diluted in water to form stock solutions of 100 or 500 mM concentrations, which were stored at 0 °C. The stock solutions were then diluted in the recording media to the final concentration of 1 mM (H150C, 1153C, 1154C, and D171C), 0.5 mM (V157C), 200 µM (V151C), or 100 µM (K173C). To avoid variations in MTS concentration due to hydrolysis, final solutions were prepared freshly just before each recording and were kept for 15 min maximum.

Intracellular cysteine accessibility to MTS reagents of the S1 segment was tested by TEVC (pHout = 7, pHin = 7.5). Intracellular accessibility was tested by patch clamp in the inside-out-state configuration (pHina = pHout = 7). The holding potential was −80 mV. Irreversible steady-state effects of MTS compounds were calculated by division of the current amplitude after application and washout of the MTS by the current measured before MTS application. State dependence of MTS reaction was tested with different protocols that vary the proportion of the MTS by the current amplitude before MTS application. State dependence of MTS constants of MTSET modification (kMTSET) were calculated by direct fitting of the current decrease during the depolarization step to +80 mV in the presence of MTSET. For all other cysteine mutants, for which MTSET induced an acceleration of channel opening, rate constants of the MTS effect at +80 mV were calculated by plotting of the amplitude of the current (time point indicated by an arrowhead in Figs. 1 and 2 and Supplementary Fig. 1) against the time spent in MTS at +80 mV. Time constants of MTS (τMTS) inhibition were obtained from the single-exponential fits of the individual curves of current versus time in MTS, and the rate constants were calculated under the assumption of a pseudo-first-order reaction: 1/τMTS = kMTSET × [MTS]. The rate constants of the MTS effect at −80 mV were approximated by the slope of a linear fit between the current amplitudes before MTS application (at −80 mV) and after MTS washout, normalized by the current amplitude after complete MTS modification at +80 mV. MTS-induced maximal inhibition was defined as the ratio of the current amplitude after MTS washout by the current measured before MTS application at the time point indicated by an arrowhead.

Modification of 153C channels by MTSET induces a shift of the G–Y relationship by approximately −60 mV, so that in many patches the modified channels are open at the −80 mV holding potential. Voltage dependence of MTSET action at position 153C (Fig. 3) was determined by measurement of the rate of current increase at the holding potential, as a readout of the number of modified channels, with constant stepping to a determined voltage for 1 s every 1 s. This method enabled us to measure the rate of channel modification even for potentials that do not fully open the unmodified channel.

VCF recordings. VCF recordings were adapted from previously described protocols19,20,23,35. Oocytes were injected for 1–2 h before being recorded with a 1 M HEPES solution at pH 7.0 to obtain ~100 mM HEPES intracellularly. Oocytes were then labeled for 45 min to 1 h in ice with 50 µM of 2-((5(6)-tetramethylrhodamine)carboxyloxamino)ethyl methanethiosulfonate (TAMRA-HEPS). After the recording, oocytes were extensively washed in the recording solution and stored in the dark at −18 °C during the course of the experiment. Chemicals were chosen from Sigma-Aldrich or Thermo Fisher Scientific.

TEVC recordings were collected with a Dagan CA-1 amplifier. Fluorescence signals were acquired through a 20×, 0.75-NA objective (Nikon) on a Nikon Diaphot inverted microscope illuminated with a 150-W xenon lamp. Light was filtered through an HQ535/50 excitation filter, an HQ610/75 emission filter and a Q565LP dichroic beamsplitter (Chroma Technology). Fluorescence intensity was measured with a Hamamatsu HC120-05 photomultiplier tube. Fluorescence signals were low-pass-filtered at 2 kHz through an eight-pole Bessel filter (Frequency Devices) and sampled at 10 kHz through a Digida-1440A controlled by pClamp 10 (Molecular Devices). Holding potential was −80 mV. Unless specified, fluorescence and current traces are the average of three recordings successively measured on the same oocyte and filtered with a boxcar filter (box width 55, approximately equivalent to a cutoff frequency of 500 Hz). Currents were leak-subtracted offline, assuming ohmic leak, with currents from potentials between −80 and −40 mV. Fluorescence signals were normalized to the baseline level to obtain AFFE values.

PCF recordings. For PCF recordings, oocytes were labeled on ice without prior HEPES injection on the day of recording with 50 µM TAMRA-HEPS in 92 mM KCl, 0.75 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES, with pH adjusted to 7.5 with KOH. Before recordings, oocytes were mechanically devitellinized under a stereoscope and placed in a recording chamber under an inverted IX71 microscope (Olympus). Giant-patch electrodes were pulled from G150TF-4 capillaries (Warner Instruments) on a P97 Micropipette Puller (Sutter) and extensively fire polished on a microforge (Narishige MF-83). Excised patches in the inside-out configuration were obtained under a 20× objective with an initial electrode resistance of 0.6–2 MΩ, depending on the pipette solution. After the excised patch was established, the objective was changed (Olympus PLAPON 60XOTIRFM NA1.45) for recording and imaging. Holding potentials were −60 or −80 mV. Recordings were performed at room temperature (22–25 °C), with an Axopatch 200B or 200A amplifier (Molecular Devices), connected via a Digidata 1440A acquisition board to a PC running pClamp 10 (Molecular Devices). Data were filtered at 2 or 5 kHz, and the sampling rate was 10 kHz. Symmetric pipette (extracellular) and bath (intracellular) solutions were the same as in patch-clamp recordings but titrated to pH 7.5. Polychrome V (TILL Photonics), attenuated by a Q565LP dichroic beamsplitter (Chroma Technology). Fluorescence intensity was measured with a Hamamatsu HC120-05 photomultiplier tube. Fluorescence signals were low-pass-filtered at 2 kHz through an eight-pole Bessel filter (Frequency Devices) and sampled at 10 kHz through a Digidata-1440A controlled by pClamp 10 (Molecular Devices). Holding potential was −80 mV. Unless specified, fluorescence and current traces are the average of three recordings successively measured on the same oocyte and filtered with a boxcar filter (box width 55, approximately equivalent to a cutoff frequency of 500 Hz). Currents were leak-subtracted offline, assuming ohmic leak, with currents from potentials between −80 and −40 mV. Fluorescence signals were normalized to the baseline level to obtain AFFE values.
Ultra, Andor Technologies). We recorded a rectangular region of interest with 4 × 4 or 8 × 8 binning in the frame transfer mode, allowing frame rates of 0.2 kHz. The spatial average of the region of interest containing the patch cone (Figs. 5a and 7a) was taken as the fluorescence signal. For fitting purposes, the signal either was left unfiltered or, for one patch, was filtered with a boxcar filter (box width 3). For display purposes, the signal was filtered with a boxcar filter of box width 7.

Data analysis and statistics. All the values in the paper represent average values. Error bars represent the s.e.m. unless otherwise noted. n represents the number of oocytes or patches tested. Data were collected on at least two different experimental days, on oocytes obtained from at least two different Xenopus animals. Before statistical testing, sample distributions were tested for normality with the Kolmogorov-Smirnov test. All Student’s and paired t tests are two sided, with samples considered to be of unequal variance.

Data were analyzed with Clampfit (Molecular Devices), Igor Pro (WaveMetrics), SigmaPlot (Systat Software) and Matlab (Mathworks) software. Activation time constants of current and fluorescence, as well as deactivation time constants of fluorescence and current traces of 249C* mutants were determined by fitting of the current and fluorescence traces with a biexponential function in the form: I (or F) = Afast × exp(−t/τfast) + Aslow × exp(−t/τslow), where τfast and τslow represent the time constants of the fast and the slow components, respectively, and Afast and Aslow the amplitudes of the fast and slow components, respectively. VCF deactivation fluorescence signal of 249C* had a fast component that matched the current kinetics (Supplementary Fig. 7d), as well as a small, very slow component that was absent in PCF experiments. We therefore took into consideration only the fast component of fluorescence recovery for 249C* and compared it to the fast component of tail current. Deactivation fluorescence and current traces of 175C* mutants were best fitted with single-exponential functions. In VCF experiments, the tail currents of D1E 175C* and 173C-Alexa had slow kinetics and a bumpy shape (possibly due to imperfect clamping) that made it difficult to fit them. To quantify the rate of tail current and fluorescence decrease for these channels, we measured the tail decay time, defined as the time necessary for current or fluorescence to decay from 90% to 10% of their initial amplitude after repolarization to −80 mV.

G-V curves were calculated from the tail-current amplitudes at −80 mV. Unless specified, F-V curves were calculated from the amplitudes of fluorescence changes at the end of each voltage step. G-V and F-V curves were fitted with the Boltzmann equation: G/Gmax (or ΔF/ΔFmax) = 1/(1 + exp(−zec0/(kT) × (V − V1/2)), where z is the number of elementary charges, e0 the elementary charge, k the Boltzmann’s constant, T the absolute temperature, and V1/2 the voltage at which half of the channels are open.

Structure illustration. Structure representations were prepared with Discovery Studio 4.0 (Accelrys).

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