Tryptophan 207 is Crucial to the Unique Properties of the Human Voltage-gated Proton Channel, hHV1

Vladimir V. Cherny  
*Rush University*

Deri Morgan  
*Rush University*

Boris Musset  
*Institute of Complex Systems 4 Zelluläre Biophysik*

Susan M. E. Smith  
*Kennesaw State University*, ssmit325@kennesaw.edu

Follow this and additional works at: [https://digitalcommons.kennesaw.edu/facpubs](https://digitalcommons.kennesaw.edu/facpubs)

Part of the [Molecular Biology Commons](https://digitalcommons.kennesaw.edu/facpubs)

**Recommended Citation**
Cherny, Vladimir V.; Morgan, Deri; Musset, Boris; and Smith, Susan M. E., "Tryptophan 207 is Crucial to the Unique Properties of the Human Voltage-gated Proton Channel, hHV1" (2015). *Faculty Publications*. 3567.  
[https://digitalcommons.kennesaw.edu/facpubs/3567](https://digitalcommons.kennesaw.edu/facpubs/3567)

This Article is brought to you for free and open access by DigitalCommons@Kennesaw State University. It has been accepted for inclusion in Faculty Publications by an authorized administrator of DigitalCommons@Kennesaw State University. For more information, please contact [digitalcommons@kennesaw.edu](mailto:digitalcommons@kennesaw.edu).
Tryptophan 207 is crucial to the unique properties of the human voltage-gated proton channel, hHV1

Vladimir V. Cherny, 1 Deri Morgan, 1 Boris Musset, 2 Gustavo Chaves, 2 Susan M.E. Smith, 3 and Thomas E. DeCoursey1

1Department of Molecular Biophysics and Physiology, Rush University, Chicago, IL 60612
2Institute of Complex Systems 4 Zelluläre Biophysik, Forschungszentrum Jülich, 52425 Jülich, Germany
3Department of Molecular and Cellular Biology, Kennesaw State University, Kennesaw, GA 30144

Part of the “signature sequence” that defines the voltage-gated proton channel (H1) is a tryptophan residue adjacent to the second Arg in the S4 transmembrane helix: RxWRxxR, which is perfectly conserved in all high confidence H1 genes. Replacing Trp207 in human H1 (hH1) with Ala, Ser, or Phe facilitated gating, accelerating channel opening by >100-fold, and closing by 30-fold. Mutant channels opened at more negative voltages than wild-type (WT) channels, indicating that in WT channels, Trp favors a closed state. The Arrhenius activation energy, $E_a$, for channel opening decreased to 22 kcal/mol from 30–38 kcal/mol for WT, confirming that Trp207 establishes the major energy barrier between closed and open hH1. Cation–π interaction between Trp207 and Arg211 evidently latches the channel closed. Trp207 mutants lost proton selectivity at pH o > 8.0. Finally, gating that depends on the transmembrane pH gradient (ΔpH-dependent gating), a universal feature of H1 that is essential to its biological functions, was compromised. In the WT hH1, ΔpH-dependent gating is shown to saturate above pH o or pH i, 8, consistent with a single pH sensor with alternating access to internal and external solutions. However, saturation occurred independently of ΔpH, indicating the existence of distinct internal and external pH sensors. In Trp207 mutants, ΔpH-dependent gating saturated at lower pH o, but not at lower pH i. That Trp207 mutation selectively alters pH i, sensing further supports the existence of distinct internal and external pH sensors. Analogous mutations in H1 from the unicellular species Karodinium veneficum and Emiliania huxleyi produced generally similar consequences. Saturation of ΔpH-dependent gating occurred at the same pH i or pH o in H1 of all three species, suggesting that the same or similar group(s) is involved in pH sensing. Therefore, Trp enables four characteristic properties: slow channel opening, highly temperature-dependent gating kinetics, proton selectivity, and ΔpH-dependent gating.

INTRODUCTION

Voltage-gated proton channels (H1) exist in diverse organisms ranging from unicellular marine species (Smith et al., 2011; Taylor et al., 2011) to humans (Ramsey et al., 2006). Their functions are equally diverse: conversion of CO2 to calcite in coccolithophores (Taylor et al., 2011), triggering the bioluminescent flash in dinoflagellates (Smith et al., 2011), and in humans participating in innate immunity (DeCoursey, 2010), B cell signaling (Capasso et al., 2010), airway acid secretion (Iovannisci et al., 2010), histamine secretion (Musset et al., 2008b), sperm motility (Musset et al., 2012) and capacitation (Lishko et al., 2010), brain damage in ischemic stroke (Wu et al., 2012), breast cancer (Wang et al., 2012), and chronic lymphocytic leukemia (Hondares et al., 2014). All known and suspected H1 to date, even in species with just 15–18% sequence identity to the human H1 (hH1), share a perfectly conserved tryptophan (Trp207 in hH1) adjacent to the second of three Arg residues in the S4 transmembrane segment (Fig S1) (DeCoursey, 2013). This Trp is part of the proposed signature sequence of the proton channel RxWRxxR (Smith et al., 2011), and is present even in several unconfirmed H1-like sequences in fungi in which the third Arg in S4 is replaced by Lys (e.g., Fusarium oxysporum, Ophiostoma piceae, and Metarhizium anisopliae). Among molecules that contain voltage-sensing domains (VSDs), only H1 and c15orf27 (whose function is unknown) contain Trp in this location (Smith et al., 2011). Here, we ask why this Trp has been conserved. We find that replacing Trp modifies four characteristic properties of hH1, revealing that it is central to the unique defining properties and functions of H1. Trp mutants opened and closed 30–100 times faster than WT, with gating kinetics less profoundly sensitive to temperature; they lost proton selectivity at high pH o; and the unique ΔpH dependence of gating was compromised. The striking

© 2015 Cherny et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.rupress.org/terms). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/).
diversity of the effects of Trp mutation indicates that this residue plays a pivotal role in the H1,1 protein.

Tryptophan is the rarest amino acid in proteins, and in membrane proteins, it is often found close to lipid head groups, preferring the interfacial environment (Killian and von Heijne, 2000; MacCallum et al., 2008). Thus, the absolute conservation of a Trp in the middle of the S4 transmembrane segment of H1,1 requires some explanation. Three other Trp residues in hH1,1 are all in the intracellular N terminus. Perhaps because both Trp and Arg residues share ambivalence by exhibiting hydrophobic mixed with polar characteristics, they interact strongly with each other (Santiveri and Jiménez, 2010). In β-hairpin peptides and in other proteins, the guanidinium group of Arg stacks against the aromatic ring of Trp via cation–π (Gallivan and Dougherty, 1999) and van der Waals interactions, stabilizing the protein structure (Tatko and Waters, 2003; Santiveri and Jiménez, 2010). The proximity of Trp and R3 (the third Arg in the S4 segment, Arg207 in mouse) in the closed structure of the mouse H1,1 (mH1,1; Takeshita et al., 2014) appears to be consistent with this type of interaction, with amino-aromatic distances <6.0 Å (Burley and Petsko, 1986). In the structure identified as a closed conformation of mH1,1 (Takeshita et al., 2014), the indole side chain of Trp205 is directed away from the pore toward the interior of the lipid bilayer, pointing downward, partially shielding the R3 side chain, which is directed down and between S4 and S3 but also has some lipid exposure. In all open-state models (Ramsey et al., 2010; Wood et al., 2012; Kulleperuma et al., 2013; Chamberlin et al., 2014), all three Arg residues of the S4 segment face the pore, but Trp still faces away from the pore. We propose that Trp stabilizes the closed hH1,1 through cation–π interaction with Arg211, and that loss of this stabilization contributes to the consequences of its mutation. A striking result was that whether Trp was replaced by Ala (hydrophobic), Ser (hydrophilic), or Phe (aromatic), H1,1 properties were changed by a quantitatively indistinguishable extent. This result suggests that the heterocyclic aromatic side chain of Trp uniquely anchors the S4 segment in the membrane.

MATERIALS AND METHODS

Gene expression

Site-directed mutants were created using the QuickChange (Agilent Technologies) procedure according to the manufacturer’s instructions. Transfection was done as described previously (Kulleperuma et al., 2013). Both HEK-293 cells and COS-7 cells were used as expression systems. We showed previously that the properties of H1,1 expressed in both cell lines were indistinguishable (Muset al., 2008a). No other voltage- or time-dependent conductances were observed under the conditions of this study. Although most mutations on hH1,1 were introduced into a Zn²⁺-insensitive background (H140A/H199A), which we have done previously as a control for endogenous H1,1 (Muset al., 2011), the level of expression of all mutants studied here was sufficiently high that contamination by native H1,1 was negligible.

Electrophysiology

In most experiments, cells expressing green fluorescent protein (GFP)-tagged proton channels were identified using inverted microscopes (Nikon) with fluorescence capability. For constructs that lacked the GFP tag, GFP was cotransfected. Conventional patch-clamp techniques were used (Kulleperuma et al., 2013) at room temperature (20–26°C). Bath and pipette solutions contained 60–100 mM buffer, 1–2 mM CaCl₂ or MgCl₂ (intracellular solutions were Ca²⁺ free), 1–2 mM EGTA, and TMAMeSO₄ to adjust the osmolality to ~300 mOsm, titrated with TMAOH. Buffers used were Homo-PIPES (Research Organics) at pH 4.5–5.0, Mes at pH 5.5–6.0, BisTris at pH 6.5, PIPES at pH 7.0, HEPES at pH 7.5, tricine at pH 8.0, CHES at pH 9.0, and CAPS at pH 10.0. Currents are shown without leak correction. To minimize pH changes caused by large H⁺ fluxes, pulses for large depolarizations in pulse families were sometimes shortened.

The reversal potential (Vrev) was determined by two methods, depending on the relative positions of Vrev and the threshold voltage for activation of the ge, Vthreshold. For constructs in which Vthreshold was positive to Vrev, the latter was determined by examining tail currents. Because hH1,1 currents were the only time-dependent conductance present, Vrev was established by the amplitude and direction of current decay during deactivation. By using this procedure, time-independent leak or other extraneous conductances do not affect Vrev (Morgan and DeCoursey, 2014). Tail currents were not observed in nontransfected cells. For mutants in which Vthreshold was negative to Vrev, it was possible to observe directly the reversal of currents activated during pulse families.

Proton current amplitude (Ige) was usually determined by fitting the rising current with a single exponential and extrapolating to infinite time. Proton conductance (gH) was calculated from Ige and Vrev measured in each solution: gH = Ige/(Vrev − Vrev). In some cases where current activation kinetics was difficult to evaluate, gH was calculated from tail current amplitudes instead of Ige.

To evaluate ΔpH dependence, it is necessary to establish the position of the gH–V relationship. For this purpose, we have adopted the voltage at which the gH is 10% of its maximal value as a function of pH (VgH,max/10), in preference to other parameters that have been used, such as the midpoint of a Boltzmann distribution or the threshold for activating detectable H⁺ current, Vthreshold. Because the gH–V relationship is steepest at low voltages, fairly large errors in estimating the maximum gH (VgH,max) produce only small errors in VgH,max/10. This parameter choice avoids the necessity of arbitrarily forcing nonsigmoidal gH–V data to fit a Boltzmann function (Muset al., 2008a) or, alternatively, the need to identify the elusive threshold of channel activation, Vthreshold, which is subjective and can be difficult when it occurs near Eγ. Nevertheless, Vthreshold remains useful as a supplemental parameter because its measurement requires minimal current flow and consequently produces negligible pH changes.

Online supplemental material

Fig. S1 shows the sequence of the S4 segment in hH1,1, kH1,1, and EhH1,1, illustrating the conserved Trp in the signature sequence that defines H1,1, RxWRxxR. Fig. S2 shows saturation of the ΔpH dependence of WT kH1,1 (from the dinoflagellate Karlodinium venecicum) and of the W176F mutant of kH,1. Fig. S3 shows the saturation of the ΔpH dependence of WT EhH1,1 (from the cocolithophore Emiliania huxleyi) and of W278X mutants of EhH1,1. The online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.201511456/DC1.
RESULTS

The ΔpH dependence of gating in hH1 saturates above pH 8

Perhaps the most remarkable property of H1 is the phenomenon of ΔpH-dependent gating. We define ΔpH, the transmembrane pH gradient, as pHo − pHi. Like other voltage-gated ion channels, H1 opens upon depolarization, but the position of the gH–V relationship is strongly and equally modulated by both pHo and pHi, shifting 40 mV for a unit change in either (Cherny et al., 1995). The set point of this relationship is positioned so that the human channel under normal conditions opens only when the electrochemical gradient is outwards. The practical consequence is that channel opening extrudes acid from the cell, which is essential to most of the functions of H1.

Fig. 1 illustrates ΔpH-dependent gating of the WT hH1. Families of currents recorded in the same cell at four pHo, with pHi 7 are shown in Fig. 1 (A–D). Channel opening is characteristically slow, especially at lower pHo. Examination of the corresponding gH–V (proton conductance–voltage) relationships derived from these currents reveals a −40-mV/U pH shift as pHo increases (Fig. 1 E). However, the shift between pHo 8 and pHo 9 is decidedly less than −40 mV. Saturation of the shift of the gH–V relationship has not previously been identified in WT H1 at either high or low pHo. A <40-mV shift between pHi 8 and 9 was noted previously and proposed to reflect the approach of pHo to the pK of a site that senses pHo (Cherny et al., 1995). This interpretation was later questioned when Vrev was found to deviate substantially from Ei at extreme pHo values (pHo 9–10), and speculatively reinterpreted as reflecting loss of pHi control at high pH, perhaps because of OH− transport in rat alveolar epithelial cells (DeCoursey and Cherny, 1997). In the present study, we will show that the attenuation of ΔpH-dependent gating constitutes genuine saturation, because it occurs at a pH where the channel is unequivocally proton selective and pHi is well established (Fig. 4).

Fig. 1 (G–J) shows WT hH1 currents at several pHi measured in an inside-out patch of membrane, a configuration that allows changing pHi. Again, activation (channel opening) is slow, increasingly so at high pHi, where the currents become smaller, presumably reflecting the rarity of permeant ions at pH 9. Corresponding gH–V relationships plotted in Fig. 1 F exhibit a 40-mV/U shift as pH increases, but also reveal that the shift appears to saturate between pHi 8 and pHi 9. Our
Mutations to Trp\(^{207}\) in hH\(_V\) compromise \(\Delta p\)H-dependent gating

We replaced the bulky Trp\(^{207}\) in the human proton channel, hH\(_V\), with Ala, Phe, or Ser, designating the mutants W207A, W207F, and W207S, respectively. All mutants generated similar voltage- and time-dependent currents. Because we could not distinguish among the properties of these three mutants, their data are combined in data summaries and termed “W207X.” Families of currents recorded in the W207A mutant at four pH\(_i\), in Fig. 2 (A–D) reveal several noteworthy differences from WT hH\(_V\). Most prominently, channel opening was two orders of magnitude faster, as will be described below (Fig. 5). Also evident is that the absolute position of the \(g_{\text{H}}–V\) relationship tended to be more negative, resulting in pronounced inward currents at lower pH\(_i\) (Fig. 2, C and D), also evident in the current–voltage relationships (Fig. 2 E). The voltage at which the \(g_{\text{H}}\) was 10\% of its maximal value, \(g_{\text{H,max}}\), in whole-cells and inside-out patches at symmetrical pH 7.0 was variable but averaged 9.8 ± 2.6 mV (mean ± SEM; \(n = 14\)) in

Figure 2. Modified pH\(_o\) sensitivity of W207A mutant hH\(_V\). Families of proton currents at several pH\(_o\) in a cell with pH\(_i\) 7.0 are illustrated (A–D). Pulses were applied in 10-mV increments up to the voltage indicated, from a holding potential of −60 (A) or −40 mV (B–D). (E) Proton current amplitudes (\(I_{\text{p}}\)) from the cell in A–D were obtained by fitting the rising current with a single exponential and extrapolating to infinite time. (F) Proton conductance (\(g_{\text{p}}\)) was calculated from the currents in E using \(V_{\text{rev}}\) measured in each solution.
WT hHv1 and −8.1 ± 3.3 mV (n = 20) in W207X mutants (P < 0.001).

More subtly, although voltage-dependent gating was shifted negatively by increases in pHo, as in all known Hv1, closer inspection reveals that the hallmark ΔpH dependence is altered in Trp207 mutants. When pHo was increased from 4.5 to 5.5 to 7.0, the ubiquitous 40-mV/U shift in the gH–V relationship (Cherny et al., 1995; DeCoursey, 2003) occurred (Fig. 2 F). However, above pHo 7.0, there was no further shift; thus, saturation of the shift occurred at ~1.5 U lower pHo than in WT (Fig. 1 E). If the mechanism by which ΔpH-dependent gating occurs involves one or more titratable groups, as has been proposed (Cherny et al., 1995), then replacement of Trp207 apparently lowers the effective pKa of this group(s).

To provide a more concise and quantitative way to evaluate ΔpH dependence, in Fig. 3 we plot the voltage at which the gH is 10% of its maximal value (VgH,max/10) as a function of pH (discussed in Materials and methods). Fig. 3 (A and B) reiterates the observation from Fig. 1 that pHo and pHi dependence of gating both change with a slope of 40 mV/U (dashed reference lines in all figures) over a wide range of pH, and both saturate between pH 8 and 9 in WT hHv1. An important additional result is that Fig. 3 (A and B) indicates that the saturation with pHo or pHi occurs independently of pHi or pHo, respectively. Thus, for example, in Fig. 3 B saturation occurred similarly above pHi 8 at either pHo 7 or 8. Evidently, saturation occurs at a particular absolute pHo or pHi, rather than at a particular ΔpH. This result is consistent with the titration of one or more specific protonation sites that sense pH on only one side of the membrane. Hence, this result contradicts the simplest mechanism of a single site with alternating access to both sides of the membrane.

Analogous plots for the Trp207 mutants (Fig. 3 C) show that their pHo dependence is fully saturated at pHo ≥7.0, at least 1 U lower than in WT, with no further shift of the gH–V relationship up to pHo 10, confirming the impression from Fig. 2. Notably, the pHi dependence did not saturate up to pHi 8 (Fig. 3 D), and thus in contrast to WT, the saturating pH differs for pHo and pHi in the Trp207 mutants. This result also speaks

Figure 3. Saturation of the ΔpH dependence of WT hHv1 (A and B) and of W207X mutants of hHv1 (C and D). The voltage at which gH is 10% maximal (VgH,max/10) is plotted as a function of pHo (A and C) or pHi (B and D), with lines connecting measurements in the same cell. In whole-cell measurements, pHi is color coded, as indicated. In inside-out patches, pHo is color coded, as indicated. For reference, the dashed gray line in each graph shows the slope of the ubiquitous 40-mV/U ΔpH shift in the gH–V relationship (Cherny et al., 1995; DeCoursey, 2003); the horizontal position of this line is arbitrary. (C) Data from 13 W207A, eight W207F, and two W207S cells. (D) Data from three W207A, one W207F, and one W207S patch. No differences were detected among the Trp replacements.
Tryptophan defines the unique properties of hHv1

its pKₐ. In any event, the presence or absence of Trp²⁰⁷ evidently modulates either the accessibility of the pH-sensing site to the external solution or the effective pKₐ of the site(s), or both.

A different analysis of the data is shown in Fig. 4. Solid red squares show that the change in Vₐ₈/₁₀ for a 1-U change in pHᵢ or pHᵦ in WT is roughly 40 mV, but it drops precipitously to ~10 mV at pHᵦ or pHᵢ 8–9. For W207X (open red squares), the shift is already depressed at pHᵦ 6–7 and is abolished (drops to ~0 mV) at higher pHᵦ (7–8, 8–9, and 9–10). In contrast, the W207X mutants exhibit no loss of ΔpH dependence up to pHᵦ 7–8, emphasizing that the W207X mutation appears to selectively alter pHᵦ but not pHᵢ sensing. This result supports the idea of distinct external and internal pH sensors.

Mutations to Trp²⁰⁷ in hHv1 facilitate channel opening

Another distinctive consequence of replacing Trp²⁰⁷ was faster channel opening, evident in Fig. 2. The turn-on of current during depolarizing pulses reflects the time course of channels opening. The rising currents were fitted with a single exponential to obtain τₐₓ, the time constant of activation (channel opening). Mean τₐₓ values plotted in Fig. 5 show that channel opening was ~100 times faster than WT for each of the Trp²⁰⁷ mutants. WT kinetics was more variable than that of any of the mutants, perhaps reflecting the stronger temperature sensitivity of WT Hv1 (DeCoursey and Cherny, 1998; Kuno et al., 2009) or variable proton depletion during the much longer pulses required to determine

against the idea that the same group might sense pH on both sides of the membrane (with alternating access), because in the Trp²⁰⁷ mutants, the pKₐ of the site(s) that sense pHᵦ and pHᵢ differ. An alternative interpretation that cannot be formally ruled out is that moving a single group to a different local environment might itself alter

Figure 4. Saturation of ΔpH-dependent gating occurs independently of loss of proton selectivity of Trp²⁰⁷ mutants. The mean ± SEM (error bars) change in Vₑ for a 1-U change in pH is plotted for WT hHv1 (closed blue circles) and for W207X (open blue circles). The shift in Vₐ₈/₁₀ in the same cells is also plotted for WT hHv1 (closed red squares) and for W207X (open red squares). Numbers of cells for both parameters are one, six, eight, and five for increasing pHᵦ in WT; one, six, and eight for increasing pHᵦ in WT; one, five, 11, six, and three for increasing pHᵦ in W207X; and one and four for increasing pHᵦ in W207X. The difference in Vₑ in W207X versus WT was significant at pHᵦ 7–8 (P < 0.02) and 8–9 (P < 0.001). The difference in Vₐ₈/₁₀ in W207X versus WT was significant at pHᵦ 6–7 (P < 0.02) and 7–8 (P < 0.0001).

Figure 5. Replacement of Trp²⁰⁷ greatly accelerates hHv1 opening. The activation time constant, τₐₓ, was obtained by fitting rising currents to a single exponential. All measurements were done at symmetrical pH 7.0 but include both whole-cell and excised inside-out patch data. Error bars represent mean ± SEM, with n = 7, 9, and 8 for W207F, W207S, and W207A, respectively; WT includes five cells and seven inside-out patches.

Figure 6. Replacement of Trp²⁰⁷ decreases the Arrhenius activation energy for hHv1 opening. The activation time constant, τₐₓ, was determined in one cell during families of pulses at various temperatures at symmetrical pH 7. The sequence is indicated in the inset. The Q₁₀ was somewhat higher at lower voltages (e.g., 4.3 at 20 mV and 3.7 at 100 mV, for the entire temperature range). As reported previously for native Hv1 (DeCoursey and Cherny, 1998), Eᵣ also increased at lower temperatures. Temperature drift during families of pulses was on the order of 1°C.
WT kinetics. Surprisingly, replacement of Trp with an aliphatic hydrophobic residue (Ala), a polar hydrophilic residue (Ser), or an aromatic residue (Phe) produced identically profound acceleration of activation. The kinetic consequences of Trp at position 207 appear to be unique and unrelated to such generic qualities as hydrophobicity, polarity, or aromaticity.

Channel-closing kinetics was examined by measuring $\tau_{\text{tail}}$, the time constant of tail current decay (deactivation). Measured at symmetrical pH 7.0 at $-40$ mV, $\tau_{\text{tail}}$ was $227 \pm 22$ ms ($n = 16$) in WT hHv1 and 29 times faster in the three Trp$^{207}$ mutants ($7.8 \pm 0.8$ ms; $n = 17$).

If the slowing of gating by Trp$^{207}$ were rate determining in WT channels, then the activation energy, $E_a$ for gating should be lower in mutants than the 30–38 kcal/mol in WT channels (DeCoursey and Cherny, 1998). Fig. 6 illustrates that this was the case. The time constant of channel opening ($\tau_{\text{on}}$) was determined by fitting rising currents with a single-exponential function in current families recorded at several temperatures. The Arrhenius activation energy, $E_a$, was calculated from $E_a = RT_1T_2/(T_2 - T_1) \ln(\tau_{\text{act},1}/\tau_{\text{act},2})$, where $R$ is the gas constant (1.9872 cal K$^{-1}$ mol$^{-1}$) and $T_1$ and $T_2$ are the lower and higher temperatures (in K) (DeCoursey and Cherny, 1998). In three experiments (two whole cell and one inside-out patch), $E_a$ determined over the entire temperature range from 11–13 to 35–37°C averaged $\sim 22$ kcal/mol. Therefore, the process involved in slow WT activation that is regulated by Trp$^{207}$ is rate limiting.

Mutations to Trp$^{207}$ in hHv1 compromise proton selectivity. Central to performing all of the functions of Hv1 is its perfect proton selectivity. Proton selectivity was evaluated by measuring the reversal potential, $V_{\text{rev}}$, at various pH. Because replacing Trp$^{207}$ shifted the $g_{H^+}$–$V$ relationship negatively, at some pH, $V_{\text{rev}}$ could be observed directly as reversal of the current during families of pulses, as illustrated in Fig. 7 (A and C). Alternatively, $V_{\text{rev}}$ can be estimated in the usual manner using tail currents (Fig. 7, E and F). Surprisingly, there was only a small shift in $V_{\text{rev}}$ (<20 mV) between pHo 8 and 9 in the W207F mutant (Fig. 7, E and F). In comparison, the same pHo change produced a nearly Nernstian (58-mV) shift in the WT hHv1 (Fig. 7, G and H). The replacement of Trp$^{207}$ compromised proton selectivity.

Fig. 8 A confirms the proton specificity of the WT hHv1. The reversal potential, $V_{\text{rev}}$, measured over a wide range of pHo (5.0–9.0) and pHi (5.5–9.0) was close to WT kinetics. Surprisingly, replacement of Trp with an aliphatic hydrophobic residue (Ala), a polar hydrophilic residue (Ser), or an aromatic residue (Phe) produced identically profound acceleration of activation. The kinetic consequences of Trp at position 207 appear to be unique and unrelated to such generic qualities as hydrophobicity, polarity, or aromaticity.

Channel-closing kinetics was examined by measuring $\tau_{\text{tail}}$, the time constant of tail current decay (deactivation). Measured at symmetrical pH 7.0 at $-40$ mV, $\tau_{\text{tail}}$ was $227 \pm 22$ ms ($n = 16$) in WT hHv1 and 29 times faster in the three Trp$^{207}$ mutants ($7.8 \pm 0.8$ ms; $n = 17$).

If the slowing of gating by Trp$^{207}$ were rate determining in WT channels, then the activation energy, $E_a$ for gating should be lower in mutants than the 30–38 kcal/mol in WT channels (DeCoursey and Cherny, 1998). Fig. 6 illustrates that this was the case. The time constant of channel opening ($\tau_{\text{on}}$) was determined by fitting rising currents with a single-exponential function in current families recorded at several temperatures. The Arrhenius activation energy, $E_a$, was calculated from $E_a = RT_1T_2/(T_2 - T_1) \ln(\tau_{\text{act},1}/\tau_{\text{act},2})$, where $R$ is the gas constant (1.9872 cal K$^{-1}$ mol$^{-1}$) and $T_1$ and $T_2$ are the lower and higher temperatures (in K) (DeCoursey and Cherny, 1998). In three experiments (two whole cell and one inside-out patch), $E_a$ determined over the entire temperature range from 11–13 to 35–37°C averaged $\sim 22$ kcal/mol. Therefore, the process involved in slow WT activation that is regulated by Trp$^{207}$ is rate limiting.

Mutations to Trp$^{207}$ in hHv1 compromise proton selectivity. Central to performing all of the functions of Hv1 is its perfect proton selectivity. Proton selectivity was evaluated by measuring the reversal potential, $V_{\text{rev}}$, at various pH. Because replacing Trp$^{207}$ shifted the $g_{H^+}$–$V$ relationship negatively, at some pH, $V_{\text{rev}}$ could be observed directly as reversal of the current during families of pulses, as illustrated in Fig. 7 (A and C). Alternatively, $V_{\text{rev}}$ can be estimated in the usual manner using tail currents (Fig. 7, E and F). Surprisingly, there was only a small shift in $V_{\text{rev}}$ (<20 mV) between pHo 8 and 9 in the W207F mutant (Fig. 7, E and F). In comparison, the same pHo change produced a nearly Nernstian (58-mV) shift in the WT hHv1 (Fig. 7, G and H). The replacement of Trp$^{207}$ compromised proton selectivity.

Fig. 8 A confirms the proton specificity of the WT hHv1. The reversal potential, $V_{\text{rev}}$, measured over a wide range of pHo (5.0–9.0) and pHi (5.5–9.0) was close to
the Nernst potential, $E_H$, shown as a dashed line. Surprisingly, Trp$_{207}$ mutants were imperfectly proton selective. Fig. 8B shows that although they were highly selective at neutral and acidic pH, at high pH$_o$ (8–10), the measured reversal potential, $V_{rev}$, deviated consistently and substantially from $E_H$.

The mean shift in $V_{rev}$ for a 1-U change in pH is plotted in Fig. 4 (blue circles). The $V_{rev}$ of WT hH$_V$1 (closed blue circles) was reasonably near $E_H$ at all pH studied, whereas $V_{rev}$ of the W207X mutants (open blue circles) was distinctly sub-Nernstian at pH$_i$ 8–10, being significantly lower than WT at pH$_i$ 7–8 and 8–9. Trp$_{207}$ mutation produces loss of proton selectivity, but only at high pH$_o$.

![Figure 8](https://example.com/figure8.png)

**Figure 8.** Proton selectivity is perfect in WT hH$_V$1 (A) but compromised in Trp$_{207}$ mutants (B). Proton selectivity is indicated by the proximity of measured values of $V_{rev}$ and the Nernst potential for H$, E_H$. Data were obtained in whole-cell (closed symbols) and inside-out patch configuration (open symbols). In whole-cell measurements, the color-coded pH$_i$ solution was in the pipette, and pH$_o$ was varied, with lines connecting measurements in each cell. In inside-out patch measurements, pH$_i$ was the pipette solution and pH$_o$ was varied. Whole-cell measurements in B include eight W207F, 12 W207A, and one W207S cell. For W207X mutants, currents in patches were too small to allow reliable estimation of $V_{rev}$.

Replacement of Trp in a dinoflagellate H$_V$1 (kH$_V$1) speeds activation

Given that replacing Trp greatly speeds activation in hH$_V$1, we were curious as to whether the same would be true in other species. To make this test rigorous, we selected an evolutionarily distant species in which the amino acid identity with hH$_V$1 is only 15%, namely *K. veneficum* (Smith et al., 2011). We made the same three substitutions to the corresponding Trp$_{176}$ in kH$_V$1 (Fig. S1): W176A, W176F, and W176S. These mutants generated proton-selective currents in the pH range explored that exhibited qualitatively similar changes when pH$_o$ was changed (Fig. 9, A vs. B, and C vs. D). Like their human counterparts, the Trp mutants activated extremely rapidly. Activation time constants were nearly two orders of magnitude faster than in the WT channel (Fig. 9E). WT closing kinetics was faster in kH$_V$1 than in hH$_V$1, and in the Trp$_{176}$ mutants, tail current decay was often too fast to distinguish reliably from capacity transients. Mirroring the pattern seen in the human Trp mutants, $\tau_{act}$ was roughly the same whether Trp$_{176}$ was replaced by Ser, Ala, or Phe (Fig. 9E). The same remarkable result obtained in both species is that Trp in the signature sequence (RxWRxxR) profoundly slows channel opening by a mechanism that other amino acids tested are unable to replicate. This result suggests a quite specific type of interaction.

The kH$_V$1 channel does exhibit $\Delta$pH-dependent gating, although its absolute voltage range of opening is 60 mV more negative than in other species (Smith et al., 2011). Therefore, it was of interest to determine whether saturation of this effect occurs. In WT kH$_V$1, saturation was observed above pH$_i$ 8.0 or pH$_o$ 8.0 (Fig. S2), similar to the pH at which saturation occurs in WT hH$_V$1. Also like hH$_V$1, Trp mutation compromised $\Delta$pH dependence, with saturation occurring at lower pH$_o$ in the W176F mutant (Fig. S2C).

Replacement of Trp in a coccolithophore H$_V$1 (EhH$_V$1) speeds activation and shifts the $g_H$–$V$ relationship negatively

To further test the generality of the roles of Trp, we produced analogous mutations in the coccolithophore, *E. huxleyi* H$_V$1 (EhH$_V$1), namely W278A, W278S, and W278F (Fig. S1). The EhH$_V$1 sequence differs drastically from hH$_V$1, with only 18% identity, as well as from kH$_V$1, with 29% identity. Fig. 10 shows that activation kinetics was also faster in the EhH$_V$1 when Trp$_{278}$ was replaced, although the effect was smaller than in the other species examined. Opening of these mutants was accelerated four- to sixfold in the positive voltage range. Another pronounced change in EhH$_V$1 mutants was a negative shift of the $g_H$–$V$ relationship, with the voltage at which the $g_H$ was 10% of $g_{H,max}$ averaging $-5.2 \pm 2.9$ (12) in WT EhH$_V$1 and $-33.4 \pm 2.3$ (14) in the mutants, a $-28\text{mV}$ shift. Some of the slowing of $\tau_{act}$ may be
pH 8.0 in WT, essentially identical to both hHV1 and kHV1. No significant change in this property was detected in the EhHV1 mutants, although the mean shift from pH 7 to pH 8 decreased from $-53 \pm 2$ mV (SEM; $n = 4$) in WT to $-43 \pm 5$ mV ($n = 4$) in mutants. The pH dependence of EhHV1 would thus be maintained reasonably well over the normal pH range experienced by coccolithophores; the pH of seawater is 7.5–8.4 (Chester and Jickells, 2012). That saturation of pH-dependent gating occurred at the same pH o and pH i in all three species suggests that the same or similar group(s) may be involved in pH sensing.

**DISCUSSION**

Activation kinetics

Cation–π interaction between Trp$^{207}$ and Arg$^{211}$ in hHV1 latches the channel closed. The most obvious effect of replacing Trp$^{207}$ with smaller amino acids was acceleration of channel opening by two orders of magnitude. The precise physical mechanism by which Trp slows WT HV1 opening can only be speculated, but several possibilities exist. In the closed mHV1 structure (78% sequence identity to hHV1), Trp is oriented toward the lipid (Takeshita et al., 2014), suggesting that hydrophobic interactions might stabilize it in this position. Hydrophobic interactions with membrane lipids have been considered for closed-state stabilization by Val$^{263}$ in the Shaker K+ channel VSD (Lacroix et al., 2013). However, the aromatic Phe, which engages in purely hydrophobic interactions (Killian and von Heijne, 2000),
and with Asn$^{210}$, although in this structure the guanidinium of R3 appears to be pointing nearly directly away from the center of the channel (Fig. 11 B). We speculate that Phe may be unable to stabilize R3 in this position, possibly because of Phe’s weaker cation–π interaction capability. The electrostatic R3–Asp interaction in closed H$_V1$ may also contribute a stabilizing function like that of the corresponding Lys$^{374}$–Asp$^{316}$ in the closed Shaker K$^+$ channel VSD (Papazian et al., 1995). In all open H$_V1$ models (Ramsey et al., 2010; Wood et al., 2012; Kulleperuma et al., 2013; Chamberlin et al., 2014), R3 has rotated to face the pore, whereas Trp still appears to face the lipid. For this kind of conformational change to occur during opening, the interactions between the Trp–Arg pair would have to be disrupted. The much more rapid opening in the W207X mutants may reflect the absence of these stabilizing interactions.

The faster activation kinetics after Trp mutation in hHV1 qualitatively resembles that seen in forced monomerization. Monomeric constructs open four to seven times faster than their dimeric counterparts (Koch et al., 2008; Tombola et al., 2008; Musset et al., 2010; Fujiwara et al., 2012). Might Trp mutation eliminate interaction at the dimer interface between Trps from each protomer that normally contribute to closed-state stabilization? This notion is contradicted by a cysteine cross-linking study indicating that the two S4 helices appear not to interact (Lee et al., 2008). On the other hand, the S4 segments are close to each other in a proposed dimer model based on the closed structure of mHV1 (Takeshita et al., 2014).
Is it possible that the primary reason for the conservation of Trp° is to slow gating? It is not immediately obvious why slow H2 activation would be evolutionarily advantageous. On the other hand, rapid opening would not confer any advantage for most of the functions proposed for H2 in mammalian cells. For example, H2 is activated in phagocytes to compensate for the inorganic activity NADPH oxidase (Henderson et al., 1987; DeCoursey et al., 2003); the latter enzyme is turned on by most stimuli only after a delay and on a time scale of seconds (DeCoursey and Ligeti, 2005). In cells in which acid extrusion via H2 occurs for the purpose of pH homeostasis or signaling, such as airway epithelia (Fischer, 2012), basophils (Musset et al., 2008b), B lymphocytes (Capasso et al., 2010), neutrophils (Morgan et al., 2009), or sperm (Lishko et al., 2010), rapid opening is less important than simply remaining open as long as necessary. The absence of inactivation is thus arguably more critical than rapid activation would be. Another possibility is that because a single HVCN1 gene codes for proton channels in a multiplicity of cell types in which H2 serves diverse purposes, the properties of the protein must be compatible with the physiology of all cells. An excitable cell might be ill-served by a rapidly activating proton channel that could interfere with the action potential.

Trp° in hH2 anchors the S4 segment and stabilizes the closed channel. By retarding channel opening by 100-fold, while slowing closing only 29-fold, Trp° might tend to produce a net stabilization of the closed state. In fact, Trp° mutants did activate at voltages 18 mV more negative than WT hH2; therefore Trp° does contribute to stabilizing a closed state. Native proton channels open almost exclusively positive to $E_h$ and thus never produce significant inward current (DeCoursey, 2003). The negative shift of the $g_{in}-V$ relationship in Trp° mutants resulted in distinct inward currents just above $V_{threshold}$, especially with inward H$^+$ gradients (e.g., Figs. 2, C–E, and 7, A and C). Substitution of Trp° thus subverts this principal feature of H2 and compromises the task of proton extrusion. One could speculate that Trp° fine tunes the voltage dependence of H2 to prevent premature opening. Because of the proximity of $V_{threshold}$ and $V_{rev}$ in WT H2 (Cherny et al., 1995; Musset et al., 2008a), without the stabilization of the closed state by Trp°, channel opening would result in proton influx. Given that mammalian cells are largely concerned with extrusion of metabolically produced acid (Roos and Boron, 1981), such a propensity would be deleterious to cell homeostasis.

In EhH2, Trp also decidedly stabilized the closed state; the $g_{in}-V$ relationship was 28 mV more negative in 4W.278X mutants than in WT. In contrast, W176X mutants in kH2 activated 25 mV more positively than WT. The atypical behavior of kH2 in this regard may reflect distinct teleological considerations. EhH2 exists to extrude acid (Taylor et al., 2011), and like hH2, must therefore be poised to open just above $E_h$. In contrast, kH2 activates 60 mV more negatively than H2 in any other species (Smith et al., 2011), which is ideal for its quite different function in dinoflagellates of mediating H$^+$ influx that triggers the flash in bioluminescent species (Fogel and Hastings, 1972). The molecular mechanism responsible for the unique kH2 voltage dependence is unknown.

Temperature dependence
The gating kinetics of H2 in several mammalian cells is extraordinarily temperature dependent, with $Q_{10}$ values of 6–9 ($E_a$ for the delay, $\tau_{act}$, and $\tau_{tail}$ were identically 30–38 kcal/mol) (DeCoursey and Cherny, 1998). The Arrhenius activation energy, $E_a$, of channel opening ($\tau_{act}$) of W207S was 20–25 kcal/mol ($Q_{10}$ of 3.5–4.0), distinctly smaller than in WT, indicating that the factors that establish the kinetics in W-free H2 have more modest $E_a$. Evidently, the perfectly conserved Trp° is the dominant contributor to the exotic temperature sensitivity of WT hH2, and replacing it lowers the $Q_{10}$ of gating into the range of most ordinary ion channels; two dozen examples are given in Table II of DeCoursey and Cherny (1998). That Trp is involved in the rate-limiting step in H2 opening is consistent with the idea that opening a closed H2 requires disrupting the cation–π interactions between Trp and Arg, and perhaps also inter-protomer Trp–Trp interactions in the dimer that stabilize closed channels.

The gating kinetics of hH2 is much slower than that of many voltage-gated channels; removing Trp eliminates this distinctive property as well. In WT H2, the activation time constant, $\tau_{act}$, is in the range of seconds at room temperature, but it plummets into the low millisecond range in Trp° mutants. Thus, in terms of both channel-opening kinetics and temperature dependence, the effect of removing Trp° is like Kryptonite, turning a Super-channel into an ordinary mortal channel.

Proton selectivity
Over a wide range of pH, the WT hH2 appears to be perfectly selective. In a previous study, WT H2 appeared to lose selectivity between pH 9 and 10 (DeCoursey and Cherny, 1997); the present study extended only up to pH 9. The Trp° mutants, however, lost selectivity at less extreme pH, beginning at pHo 8 (Figs. 4, 7, E and F, and 8 B). It is unlikely that this loss of proton selectivity reflects a direct participation of Trp in the selectivity mechanism. It is well established that an Asp in the S1 helix is crucial to proton selectivity (Musset et al., 2011; Smith et al., 2011). This Asp can be relocated from position 112 to 116 in hH2 without loss of proton selectivity, but charge compensation by interaction with
one or more S4 Arg appears essential (Morgan et al., 2013). Recently, quantum mechanical calculations demonstrated an explicit mechanism by which Asp–Arg interaction is sufficient to produce proton-selective conduction without requiring contribution from the rest of the protein beyond providing a scaffold and focusing aqueous access to the selectivity filter (Dude et al., 2015). If Trp helps anchor the S4 helix in the membrane, its removal may allow sufficient intramolecular movement to disrupt the Asp–Arg interaction that is critical to proton-selective conduction. That the loss of selectivity manifests only at high pHs may reflect deprotonation of a cationic group that stabilizes the open channel.

Trp^{207} is essential for normal ΔpH-dependent gating

Perhaps the most striking consequence of Trp mutation is the weakening of ΔpH-dependent gating, a quintessential feature that provides the basis for H2A function in all cells. In all species, and even among all known H2A mutations described to date (Ramsey et al., 2010; Musset et al., 2011; DeCoursey, 2013), the gi–V relationship shifts a roughly 40-mV/U change in ΔpH over a wide range of pHs and pHi. With the single exception of the dinoflagellate K. veneficum (Smith et al., 2011), this behavior results in H2A opening only when the electrochemical gradient is outwards, so that H2A extrudes acid. The mechanism of ΔpH-dependent gating remains one of the most elusive unsolved mysteries regarding H2A. The first and only explicit model of ΔpH-dependent gating (but see Villalba-Galea, 2014) postulated titratable sites that were alternatively accessible to external or internal solutions (Cherny et al., 1995). A systematic attempt to identify which site(s) was (were) involved revealed no single ionizable residue whose mutation to a non-ionizable residue abolished this phenomenon (Ramsey et al., 2010). Rather than protonation of a site, interaction of protonated water with the Arg residues in the S4 helix was suggested to effect ΔpH-dependent gating (Ramsey et al., 2010). The possibility remained that multiple titratable sites are involved, an appealing idea because of the wide pH range over which the gi–V relationship shifts according to the 40-mV rule. If one or more titratable sites are involved, the effect of pH might be expected to saturate. We demonstrate here that saturation of ΔpH-dependent gating does occur above pH 8.0 in WT H2A (Fig. 3), consistent with previous observations in native rat proton currents (Cherny et al., 1995). Correspondingly, the regulatory sites proposed in our model were assigned a pKa of 8.5, which was assumed to be same for access from internal or external solutions (Cherny et al., 1995).

Although distinct ΔpH-dependent gating persists in H2A Trp^{207} mutants, the pH at which saturation occurred dropped to pH7 but did not change for pH.

Evidently, mutation of Trp^{207} lowers the apparent pKa of the putative external site(s) without changing internal pH sensing. Despite not being titratable itself, Trp is evidently a key component in this mechanism, and may regulate the accessibility of another pH-sensing site, evidently increasing its effective pKa. Because the same kind of pKa shift occurs in both kH2A and hH2A, the putative titratable group(s) may be conserved in these species. In the closed mH2A crystal structure (Takeshita et al., 2014) Arg^{320} (R3) is twisted away from the pore into a hydrophobic pocket of the protein. If R3 is directed away from the aqueous pore, its pKa may be decreased substantially (Kim et al., 2005) and could thus be a candidate pH regulatory site. However, mutation of R3 did not eliminate ΔpH-dependent gating (Ramsey et al., 2010). Furthermore, experimental evidence indicates that Arg remains charged even inside proteins (Harms et al., 2011). Rather than viewing these effects as a change in effective pKa, it is equally possible that mutation might alter the accessibility to protons of a site buried within the membrane electric field, by changing the effective dielectric constant in the pathway or the voltage drop to reach the site.

The results of this study indicate that both external and internal pH sensing may be accomplished by titratable groups with similar effective pKa in WT H2A and in two unicellular marine species: kH2A and EhH2A. Furthermore, the Trp^{207} mutation in H2A (or the Trp^{207} mutation in kH2A) selectively lowered the apparent pKa for external but not internal pH, there appear to be distinct internal and external pH-sensing sites. This result speaks against the idea that a single site might alternatively sense pHs and pHi (Cherny et al., 1995), and indicates that distinct internal and external sensors (that must nevertheless interact with each other) are involved.

The kinetics of ion channel gating is often critical to their specialized function. The activation kinetics of voltage-gated Na+ and K+ channels is tunable by specific residues in S2 and S4 transmembrane segments (Lacroix et al., 2013). Here, we show that the highly conserved tryptophan residue in the S4 signature sequence of H2A (RxWRxRR) is responsible for the characteristically slow kinetics of hH2A. By stabilizing the closed state and optimizing ΔpH sensing, Trp fine-tunes the threshold for channel opening, which in most species is just positive to EFa. Trp thus acts as a mechanism for adjusting the ΔpH-dependent gating that is prerequisite to the functions of H2A in all species.

We appreciate the generous gift of the EhH2A construct by Alison Taylor (University of North Carolina, Chapel Hill, NC) and Colin Brownlee and Glen Wheeler (Marine Biological Association of the UK, The Laboratory, Plymouth, UK). The authors appreciate helpful discussions with Artem Ayuyan (Rush University, Chicago, IL) and Valerij Sokolov (Frumkin Institute of Physical Chemistry and Electrochemistry of the Russian Academy of Sciences, Moscow, Russia).
This work is supported by US National Science Foundation award MCB-0943362 and US National Institutes of Health (NIH) grant GM102336 (to T.E. DeCoursey and S.M.E. Smith). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

The authors declare no competing financial interests.

Merritt C. Maduke served as editor.

Submitted: 11 June 2015
Accepted: 18 September 2015

Note added in proof. A recent EPR spectroscopy study of hHV1 (Li et al. 2015. The resting state of the human proton channel dimer in a lipid bilayer. Proc. Natl. Acad. Sci. USA. In press) showed that the dimer interface includes the top of S1 and the lower part of S4.

REFERENCES
Tryptophan defines the unique properties of hHV1

Published October 12, 2015


