Exotic properties of a voltage-gated proton channel from the snail *Helisoma trivolvis*

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Voltage-gated proton channels, H₄₁, were first reported in *Helix aspersa* snail neurons. These H⁺ channels open very rapidly, two to three orders of magnitude faster than mammalian H₄₁. Here we identify an H₄₁ gene in the snail *Helisoma trivolvis* and verify protein level expression by Western blotting of *H. trivolvis* brain lysate. Expressed in mammalian cells, HtHV₁ currents in most respects resemble those described in other snails, including rapid activation, 476 times faster than hHV₁ (human) at pH 7, between 50 and 90 mV. In contrast to most H₄₁, activation of HtHV₁ is exponential, suggesting first-order kinetics. However, the large gating charge of ~5.5 eₒ suggests that HtHV₁ functions as a dimer, evidently with highly cooperative gating. HtHV₁ opening is exquisitely sensitive to pHₒ, whereas closing is nearly independent of pHₒ. Zn²⁺ and Cd²⁺ inhibit HtHV₁ currents in the micromolar range, slowing activation, shifting the proton conductance–voltage (gₜ-V) relationship to more positive potentials, and lowering the maximum conductance. This is consistent with HtHV₁ possessing three of the four amino acids that coordinate Zn²⁺ in mammalian HV₁. All known HV₁ exhibit ΔpH-dependent gating that results in a 40-mV shift of the gₜ-V relationship for a unit change in either pHₒ or pHi. This property is crucial for all the functions of HV₁ in many species and numerous human cells. The HtHV₁ channel exhibits normal or supernormal pHₒ dependence, but weak pHi dependence. Under favorable conditions, this might result in the HtHV₁ channel conducting inward currents and perhaps mediating a proton action potential. The anomalous ΔpH-dependent gating of HtHV₁ channels suggests a structural basis for this important property, which is further explored in this issue (Cherny et al. 2018. *J. Gen. Physiol*. https://doi.org/10.1085/jgp.201711968).

Introduction

Voltage-gated proton channels, H₄₁, remain relative newcomers to the ion channel family. Although the idea of a depolarization-activated proton-selective ion channel was proposed in 1972 by J. Woodland Hastings and colleagues (Fogel and Hastings, 1972), the first voltage-clamp study that established the existence of this channel type occurred a decade later in the snail *Helix aspersa* (Thomas and Meech, 1982). An H₄₁ gene was not identified until 2006 (Ramsey et al., 2006; Sasaki et al., 2006). Strong interest in this channel has arisen for two main reasons. First, its structure, with just four transmembrane helices, closely resembles the voltage-sensing domain of other voltage-gated ion channels, making it a unique model for voltage-gating mechanisms. By combining voltage sensing, gating, and conduction into a single module, H₄₁ uniquely provides a direct readout of its gating state. Second, exceedingly diverse functions have been identified for H₄₁ in many species and in many human tissues (DeCoursey, 2010).

The first systematic voltage-clamp characterization of voltage-gated proton currents was in *Lymnaea stagnalis* snail neurons (Byerly et al., 1984). When mammalian proton currents were identified a decade later (DeCoursey, 1991), the most obvious difference was that H₄₁ in snails activated two to three orders of magnitude faster. Here, we investigate the properties of the *Helisoma trivolvis* snail H₄₁ gene product. We searched a transcriptome of *H. trivolvis* and found a putative HtHV₁; we then cloned the gene from a cDNA pool constructed from *H. trivolvis* brain tissue. We find many similarities to native proton currents studied in situ in other snail species, including rapid gating kinetics and other significant differences from mammalian H₄₁. HtHV₁ currents differ from mammalian H₂₁ in having exponential (vs. sigmoid) activation, similarity of τₐct and τᵦct at overlapping voltages, and maximal time constants near the midpoint of the proton conductance–voltage (gₜ-V) relationship, all features suggestive of simple first-order gating kinetics expected of a monomeric protein. However, the existence of an extensive coiled-coil motif in the C terminus together with steep voltage dependence suggests “cooperative” gating of a dimeric protein.
inhibition of HtHV$_1$ by Zn$^{2+}$ and Cd$^{2+}$ is explained by conservation of three of four members of the Zn$^{2+}$-binding site (Takeshita et al., 2014). The most remarkable property of the HtHV$_1$ channel is that its sensitivity to pH$_i$ is anomalously weak. The voltage-gating mechanism of all H$_2$V$_1$ identified to date is unique in being nearly equally responsive to pH$_o$ and pH$_i$, such that a one-unit change in either shifts the $g_{H-V}$ relationship by 40 mV. This “rule of forty” (DeCoursey, 2013) has the biologically crucial effect of ensuring that H$_2$V$_1$ channels open only when there is an outward electrochemical gradient for H$^+$. In other words, H$_2$V$_1$ channels open only when doing so will result in acid extrusion from cells. Extensive mutation of HtHV$_1$ has failed to produce any significant violation of the rule of forty (Ramsey et al., 2010; DeCoursey, 2016). In this issue, Cherny et al. identify a single amino acid difference between HtHV$_1$ and hHV$_1$ that appears to be responsible for the anomalous ΔpH dependence of the snail channel.

**Materials and methods**

**Snail tissue**

*H. trivolvis*, a pulmonate snail (order: Basommatophora; family: Planorbidae) from an albino stock maintained and continuously bred in aquaria at Georgia State University, was used for experiments. Snails were originally caught in the wild and introduced as an experimental model animal by S.B. Kater (Kater, 1974).

**Gene cloning, mutagenesis, antibody synthesis, and Western blotting**

Basic Local Alignment Search Tool searches of a transcriptome from *H. trivolvis* (unpublished data) yielded a hit that matched the criteria for an H$_2$V$_1$ sequence (Smith et al., 2011). Brains were dissected from *H. trivolvis* (Cohan et al., 2003), RNA was extracted from brain tissue using the RNeasy kit (Qiagen), and a cDNA pool was constructed using the SuperScript III kit (Life Technologies) according to the manufacturer’s instructions. Primers designed against the transcriptome hit were used to clone the putative HtHV$_1$ coding sequence; the sequence was confirmed by commercial sequencing (SourceBio Science). This coding sequence was subcloned into eukaryotic expression vector pCA-IRESeGFP. Site-directed mutagenesis of HtHV$_1$ was performed and sequence verified commercially (Genewiz). Antibody was raised in rabbit to a synthetic peptide (RSPSDHGEGFEEPLC) based on the predicted HtHV$_1$ epitope and affinity purified (GenScript) with a final concentration of 0.904 mg/ml. Total lysate was prepared from *H. trivolvis* brains that had been stored whole in Qiagen RLT buffer at ~80°C for 12 min. Brains were thawed and triturated briefly on ice; the lysate was cleared by centrifugation at 10,000 × g for 5 min. Proteins from *H. trivolvis* brain lysate were separated by SDS-PAGE, Western blotted, and probed with anti-HtHV$_1$ antibody (diluted 1:10,000 in blocking buffer) either alone or preincubated with 1,000-fold molar excess of synthetic peptide corresponding to the epitope.

**Electrophysiology**

HEK-293 cells were grown to ~80% confluence in 35-mm culture dishes. HEK-293 cells were transfected with 0.4–0.5 µg cDNA using Lipofectamine 2000 (Invitrogen) or polyethylenimine (Sigma). Plasmids that did not include GFP were cotransfected with GFP. After 24 h at 37°C in 5% CO$_2$, cells were trypsinized and replated onto glass coverslips at low density for patch-clamp recording. We selected green cells under fluorescence for recording. Because HEK-293 cells often have small endogenous H$_2$V$_1$ currents (Müssel et al., 2011), cells that exhibited small currents suspected to be native were exposed to 1 µM Zn$^{2+}$, which has generally weaker effects on HtHV$_1$ (20% slowing of $\tau_{act}$, ~5 mV shift of the $g_{H-V}$ relationship, and a 24% decrease in $g_{H,max}$ in three to four cells at pH$_o$ 7) than on HtHV$_1$ (more than a twofold slowing of $\tau_{act}$, ~20 mV shift of the $g_{H-V}$ relationship; Müssel et al., 2010b). Cells determined on this basis to exhibit native currents were excluded from the study.

Micropipettes were pulled using a Flaming Brown automatic pipette puller (Sutter Instruments) from Custom 8520 Patch Glass (equivalent to Corning 7052 glass; Harvard Apparatus), coated with Sylgard 184 (Dow Corning Corp.), and heat polished. Microelectrodes were prepared from H. trivolvis brains that had been stored whole at −80% confluence in 35-mm culture dishes. HEK-293 cells were grown to ~80% confluence in 35-mm culture dishes. HEK-293 cells were transfected with 0.4–0.5 µg cDNA using Lipofectamine 2000 (Invitrogen) or polyethylenimine (Sigma).
Results
HtHV1 is a voltage-gated proton-selective channel
The gene coding for a putative voltage-gated proton channel was identified based on criteria established previously, namely the presence of four transmembrane helices homologous to S1–S4 of voltage sensor domains with an Asp in the middle of the S1 transmembrane helix and the RxWRxxR motif in S4 (Musset et al., 2011; Smith et al., 2011). We cloned the putative HtHV1 gene from a cDNA pool of brain tissue, verifying that this gene is expressed at the RNA level. Protein level expression was verified by Western blotting of H. trivolvis brain lysate probed with a commercially raised antibody to a synthetic peptide based on a HtHV1 epitope (Fig. 1, inset). The single protein detected ran at ~50 kDa, somewhat larger than the predicted size of 40 kDa. Glycosylation at the five putative N-glycosylation sites in the S1–S2 linker could account for this discrepancy, given that N-linked oligosaccharides range from 1,884 to 2,851 D (Imperiali and O’Connor, 1999). Excess synthetic peptide abolished the binding of antibody to brain lysate, establishing the specificity of the antibody. The antibody did not significantly bind to two proteins that do not contain the epitope: human glutathione S-transferase and luciferin binding protein from Lingulodinium polyedrum.

The HtHV1 channel protein (Fig. 1) is substantially larger than the human hHV1, with 360 amino acids (hHV1 has 273). Much of this excess resides in the S1–S2 extracellular linker with 73 residues (vs. eight in hHV1), which contains five potential N-glycosylation sites (vs. 0 in hHV1). Focusing on the transmembrane regions, HtHV1 has charged amino acids nearly identical to those of hHV1. One exception is at the outer end of the S1 helix, where hHV1 has basic Lys225 but snail HtHV1 has acidic Glu120. There is extensive predicted coiled-coil in the C terminus: 36 residues (positions 289–324) with 90% stringency, and 28 residues (294–321) with 99% stringency according to MARCOIL (Delorenzi and Speed, 2002). HtHV1 in several species have been shown to exist as dimers, largely because of coiled-coil interactions in the C terminus (Koch et al., 2008; Lee et al., 2008; Tombola et al., 2008).

The HtHV1 gene was transfected into HEK-293 cells. Under voltage clamp, transfected cells displayed depolarization-activated currents. The selectivity of these currents was established by measuring the reversal potential, V_reversal over a range of pH, and pH values (Fig. 2). The measured values of V_reversal are close to the Nernst potential for H⁺, E_H⁺, shown as a dashed line. Clearly, the HtHV1 channel is highly proton selective over the pH range studied.

HtHV1 gating is rapid with unusual voltage dependence
A family of currents generated by HtHV1 at symmetrical pH 6.0 is illustrated in Fig. 3 A. The currents activate rapidly with depolarization, and activation becomes much faster at higher voltages. Although HtHV1 currents in all species activate more rapidly at more positive voltages, the τ_open of HtHV1 currents (solid and open red squares in Fig. 3 C) exhibits noticeably steeper voltage dependence. The maximum slope of the τ_open-V relationship in seven cells at pH 6 was 13.0 ± 3.4 mV/e-fold change in τ_open (mean ± SD). In several mammalian H/V, τ_open changes e-fold in 40–72 mV (DeCoursey, 2003). Channel closing in HtHV1 was also steeply voltage dependent (Fig. 3 B and blue diamonds in Fig. 3 C), with τ_tail changing e-fold in 14.2 ± 1.9 mV in six cells. In mammalian cells, the slope is typically much flatter, 26–44 mV/e-fold change in τ_tail (DeCoursey, 2003).

A remarkable feature of HtHV1 is that at intermediate voltages where the measurements overlap, the time constants of H⁺ current turn-on (τ_open) and deactivation (τ_tail) essentially superimpose (Fig. 3 C). This behavior is suggestive of simple first-order kinetics, such as a two-state system:

\[
\text{Closed} \xrightarrow{\alpha} \text{Open} \]

(Scheme 1)

in which α is the rate of channel opening and β is the rate of channel closing, and the time constant t is \((\alpha + \beta)^{-1}\) (Hodgkin and Huxley, 1952). Another feature suggestive of first-order kinetics is evident in the gHV-V relationship from this cell (Fig. 3 D). The voltage at which the gHV is half-maximal is ~40 mV, where the time constants are maximal (Fig. 3 C). However, the limiting slope of the gHV-V relationship in Fig. 3 D, i.e., the slope of the most negative values obtained, indicates a gating charge of ~6 e⁻. The mean gating charge in 18 limiting slope measurements was 5.5 ± 0.9 e⁻ (mean ± SD). Because the range of gHV values resolved did not exceed three orders of magnitude, these gating charge estimates should be considered lower limits. In most species, cooperative gating of the dimeric H/V channel doubles the gating charge from 2–3 to 4–6 e⁻ (Gonzalez et al., 2010, 2013; Fujiwara et al., 2012).

Mean gating kinetics determined at symmetrical pH 7.0 is shown in Fig. 4 A. As was also seen at pH 6.0 (Fig. 3 C), at voltages where τ_open and τ_tail overlap, they have similar values, suggestive of first-order gating kinetics. In the first description of proton currents in snail neurons, the activation time to half-peak current was 25 ms or less at pH 7 (Byerly et al., 1984). With this in mind, the activation kinetics of HtHV1 is quite similar to that reported in neurons from L. stagnalis. When proton currents were first identified in mammalian species, they were found to be radically slower (DeCoursey, 1991; Bernheim et al., 1993; DeCoursey and Cherny, 1993; Demaurex et al., 1993; Kapus et al., 1993). The activation kinetics of HtHV1 is two to three orders of magnitude faster than that of hHV1 (Fig. 4 B), averaging 476 times faster between 50 and 90 mV at pH 7.

HtHV1 is sensitive to inhibition by external Zn²⁺ and Cd²⁺
The polyvalent metal cations Zn²⁺ and Cd²⁺ were among the first H/V inhibitors identified (Thomas and Meech, 1982; Mahaut-Smith, 1989b). Zn²⁺ in particular has been used widely on H/V identified in new species and remains the most potent inhibitor (Cherny and DeCoursey, 1999). Fig. 5 illustrates the effects of 100 μM Zn²⁺ or Cd²⁺ on HtHV1 currents. Three main effects are evident: the current amplitude is reduced, the current activates more slowly (scaled currents in Fig. 5 D), and the gHV-V relationship is shifted positively along the voltage axis. These three parameters are interrelated in that a positive shift of the...
The mean changes in these three parameters produced by 10 or 100 µM of the two metals are summarized in Fig. 5E. These three effects of polyvalent metal cations have been observed for HV1 from many species. As in rat HV1 (Cherny and DeCoursey, 1999), Zn²⁺ is more potent than Cd²⁺ in HtHV1. Focusing on the three main effects, HtHV1 was more sensitive, similar to, or less sensitive than human HV1 (hHV1). The reduction of gH,max is glaringly obvious for HtHV1, whereas in mammalian HV1 this effect is small and difficult to detect because of the interrelatedness of the three effects (Cherny and DeCoursey, 1999).

Zn²⁺ at 10 µM slows τact by four- to fivefold in both hHV1 (Musset et al., 2010b) and HtHV1 (Fig. 5). In contrast, the shift of the gH-V relationship by Zn²⁺ is far more profound in human hHV1, with a 20-mV shift produced by 1 µM Zn²⁺ (Musset et al., 2010b) compared with a 12-mV shift by 10 µM Zn²⁺ in HtHV1 (Fig. 5).

Unique ΔpH dependence of HtHV1 gating

Families of proton currents generated by the H. trivolvis proton channel gene product, HtHV1, in a cell studied at four pH values with pH 6 are illustrated in Fig. 6 (A–D). The currents activate with depolarization, and activation becomes much faster at higher voltages. Both voltage dependence and kinetics were

**Figure 1.** The HtHV1 proton channel sequence. Topology of the HtHV1 channel, with transmembrane regions defined by alignment with those determined for hHV1 by electron paramagnetic resonance (Li et al., 2015). Amino acids in transmembrane regions are color coded as follows: red, acids; blue, bases; brown, amines; purple, aromatics; green, hydroxyls; and orange stars, putative (extracellular) glycosylation sites. Noteworthy are D107 in S1, which presumably confers H⁺ selectivity, and the RxWRxxR motif in S4, both of which are conserved universally in all known HV1. Sequence in black is the epitope used to generate antibody. Inset shows Western blots of H. trivolvis brain, confirming the presence of HtHV1 protein. Drawn with TOPO2 (http://www.sacs.ucsf.edu/TOPO2/).
exquisitely sensitive to pHo. At higher pHo, the proton conductance, gH, turned on at more negative voltages and turned on much more rapidly (note the different time bases). Fig. 6 (E–H) shows deactivation kinetics at each pHo during tail current measurements in this cell. Channel closing becomes much more rapid at more negative voltages.

In Fig. 6 I, time constants of H+ current turn-on (activation, τact) and turn-off (deactivation, τtail) from the same cell are plotted. Several intriguing features emerge. Unlike HtHV1 in other species, at intermediate voltages where τact and τtail overlapped, they were of similar magnitude (as was seen in Figs. 3 and 4). Because this behavior suggests first-order kinetics (Scheme 1), the data in Fig. 6 I were analyzed in this way, and the expressions for each rate constant are given on the graph in the following form:

\[ \alpha(V) = \alpha e^{V/k_{\alpha}} \]  
\[ \beta(V) = \beta e^{-V/k_{\beta}}. \]

The voltage dependence of τact is steep (small k\( \alpha \)) and appears to become steeper at lower pHo. The voltage dependence of channel closing, τtail, is also steep (small k\( \beta \)) but appears to be independent of pHo. To a first approximation, β is independent of pHo, whereas α is markedly influenced by pHo. Mean values for the rate constants are plotted in Fig. 7. Confirming the impression from Fig. 6 I (where k\( \alpha \) was 11.4, 18, 26, and 30 mV at pHo 5, 6, 7, and 8), k\( \alpha \) increased with pHo, and k\( \beta \) was pH independent. But by far the strongest effect of pH is that increasing pHo massively increases the opening rate constant α, which increased more than an order of magnitude per unit increase in pHo. Stated differently, protonation at the external face of the HtHV1 channel strongly inhibits channel opening. More subtly, it is evident that for any given pHo, α is higher and β is lower at pH 6 than at pH 7; hence, lower pH both promotes opening and slows closing.

The gH–V relationships from the cell in Fig. 6 (A–I) are plotted in Fig. 6 J. Like other H\( \alpha \), HtHV1 exhibits robust pHo-dependent shifts with increasing pHo shifting the gH–V relationship negatively. The shifts for pHo 5 → 6 and 6 → 7 are closer to 50 than 40 mV, indicating that HtHV1 exceeds the rule of forty for changes in pHo. To reconstruct gH–V relationships using the simple first-order assumption (Scheme 1 and Eqs. 1 and 2), which predicts that Popen = α/(α + β), the solid curves in Fig. 6 J were drawn from the rate constant equations in Fig. 6 I scaled by gH,max. Their limiting slope at negative voltages is shallower than observed. Squaring the Popen–V relationships, as in the classic Hodgkin–Huxley \( n^2 \) approach (Hodgkin and Huxley, 1952), produces the steeper dashed curves, which better approximate the data. Without pushing the model too far, we conclude that it is probable that, like several other H\( \alpha \) (Gonzalez et al., 2010; Musset et al., 2010b; Tombola et al., 2010; Fujiwara et al., 2012), HtHV1 functions as a dimer in which both protomers must activate before either one conducts.

The effects of changes in pHi were explored in inside-out patches, as illustrated in Fig. 8. The resolution was limited somewhat by the typically small current amplitude combined with...
Figure 3. Strong voltage dependence of HtHv1 gating kinetics. (A) A family of proton currents at pH_e of 6 and pH_i of 6 in a HEK-293 cell transfected with HtHv1. Pulses were applied in 10-mV increments up to 100 mV from a holding potential, V_{hold} = −40 mV. (B) Tail currents in the same cell elicited by a prepulse to 80 mV, in 10-mV increments from −60 to 40 mV. (C) Gating kinetics in the same cell. The time constant of channel opening, τ_{act} (red squares) was obtained from single exponential fits to rising currents. Tail current (deactivation, channel closing) time constants, τ_{tail}, were also from single exponential fits. Open squares and dashed lines show τ_{act} from a second family. (D) The proton conductance was calculated from the extrapolated single exponential fits of currents and the measured V_{rev}.
rapid activation kinetics at some pH. Activation kinetics could be resolved at low but not at high pHi. For example, at pHi 8 (Fig. 8 C), inward current is clearly activated, but the kinetics is ambiguous. Nevertheless, it is evident in Fig. 8 D that activation kinetics depends only weakly on pHi, in stark contrast to the strong dependence seen for pHo (Figs. 6 and 7), and in contrast to mammalian H+1, in which lowering pHi speeds activation fivefold/unit (DeCoursey and Cherny, 1995; Villalba-Galea, 2014).

Deactivation kinetics was poorly resolved in most patches. The most surprising feature (Fig. 8 E) is that the heretofore universal rule of forty governing ΔpH-dependent gating is violated by HtH+1. Changing pHi shifts the gH-V relationship of HtH+1 by just 20 mV/unit or less. The aberrant behavior of HtH+1 provides clues to the mechanism of ΔpH-dependent gating.

Fig. 9 summarizes the ΔpH dependence of HtH+1. For a variety of reasons discussed elsewhere (Cherny et al., 2015), we
have adopted $V_{(g_{H,max}/10)}$, the voltage at which the $g_{H}$ is 10% of its maximal value, as a parameter to define the position of the $g_{H}$-$V$ relationship. We find this preferable to other parameters that have been used for this purpose, such as the midpoint of a Boltzmann curve (which frequently does not fit the data well or is ill determined) or the threshold voltage at which current is first detectable (which is arbitrary, depends on the signal-to-noise ratio, and is particularly difficult to resolve when it occurs near $V_{rev}$, as frequently occurs in HtH1). It is evident in Fig. 9 that when pH$_H$ < 7, changes in pH$_H$ shift $V_{(g_{H,max}/10)}$ by more than 40 mV/unit (for reference, this slope is shown as a dashed green line in Fig. 9). H$_{H}$1 in two other species (coccolithophore EhH$_{H}$1 and insect NpH$_{H}$1) also exhibit shifts with pH$_H$ greater than 40 mV/unit (Cherny et al., 2015; Chaves et al., 2016). At pH$_H$ higher than 7, the shift decreases, which may reflect saturation of the response caused by the ambient pH approaching the pK$_a$ of a critical titratable group. Saturation of pH dependence has been observed previously in hH$_{H}$1 at pH > 8 (Cherny et al., 2015).

The most striking result in Fig. 9 is the data for changes in pH$_I$ (dark red diamonds), which reveal that the position of the...
Figure 6. **Exquisite sensitivity of HtHV1 gating kinetics to pHo.** (A–D) Families of proton currents at pHo 5, 6, 7, and 8 with pH 6 in a HEK cell transfected with HtHV1. Pulses were applied in 10-mV increments from a holding potential, Vhold = −40 mV (A and B), −60 mV (C), or −90 mV (D) up to the voltage indicated. Note the different time calibrations. (E–H) Tail current measurements in the same cell at each pHo. Tail currents were recorded in 10-mV increments over the voltage range indicated. Vhold is the same as in the top row, and the prepulse voltage is indicated. Current calibration in F also applies to G and H. (I) Dependence of gating kinetics on pHo. At each pHo value, the solid symbols show τact from single exponential fits of currents during activation, and the half-filled symbols or inverted triangles show deactivation kinetics (τtail) during tail currents. Note that τact and τtail overlap, consistent with simple first-order kinetics, and both have steep voltage dependence. The curves are drawn from the rate constant equations given on the graph in I, where τ = 1/(α + β). (J) gH−V relationships measured in the same cell. Solid curves show Popen−V relationships calculated from the rate constants defined by the equations in I (Popen = α/(α + β), scaled by gH,max). The dashed curves show the prediction of a Hodgkin–Huxley n2 model (Popen = [α/(α + β)]2, scaled by gH,max).
The $g_{51}$-$V$ relationship depends only weakly on $pH_i$. There is no clear indication of saturation, although the slope appears to increase at larger $\Delta pH$ (i.e., lower $pH_i$). This is qualitatively like the whole-cell $pH_o$ response, which is steepest at low $pH_o$ and saturates at high $pH_o$. Over the entire $\Delta pH$ range, the mean slope is only 15.3 mV/unit change in $pH_i$. $H_{tHV1}$ is the first $H_{V1}$ in which such weak $\Delta pH$ dependence has been identified.

**Discussion**

The rapid kinetics of $H_{tHV1}$ resembles that of other snail proton channels but differs from mammalian $H_{V1}$

The snail $H_{v1}$, $H_{tHV1}$, exhibits all of the major features of $H_{V1}$ in all species studied thus far. It is highly proton selective and it is voltage gated, opening with depolarization, and opening more rapidly at more positive voltages. Furthermore, its voltage dependence is strongly modulated by $pH$, such that increasing $pH_o$ or decreasing $pH_i$ shifts the $g_{51}$-$V$ relationship negatively, in what has been called $\Delta pH$-dependent gating (Cherny et al., 1995). Beyond these qualitative similarities, however, $H_{tHV1}$ differs markedly from $H_{V1}$ in humans and other mammalian species. The main differences include very rapid activation kinetics, steeply voltage-dependent activation kinetics, activation in a more negative voltage range, exponential rather than sigmoid activation, and distinctly aberrant $\Delta pH$ dependence. These properties are discussed below.

The first voltage-gated proton channels to be characterized by voltage clamp were in neurons from the snails $L. stagnalis$ (Byerly et al., 1984), $H. aspersa$ (Thomas and Meech, 1982; Mahaut-Smith, 1989b), and $Helix pomatia$ (Doroshenko et al., 1986). All activated rapidly, with time constants, $\tau_{act}$, of a few milliseconds. When mammalian proton currents were identified, the most obvious difference was much slower activation, with $\tau_{act}$ in the range of seconds (DeCoursey, 1991; Bernheim et al., 1993; Demaurex et al., 1993; Kapus et al., 1993) or even minutes (DeCoursey and Cherny, 1993). A more subtle difference was that mammalian $H_{V1}$ activate with a distinct delay, whereas snail $H_{V1}$ activate exponentially. We show here that the $H_{tHV1}$ channel shares both properties with other snail $H_{V1}$. Byerly et al. (1984) reported half-times for activation of less than 25 ms for proton currents in $L. stagnalis$ neurons at $pH_o 7.4$, as observed here at $pH_o 7$ (Fig. 4 A).

Paradoxically, some aspects of gating in $H_{tHV1}$ suggest a simple first-order transition between closed and open states

Activation and deactivation time constants in $H_{tHV1}$ are of similar magnitude at voltages where they overlap. This property is typical of a simple first-order system (Scheme 1). In mammalian $H_{v1}$ (DeCoursey, 1991; Cherny et al., 1995, 2001; DeCoursey and Cherny, 1996, 1997; Cherny and DeCoursey, 1999; Schilling et al., 2002), activation tends to be slower than deactivation. This asymmetrical behavior is typical of cooperatively gated multimeric channels (Hodgkin and Huxley, 1952; Hille, 2001), because all subunits must activate before the channel conducts, whereas only one subunit needs to deactivate to close the pore. In rat $H_{v1}$, deactivation was rapid, $pH_o$ independent, and weakly voltage dependent at large negative voltages (Cherny et al., 1995). However, near the threshold voltage for $g_{51}$ activation, a second slower component of $\tau_{tail}$ appeared that was $pH_o$ dependent and of comparable magnitude to $\tau_{act}$. Also suggestive of a first-order system in $H_{tHV1}$ is that $\tau_{act}$ and $\tau_{tail}$ were slowest at the midpoint of the $pH$ range.
Figure 8. Gating of proton currents in HtHV1 in an inside-out patch at several pHi values. (A–C) Current families at pH 5, 6, and 8 in a patch with pHo 7. Pulses were applied in 10-mV increments up to the voltage indicated, from \(V_{\text{hold}} = -90\) mV (A) or -70 mV (B and C). (D) Activation kinetics appears to be nearly independent of pHi. (E) The dependence of the \(g_{\text{H}}-V\) relationship on pHi is weaker than in all other known H V1. Currents were determined by extrapolation of single exponential fits for rising currents, or from steady-state currents when kinetics was inscrutable (e.g., at pH 8).
The $g_{\text{H}}$--$V$ relationship (Figs. 3 and 6). Finally, activation kinetics was well described by a single exponential and could not be fitted reasonably with a higher-order function.

There is general agreement that the $H_V^1$ dimer in several species gates “cooperatively,” but it is less clear what this word means; in drug binding, cooperativity can be produced by quite different mechanisms (Colquhoun, 1973). One sense is that, like the Hodgkin–Huxley model, multiple subunits must move before the channel can conduct. Another sense is that, like oxygen binding to the four hemes of hemoglobin, the movement of one $H_V^1$ protomer promotes the movement of the other. The sigmoid activation kinetics of $H_V^1$ in several species (Gonzalez et al., 2010; Musset et al., 2010b; Tombola et al., 2010; Fujiwara et al., 2012) appears to reflect that both protomers must undergo a conformational change before either can conduct (first sense; Gonzalez et al., 2010) or highly cooperative gating (second sense; Tombola et al., 2010). When $H_V^1$ is forced to exist as a monomer, by splicing it with the N terminus of Ciona intestinalis voltage-sensing phosphatase or by truncating the C terminus, the current turns on exponentially and five to seven times faster than with the WT dimeric protein (Koch et al., 2008; Musset et al., 2010a,b; Tombola et al., 2010; Fujiwara et al., 2012) appears to reflect that both protomers must undergo a conformational change before either can conduct (first sense; Gonzalez et al., 2010) or highly cooperative gating (second sense; Tombola et al., 2010). When $H_V^1$ is forced to exist as a monomer, by splicing it with the N terminus of Ciona intestinalis voltage-sensing phosphatase or by truncating the C terminus, the current turns on exponentially and five to seven times faster than with the WT dimeric protein (Koch et al., 2008; Musset et al., 2010a,b; Tombola et al., 2010; Fujiwara et al., 2012). The dimerization of $H_V^1$ in several species appears strongly dependent on coiled-coil interactions in the C terminus (Koch et al., 2008; Lee et al., 2008; Tombola et al., 2008; Li et al., 2010). $H_T^1$ has extensive predicted coiled-coil in its C-terminal region. This complicates the interpretation for $H_T^1$, because the exponential activation and the apparently first-order kinetics suggest monomeric behavior. One explanation might be that $H_T^1$ exists in the membrane as a dimer because of the coiled-coil region, but the coupling between C terminus and S4 segment is dysfunctional, as can be achieved experimentally by introducing a flexible linker between S4 and the C terminus (Fujiwara et al., 2012). However, this appears unlikely to be the case, because the apparent gating charge of $H_T^1$ is nearly 6 $e_0$ ($5.5 \pm 0.9$, mean $\pm$ SD in a sample of 18 $g_{\text{H}}$--$V$ curves), based on the limiting slope of the $g_{\text{H}}$--$V$ relationship. Monomeric $H_V^1$ typically exhibit gating charge roughly half that of the dimer, 2–3 versus 4–6 $e_0$. When the coupling between the C terminus and the S4 helix was disrupted by a flexible linker, the gating charge was halved (Fujiwara et al., 2012). Given that $H_T^1$ has charged amino acids in its transmembrane regions similar to those of other $H_V^1$, we assume that its gating charge has analogous origins. The $g_{\text{H}}$--$V$ relationships in Fig. 6 also are compatible with Hodgkin–Huxley-type gating. One possibility is that a concerted rate-limiting step in opening occurs late, presumably after the conformational changes in each monomer (Gonzalez et al., 2010; Musset et al., 2010b; Villalba-Galea, 2014). The voltage-dependent movement of monomers may be so rapid in $H_T^1$ that the concerted opening step becomes rate limiting. Another speculative explanation for its exponential activation is that $H_T^1$ enjoys tighter coupling between protomers than $H_V^1$ in other species; in essence, both S4 helices move together.

**The gating of $H_T^1$ depends steeply on voltage**

Perceptibly different from mammalian $H_V^1$, the activation kinetics of snail $H_V^1$, $H_T^1$, is more steeply voltage dependent, giving
a family of currents a distinctive gestalt (Fig. 3A). In HtH1, $\tau_{act}$ decreased e-fold in 13.8 mV, in contrast to several mammalian H1, where $\tau_{act}$ changes e-fold in 40–72 mV (DeCoursey, 2003). In addition, $\tau_{act}$ increased e-fold in 14.0 mV in HtH1, compared with a slope typically 26–44 mV/e-fold change in $\tau_{act}$ in mammalian cells (DeCoursey, 2003). The steeply voltage-dependent gating kinetics of HtH1 is strikingly reminiscent of voltage-gated $K^+$ channel behavior (Cahalan et al., 1985).

Gating kinetics in HtH1 is strongly dependent on pH

Byerly et al. (1984) noted that activation kinetics in snail LsH1 slowed at lower pH, more than could be accounted for by the shift of the $g_{H^{-}}$-V relationship. This is clearly true of HtH1 as well. The $\tau_{act}$-V relationship shifts positively with lower pHo (Fig. 61), but its maximum increases by roughly an order of magnitude per unit decrease in pHo. In stark contrast, in rat H1, the $\tau_{act}$-V relationship mainly shifted along the voltage axis with changes in pHo, with little change in kinetics. However, the $\tau_{act}$-V relationship in rat was strongly affected by pHi, slowing fivefold per unit increase in pHi (DeCoursey and Cherny, 1995). In one study of human hH1, gating was described by three exponentials with activation generally faster at lower pH, and deactivation faster at higher pHi (Villalba-Galea, 2014). Qualitatively similar results were reported in mouse macrophages, but the largest change was a two- to threefold slowing of $\tau_{act}$ for a 1.5-unit increase in pHi, or a 2.1-unit decrease in pHo (Kapus et al., 1993). An insect H1, NpH1, however, exhibited nearly as strong pHo dependence as found here in HtH1 (Chaves et al., 2016). In contrast to the strong dependence of activation kinetics on pHi in rat (DeCoursey and Cherny, 1995), in HtH1, $\tau_{act}$ was in a similar range at all pHi values from 5 to 8. These differences in gating kinetics among species may make it challenging to produce a single universal model that describes the voltage and pH dependence of gating in all species.

Metal binding site in HtH1

HtH1 was moderately sensitive to inhibition by Zn$^{2+}$, the classic (Thomas and Meech, 1982; Mahaut-Smith, 1989a) and still most potent (Cherny and DeCoursey, 1999) H1 inhibitor. Somewhat weaker effects were observed for Cd$^{2+}$ (Fig. 5). The principal effects of Zn$^{2+}$ on mammalian H1 are a slowing of activation, a positive shift of the $g_{H^{-}}$-V relationship, and possibly a reduction of the maximum H$^+$ conductance, $g_{H_{\text{max}}}$ (Cherny and DeCoursey, 1999). These effects are also observed in HtH1, but the decrease in $g_{H_{\text{max}}}$ is much more obvious, whereas the shift of the $g_{H^{-}}$-V relationship is substantially weaker in HtH1 than in hH1. Thus, the $g_{H^{-}}$-V relationship in human hH1 is shifted more by 1 mM Zn$^{2+}$ (Musset et al., 2010b) than HtH1 is shifted by 10 mM Zn$^{2+}$ (Fig. 5).

In mammalian H1, Zn$^{2+}$ binds mainly to two His: His$^{140}$ and His$^{195}$ in hH1 (Ramsey et al., 2006; Musset et al., 2010b). Surprisingly, when mH1 was crystallized, it contained a Zn$^{2+}$ atom, coordinated by the corresponding two His with contributions from two acids, Glu$^{115}$ and Asp$^{119}$, given in Table 1 (Takeshita et al., 2014). Mutation of both acids simultaneously decreases Zn$^{2+}$ affinity of mH1, but neutralizing either alone does not (Takeshita et al., 2014). As indicated in Table 1, three of these four corresponding residues are conserved in HtH1: Glu$^{114}$, Glu$^{118}$ (conservatively replacing Asp), and His$^{201}$, with Val$^{254}$ replacing the second His. Consistent with the partial conservation of the mammalian Zn$^{2+}$ site, Zn$^{2+}$ was generally less potent in HtH1 but still quite effective. The main difference in the presumed Zn$^{2+}$ binding residues in HtH1 is the lack of His$^{193}$. One might therefore speculate that His$^{193}$ in human hH1 is important in Zn$^{2+}$ shifting the $g_{H^{-}}$-V relationship positively. Evidently, when the binding site includes His$^{193}$ located in the external S2–S3 linker, Zn$^{2+}$ binding biases the membrane potential more effectively. Coordination by four amino acids is more typical of a structural Zn$^{2+}$ binding site, whereas catalytic Zn$^{2+}$ binding sites usually have three amino acids and one water as a ligand (Auld, 2001). Of interest is a study showing that the metal transport site of ZnT transporters is selective for Zn$^{2+}$ over Cd$^{2+}$ when the four ligands are 2 His + 2 acids, but cannot discriminate the two metals with 1 His + 3 acids (Hoch et al., 2012). The HtH1 channel has 1 His + 2 acids and is moderately selective for Zn$^{2+}$ over Cd$^{2+}$. As shown in Table 1, the NpH1, CIH1, Cph1, Sph1, and DrH1 channels share a 1 His + 3 acids scheme and are much less sensitive to Zn$^{2+}$ than mammalian H1 ($Cd^{2+}$ was not tested) and generally less sensitive than HtH1. Intriguingly, the D145H mutation in NpH1 results in 2 His + 2 acids, which markedly increases its Zn$^{2+}$ sensitivity (Chaves et al., 2018). Empirically, Table 1 indicates that the configurations of H1 for Zn$^{2+}$ binding to H1 in order of decreasing efficacy are: 2 His + 2 acids > 1 His + 2 acids > 1 His + 3 acids. It appears that the 1 His + 3 acids motif is somewhat less favorable for Zn$^{2+}$ binding than 1 His + 2 acids as found in HtH1, which seems paradoxical, because the 1 His + 3 acids motif has four ligands instead of three, possibly plus water. Perhaps geometrical factors can be more important than the number of ligands.

The $g_{H^{-}}$-V relationship of HtH1 depends more on pH and less on pHi than H1 in other species

A unique property of H1 is that its voltage-dependent gating is strongly modulated by pH in a manner called $\Delta$pH dependence (Cherny et al., 1995). The $g_{H^{-}}$-V relationship is shifted equally by increasing pH or decreasing pH, by −40 mV/unit change, thus responding to the pH gradient ($\Delta$pH) rather than to the absolute pH (Cherny et al., 1995). The practical consequence is that H1 opens only when the electrochemical gradient for H$^+$ is outward, such that when the channel opens it will always extrude acid from the cell (Doroshenko et al., 1986; DeCoursey and Cherny, 1994). To a rough approximation, all H1 appear to shift by 40 mV/unit at all pH values (DeCoursey, 2003). Until recently, the rare exceptions to this rule of forty were ignored as anomalies, perhaps reflecting difficulties of the measurements, in particular with control over pH (DeCoursey and Cherny, 1997). However, measurements explicitly addressing this point revealed that the $\Delta$pH-dependent gating of hH1, kH1, and EhH1 does indeed deviate by saturating at high pH, namely above pH 8 or pH 8 (Cherny et al., 2015). Byerly et al. (1984) reported little shift between pH 7.4 and 8.4 in L. stagnalis, and this observation is consistent with the saturation at high pH observed here for HtH1 (Fig. 9). The slope in HtH1 begins to decrease above pH 7 (Fig. 9), suggesting that saturation begins at lower pH, than in
hHV1. Saturation of ΔpH-dependent gating suggests that pH is approaching the effective pKᵣ of one or more titratable groups that sense pHₒ. Given this interpretation, the effective pKᵣ is roughly 1 unit lower in HtHV1 than in hHV1.

Another deviation from the rule of forty is that the gᵥ₋V relationship in HtHV1 shifted ~60 mV/unit change in pHₑ between pHₑ 5 and 7 (Fig. 9), well above the classic value of 40 mV/unit change in pHₑ. (Cherny et al., 1995). This unusual property is shared by several disparate species, including other snails. In H. pomatia, the shift was 63 mV from pHₑ 7.5 to 6.6 (Doroshenko et al., 1986). In L. stagnalis, the shift was 46 mV from pHₑ 7.4 to 6.4 (Byerly et al., 1984). Changes in pHₑ in a coccolithophore EhHV1 produced shifts of ~50 mV/unit (Cherevko et al., 2015). An insect Hᵥ₁ (NpHV1) shifts 54 mV/unit change in pHₑ (Chaves et al., 2016).

More dramatically, changes in pHᵥ₁ produced much smaller shifts of the gᵥ₋V relationship in HtHV1 than the 40 mV in mammalian species (Cherny et al., 1995). The mean shift in HtHV1 between pHₑ 5 and 9 was only 15.3 mV/unit (Fig. 9). This is in remarkable agreement with the 15 mV/unit reported in the snail LsHV1 between pHₑ 5.9 and 7.3 (Byerly et al., 1984). Meech (2012) recently emphasized the stronger effects of pHₑ over pHᵥ₁ after reanalyzing old data. However, in another snail, H. pomatia, HpHV₁ apparently shifted normally, roughly 30–50 mV/unit change in pHₑ (Doroshenko et al., 1986), so on this point it is not possible to generalize about molluscan Hᵥ₁.

The ΔpH dependence of mammalian Hᵥ₁ results in only outward H⁺ currents under most circumstances, which is crucial to many if not all of its functions (DeCoursey, 2003). One striking consequence of the anomalous ΔpH dependence of HtHV1 and perhaps of other snail Hᵥ₁ is that inward currents are readily observed at certain ΔpH. Even at symmetrical pH, there are often inward currents. More conspicuously, because of the weak dependence on pHᵥ₁, an inward pH gradient (ΔpH < 0) produces inward currents over an extensive voltage range (e.g., Fig. 8C). Inward currents would affect neuronal excitability by providing a depolarizing current. They at first appear incompatible with an early proposal that proton currents in snail neurons function to extrude protons that enter via Ca²⁺/H⁺ exchange after each Ca²⁺-mediated action potential (Ahmed and Connor, 1980; Thomas and Meech, 1982; Byerly et al., 1984), but under normal conditions of an outward H⁺ gradient, inward currents would likely not be activated. Nevertheless, the possibility arises that Hᵥ₁ might mediate action potentials in molluscan neurons under certain conditions, although Ca²⁺ channels are thought to be primarily responsible (Hagiwara and Byerly, 1981). Hᵥ₁ appears to mediate action potentials in bioluminescent dinoflagellates (Fogel and Hastings, 1972; Smith et al., 2011; Rodriguez et al., 2017).

As L. stagnalis and H. trivolvis snails live in similar habitats, we expect that their proton channels should function similarly. This view is supported by the similarity of the LsHV₁ sequence to that of HtHV₁, especially in the S2/S3 region that we have identified for its importance in pHᵥ₁ sensing in the accompanying paper (Cherny et al., 2018). In that paper, we identify a single amino acid difference between hHV₁ and HtHV₁ that appears largely responsible for the difference in pHᵥ₁ sensing.
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