Histidine$^{168}$ is crucial for ΔpH-dependent gating of the human voltage-gated proton channel, hHV$_1$

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We recently identified a voltage-gated proton channel gene in the snail Helisoma trivolvus, HtHV$_1$, and determined its electrophysiological properties. Consistent with early studies of proton currents in snail neurons, HtHV$_1$ opens rapidly, but it unexpectedly exhibits uniquely defective sensitivity to intracellular pH ($pH_i$). The $H^+$ conductance ($g_{H}$)-$V$ relationship in the voltage-gated proton channel (H$_V$,1) from other species shifts 40 mV when either $pH_o$ or $pH_i$ (extracellular pH) is changed by 1 unit. This property, called ΔpH-dependent gating, is crucial to the functions of H$_V$,1 in many species and in numerous human tissues. The HtHV$_1$ channel exhibits normal $pH_o$ dependence but anomalously weak $pH_i$ dependence. In this study, we show that a single point mutation in human hHV$_1$—changing His$^{168}$ to Gln$^{168}$, the corresponding residue in HtHV$_1$—compromises the $pH_i$ dependence of gating in the human channel so that it recapitulates the HtHV$_1$ response. This location was previously identified as a contributor to the rapid gating kinetics of H$_V$,1 in Strongylocentrotus purpuratus. His$^{168}$ mutation in human H$_V$,1 accelerates activation but accounts for only a fraction of the species difference. H168Q, H168S, or H168T mutants exhibit normal $pH_o$ dependence, but changing $pH_i$ shifts the $g_{H}$-$V$ relationship on average by <20 mV/unit. Thus, His$^{168}$ is critical to $pH_i$ sensing in hHV$_1$. His$^{168}$, located at the inner end of the pore on the S3 transmembrane helix, is the first residue identified in H$_V$,1 that significantly impairs $pH$ sensing when mutated. Because $pH_i$ dependence remains intact, the selective erosion of $pH_o$ dependence supports the idea that there are distinct internal and external pH sensors. Although His$^{168}$ may itself be a $pH_i$ sensor, the converse mutation, Q229H, does not normalize the $pH_i$ sensitivity of the HtHV$_1$ channel. We hypothesize that the imidazole group of His$^{168}$ interacts with nearby Phe$^{165}$ or other parts of hHV$_1$ to transduce $pH_i$ into shifts of voltage-dependent gating.

Introduction

Voltage-gated proton channels, H$_V$,1, possess several distinctive or unique properties (DeCoursey, 2015). They are exquisitely proton selective with single-channel currents in the femtomolar range (Cherny et al., 2003). Perhaps because the conduction pathway is narrow and lacks a continuous water wire (Kulleperuma et al., 2013; Morgan et al., 2013; Chamberlin et al., 2014; Dudev et al., 2015), no high-affinity blockers exist that act by simple occlusion of the pore. The most potent inhibitor is Zn$^{2+}$, which binds competitively with protons to two histidines in the external vestibule of human H$_V$,1 (hHV$_1$) and interferes with channel opening (Cherny and DeCoursey, 1999; Takeshita et al., 2014). Although controversial (Bennett and Ramsey, 2017; DeCoursey, 2017), the conduction pathway includes an aspartate that appears to be obligatorily protonated and deprotonated during permeation (DeCoursey, 2003; Musset et al., 2011; Smith et al., 2011; Dudev et al., 2015; van Keulen et al., 2017). Consequently, $H^+$ conduction has anomalously large temperature dependence (DeCoursey and Cherny, 1998; Kuno et al., 2009) and deuterium isotope effects (DeCoursey and Cherny, 1997) when compared with other channels. H$_V$,1 in many species are dimers (Koch et al., 2008; Lee et al., 2008; Tombola et al., 2008), but monomeric constructs also function with fairly subtle differences from the dimer (Koch et al., 2008; Tombola et al., 2008; Musset et al., 2010).

Perhaps the most unusual property of H$_V$,1 is ΔpH-dependent gating, a unique mechanism that is essential to all its functions. Although H$_V$,1 is a voltage-gated ion channel, the voltage at which all known H$_V$,1 open is strongly dependent on pH. Increasing pH$_o$ or decreasing pH$_i$ shifts the $H^+$ conductance ($g_{H}$)-$V$ relationship negatively by ~40 mV/unit. At symmetrical pH ($pH_o = pH_i$), the threshold for activation in most species is 10–20 mV (Cherny et al., 1995; DeCoursey, 2013). The combination of these properties ensures that under almost all conditions encountered by living cells, H$_V$,1 opens only when the electrochemical gradient for $H^+$ is outward, so that opening H$_V$,1 channels results in...
acid extrusion from cells (DeCoursey, 2003). Functions of H20 beyond acid extrusion, such as compensating electrically for the electrogenic activity of NADPH oxidase (Henderson et al., 1987; DeCoursey et al., 2003; DeCoursey, 2010), also require ΔpH-dependent gating to enable H20 to open appropriately (Murphy and DeCoursey, 2006).

The phenomenon of ΔpH-dependent gating seems to be remarkably robust. Despite at least 171 different H20 mutants having been characterized electrophysiologically (DeCoursey et al., 2016), mutation at only one position, Trp207 (in hH20 numbering), significantly altered ΔpH-dependent gating (Cherny et al., 2015). Mutation to this Trp resulted in premature saturation of the response to pHo but did not affect pHi responses. Recently identified H20 in a few species (Cherny et al., 2015; Chaves et al., 2016), including the voltage-gated proton channel gene in the snail Helisoma trivolvis (HtH20, Thomas et al., in this issue), have an exaggerated pHo response, shifting more than 40 mV/unit. Evaluation of three dozen hH20 mutants revealed shifts of the absolute voltage dependence over a 270-mV range, but every mutant still exhibited an ∼40-mV/pH unit shift from whatever its starting voltage was (Ramsey et al., 2010). We were therefore surprised to find that despite a robust pHo response, the WT HtH20 channel has anomalously weak pHi dependence, with the gH-V relationship shifting just 20 mV/unit or less (Thomas et al., 2018). While testing several possible explanations for the rapid gating of snail HtH20, we serendipitously discovered that replacement of a single amino acid, His168, in the human channel nearly abolishes its pHi dependence. We conclude that His168 is a crucial component of pH-sensing in hH20.

**Materials and methods**

Electrophysiological and mutagenesis methods for HtH20 are described in the accompanying article (Thomas et al., 2018); mutagenesis of hH20 was described previously (Musset et al., 2010). A triple mutant of hH20 (H167N/H168V/K168N) was supplied by I. Scott Ramsey and David E. Clapham (Harvard Medical School, Boston, MA). Statistical comparison of groups was done by using Student’s t test.

**Sequence analysis**

46 full-length H20 sequences, including 11 electrophysiologically confirmed and 13 high-confidence sequences from protists—previously shown to be more diverse than animal H20 (Smith et al., 2011)—were aligned with MAF (Katoh and Standley, 2013). Sequences were trimmed to the voltage-sensing domain as described previously (Smith et al., 2011). Sequences that were not confirmed electrophysiologically were excluded, and empty

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**Figure 1.** Alignment of H20 S2–S3 linker and nearby regions (E153–D185 in hH20). H20 from several species were characterized as activating rapidly (green background: HtH20, LsH20, and SpH20) or slowly (pink background: HH20, mH20, OcH20, RhH20, and GgH20) based on available electrophysiological data. Unshaded species exhibit intermediate kinetics. In hH20 numbering, the asterisk (*) indicates the position corresponding to the throttle histidine H168; the caret (^) indicates the position corresponding to F165. The S2–S3 linker in hH20 encompasses K157–F165 (Li et al., 2015).

**Figure 2.** His168 mutants are proton selective. Mean ± SEM values for the reversal potential (Vrev) are plotted for 12 H168Q, 9 H168T, and 6 H168S cells or patches. The linear regression slope for each mutant is −52.6, −53.2, and −56.3 mV/unit change in ΔpH, respectively. For comparison, the gray dashed line shows the Nernst potential, E0, expected for perfect H+ selectivity.
columns removed from the alignment. The activation (opening) kinetics of electrophysiologically confirmed sequences were characterized as fast (H. trivolvis, HtHV1; Lymnaea stagnalis, LsHV1; and Strongylocentrotus purpuratus, SpHV1) or slow (Homo sapiens, hHV1; Mus musculus, mHV1; Oryctolagus cuniculus, OcHV1; Rattus norvegicus, RnHV1; and Cricetulus griseus, CgHV1); sequences with intermediate or unknown kinetics were removed. To identify sequence sites that distinguish sequence subfamilies, the alignment was submitted to the SPEER server (Chakraborty et al., 2012) with either user-defined (two subfamilies) or automated subgrouping and the following parameters: single-submission eight sequences, Relative Entropy term, PC Property Distance term, and Type. The same alignment but in randomized sequence order was also submitted with the automated subgrouping parameter and all other parameters as above. Sites corresponding to His167 and His168 in hHV1 were identified as appearing in the top 10 sites identified in all analyses and having a P value of <0.05 in at least one analysis.

Results
Is His168 responsible for the slow gating kinetics of hHV1?
In our companion article (Thomas et al., 2018), we reported the identification of an H1 in a snail H. trivolvis (HtHV1) that exhibited rapid activation kinetics as reported for native proton currents in other snails (Byerly et al., 1984). We performed an analysis of subfamily determining sites to distinguish H1 sequences with fast versus slow gating kinetics. Species categorized as having fast gating included snails, HtHV1 (Thomas et al., 2018) and L. stagnalis, LsHV1 (Byerly et al., 1984); and sea urchin, S. purpuratus, SpHV1 (Sakata et al., 2016) and are shaded green in Fig. 1. Species with slow gating are human, hHV1 (Bernheim et al., 1993; DeCoursey and Cherny, 1993; Demaurex et al., 1993); mouse, mHV1 (Kapus et al., 1993); rabbit, O. cuniculus, OcHV1 (Nordström et al., 1995); rat, R. norvegicus, RnHV1 (DeCoursey, 1991); and Chinese hamster, C. griseus, CgHV1 (Cherny et al., 1997) and are shaded pink in Fig. 1. This analysis pointed to two residues corresponding to His167 and His168 in hHV1 numbering; these residues are in the intracellular S2/S3 loop, extending into the S3 transmembrane helix. Sakata et al. (2016) found that substituting either Ser or Thr for His168 in mHV1, which corresponds to His168 in hHV1 (Fig. 1, asterisk), accelerated activation kinetics by an order of magnitude, raising the possibility that His in this position governs activation kinetics, acting as a “throttle.” The same substitutions at the neighboring His167 had no effect. For these reasons we focused our experiments on the S2/S3 loop area.

Similar to Sakata et al. (2016), we engineered mutations of His168, replacing it with Ser (H168S) or Thr (H168T). Because activation of snail HtHV1 is distinctly faster than that of sea urchin SpHV1, we reasoned that replacing His168 with Gln, which is at the corresponding position in HtHV1 (H168Q), should cause even faster kinetics. As a control, we replaced the neighboring His167 in hHV1 with Lys (H167K), which occupies this position in HtHV1 (Fig. 1, asterisk), accelerated activation kinetics by an order of magnitude, raising the possibility that His in this position governs activation kinetics, acting as a “throttle.” The same substitutions at the neighboring His167 had no effect. For these reasons we focused our experiments on the S2/S3 loop area.

Robust proton selective currents were observed in all mutants. Results from H168S, H168T, and H168Q mutants
were indistinguishable, and we thus refer to all such mutants as H168X. Fig. 2 shows that the reversal potential \(V_{\text{rev}}\) in the three H168X mutants was close to the Nernst potential, confirming proton selectivity.

Fig. 3 illustrates the effects of these mutations on channel-opening kinetics. Replacing His\(^{168}\) with any of the three amino acids (Ser, Thr, or Gln) produced a 10-fold speeding of the activation (opening) time constant \(\tau_{\text{act}}\) in the positive voltage range, much like the analogous effect in mHV1 (Sakata et al., 2016). Mutating the neighboring His\(^{167}\) had no clear effect. That the effects of the three substituents (Ser, Thr, or Gln) were identical suggests that the speeding of activation is caused by removing the imidazole group of His and is not caused by introducing other side chains at this position. Thus, the hydroxyl group of Thr or Ser was no more or less effective than the amide group of Gln. Presumably, His\(^{168}\) interacts specifically with other parts of the hHV1 channel in a manner that results in slower activation. Nevertheless, despite the distinct acceleration caused by His\(^{168}\) removal, \(\tau_{\text{act}}\) remained much slower than in the WT snail channel HtHV1.

**His\(^{168}\) mutation alters \(\Delta pH\)-dependent gating of hHV1**

The His\(^{168}\) mutants exhibited normal responses to changes in pH\(_o\). Families of currents in a cell expressing the H168Q mutant are shown in Fig. 4, A–C. The currents appear qualitatively like WT hHV1 currents, although with somewhat faster activation. Changing pH\(_o\) from 5 to 6 to 7 shifted the H\(^+\) current \(I_h\)–V (Fig. 4 D) and \(g_h\)–V relationships (Fig. 4 E) by \(-40\) mV/unit. This behavior conforms to the classical “rule of forty” observed for H\(_1\) in all species thus far examined (DeCoursey, 2013), except for HtHV1 as described in the companion article (Thomas et al., 2018).

In contrast, mutation of His\(^{168}\) to Ser, Thr, or Gln greatly weakened the sensitivity of the mutant channels to pH\(_i\). Fig. 5, A–E shows currents in an inside-out patch with H168T at pH\(_o\) 7 and pH\(_i\) ranging from 5 to 9. Although the \(I_h\)–V relationship does shift positively at higher pH\(_i\) (Fig. 5 F), the shift is distinctly \(-40\) mV, especially at high pH\(_i\), with the result that there are inward currents over a wide voltage range. Inward currents in WT hHV1 are rarely observed and occur mainly with large outward pH gradients (Musset et al., 2008). It is evident from the \(g_h\)–V relationships (Fig. 5 G) that the shifts are \(\pm 20\) mV/unit over the entire pH range studied.

Measurements of the position of the \(g_h\)–V relationship in several cells and patches are summarized in Fig. 6 A, quantified as \(V(g_h,\text{max}/10)\), the voltage at which \(g_h\) reached 10% of \(g_h,\text{max}\) its maximal value. Changes in pH\(_i\) for the H168X mutants (Fig. 6 A, blue circles) produce a slope of \(-40\) mV/unit up to pH\(_o\) 8 (\(\Delta pH = 1\)). This behavior is identical to the WT hHV1 response (Cherny et al., 2015). Data from inside-out patches (Fig. 6 A, dark red diamonds) reveal that the H168X mutants exhibit a greatly attenuated pH\(_i\) response. The entire curve has a mean slope of only 16.1 mV/unit. However, the relationship appears steeper at high \(\Delta pH\). Omitting the point at \(\Delta pH = 2\) (pH\(_o\) 7, pH\(_i\) 5), the mean slope drops to 12.6 mV/unit.

The behavior of the H168X mutants strikingly resembles that of WT HtHV1, as illustrated in Fig. 6 B. The data for HtHV1 from the companion article (Thomas et al., 2018) are replotted here (Fig. 6 B, open symbols and dotted lines) for comparison.

Figure 4. **Gating of His\(^{168}\) mutants has normal pH\(_o\) dependence.** (A–C) Families of currents in a cell expressing H368Q with pH\(_o\) 6 and pH\(_i\) 5, 6, and 7, with pulses in 10-mV increments up to the voltage indicated. Holding potential, \(V_{\text{hold}}\) was −40 mV (A and B) or −60 mV (C). (D and E) Proton current–voltage curves (D) and \(g_h\)–V relationships (E) from the families in A–C exhibit a normal 40-mV shift/unit change in pH\(_o\).
Comparison of pH$_o$ responses reveals that HtHV1 has an exceptionally steep ΔpH dependence at pH$_o$ 7 or less, roughly 60 mV/unit. The response to pH$_o$ for H168X mutants (Fig. 6B, shaded blue circles) is a 40-mV/unit shift, which is normal for human hHV1 (Cherny et al., 2015). The response of H168X mutants to pHi changes (Fig. 6B, red diamonds) is notably weak and closely parallels the response of WT snail HtHV1. With respect to ΔpH-dependent gating, the mutation of a single amino acid, His168, effectively converts the human channel into a snail channel.

The immediate neighbors of His168 do not appear to contribute

Located next the His168 that plays a critical role in pHi sensing is another His, His167. We wondered whether this nearby position might share the effect. However, the single mutation H167K did not impair pHi sensing. This specific replacement was selected because HtHV1 has Lys at the corresponding position (Fig. 1). Fig. 7 illustrates H$^+$ currents in an inside-out patch expressing the H167K mutant at pH 6, 7, and 8. The gH-V relationship shifted normally (Fig. 7E). Mean data from three patches with H167K are plotted in Fig. 8, which shows that the pH$_i$ response of this mutant is indistinguishable from that of WT hHV1. Furthermore, Fig. 3 showed that the H167K mutation did not alter activation kinetics. Evidently, His168 but not His167 is located precisely where it can strongly influence gating in response to pH$_i$.

We also tested a triple mutant, in which three consecutive titratable amino acids at the inner end of the S3 helix were replaced by neutral residues (H167N/H168V/K169N). In inside-out patches with pH$_o$ 7.5, studied at pHi 5.5, 6.5, 7.5, and 8.5, the sensitivity to pHi was weak, like that of the H168X single mutants. The mean slope by linear regression of the relationship between ΔpH and V(gH,max/10) in seven patches was 20.1 mV/unit, similar to 16.1 mV/unit in H168X (Fig. 6), suggesting that the amino acids neighboring His168 were inactive. This triple mutant was examined previously, and normal ΔpH dependence was reported (Ramsey et al., 2010). However, in that study only pH$_o$ was varied, not pH$_i$. The triple mutant, like all single His168 mutants, exhibits normal pH$_o$ dependence but abnormally weak pH$_i$ dependence.

Possible interaction between H168 and F165

Data on pHi sensitivity are somewhat less abundant than for pH$_o$ sensitivity, and much is derived from native tissues. Species with H$_i$1 that have been shown to exhibit classical rule of forty
behavior for changes in pH include human (Demaurex et al., 1993; Gordienko et al., 1996; Schrenzel et al., 1996; Schilling et al., 2002), mouse (Kapus et al., 1993; Szteyn et al., 2012), Rana pipiens (Gu and Sackin, 1995), rat (Cherny et al., 1995; DeCoursey and Cherny, 1995), Karlodinium veneficum (Cherny et al., 2015), Emiliania huxleyi (Cherny et al., 2015), and Lingulodinium polyedrum (Rodriguez et al., 2017). The only violators of the rule thus far are the snails H. trivolvis (Thomas et al., 2018) and L. stagnalis (Byerly et al., 1984). Another intriguing exception is a short isoform of hHv1 expressed in sperm that lacks the first 68 amino

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Figure 6. The His168 mutation recapitulates the anomalous ΔpH dependence of HtHV1. (A) The effect of pHo and pHi on the position of the $g_{HV}$-V relationship in the three His168 mutants combined (mean ± SEM) is plotted. The position of the $g_{HV}$-V relationship was defined in terms of $V(g_{HV,max})/10$. The dashed green line shows a slope of 40 mV/unit for reference. The dependence of these mutants on pHo is normal, whereas their pHi response is greatly attenuated. Linear regression on all points (ignoring the obvious nonlinearity) gives a slope of 38.9 mV/unit change in pHo and 16.1 mV/unit change in pHi. (B) The same data are replotted (shaded symbols) along with the analogous measurements for HtHV1 (open symbols) taken from Fig. 9 of the companion article (Thomas et al., 2018). In whole-cell measurements, pHo was varied with pHi 7 (blue symbols). When pHi was varied by using inside-out patches with pHo 7, there was very little shift of the $g_{HV}$-V relationship (red symbols). Numbers of cells for increasing ΔpH in His168 mutants for pHi 7 are 3, 10, 11, and 5 and for pHo 7 are 6, 10, 14, 11, and 4.
His168 is an internal pH sensor of hHV1.

Although in the available crystal structure of HtHV1 (Takeshita et al., 2014) this area did not have sufficient electron density to be visualized, molecular models of HtHV1 (Kulleperuma et al., 2013; Li et al., 2015) indicate that Phe165 could plausibly interact with the nearby His168 through π-stacking or cation-π interactions.

To test the idea that His168 interacts with Phe165, we individually mutated Phe165 and as a control, Phe166. F166L produced robust currents, and as shown in Fig. 8, pHi sensing of F166L was essentially normal and similar to that of WT hHV1. F165H and F165A (Fig. 8, F165X) produced currents that activated in a more negative voltage range than WT. To enable comparison of the slopes, 13.8 mV has been added to these data, so they superimpose on WT at ΔpH = 1. The pHi sensitivity of F165A and F165H was closer to WT than to that of the H168X mutants. Finally, the double mutant F165L/H168Q was also close to the single H168X mutant data (unpublished data). Although these results do not rule out the possibility of interaction between His168 and Phe165, it appears that His168 is distinctly more critical for pHi sensing.

The converse mutation Q229H fails to impart human characteristics to the H. trivolis channel

The single point mutation H168X (X = T, S, Q) profoundly alters the gating and pH sensing of the human channel hHV1, accelerating activation 10-fold and greatly attenuating the pHi response. We next considered whether the converse mutation (Q229H) would impart humanoid characteristics to HtHV1. In short, this mutation had no discernable effect on the gating kinetics or pH sensitivity of HtHV1 (Fig. 9). The Q229H mutation did not slow activation; at +40 mV at pHo 7, τact was 11.8 ± 1.4 ms (n = 17) in WT HtHV1 (Fig. 3) and 10.0 ± 2.0 ms (n = 8) in Q229H (P = 0.53). We assessed pHo sensitivity in whole-cell experiments. Like WT HtHV1, activation kinetics was extremely sensitive to pHo, whereas the deactivation or tail current (closing) time constant appeared to be pHi independent. Like WT, changes in pHo produced supernormal shifts in the position of the gH-V relationship, averaging 57 mV/unit between pHo 5 and 7 (Fig. 10), paralleling the WT response although the absolute voltages appear somewhat more negative. Similarly, the Q229H mutation did not discernably change the weak response of HtHV1 to changes in pHi (Figs. 9 E and 10). In three inside-out patches studied at pHi 5, 6, 7, and 8, the slope of the mean dependence of V(gH,max/10) on ΔpH was 18 mV/unit by linear regression. It is clear that the extraordinary changes to the pHi response of the human channel resulting from His168 mutation reflect interactions that are not confined to this single amino acid and therefore are not easily transferred.

Discussion

Is the human hHV1 channel slow because of the throttle residue, His168?

The sea urchin proton channel, SpHV1, exhibits rapid activation kinetics 20–60 times faster than the mouse mHV1. Sakata et al. (2016) investigated the structural basis for this property and, by creating progressively smaller chimerae with mHV1, finally identified a single amino acid (Ser) at the inner end of the S3 transmembrane segment that appeared largely responsible. Introducing Ser into mHV1 (H164S) accelerated mouse activation kinetics of the N terminus and has weakened pHi sensing (Berger et al., 2017). Inspection of sequences (Fig. 1) for which pHi sensitivity has been measured directly (HtHV1, LsHV1, EhHV1, kHV1, LpHV1, hHV1, mHV1, and RnHV1) shows that Leu substitutes for Phe at the Phe165 position (human numbering) in two species with rapid gating and attenuated pHi responses, HtHV1 and LsHV1.
by more than an order of magnitude. In mHV1, H164T produced speeding similar to H164S, suggesting that the hydroxyl group is important (Sakata et al., 2016). Intriguingly, however, the converse mutation, and even substituting the entire S3 helix from mHV1 into SpHV1, failed to slow the kinetics of the latter.

We engineered mutations of human hHV1 at His168 (the position that corresponds to His164 in mHV1), replacing the His with Ser (H168S), Thr (H168T), or Gln (H168Q). In most respects, the results resembled those in mHV1: mutations at His168 accelerated activation by about one order of magnitude (Fig. 3). Because HtHV1 has Gln at the corresponding position and HtHV1 is distinctly faster than SpHV1, we expected that Gln substitution might produce faster activation; however, activation kinetics of H168Q were very similar to those of H168S and H168T, all of which remained much slower than that of WT HtHV1 (Fig. 3).

Because the three mutants behaved indistinguishably, we conclude that the effects result from the removal of the imidazole side chain of His168 and not from the side chains that replace it.

The precise location of His168 is important. The His immediately adjacent to His168 does not share its impact on gating kinetics. Sakata et al. (2016) replaced His166 in mHV1 with the SpHV1 residue at the corresponding position (H163R); this mutation had little effect on kinetics of mHV1. We replaced His167 in hHV1 with Lys, which occupies the corresponding position in HtHV1. In neither species was activation kinetics appreciably affected (Fig. 3).

Also, similar to the results in mHV1, the converse mutation substituting the hHV1 residue for the HtHV1 throttle residue, Q229H, did not slow HtHV1 kinetics. Therefore, the mechanism by which His168 retards activation must require interaction with other parts of the protein that are absent in Ht1 from species with rapid gating.

**His168 in hHV1 is crucial for pH sensing**

In searching for a mechanistic explanation for the rapid gating kinetics of HtHV1, we serendipitously identified a histidine near the inner end of the channel that is crucial for pH sensing. Transferring the Gln229 from the snail channel HtHV1 to replace His168 in the human channel hHV1 (Fig. 1) reproduced the impoverished pH sensing of HtHV1 almost exactly (Fig. 6 B). Because three different substituents (Ser, Thr, and Gln) had indistinguishable effects (Figs. 3 and 6), it appears that a specific property of His in this position is involved. Because the converse mutation (Q229H) did not transfer human-like pH sensing (or activation kinetics) to HtHV1 (Fig. 9), His168 must perform pH sensing by interacting with its immediate environment in the human channel. This conclusion is reinforced by the fact that three unicellular species that lack His at this position (kHV1, LpHV1, and EhHV1; Fig. 1) exhibit normal pH sensitivity (Cherny et al., 2015; Rodriguez et al., 2017). Replacing the neighboring His229 had no effect, and mutating both His167 and Lys169 (HHK→NVN) had no effect beyond that of His168 alone, indicating that the specific location at position 168 is critical.

When the ΔpH dependence of H21 gating was discovered (Cherny et al., 1995), the simplest model that could reproduce the entire phenomenon postulated a single pH sensor with alternating access to both internal and external solutions. An expanded model with additional complexity includes distinct external and internal pH sensors. A “counter-charge” model has been suggested in which protonation disrupts charge-pair interactions,
so that low pH$_i$ promotes opening and low pH$_o$ promotes closing (DeCoursey, 2018). A different kind of proposal for pH sensing does not invoke titratable pH sensors; instead, protonated waters in H$_V$I communicate information about local pH through electrostatic interactions with the voltage-sensing Arg residues in the S4 helix (Ramsey et al., 2010). Recently, the first mutation in H$_V$I documented to compromise pH sensing was reported (Cherny et al., 2015). In these Trp mutants, pH$_o$ but not pH$_i$ sensitivity was affected, suggesting that separate pH$_o$ and pH$_i$ sensors exist in H$_V$I. Intriguingly, like the H168X mutants, these W207X mutants also dramatically accelerated hH$_V$I gating kinetics, and HtH$_V$I itself has extremely rapid gating.

That HtH$_V$I has normal pH$_o$ sensitivity but anemic pH$_i$ sensitivity suggests that its ΔpH dependence is accomplished by distinct external and internal pH sensors. That the H168X mutation in hH$_V$I selectively attenuates its pH$_i$ sensitivity without affecting its pH$_o$ sensitivity further supports the existence of distinct external and internal pH sensors.

To search for a structural basis for this species difference in pH$_i$ sensing, we compared the sequences of H$_V$I in species in which pH$_i$ responses have been documented to obey the rule of forty (human, mouse, rat, K. veneficum, E. huxleyi, and L. polyedrum; excepting R. pipiens for which we could find no sequence), to the sequence of HtH$_V$I with its anomalously weak

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**Figure 9.** The Q229H mutation does not restore human-like activation kinetics or pH$_i$ sensitivity to HtH$_V$I. (A–D) Families of currents in an inside-out patch with pH$_o$ 7 in the pipette solution in 10-mV increments up to the voltage shown from $V_{	ext{hold}} = -80, -60, -40, -40$ mV, at pH 5, 6, 7, and 8, respectively. (E) Currents from this patch converted to $g_H$ produce $g_H - V$ relationships that shift much less than 40 mV/unit. Corresponding measurements in WT HtH$_V$I are plotted in Fig. 5 G.
sensitivity to pH. We noticed a striking substitution in HtHV1, in which Phe165 (human numbering; Fig. 1) is replaced by Leu. This position is very highly conserved: in the alignment of 46 HV1 sequences that includes vertebrates, invertebrates, and protists, Phe is replaced by the conservative Tyr five times, by Leu twice, and by Met once. In this context, it is important to note that the protist HV1 sequences are more diverse than all animal sequences (Smith et al., 2011), highlighting the very high conservation of Phe at this position. The two snail species in which Leu replaces Phe (HtHV1 and LsHV1) both have rapid gating and weak pHi sensitivity (Fig. 1). In structural models of HV1 (Kulleperuma et al., 2013; Li et al., 2015), Phe165 is in a position in which it could plausibly interact with His168, through π-stacking or cation-π interactions. Reasoning that such an interaction might be part of the pH-sensing mechanism, we replaced Phe165 in hHV1 with Ala or His to attempt to disrupt the interaction. Both F165A and F165H exhibited pHi sensing intermediate between WT and the H168x mutants but closer to WT hHV1. The neighboring F166L mutation did not affect pHi sensing (Fig. 8). For geometrical reasons, we consider it more likely that His168 could interact with Phe165 than with Phe166 because the latter would be constrained by proximity. Intrahelical i, i + 2 side-chain–side-chain contacts are statistically improbable (Walther and Argos, 1996). That Phe165 mutants were activated at more negative voltages than WT (Fig. 8) is consistent with F165-H168 interaction impeding hHV1 opening. That the insertion of His into the corresponding position in HtHV1 did not enhance pHi sensitivity indicates that the mere presence of His at this location is not sufficient, but that to affect pHi sensing requires additional interaction. Evidently, and perhaps surprisingly, the intracellular S2–S3 linker is of central importance in sensing pHi in human hHV1. Furthermore, the selective effect of mutations in this region on pHi sensing while leaving pHo sensing intact supports the idea that ΔpH-dependent gating of H1V1 relies on independent internal and external pH sensors.

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