Hydrophobic gasket mutation produces gating pore currents in closed human voltage-gated proton channels

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The hydrophobic gasket (HG), a ring of hydrophobic amino acids in the voltage-sensing domain of most voltage-gated ion channels, forms a constriction between internal and external aqueous vestibules. Cationic Arg or Lys side chains lining the S4 helix move through this “gating pore” when the channel opens. S4 movement may occur during gating of the human voltage-gated proton channel, hHV1, but proton current flows through the same pore in open channels. Here, we replaced putative HG residues with less hydrophobic residues or acidic Asp. Substitution of individuals, pairs, or all 3 HG positions did not impair proton selectivity. Evidently, the HG does not act as a secondary selectivity filter. However, 2 unexpected functions of the HG in hHV1 were discovered. Mutating HG residues independently accelerated channel opening and compromised the closed state. Mutants exhibited open–closed gating, but strikingly, at negative voltages where “normal” gating produces a nonconducting closed state, the channel leaked protons. Closed-channel proton current was smaller than open-channel current and was inhibited by 10 μM Zn2+. Extreme hyperpolarization produced a deeper closed state through a weakly voltage-dependent transition. We functionally identify the HG as Val150, Phe150, Val177, and Val178, which play a critical and exclusive role in preventing H+ influx through closed channels. Molecular dynamics simulations revealed enhanced mobility of Arg108 in mutants exhibiting H+ leak. Mutation of HG residues produces gating pore currents reminiscent of several channelopathies.

HV CN1 | voltage-sensing domain | voltage gating | ion channels | protons

Voltage-gated proton channels (hHV1) exist in phylogenetically disparate species where they perform even more disparate functions, from calcification in coccolithophores (1) and mediating action potentials in bioluminescent dinoflagellates (2, 3), to numerous functions in various human tissues (4) such as compensating for electron flux in phagocytes (5–10) and enabling sperm capacitation (11). Identification of the gene (12, 13) revealed that HVT1 has 4 transmembrane helices, S1 to S4, and is homologous to the voltage-sensing domains (VSDs) present in most voltage-gated ion channels, voltage-sensing phosphatases, and TMEM266 (14). Unlike VSDs of other channels that sense voltage and cause a separate pore to open or close, HVT1 itself conducts protons (15), producing a direct readout of its gating state and making it a unique system for studying gating mechanisms.

Cysteine scanning studies of the aqueous accessibility of residues on the S4 helix of K+ channels revealed that VSDs contain 2 aqueous vestibules that are separated by a relatively short isthmus, termed the hydrophobic gasket (HG), within which S4 residues are inaccessible from either side of the membrane (16–18). In Shaker K+ channels, replacing each of the first 4 Arg in S4 individually with His further revealed that in each case anomalous proton transfer, by carrier or channel mechanisms, occurred within a specific voltage range (19–21). These results suggested that a proton, presumably as a hydronium ion, can access the HG at the center of the “gating pore” where the imidazole group of His accepts the proton, perhaps rotates, and then protonates a water molecule on the distal side.

Several amino acids contributing to the HG in Shaker K+ channels were identified, including I237 on S1 and F290 on S2 (22, 23); these positions correspond to Val150 and Phe150 in hHV1. The same highly conserved Phe in S2 together with 2 conserved acidic groups (Asp174 and Glu153 in hHV1) was proposed to act as a “charge transfer center” that temporarily stores each cationic group (Arg or Lys) in the Shaker S4 segment as it moves from the internal vestibule to the external vestibule (24). That hHV1 shares the architectural feature of a short hydrophobic barrier is suggested by the R205H mutant in which the His shuttles protons into the cell at negative voltages (25), and by efficient proton permeation through open channels.

The Ciona intestinalis voltage-sensing phosphatase (CiVSP) is more closely related to HVT1 phylogenetically than are K+ channels (3). Crystal structures of CiVSP were determined in both “down” and “up” positions, corresponding with closed and

Significance

A large family of membrane proteins, voltage-gated ion channels, regulate a vast array of physiological functions in essentially all life forms. How these molecules sense membrane potential and respond by creating ionic conduction is incompletely understood. The voltage sensors of these channels contain a “hydrophobic gasket,” a ring of hydrophobic amino acids near the center of the membrane, separating internal and external aqueous solutions. Although voltage-gated proton channels, HVT1, resemble voltage-sensing domains of other channels, they differ fundamentally. On depolarization, HVT1 conducts protons, whereas other voltage-sensors open a physically distinct pore. We identify Val150, Phe150, Val177, and Val178 as the hHV1 hydrophobic gasket. Replacement with less hydrophobic amino acids accelerated channel opening and caused proton-selective leak through closed channels.

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open states (26). Li et al. (27) also studied hHv1 using electron paramagnetic resonance (EPR) and produced closed and open models of hHv1 based on the CiVSP structures. These studies identified 3 conserved hydrophobic amino acids (Val109, Phe150, and Val178) (28) as plausible members of the HG in hHv1. The positions of S1 and S4, but not S2 and S3, in the hHv1 models corresponded well with the single existing crystal structure of H2v1 in a presumed closed state. In the chimeric molecule successfully crystallized (29), the cytoplasmic ends of S2 and S3 segments of the mouse channel were replaced with the corresponding section of CiVSP. Li et al. (27) concluded that if S3 in the mH2v1 crystal were shifted upward (toward the extracellular surface) and S2 downward by 1 helical turn, the structure and model would match. A significant consequence is that, in the mH2v1 crystal structure, the third HG residue is predicted to be Phe128, not Val178 (both with hHv1 numbering). Fig. 1 illustrates the location of the putative HG residues in the homology model and crystal structure of H2v1 in relation to other important amino acids, including Asp112 that is required for proton selectivity (30) and Arg208, the central of 3 Arg in the S4 helix that contribute to gating charge (31).

Here, we address both the identity of amino acids contributing to, and the functional importance of, the HG in hHv1 by replacing several amino acids in this region with neutral but smaller and less hydrophobic ones, or with Asp, which may be charged. The results reaffirm that hHv1 contains a narrow barrier separating internal and external solutions like other VSDs, but in addition the HG ensures the occlusion of protons in the closed channel. We mutated both Val178 and Phe150 to determine which is more likely to be a member of the HG by exploiting our discovery of distinctive effects of mutating Val109 and Phe150. This approach identified Val178 and another HG residue, Val112.

**Results**

**Experimental Strategy.** Molecular dynamics (MD) simulations of a homology model of the open state of hHv1 (33) were performed, and mutations designed to compromise the integrity of the HG were proposed for experimental testing. Increased pore hydration and a lowered barrier in the potential of mean force for the movement of Na⁺ along the channel suggested that some of these mutants might be permeable to Na⁺ in the open state (SI Appendix, Fig. S1 and Table S1). When these mutants were tested experimentally, the channel did not conduct Na⁺ (Table 1) but was discovered to be leaky to H⁺ in the closed state.

In order to evaluate the role and importance of the HG in hHv1, we mutated the putative HG residues themselves (V109, F150, and V178) as well as several nearby hydrophobic amino acids (Fig. 1). Specifically, L108 and V177 neighbor putative HG positions; I105, L147, and F182 are 1 tier below or above the HG in our model. We mutated one or more HG residues to less hydrophobic and smaller amino acids, or to the negatively charged Asp. The background was the full-length wild-type (WT) hHv1, which assembles as a dimer. Distinctive features common to the last 10 constructs in Table 1 are discussed using F150D and V109D as examples. The similar phenomenology of these mutants reinforces the idea that their behavior is a consequence of decreased hydrophobicity.

**Lower Hydrophobicity at the HG Did Not Impair H⁺ Selectivity.** For each construct, voltage-clamp current families were collected at several pH to evaluate the general effects of the mutation. Essentially all hHv1 mutants studied were strongly proton selective (Table 1). Selectivity was assessed by determining the reversal potential, \( V_{rev} \), at several pH. For most mutants in the upper half of the table, changes in pHₗ (in whole-cell studies) or pHₑ (in inside-out patch studies) shifted \( V_{rev} \) by ≥50 mV/unit, which indicates H⁺ selectivity. Imperfect pH control accounts for deviations from the Nernst potential for H⁺ (34, 35). We specifically examined possible Na⁺ permeability. Changing the predominant cation from TMA⁺ to Na⁺ shifted \( V_{rev} \) by only a few millivolts, within the range of liquid junction potentials, which we consider negligible. Replacing TMA⁺ with Na⁺ should shift \( V_{rev} \) by

![Fig. 1.](image_url)
17.5 mV if $P_H/P_{Na}$ were 10⁶ and by 2.4 mV if $P_H/P_{Na}$ were 10⁷, calculated with the Goldman–Hodgkin–Katz voltage equation. We cannot rule out Na⁺ permeation altogether; we simply cannot measure it.

Increasing the Hydrophilicity of the HG Compromises the Impermeability of the Closed Channel. Increasing the hydrophilicity of the HG impaired the ability of hH₃.1 channel closure to occlude proton leak through the channel. Fig. 2 illustrates this dramatic consequence in the F150D mutant. Three families of currents in the same cell are shown, nominally at pH₁ and pH₂. The family in Fig. 24 generally resembles WT hH₃.1 currents, but with some differences. Time-dependent turn-on of outward current appears at 0 mV and above (~20 mV earlier than WT). Most remarkably, the cell appears leaky, with a substantial time-independent current at $V_{hold}$ and with large instantaneous jumps at the start of each depolarizing pulse. Four kinds of evidence support the idea that this “leak” conductance ($g_{H,closed}$) is carried largely or entirely by protons through a closed state of the mutant proton channel. First, it was inhibited by Zn²⁺. Zn²⁺ has no effect on leak current in cells transfected with WT hH₃.1 (36). Fig. 2B and C illustrates the inhibition of both open and closed F150D channels by 1 μM Zn²⁺ and 10 μM Zn²⁺. The currents during pulses to +80 mV superimposed in Fig. 2D illustrate that the holding current at −40 mV, and the outward currents appear similarly sensitive to inhibition by Zn²⁺. We interpret the inward current at −40 mV as closed-channel current, because time-dependent activation of proton current was evident only at 0 mV or more positive (Fig. 2A). We interpret the instantaneous jump in outward current during depolarizing pulses as current flowing through conducting closed channels, which is followed by a slow rise in current that we attribute to channel opening in a relatively normal time-dependent manner.

Second, the inward current at the holding potential $V_{hold}$ lowered pH₃, consistent with proton-selective leak current. When inward current at $V_{hold}$ was increased by removing Zn²⁺ (Fig. 2E) or lowering pH₂ (Fig. 3), the test pulse current amplitude increased over several minutes as the greater proton influx lowered pHi. This phenomenon made quantitative study of the properties of mutants displaying $g_{H,closed}$ quite difficult, because essentially all pH₁ properties depend strongly on pH. In general, constitutive $g_{H}$ will act to dissipate the applied pH gradient. Because we typically held the membrane at negative voltages (to close the channels), the presence of a closed-channel $g_{H}$ will tend to drive the Nernst potential for H⁺, $E_{rev}$, toward $V_{hold}$. At symmetrical pH₃, 7, pH₃ is nominally 0 mV, but continuous H⁺ influx at $V_{hold}$ will lower pH₃. Indeed, in 4 cells expressing F150D, the holding current at −40 mV averaged −49 ± 10 pA (mean ± SEM) and $V_{rev}$ was −10.8 ± 1.6 mV. Measured by tail currents, $V_{rev}$ became more negative when $V_{hold}$ was more negative, because there was a larger steady H⁺ influx. Fig. 2E illustrates the mutable nature of pH₃ in these experiments. The record begins at symmetrical pH 7 (nominal) in the presence of 10 μM Zn²⁺, which had greatly reduced both inward and outward currents. Application of pH₃ solution with EGTA to this cell relieved the current to −40 mV (Fig. 2D) and by 2.4 mV if $P_H/P_{Na}$ continually dissipated the pH gradient even if the channel is perfectly proton selective, to an extent dependent on geometry and other factors.

**Table 1. Closed channel conductance and selectivity of HG mutants**

| Mutant | $g_{H,closed}$ | $g_H$(O) pH 7 | $\Delta V_{rev}$ pH 7−6 | $\Delta V_{rev}$ pH 7−8 | $\Delta V_{rev}$ pH 6−5 | $\Delta V_{rev}$ TMA⁺−Na⁺ |
|-------|----------------|--------------|-----------------|----------------|----------------|------------------|
| I105G | No             | 0            | 56.7 ± 1.6 6   | 53.7 ± 3.5 5  | 1.8 ± 0.8 3  | —                |
| I105D | No             | 0            | 49.4 ± 2.3 5   | 49 1 60 1 | 1.8 ± 0.8 3  | —                |
| L108T | No             | 0            | 53 (1)         | 55.5 ± 3.5 2  | 2.4 (1)       | —                |
| V109T | No             | 0            | 52.8 ± 1.1 4   | 55.7 ± 1.9 3  | 1.6 (1)       | —                |
| V109A | No             | 0            | 56.5 ± 1.0 4   | 54.0 ± 6.0 2  | 1.1 ± 1.3 3  | —                |
| L147T | No             | 0            | 51.3 ± 3.0 3   | —              | —              | —                |
| L147D | No             | 0            | 50.7 ± 1.7 3   | 53 (1)         | 0.2 ± 0.4 5  | —                |
| F150A | No             | 0            | 47.5 ± 1.5 2   | 52 (1)         | −0.5 ± 0.8 4  | —                |
| V178T | No             | 0            | 49 (1)         | —              | —              | —                |
| F182A | No             | 0            | 55.8 ± 1.1 4   | 50.1 ± 3.0 2  | 45           | −0.9 ± 1.1 3  | —                |
| V177T | No             | 0            | 47.0 ± 4.0 3   | 53.8 ± 2.5 5  | −0.3 ± 1.0 3  | —                |
| V177D | Yes            | 0.103 ± 0.018 (4) | 55.3 ± 3.7 3  | 0.5 ± 0.2 3  | —              | —                |
| V178A | Yes            | 0.017 ± 0.010 (5) | 50.2 ± 1.4 4  | 47 (1)         | 0.6 ± 0.7 3  | —                |
| V109A/F150A | Yes | 0.164 ± 0.044 (6) | 49.7 ± 3.8 3 | 34 (1)         | 0.8 ± 1.1 4  | —                |
| V109A/V178A | Yes | 0.028 ± 0.012 (7) | 49.9 ± 1.3 4 | 0.8 ± 0.8 3  | —              | —                |
| F150A/V178A | Yes | 0.144 ± 0.015 (3) | 44.5 ± 0.5 2  | 70 (1)         | −0.6 ± 0.6 3  | —                |
| V178D | Yes            | 0.036 ± 0.019 (4) | 57.3 ± 1.4 4  | 50.7 ± 2.7 3  | 55 (1)        | 0.6 ± 1.2 6  | —                |
| V109A/F150AV178A | Yes | 0.35 ± 0.05 (9) | 44.0 ± 2.8 5  | 36.3 ± 4.9 3  | 29.7 ± 11.9 (3) | −0.3 ± 0.4 4  | —                |
| V109D | Yes            | 0.40 ± 0.06 (5) | 59 (1)         | —              | 3.0 ± 0.9 3  | —                |
| F150D | Yes            | 0.38 ± 0.09 (4) | 39.3 ± 3.5 3  | 45 (1)         | 39 ± 6 2   | 1.2 ± 1.4 4  | —                |
| F150D/R211G | Yes | 0.67 ± 0.04 (6) | 39.6 ± 8.2 5  | 33.3 ± 8.4 (4) | 41.0 ± 6.4 (4) | 1.8 ± 0.4 5  | —                |

The existence of $g_{H,closed}$ was defined as consistent reversible changes in the holding current of >1 pA when pH was changed or with addition of Zn²⁺ or both. Absolute values of $V_{rev}$ changes with pH are given for both whole-cell and inside-out patch measurements. Mutants with large $g_{H,closed}$ tend to have smaller and more variable $V_{rev}$ changes with pH because proton leak at $V_{hold}$ continually dissipated the pH gradient even if the channel is perfectly proton selective, to an extent dependent on geometry and other factors. Parameter $g_H(O)$ is the ratio of closed to open $g_H$ (Materials and Methods). Monovalent cation substitutions were done at pH 7/7, and measured liquid junction potentials have been corrected.
in whole-cell studies, consistent with changes pH− was decreased in cells with solutions at pH 7. Zn in nontransfected HEK-293 cells. We conclude to 6.5 hyperpolarized the membrane by a nearly was g or Na 5.5) increased the holding decreased at higher pHo in whole-cell studies, consistent with protons being a major charge carrier. Figs. 2E and 3 illustrate this phenomenon in F150D and V109D mutants, respectively. The manifestations in inside-out patches were inverted, and the inward current at Vhold became smaller when pHi was lowered. In cells lacking hHv1 or with WT hHv1, the leak current at Vhold did not change with pHc. When pHo was decreased in cells with V109D, closed-channel proton currents increased. Lowering pHo from 5.5 to 4.5 (with nominally pH i 5.5) increased the holding current at −40 or −60 mV by a factor 3.0 ± 0.2 in 5 cells (mean ± SEM), as illustrated in Fig. 3. However, correcting for the change in driving force decreases this difference. Evidently gH, closed increases as pHo is lowered considerably less than in proportion to the proton concentration.

Finally, if HG mutant channels conduct protons selectively in both open and closed states, and any other conductances present are negligibly small, then the membrane should act as a pH electrode. This prediction is borne out in Fig. 4, which shows the effects of pH changes on membrane potential in a cell with V109D channels studied under current-clamp conditions. Beginning with symmetrical pH 5.5 solutions, the membrane potential was near 0 mV. Changing pHo to 4.5 resulted in a rapid 40-mV depolarization that was reversed upon return to pH 5.5. Increasing pHo to 6.5 hyperpolarized the membrane by a nearly Nernstian amount (∼58 mV/unit pH), showing that the membrane conductance is overwhelmingly proton selective. Returning to pH 5.5 resulted in rapid depolarization to ∼0 mV. Replacing CH3SO3− with Cl− produced only a transient blip, indicating negligible anion permeability. Replacing TMA+ with Na+ or K+ had similarly minor effects. That the membrane potential of this cell approached the Nernst potential for protons (E0H) and was minimally affected by other ions indicates that the conductance was proton selective. If a small fraction of channels remain open at Vrev (0 mV at symmetrical pH), the membrane potential changes most likely reflect proton conductance through a mixture of open and closed channels.

We tested the proton selectivity of closed channels by comparing the holding current in TMA+ or Na+ solutions at pH 7. Na+ sometimes produced small increases in the holding current in some mutants, but these were insensitive to Zn2+, which blocked gH, closed (Fig. 2). Furthermore, we sometimes saw similar effects of Na+ in nontransfected HEK-293 cells. We conclude that closed hHv1 channels are not detectably permeable to Na+.

**Estimating the Conductance of Closed Channels.** Given that the existence of gH, closed changes pHi and consequently Vrev, accurate conductance estimates are improbable. However, in cells in which 10 to 100 μM Zn2+ was applied (e.g., Fig. 2), typically up to 90% of the holding current was eliminated. The error is thus not inordinate. Rough estimates were obtained at symmetrical pH 7.0 in 2 ways (Materials and Methods). The holding current, usually at −40 or −60 mV, was considered to reflect mainly gH, closed. The time-independent (or weakly time-dependent) currents at negative voltages reversed at very nearly the same
was substituted
- reveal that, following pulses in
one-third of the open
relationship due to the
was now 4.86. Thus,
to 4.5 (Fig. 5
- shifts the
Closed
vol. 116
shifted 49 mV when
lowered pH
solutions, with membrane potential recorded
based on traditional
5.5. Lowering pH
of open channels is
relationship of all known proton
V
has a
was
was changed. When pH
was near
appears to activate at somewhat more negative voltages.
5.5, the test current during
transitions are kinetically distinct from the
Membrane potential response to pH changes in a cell with the
or K
18955
determined by tail currents in this cell
were closed. The well-spaced currents at positive
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SI Appendix
provides additional
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force) as well as the expected positive shift of the
ΔpH dependence of gating (37). However, during subsequent pulses the test
current increased progressively as the H
influx that occurs continuously at
V
hold lowered pH
and reversed these 2 changes, resulting in both
E
and the
gate-V
relationship shifting negatively over the course of several minutes.
Eventually, the test current at 20 mV is even larger at pH4.5 than it was at
nominally symmetrical pH, presumably indicating that the true pH
has dropped lower than it was initially at pH4.5. Lowering pH increases g
of WT
H
1 channels by roughly 2-fold/unit in most whole-cell studies of voltage-
gated proton channels (38). Upon return to pH5.5, the test current during
the first pulse is much larger than it was previously in the same solution,
reflecting the lower pH.
The holding current at ~60 mV rapidly approaches
0 pA, showing that the true pH gradient shortly after the bath change was
roughly pH4.5, pH 4.5, and thus E
was near V
hold at ~60 mV. Gradually,
H
directly extruded by the large outward test pulse currents and decreased H
influx at V
hold restores pH4 toward its previous value.

Fig. 3. Proton influx through closed V109D channels lowers pH. Whole-cell currents were recorded initially in symmetrical pH 5.5 solutions, with the membrane held at ~60 mV and 2 ± test pulses to 20 mV applied every 30 s. The arrows indicate when pH4 was changed. When pH4 was lowered from
5.5 to 4.5, the holding current increased immediately, reflecting proton influx through closed (C1) channels. The test pulse current at 20 mV decreased at first,
reflecting the positive shift of E
which (decreases or even inverts the driving
force) as well as the expected positive shift of the
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H
1 channels by roughly 2-fold/unit in most whole-cell studies of voltage-
gated proton channels (38). Upon return to pH5.5, the test current during
the first pulse is much larger than it was previously in the same solution,
reflecting the lower pH.

Voltage as the time-dependent “normal” g
based on traditional
tail current measurements, consistent with both being largely or
entirely proton selective. In mutants with large g
closed there was an instantaneous jump upon depolarization, which we as-
scribe to closed-channel conductance, followed by a slower time-
dependent increase in current, which we attribute to the normal
opening process.
We assume that the time course reflects the
increasing proportion of open channels, and the final current
reflects the fully open-channel conductance.

The amplitude of g
closed relative to the g
of open channels is given in Table 1 as g
4θ(C/O). Replacing individual HG residues with Asp produced detectable g
closed only for V178A. However, all 3 double Ala mutants (V109A/F150A, V109A/V178A, and F150A/V178A) leaked protons when closed, and the triple Ala
mutant had a large leak with g
closed one-third of the open
conductance. Each HG residue replaced by Asp produced distinct g
Closed.

A Deeper Closed State Revealed in V109D. Fig. 5A shows a typical family of V109D currents at symmetrical pH 5.5/5.5. From
V
hold = ~60 mV pulses to more negative voltages elicited slowly
decaying inward currents. A superficial interpretation might be
that these are “tail currents” due to a population of channels open at ~60 mV that close at more negative voltages. Consistent with this idea, repolarization to ~60 mV results in slowly activ-
vating inward currents, which are more obvious in Fig. 5B. Above
about ~30 mV, a proton conductance with more normal appearance begins to turn on. Its activation is faster than WT H4V1, and
the g
appears to activate at somewhat more negative voltages. For reasons discussed above, we suspect that the apparently closed channels still conducted proton current, even at ~60 mV. One consequence is that V
rev determined by tail currents in this cell
was ~28 mV. The continuous proton influx at V
hold lowered pH4 well below the nominal pH 5.5 of the pipette solution to ~5.02.

Lowering pH4 shifts the g
-V
relationship of all known proton
channels by ~40 mV to more positive voltages (4, 37, 38). We therefore lowered pH4 to 4.5 (Fig. 5B), to ensure that all of the channels at V
hold were closed. The well-spaced currents at positive voltages now appear to activate about 40 mV more positively, with increased spacing above +10 mV, but the inward (proton-
selective) holding current at ~40 mV was increased, and the decaying inward currents during pulses to more negative voltages were more pronounced. Both from time-dependent relaxation of
tail currents and the reversal of total current, V
rev was ~21 mV, indicating that pH4 was now 4.86. Thus, V
rev shifted 49 mV when
H4 was lowered from 5.5 to 4.5, and ΔpH shifted by 0.84 units. The V
rev shift was thus 58.3 mV/unit (49 mV/0.84 units) indicating perfect proton selectivity. Examination of the tail currents seen
upon repolarization at both pH4 reveal that, following pulses in the
negative voltage range, the currents exhibit a monoeXponential
time course. Following depolarizing pulses that activate normal
time-dependent outward currents, the tail currents are distinctly
double exponential. The amplitudes of each kinetic component as well as their sum are plotted in Fig. 5C. The fast tail currents are associated with activation of the normal g
and are steeply voltage dependent. The slow tail currents evidently reflect a slow gating process between closed but conducting channels and a deeper
closed state that appears not to conduct detectably.

The behavior of V109D in Fig. 5 suggests the following gating transitions:

\[
\text{Closed}_2 \xrightarrow{\text{slow}} \text{Closed}_1 \xrightarrow{\text{slow}} \text{Open}.
\]

The Closed2 (C2) state does not conduct or conducts negligibly. Closed1 (C1) and Open (O) both conduct protons but O has a higher conductance. The transition between C2 and C1 has very weak voltage dependence. The C1→O transition has strong volt-
age dependence that presumably reflects movement of most of
the gating charge.

The experiment in SI Appendix, Fig. S2 provides additional evidence that C2→C1 transitions are kinetically distinct from the
normal opening transition. Activation kinetics and amplitude depend on prepulse voltage. Similar behavior was observed in many
cells expressing V109D, both at pH 7 and at pH 5.5. According to
[1], the most negative pre pulses produce C2→C1→O transitions,

![Fig. 3](image-url)

**Fig. 3.** Proton influx through closed V109D channels lowers pH. Whole-cell currents were recorded initially in symmetrical pH 5.5 solutions, with the membrane held at ~60 mV and 2 ± test pulses to 20 mV applied every 30 s. The arrows indicate when pH4 was changed. When pH4 was lowered from 5.5 to 4.5, the holding current increased immediately, reflecting proton influx through closed (C1) channels. The test pulse current at 20 mV decreased at first, reflecting the positive shift of Eh which (decreases or even inverts the driving force) as well as the expected positive shift of the gate-V relationship due to the ΔpH dependence of gating (37). However, during subsequent pulses the test current increased progressively as the H influx that occurs continuously at Vhold lowered pH and reversed these 2 changes, resulting in both Eh and the gate-V relationship shifting negatively over the course of several minutes. Eventually, the test current at 20 mV is even larger at pH4.5 than it was at nominally symmetrical pH, presumably indicating that the true pH has dropped lower than it was initially at pH4.5. Lowering pH increases gH of WT H1 channels by roughly 2-fold/unit in most whole-cell studies of voltage-gated proton channels (38). Upon return to pH5.5, the test current during the first pulse is much larger than it was previously in the same solution, reflecting the lower pH. The holding current at ~60 mV rapidly approaches 0 pA, showing that the true pH gradient shortly after the bath change was roughly pH4.5, pH 4.5, and thus Eh was near Vhold at ~60 mV. Gradually, H directly extruded by the large outward test pulse currents and decreased H influx at Vhold restores pH4 toward its previous value.

![Fig. 4](image-url)

**Fig. 4.** Membrane potential response to pH changes in a cell with the V109D mutant confirms proton selectivity. The initial condition was symmetrical pH 5.5/5.5. From Vhold = ~60 mV pulses to more negative voltages elicited slowly decaying inward currents. A superficial interpretation might be that these are “tail currents” due to a population of channels open at ~60 mV that close at more negative voltages. Consistent with this idea, repolarization to ~60 mV results in slowly activating inward currents, which are more obvious in Fig. 5B. Above about ~30 mV, a proton conductance with more normal appearance begins to turn on. Its activation is faster than WT H4V1, and...
but more positive prepulses result in mostly \( C_1 \rightarrow O \) transitions. SI Appendix, Fig. S2 confirms that the slow decay of current at large negative voltages reflects transitions unrelated to the \( C_1 \leftrightarrow O \) transition, presumably \( C_1 \rightarrow C_2 \).

**Decreasing Hydrophobicity Outside the HG Does Not Impair Selectivity or Induce \( g_{H,Closed} \)**. Table 1 indicates whether each mutant exhibited closed-channel \( g_H \), and its magnitude relative to the open-channel \( g_{H,Closed} \). Mutations expected to decrease the hydrophobicity at positions I105, L108, L147, F150, V177, V178, or F182 did not detectably impair \( H^+ \) selectivity. Of the positions tested, the only mutations that exhibited closed-state conductance were of the predicted HG residues, V109, F150, and V178, as well as V177. Because Val\(^{178} \) is the central of 5 consecutive valines, and Val\(^{177} \) obliquely faces the pore, we suspect that V177 and V178 act interchangeably. Convincing \( g_{H,Closed} \) was not observed for another HG neighbor (L108T) or for positions 1 helical turn above (F182A) or below the HG (L105G, L105D, L147D).

**Is Phe\(^{182} \) Rather than Val\(^{178} \) Part of the HG?** Homology models identify Val\(^{178} \) as part of the HG (Fig. 1), but in the crystal structure of mH\(_1\), this position appears to be occupied by Phe\(^{182} \) (Phe\(^{182} \) in hH\(_1\)) (29). If the crystal structure is closer to reality than the homology models, Phe\(^{182} \) mutants might be expected to share the properties of mutations at the other 2 elements of the HG. Unfortunately, F182D mutants did not produce detectable currents, despite the transfected cells being green, indicating likely membrane expression. The lack of current in F182D may indicate that it does not face the pore in hH\(_1\), consistent with the homology model but not the crystal structure (Fig. 1). We then tried F182A, because a neutral substituent might be less disruptive than introducing a charge. Currents were detected and were proton selective, but convincing \( g_{H,Closed} \) was not detected (Table 1). In contrast, V178A, V178D, and all double or triple Ala mutants that included Val\(^{178} \) exhibited \( g_{H,Closed} \). That V177D also exhibits \( g_{H,Closed} \) further supports the role of its neighbor Val\(^{182} \) in the HG, because both are at a similar “height” in the membrane. Combined, these results strongly support the idea that Val\(^{182} \) (in concert with Val\(^{178} \)) and not Phe\(^{182} \) completes the HG in hH\(_1\).

**The Double Mutant F150D/R211G.** Our open-channel model of hH\(_1\) exhibited frequent interaction between F150 and R211 in the HG region, at the peak energy barrier for cation permeation (33). We therefore tested a double mutant, F150D/R211G, but it retained high proton selectivity (Table 1). Like F150D alone, this double mutant exhibited \( g_{H,Closed} \).

**Increasing the Hydrophilicity of the HG Region Facilitates Channel Opening.** Fig. 6 illustrates that most of the mutations intended to make the HG less hydrophobic accelerated channel opening. Mutation to Ala significantly accelerated activation in F150A, but mutations to Asp had greater impact. Each single Asp (V109D, F150D, V178D) mutant opened substantially faster than WT. The ubiquity of faster activation suggests that, in WT channels, the HG acts to retard channel opening. The speeding of activation and induction of \( g_{H,Closed} \) appear to be independent and mechanistically unrelated consequences of HG mutation. There was no correlation between the magnitude of \( g_{H,Closed} \) in each mutant and the speeding of \( \tau_{act} \) (\( R^2 = 0.19 \) and 0.17 for linear regression on +40- or +60-mV data, respectively). Notably, some mutations (e.g., I105G, I105D, and F150A) profoundly accelerated activation, but did not produce \( g_{H,Closed} \). Conversely, V178A, V109A/F150A, and V109A/V178A exhibited \( g_{H,Closed} \) without a significant decrease in \( \tau_{act} \).

Further evidence that reducing hydrophobicity of the HG region promotes channel opening is a substantial negative shift of the \( g_{H,Closed}^{-} \)relationship in I105A and I105D (SI Appendix, Fig. S3). On average, the voltage at which the \( g_H \) was 10\% maximal (39) shifted from 14.1 ± 3.4 mV (mean ± SEM) in 7 cells transfected with WT hH\(_1\) to −13.3 ± 3.6 mV (\( n = 7 \)) in I105G mutants and −39.9 ± 8.3 mV (\( n = 5 \)) in I105D (\( P < 0.0001 \) for both). We did not attempt to quantify \( g_{H,Closed}^{-} \)data for other mutants, because the additional time- and voltage-dependent \( g_{H,Closed} \) process [1] made unambiguous interpretation difficult. Other constructs did not appear as obviously shifted as I105H mutants, however. That I105H mutation produced a large negative shift without producing \( g_{H,Closed} \) further indicates that the 2 key functions of the HG, namely preventing proton influx through closed channels and retarding channel opening, are mechanistically independent.

**The HG Prevents the Guanidinium of Arg\(^{208} \) from Moving Above Residue 150 in the Closed State.** To investigate the molecular basis for closed-state proton leakiness in these HG mutants, extensive
Fig. 6. Many HG mutants have faster activation kinetics. Mutants without detectable $g_{\text{Cl,open}}$ are labeled in green, and those with $g_{\text{Cl,open}}$ are red. Time constants of activation ($\tau_{\text{act}}$) were determined by single- or occasionally by double-exponential fits to increasing currents. Plotted are mean ± SEM $\tau_{\text{act}}$ at 60 mV (light gray) and 40 mV (dark gray) in each mutant. When 2 exponentials were required to fit the currents reasonably, the slower one is plotted here. Significant differences from $\tau_{\text{act}}$ in WT hH$\alpha$1 indicated by * $P < 0.05$, ** $P < 0.01$ by Student's t test. SI Appendix, Table S2 gives statistical details of these studies. An error bar for V109T is truncated.

simulations totaling 6.5 $\mu$s were performed using the spectroscopically and biochemically constructed model of the resting state of hH$\alpha$1 by Li et al. (27). The effect of these mutations on the size and nature of the HG was analyzed by computing the average hydration along the channel (SI Appendix, Fig. S4). Increased pore hydration relative to WT was observed in the intracellular vestibule ($-0.9 < z < -0.5$ nm) in single aspartate mutants; however, little to no change was seen in leaky alanine mutants. Therefore, we conclude that H$^+$ leakiness in the closed state is not due to increased hydration of the HG.

We then monitored the position of the guanidinium group of Arg$^{208}$ relative to the side chain of residue 150 versus the position of the S4 helix center of mass relative to the rest of the channel (Fig. S1 to S3, and SI Appendix, Fig. S5). Consistent with the gating charge distribution of the closed state, the guanidinium group remained below Phe$^{150}$ most of the time in the systems considered. However, transient excursions (~2 to 3 Å) of the guanidinium group of Arg$^{208}$ above residue 150 were observed in single threonine mutants (Fig. 7B). These excursions became more likely in single alanine mutants (Fig. 7C). In double- and triple-alanine HG mutants, 2 distinct metastable conformational states were sampled in which the guanidinium group of Arg$^{208}$ is positioned either ~1 Å above or ~2 Å below residue 150 (Fig. 7D). Finally, a third conformation was populated in single aspartate mutants with the side chain of Arg$^{208}$ positioned 2 Å above Phe$^{150}$ (Fig. 7E). At most, the upward shift of the Arg$^{208}$ side chain is correlated to an 0.5- to 1-Å upward shift of the S4 helix, falling well short of the >4-Å S4 translation to the activated state in other homologous voltage sensing domains (26, 40). In our open-state model, Arg$^{208}$ faces Asp$^{12}$ (33). Importantly, dynamic fluctuations of the guanidinium group of Arg$^{208}$ past residue 150 were significant and metastable in all leaky mutants but were transient and occurred very rarely in the WT channel (Fig. 7A) or in nonleaky mutants (Fig. 7 B and C).

Taken together, our simulation results suggest that the HG acts as a hydrophobic and steric barrier that prevents the Arg$^{208}$ guanidinium from slipping above the gating charge transfer center in the resting state.

Discussion

The HG Retards Channel Opening in WT hH$\alpha$1. Many of the HG mutants studied activated about an order of magnitude faster.

Fig. 7. Conformational fluctuations of WT and HG mutants of the resting state of hH$\alpha$1 from MD simulations. The axial position of the guanidinium group of Arg$^{208}$ relative to the side chain of residue 150 is shown versus the axial position of the center of mass of the S4 helix relative to the center of mass (CoM) of the rest of the channel (helices S1 to S3). (A) WT hH$\alpha$1 samples a single basin (α), with the position of the guanidinium group remaining below residue 150 (dashed line). (B) Rare and transient excursions of the guanidinium group above residue 150 are observed in the (nonleaky) threonine HG mutants. (C) These excursions become more likely in single alanine mutants, including leaky V178A, and (D) populate a distinct conformational state (β) in double- and triple-alanine mutants. (E) Finally, aspartate mutants sample 3 distinct basins, 2 of which are located at or above residue 150 (α and β). Representative snapshots of the single basin in WT (α) and the 3 basins (α, β, γ) in F150D are shown below the distributions. S1 (red), S2 (yellow), S3 (green), and S4 (blue) helices are shown as ribbons with side chains labeled and colored based on their residue type: hydrophobic (cyan), acidic (red), and basic (blue), with pore-associated water molecules (black). Individual distributions are included in SI Appendix, Fig. S5.
than WT hHv1 (Fig. 6). This result is consistent with the report that, in V109A, F150A, and V178A, the $g_{H^+} - V$ relationship is shifted negatively by 10 to 27 mV (41); and the present observation that activation of I105A and especially I105D are shifted dramatically toward negative values. Together, these findings indicate that the HG in WT channels impedes channel opening. Mutations in the HG region did not affect activation kinetics equally. Although most Asp mutations (I105D, V109D, F150D, V177D, and V178D) strongly accelerated opening, among the single neutral mutants only F150A (42) and I105G did. There was no correlation between the speeding of activation and the magnitude of $g_{H^+\text{closed}}$, which shows that these consequences have different mechanisms. The proximity of Ile105 to the HG is evidently sufficient that its mutation speeds activation, but without producing $g_{H^+\text{closed}}$. We conclude that activation kinetics is influenced by the overall hydrophobicity of the region, whereas inducing $g_{H^+\text{closed}}$, requires decreased hydrophobicity specifically in the locations occupied by Val109, Phe150, Val177, and Val178. Surprisingly, the HG in the Shaker $K^+$ channel VSD appears to have the opposite effect on gating. Replacing Ile237 (which corresponds with Val109 in hHv1) with less hydrophobic amino acids promoted the closed state, shifting $g-V$ relationships positively (23).

Teleologically, that the HG resists channel opening may serve to ensure that hHv1 opens at appropriate voltages. The ΔpH dependence of its gating means that, under all conditions, hHv1 is poised to open just above $E_R$, when there is an outward electrochemical gradient for protons. Opening at more negative voltages would allow proton influx, which in most situations in most cells is deleterious.

Reducing the Hydrophobicity of the HG Produces Closed-State Proton Conduction. The most dramatic and consistent effect of reducing the hydrophobicity of HG residues was to produce a closed-channel proton-selective leak conductance. The conductance of closed channels, $g_{H^+\text{closed}}$, was smaller than that of the open channel, $g_{H^+}$, but substantial, and dependent on many factors, especially $p_{H^+}$. The magnitude of $g_{H^+\text{closed}}$ increased with the number of substitutions to the HG. The single-mutant V178A had distinct but tiny $g_{H^+\text{closed}}$ averaging only 1.7% of the open $g_{H^+}$. All 3 double Ala mutants produced moderate $g_{H^+\text{closed}}$ 3 to 16% of $g_{H^+}$ (Table 1). The triple-mutant V109A/F150A/V178A produced robust $g_{H^+\text{closed}}$ (35% of $g_{H^+}$) comparable to that seen in V109D or F150D. We conclude that the HG in H1 ensures that the closed channel is fully occluded—that no ions, not even protons, are allowed into the cell.

The HG mutants still opened and closed in a voltage- and time-dependent manner, but in a voltage range negative to that producing normal time-dependent gating, closed channels allowed continuous proton influx. It might be argued that these mutations disrupt the integrity of the protein or disturb gating, and that consequently the proton leak is not very meaningful, simply indicating a broken channel. Three observations argue against this interpretation. First, these mutants still exhibited voltage- and time-dependent gating, suggesting that the gating mechanism was not grossly altered. Second, the strong proton selectivity of the closed-channel leak conductance suggests that the selectivity filter remained intact. Proton-selective conduction occurs only under the specific conditions of an appropriately juxtaposed Asp in S1 and Arg in S4 (43–45), and thus requires integrity of the selectivity filter of mutant proteins. Finally, potent Zn$^{2+}$ inhibition of depolarization-activated H$^+$ current suggests that the Zn$^{2+}$ binding site remained largely intact. In WT hHv1, Zn$^{2+}$ is coordinated tetrahedrally by His190, His193, Asp123, and Glu119 (residues residing on S1, S2, and the S3–S4 linker) based on mutation studies (12, 36), IR spectroscopy (46), and on the X-ray structure of the hHv1 crystal, which included a Zn$^{2+}$ atom at its external binding site (29). That Zn$^{2+}$ still potently inhibits both closed- and open-channel currents supports the idea that the HG mutants were globally functionally intact.

Which Amino Acids Comprise the HG? Homology models identify Val178 as part of the HG (Fig. 1), analogous to Ile105 in CiVSP or Ile237 in Shaker (28, 47). However, in the crystal structure of hHv1, this position appears to be occupied by Phe78 (Phe152 in hHv1), in a region called the inner hydrophobic layer (29). A subsequent EPR study of hHv1 concluded that in the crystal structure of hHv1, replacing a 25-amino acid region of S2 through S3 with amino acids spliced from CiVSP resulted in a register shift of 1 turn of the helix up for S2 helix and down for S3 (27). When the crystal structure is “corrected” for these shifts, Val178 aligns with Phe152 and Val109 to complete the HG (27), as shown in Fig. 1B. Given this ambiguity, we mutated Phe142 in hHv1. If the crystal structure is closer to reality than the homology models, Phe142 mutants might be expected to share the manifestations of mutations to the other 2 HG elements. The F182D mutant did not produce detectable currents, despite the transfected cells being green, indicating likely membrane expression. In a previous study, we introduced Asp at each of 11 contiguous locations along the S1 transmembrane helix and observed current only with Asp at the 3 positions judged to face the pore in our homology model (43). Although other explanations are possible, the lack of current in F182D may indicate that it does not face the pore in hHv1, consistent with homology model predictions (27). The less obstructive F182A mutant generated proton-selective currents but exhibited no $g_{H^+\text{closed}}$. We conclude that Val178 and not Phe142 is part of the HG.

To further define the location of the HG, we mutated hydrophobic residues that face the pore in the model and reside 1 helical turn above (Leu147, Phe152) or below (Ile105) the putative HG. All remained proton selective and none exhibited detectable $g_{H^+\text{closed}}$. It is surprising that L147T or L147D did not, because at the corresponding Shaker position, I287H does leak protons (22), but position 147 in S2 of hHv1 is at the level of Asp152, the selectivity filter (30), which has no parallel in the Shaker VSD. We mutated Val177, which neighbors Val178, the central member of a string of 5 valines. In a closed hHv1 model (27), Val178 faces the pore directly, but with a small rotation Val177 would face the pore. V177D produced a distinct $g_{H^+\text{closed}}$ with faster activation. Evidently decreasing the hydrophobicity of either Val178 or Val177 compromises the closed channel. Taken together, these results support the identification of the HG in hHv1 as Val109, Phe150, Val177, and Val178.

What Is the Proton-Selective Pathway through Closed HG Mutant Channels? One might imagine that decreased hydrophobicity in the HG region allows a more continuous water wire or, in the case of Asp mutants (V109D, F150D, V177D, and V178D), may directly create a proton transfer site. MD simulations in the closed-channel model (53) contain 2 hydrophobic regions. However, the proximity of Ile105 to the HG is evidently sufficient that its mutation speeds activation, but without producing $g_{H^+\text{closed}}$. We conclude that activation kinetics is influenced by the overall hydrophobicity of the region, whereas inducing $g_{H^+\text{closed}}$, requires decreased hydrophobicity specifically in the locations occupied by Val109, Phe150, Val177, and Val178.
to preclude H⁺ conduction in closed hH1V1 channels, and furthermore, that other bottlenecks are not.

The fact that closed hH1V1 channels conduct protons if the HG is made less hydrophobic, even by single point mutations, is consistent with the view that the physical barrier separating protons from one side of the closed channel to the other is relatively thin. Also consistent with this view is the observation that the R205H mutant of hH1V1 can conduct protons at negative voltages where the channel is presumably closed (25). A crucial property of the HG in other channels is that most of the membrane potential drops across this relatively short distance (~4 to 10 Å) (17–21, 54–56). Consequently, charged groups that move through the HG essentially transfer their charge from intracellular to extracellular while moving across only a fraction of the membrane thickness.

Steep voltage dependence can thus be achieved with limited physical movement, illustrating the parsimony of natural selection. Like other VSDs, hH1V1 has a focused electric field that enables gating with minimal motion of the protein.

It is clear that a conserved Asp in the middle of the S1 transmembrane helix is crucial for the H⁺ selectivity of hH1V1, because mutations that neutralize Asp result in anion permeability in 3 species (3, 30, 57). In most open-state homology models, Asp interacts almost continuously with one or more Arg in S4 (33, 43, 45, 53, 58), while quantum mechanical calculations on a reduced model of the HG selectivity filter showed that Asp–Arg interaction can explain both proton permeation and proton selectivity (44). That 

\[ \text{Arg}_{112} \] is proton selective supports the idea that the sole selectivity filter, comprising Asp^{112} in S1 interacting with one or more Arg from S4, remains intact in the (C₁) closed state of hH1V1.

**H⁺ Leakiness in HG Mutants Is Due to a More Open-Like Conformation in the Resting State.** Consistent with the above considerations on the nature and the thickness of the barrier opposing proton permeation in the resting state of the channel, the HG mutants in which the guanidinium group of Arg^{208} was most likely to slip past residue 150 correspond to the experimentally determined H⁺ leaky mutants (Fig. 7 and SI Appendix, Fig. S5). In this compromised conformational state, the channel topologically resembles the activated state in that Arg^{208} faces the extracellular vestibule of the pore, with a gating charge distribution intermediate between that of the closed state, in which Arg^{208} sits below Phe^{149} in the intracellular vestibule, and that of the open state, in which Arg^{208} is near Asp^{112} in the extracellular vestibule. These results suggest that H⁺ leakiness in HG mutants is a result of the inability of the gasket to prevent the guanidinium group of Arg^{208} from crossing the charge transfer center in a process where reduced steric interactions and new charge–charge interactions compromise the integrity of the HG.

**Why Does Zn²⁺ Inhibit Closed-Channel H⁺ Current?** The bulk of evidence supports the idea that Zn²⁺ inhibits WT hH1V1 currents by binding to the closed WT hH1V1 channel and preventing opening (59–61). In a recent proposal, protons permeate hH1V1 by binding consecutively to 3 sites, each with 2 acidic groups; the internal site includes E153 and D174, the central site D112 and D185, and the outer site E119 and D123 (62). Although the amino acids most critical for Zn²⁺ binding to mammalian Kᵥ1 are His^{140} and His^{193} (12, 29, 36), the crystal structure of the closed mKᵥ1 also implicates E119 and D123 in Zn²⁺ coordination (29). Thus, both acids comprising the external proton binding site (62) would be busy binding Zn²⁺, and unavailable to shuttle protons out of the pore. Because E119 and D123 are both on S1, their relative positions would likely be uninfluenced by opening or closing of the channel. Although decreased hydrophobicity of HG mutants allows proton permeation through the HG constriction in closed (C₁) channels, the proton might still need the outer pair of acids to complete its journey. Despite the attractiveness of this mechanism, we cannot rule out an alternative possibility that Zn²⁺ inhibits closed-channel H⁺ current by the simple fact of binding in the outer vestibule and occluding the proton pathway by electrostatic repulsion and steric obstruction.

**Implications for Gating Mechanisms.** We present strong evidence for at least 2 distinct closed states, consistent with many previous studies proposing multiple gating states (31, 37, 42, 63–67). That the shallow C₁ closed state conducts protons in HG mutants uniquely enables distinguishing this state electrophysiologically. Because a variety of mutations produced similar phenomenology, it appears that the gating mechanism itself was not grossly altered; hence, the C₁ state likely exists in WT channels. At voltages negative to the normal gating process, larger hyperpolarization produced a slow tail current indicating a second closing step (Fig. 5). The voltage dependence of this (C₁ ↔ C₂) gating process is extremely weak. Similarly, Cherny et al. (37) observed a steeply voltage-dependent closing process in rat hH1V1 near threshold voltages, with a weakly voltage-dependent component at more negative voltages. Several previous studies have concluded that most of the gating charge moves between closed states, prior to the opening transition (25, 65–67). The weak voltage dependence of C₁ ↔ C₂ transitions and the steep voltage dependence of the C₁ ↔ O step (Fig. 5 and SI Appendix, Fig. S2) observed here appear to contradict this conclusion, although the C₁ ↔ O step may encompass several substrates of a more complete model. An intriguing possibility is that the deep closed-state C₂ occurs when extreme hyperpolarization traps Arg^{208} below the HG where it resides in WT hH1V1 (Fig. 7A).

Many if not most gated ion channels contain a hydrophobic gate that undergoes sharp wetting and dewetting transitions resulting from conformational changes upon opening and closing, respectively (53, 68–71). Whether or not the HG functions as a hydrophobic gate in hH1V1 is unclear. First, hydrophobic gates tend to extend over distances of 1 to 1.5 nm, longer than the length of the HG. Second, we did not find systematic differences in hydration between HG mutants exhibiting proton permeation or not (SI Appendix, Fig. S4). Previously, we found hydration to be indistinguishable in H⁺-selective, anion-selective, and non-conducting hH1V1 mutants (43). It is possible that the HG widens upon opening (53) or that open and closed states differ in side-chain orientations (27). In light of the ΔpH dependence of H₁ activation (37), protonation of internal residues at low pH, or deprotonation of external residues at high pH, might alter the channel conformation in a way that permits proton permeation. Answering these questions will require detailed comparisons of open and closed states of the channel.

**Implications for Genetic Disease.** Intriguingly, episodic ataxia in the human Kᵥ1.1 channel is associated with mutations at positions corresponding to Val^{109} (72) and Val^{178} (73) in hH1V1. We predict that an individual with mutations to HG residues in hH1V1 might exhibit closed-channel proton current. We and others (22, 25) have noticed that mutations that produce constitutive proton leak seem to decrease the vitality and longevity of cells that express these channels. Specific mutations to the VSD of other voltage-gated ion channels produce “omega currents” or “gating pore currents” in which the VSD becomes permeable to protons (74) or simply to cations in general. These gating pore currents are associated with a variety of hereditary diseases that mostly afflict excitable cells, nerve and muscle (75). The COSMIC database (76) has an entry for an F150C mutation in the HVNC1 gene from a 70-y-old male with bladder carcinoma, raising intriguing possibilities for future study.

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Materials and Methods

MD Simulations. MD methods are provided in SI Appendix.

Gene Expression. Site-directed mutants were created using the Stratagene QuickChange (Agilent) procedure according to the manufacturer’s instructions. Transfection into HEK-293 cells was done as described (33). The following mutants were produced by GenScript: L147D, F150A, F150D, R211G, 11. P. V. Lishko, I. L. Botchkina, A. Fedorenko, Y. Kirichok, Acid extrusion from human neutrophils in conditions of extracellular alkalization and alkaline washouts. Biophys. J. 101, 1802–1807 (2011).

Electrophysiology. In most experiments, cells expressing GFP-tagged proton channels were identified using Nikon inverted microscopes with fluorescence capability. For constructs that lacked the GFP tag, GFP was cotransfected. Conventional patch-clamp techniques were used (33) at room temperature (20 to 26 °C). Bath and pipette solutions contained 60 to 100 mM buffer, 1 to 2 mM CaCl₂ or MgCl₂ (intracellular solutions were Ca²⁺-free), 1 to 2 mM EGTA, and TMAMeSO₄ to adjust the osmolality to ~300 mosM, titrated with TMAOH. Buffers used were Homopipes at pH 4.5 to 5.0, Mes at pH 5.5 to 6.0, BisTris at pH 6.5, Pipes at pH 7.0, Heps at pH 7.5, and Tricine at pH 8.0. Currents are shown without leak correction. To minimize pH changes due to large currents, pulses for large depolarizations in pulse families were sometimes shortened. Reversal potentials (Vrev) were determined by 2 methods, as described previously (77).

Closed-Channel Conduction. Proton current amplitudes (irev) were usually determined by fitting the rising current with a single exponential and extrapolating to infinite time. Proton conductance (grev) was calculated from irev and Vrev measured under the conditions of this study. Most mutations were introduced into the WT background. In a few cases, mutations were introduced into a Zn²⁺-resistant background (H140A/ H153A), which was used previously to distinguish the mutant channels from the endogenous H₁ (30). In most cases, the level of expression of all mutants studied here was sufficiently high that contamination by native H₁ was negligible.

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