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An aqueous H⁺ permeation pathway in the voltage-gated proton channel Hv1

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Abstract

Hv1 voltage-gated proton channels mediate rapid and selective transmembrane H⁺ flux and are gated by both voltage and pH gradients. Selective H⁺ transfer in membrane proteins is commonly achieved by Grotthuss proton 'hopping' in chains of ionizable amino acid side chains and intraprotein water molecules. To identify whether ionizable residues are required for proton permeation in Hv1, we neutralized candidate residues and measured expressed voltage-gated H⁺ currents. Unexpectedly, charge neutralization was insufficient to abrogate either the Hv1 conductance or coupling of pH gradient and voltage-dependent activation. Molecular dynamics simulations revealed water molecules in the central crevice of Hv1 model structures but not in homologous voltage-sensor domain (VSD) structures. Our results indicate that Hv1 most likely forms an internal water wire for selective proton transfer and that interactions between water molecules and S4 arginines may underlie coupling between voltage- and pH-gradient sensing.

Voltage-gated cation channels typically achieve rapid yet selective transmembrane ion flux by coordinating the dehydrated permeant ion in a tetrameric selectivity filter structure. In the superfamily of voltage- and ligand-gated cation channels, each six transmembrane-spanning channel protomer contains a voltage-sensor domain (VSD) that serves to regulate the opening and closing of the central ion-conducting pore that is structurally distinct from the pore domain^{1–3}. The human *Hvcn1* allele encodes a 273-residue VSD protein, Hv1, that is

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sufficient to reconstitute the characteristic proton selectivity and voltage- and pH gradient-dependent gating of native voltage-gated H⁺ conductances^{4–7}. N- and C-terminal truncations alter channel kinetics and voltage dependence but do not abolish H⁺ current, indicating that the minimal (~175-residue) monomeric VSD of Hv1 is sufficient to form a H⁺-selective permeation pathway^{8,9}.

Grotthuss-type H⁺ transfer in hydrogen bonded chains (HBCs) accounts for the anomalously high mobility of protons in aqueous solution, H⁺ transfer in ice and H⁺-selective transport in a variety of proton transporters and channels^{6,10–12}. Evidence for HBC proton transfer via transient protonation of residue side chains has been presented for several H⁺ transport proteins, including bacteriorhodopsin and H⁺-ATPases^{6,11,12}. In voltage-gated proton channels, transmembrane H⁺ flux is faster than the calculated rate of H₃O⁺ diffusion in water, yet Hv1 remains exquisitely proton selective ($P_H / P_{Na} > 10^6$) (ref. 6). These and other data suggested that H⁺ transfer in Hv1 voltage-gated proton channels occurs by the Grotthuss mechanism^{6,13}. We therefore hypothesized that if H⁺ permeation in Hv1 requires the titration of at least one ionizable residue side chain to complete the HBC on which protons move through the open channel, then neutralizing mutations of crucial ionizable residues would be expected to abrogate expressed H⁺ currents. However, our results indicate that proton flux through Hv1 does not require the explicit titration of residue side chains, arguing instead that H⁺ transfer occurs in a water wire within the central crevice of the VSD.

RESULTS

Neutralizing mutations in Hv1 conduct voltage-gated currents

To determine whether H⁺ permeation in Hv1 requires residues that could serve to complete an HBC for Grotthuss-type H⁺ transfer, we first identified candidate H⁺-transfer residues by comparing orthologous Hv1 residue sequences from human, mouse and *Ciona intestinalis*, each of which is sufficient to reconstitute voltage-gated proton channel activity in heterologous expression systems^{4,5,7}. We then systematically mutated conserved candidate ionizable residues in the Hv1 VSD to a nonionizable residue, typically alanine (Fig. 1a and Supplementary Fig. 1) and measured H⁺ currents in HEK cells expressing mutant channels 18–24 h after transfection.

Arginine residues located at every third position along the S4 helix of voltage-gated cation channels and voltage-sensitive phosphatases (Supplementary Fig. 1) sense the transmembrane electrical field and drive conformational changes that are associated with channel gating and enzyme activation^{1–3}. Arginine residues in S4 must remain charged to sense membrane potential and deprotonation ($pK_a \sim 12$) is unlikely in the physiological pH range, suggesting that H⁺ permeation in Hv1 is not likely to require the titration of these residues. As expected, we found that alanine substitution for arginine at each of the three conserved S4 positions in Hv1 does not interfere with the expression of robust voltage-dependent H⁺ currents^{4,5} (Supplementary Table 1).

The Shaker Kv channel VSD generates a H⁺-selective conductance only when S4 arginine residues are mutated to histidine¹⁴. To determine whether Hv1 uses a histidine-based H⁺ shuttle mechanism, we simultaneously neutralized pairs of histidine residues that are

predicted to be accessible to either the extracellular (His140 and His193) or intracellular (His167 and His168) environments. We previously showed that the neutralization of the two extracellular histidine residues abolished the sensitivity of Hv1 to Zn^{2+} but only modestly affected the voltage dependence of expressed proton currents⁴ (Supplementary Fig. 2a and Supplementary Table 1). Simultaneous mutation of the two intracellular histidines also produced only small changes in Hv1 currents (Supplementary Fig. 2a and Supplementary Table 1). Our data therefore argue against the possibility that proton permeation in Hv1 is mediated by a histidine shuttle or transporter mechanism. The remaining pool of conserved candidate HBC residues in the Hv1 VSD includes seven acidic (aspartate and glutamate) and three basic (lysine) residues (Fig. 1a and Supplementary Fig. 1). Substitution for a neutral residue at each of these candidate positions also failed to abrogate expression of voltage-dependent H^+ currents (Fig. 1, Supplementary Fig. 2 and Supplementary Table 1).

We estimated the effect of mutations on the voltage dependence of Hv1 activation by measuring the voltage at which a detectable tail current was elicited (V_{thr})⁷ in the absence of a pH gradient ($\text{pH} = 0$, where $\text{pH} = \text{pH}_o - \text{pH}_i$). V_{thr} ranged from -135 mV (D174N) to $+77$ mV (R211A) in singly mutated Hv1 channels compared to $+7$ mV for WT Hv1 (Figs. 1 and 2 and Supplementary Table 1). Whereas the neutralization of certain residues had only a small (E119A, D123A, K125A, S143A, S181A) or negligible (K157A, R205A, R208A, K221A) effect on Hv1 voltage dependence, other positions were substantially more sensitive to mutagenesis (Fig. 1 and Supplementary Table 1). Residues where single mutations caused the largest positive shifts in V_{thr} were located in S1 (D112A, $V_{\text{thr}} = 66 \pm 3$ mV), S3b (D185A, $V_{\text{thr}} = 65 \pm 3$ mV) and S4 (R211A, $V_{\text{thr}} = 77 \pm 4$ mV). Large negative shifts in V_{thr} resulted from mutations of residues in S2 (E153N, $V_{\text{thr}} = -110 \pm 8$ mV; E153A, $V_{\text{thr}} = -48 \pm 4$ mV) and S3a (D174N, $V_{\text{thr}} = -135 \pm 6$ mV; D174A, $V_{\text{thr}} = -104 \pm 7$ mV). Removal of the N-terminal 96 residues (N) did not alter V_{thr} , but insertion of a premature stop codon after Lys221, which deletes the C-terminal 51 residues (C), caused V_{thr} to shift $+15$ mV (Supplementary Table 1).

To address the possibility that nearby residues might functionally compensate for one another in singly mutated Hv1 channels, we created a number of double mutations of residues that were predicted to lie in close proximity. As with single mutations, we observed a range of effects on V_{thr} , but all double mutations that we tested were tolerated. Hv1 contains several acidic residues in S2 and S3 that are predicted to face the cytoplasm (E153, E164, E171 and D174A). E164A E171A showed WT-like voltage dependence (Supplementary Fig. 2a and Supplementary Table 2), and combining E171A with D174A did not appreciably shift V_{thr} compared to D174A alone (Supplementary Table 1). Single and double neutralizing or charge-conserving mutations at Glu153 and Asp174 significantly shifted voltage-dependent activation toward negative potentials (Supplementary Table 1). A similar negative shift in voltage-dependent activation was reported for E153C in dimeric Hv1 channels, but the effect of this mutation is absent in ‘monomerized’ channels¹⁵. Visual inspection of cell morphology and GFP fluorescence suggested that relatively few cells survived after expressing double neutralizing mutations (E153A D174A and E153N D174N); mutant channels were largely retained in intra-cellular compartments, and the majority of transfected cells did not express measurable current. However, we were able to

measure voltage- and pH_o -sensitive currents in some transfected HEK 293T cells, but small current amplitudes and slow deactivation kinetics precluded an accurate determination of the shift in voltage dependence (estimated $V_{\text{thr}} \sim -100$ mV for E153A D174A and E153N D174N).

Several Hv1 triple mutations of candidate ionizable residues also retained H^+ channel function. Simultaneous neutralization of three consecutive conserved basic residues in the S2-S3 linker (His-His-Lys (HHK) motif, Supplementary Fig. 1) was almost without effect (Supplementary Table 1 and Supplementary Fig. 2a), but D112N E153N D174N and E153A D174A K221A channels behaved similarly to double mutations of the same residues (estimated $V_{\text{thr}} \sim -100$ mV). In S4, both R205A R208A ($V_{\text{thr}} = 135 \pm 15$ mV) and R205A R211A ($V_{\text{thr}} = 103 \pm 13$ mV) supported H^+ currents (Supplementary Fig. 2b and Supplementary Table 1), but we were unable to measure currents in cells expressing the S4 triple mutant R205A R208A R211A.

The dependence of V_{thr} on the pH gradient over a wide range of absolute pH_i and pH_o is a defining feature of voltage-gated proton channels⁶. We found that the slope of the V_{thr} -pH relation (~ 40 mV per pH unit for both expressed Hv1 (refs. 4,5,7) and native voltage-gated H^+ conductances¹³) is generally insensitive to charge-neutralizing mutations (Fig. 2). The V_{thr} -pH relation appeared to deviate from WT in only two mutant constructs, N and R211A (Supplementary Fig. 2 and Supplementary Table 1). N-terminal deletion caused the V_{thr} -pH slope to decrease somewhat (-28 ± 2 mV per unit pH), whereas R211A caused the slope to increase (-53 ± 7 mV per unit pH). Allosteric coupling of transmembrane pH- and voltage-gradient sensing was therefore maintained in each of the functional Hv1 mutations that we tested. C-terminal truncation of mouse Hv1 (VSOP) after the second arginine in S4 also failed to abrogate H^+ selectivity¹⁶. Conservation of the hallmark voltage-pH gradient coupling, in the face of structural perturbations that otherwise elicit large changes in voltage and pH sensitivity, suggests that the fundamental biophysical mechanisms underlying H^+ selectivity and V_{thr} -pH coupling remain intact in Hv1 mutant channels that express measurable currents.

An aqueous crevice in Hv1 model structures

To gain structural insight into the H^+ permeation pathway in Hv1, we constructed homology models based on the KvAP¹⁷ and Kv1.2-2.1 chimera¹⁸ X-ray crystal structures. We then subjected the Hv1 homology models to sequential coarse-grained and atomistic molecular dynamics simulations on hydrated systems containing solvated ions and 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) lipids. Because crystallization conditions (~ 0 mV) favor the thermodynamically stable ‘up’ state of the VSD, our Hv1 homology models are likely to represent a conducting state of the channel. Rather than arbitrarily positioning the protein into a pre-equilibrated bilayer, we allowed protein and lipids to self-assemble during coarse-grained simulation in the presence of water and ions for 320 ns¹⁹. We subsequently converted the equilibrated coarse-grained models to an atomistic system by superimposition of C α atom to particle coordinates and least-square fitting for the protein and by a fragment-based approach for the lipids (see Online Methods). We ran molecular dynamics simulations (20 ns) on POPC bilayer-embedded Hv1 structures in the presence of explicit water and

ions (Fig. 3). A similar *in silico* approach to the structural determination of $\beta 2$ adrenoreceptors that combined homology modeling with molecular dynamics simulation was shown to reproduce key dynamic features and ligand-binding properties of the experimental structures²⁰.

As expected, the Hv1 model structures are defined by four helical transmembrane segments (S1–S4) arranged in a typical VSD protein fold (Fig. 3a). Molecular dynamics simulation results indicate that residues that are required for Hv1 sensitivity to bath-applied Zn^{2+} (His140 and His193)⁴ are extracellularly exposed in the Hv1 model (Supplementary Fig. 3c). However, consistent with another recently reported Hv1 homology model structure, His140 and His193 remain separated by ~ 20 Å during our atomistic molecular dynamics simulations, suggesting that Zn^{2+} binding is mediated by adjacent subunits in a dimeric channel complex²¹ or that monomeric channels bind Zn^{2+} via His140 and His193 in a distinct, perhaps closed, conformational state. A noteworthy feature of the model is the organization of conserved polar and charged residue side chains into external (Asp112, Ser143, Ser181, Asp185, Arg211) and internal (Glu153, Lys157, Glu171, Asp174 and Lys221) clusters (Fig. 3c and Supplementary Fig. 4). During the molecular dynamics simulation, we observed side chains from residues in the intra- and extracellular clusters to protrude into the central crevice and participate in hydrogen bonding and electrostatic interaction networks with one another and with water molecules (Fig. 3 and Supplementary Fig. 4). Asp112, Arg211 and Asn214 are positioned near the point of maximum constriction between the internal and external residue clusters and aqueous cavities (Fig. 3c), suggesting that these residues may lie near the center of an electrical field¹⁴. Arg205 and Arg208 (S4) interact primarily with lipid head groups and solvent during molecular dynamics simulations, but Arg211 interacts mainly with water and other residues in the external cluster rather than lipids (Supplementary Fig. 3a,b).

The existence of intra- and extracellular residue clusters (Fig. 3c) suggests that residues within each of the two clusters might have discrete functional roles in Hv1, perhaps serving to functionally polarize the VSD. Consistent with this notion, we observed a pattern in the effects of point mutations on voltage-dependent H^+ currents: mutations of predicted internally exposed residues shifted V_{thr} negatively, whereas mutations in the external cluster resulted in positive V_{thr} shifts (Fig. 1 and Supplementary Table 1). For example, R211A (S4) shifted $V_{\text{thr}} > +70$ mV relative to WT Hv1 (Supplementary Table 1). A further positive shift in V_{thr} was seen in the doubly mutated R205A R208A and R205A R211A constructs (Supplementary Fig. 2b and Supplementary Table 1). Mutations of nearby extracellular acidic residues Asp112 (S1) and Asp185 (S3b) also caused large rightward shifts in V_{thr} (Fig. 1 and Supplementary Table 1), and the D112N D185A double mutant of these residues resulted in an additive rightward shift in V_{thr} compared to either single mutant (Supplementary Fig. 2 and Supplementary Table 2). In contrast, both neutralizing and charge-conserving mutations of the intracellular acidic residues Glu153 (S2) and Asp174 (S3a) resulted in more negative V_{thr} values (Fig. 1 and Supplementary Table 1). The correspondence between our *in vitro* and *in silico* results indicates that our Hv1 homology models represent functionally relevant structures. Our Hv1 homology model is also consistent with recent Hv1 residue-accessibility studies^{16,22}.

Water molecules in the central Hv1 crevice could form an aqueous proton wire if they effectively bridge the intra- and extracellular water-filled cavities in the Hv1 open state (Fig. 4a), as shown by molecular dynamics simulation of Hv1-like mutations introduced into the KvAP VSD *in silico*²³. Water density calculations (measured over the last 10 ns of atomistic molecular dynamics simulations) reveal a continuous column of water molecules in the Hv1 central crevice (Figs. 3 and 4). Water molecules in the Hv1 crevice appear well coordinated by hydrogen bonding with multiple partner residues in molecular dynamics simulations (Supplementary Fig. 4a,c). Hv1 mutations that remove side chain hydroxyls, which could help create a hydrophilic pocket for water in the central Hv1 crevice (Fig. 3), did not abrogate expression of H⁺ current, similar to our results with charged residue mutations (Supplementary Fig. 2a and Supplementary Table 1). Unlike Hv1, simulated water densities in X-ray structures for Kv and related channel VSDs (Kv1.2-2.1 chimera, Kv1.2, KvAP, Mlotik) and a NaChBac homology model showed large discontinuities in central-crevice water residency (Fig. 4 and Supplementary Fig. 5), suggesting that an organized water column that is simultaneously accessible to both intra- and extracellular water is a specific property of the Hv1 structure.

DISCUSSION

The main experimental result of this study is that, contrary to our expectation for Grotthuss H⁺ transfer requiring side chain titration of at least one residue, each of the neutralizing Hv1 single point mutants that we tested still generated unambiguous voltage- and pH gradient-sensitive H⁺ currents (Figs. 1 and 2). Consistent with previous results^{8,9}, we found that deletion of the Hv1 N or C terminus alters channel kinetics but does not abolish H⁺ current (Supplementary Table 1), indicating that the terminal amino and carboxyl groups are not required to form an HBC for proton transport. The most straightforward interpretation of our data is that explicit side chain titration is not necessary for transmembrane H⁺ permeation in Hv1. Grotthuss-type proton transfer in Hv1 is therefore most likely to occur in a network of protein-associated water molecules that bridges the intra- and extracellular milieus in the channel's open state.

The hypothesis that the proton conductance in Hv1 occurs in a water wire is further supported by our molecular modeling and simulation studies. Molecular dynamics simulations of Hv1 open-state homology models in the presence of explicit water and counterions revealed a region of prominent water density within the central crevice of the Hv1 VSD (Figs. 3 and 4). The absence of similar water densities in parallel molecular dynamics simulations of Kv X-ray structures and homologous VSD model structures indicates that the central water density observed in Hv1 is a specific property of the Hv1 protein and not merely a consequence of the simulation parameters. Hydrogen bonds between and among waters and specific residue side chains observed during molecular dynamics simulation appear to stabilize water occupancy, suggesting that Hv1 contains a network of intraprotein water molecules that do not readily exchange with the bulk. Evidence for H⁺ transfer via protein-associated waters was reported previously for other membrane proteins^{12,24–26}. Hv1 thus represents a specific example in which waters, in addition to residues, are essential for H⁺ channel function. The functional relevance of water

molecules in other VSD-containing proteins, from ion channels to phosphatases, remains unknown.

An important caveat to our interpretation is the possibility that Hv1 contains more than one H^+ transfer residue, such that functional compensation is provided by another ionizable residue in singly mutated channels. However, even combined double and triple mutations of residues that markedly altered Hv1 voltage dependence still generated measurable H^+ currents, and every functional Hv1 mutation that we tested showed the characteristic sensitivity of channel opening to both voltage and the pH gradient, suggesting that fundamental mechanisms of gating and H^+ permeation are not perturbed in the mutant Hv1 channels examined here. A rigorous test of the water-wire mechanism in Hv1 would involve the simultaneous neutralization of all candidate ionizable residues, but given the roles of these residues in maintaining VSD stability and function^{1,3,27}, it seems improbable that such a mutant protein would be functionally expressed. Indeed, although two of the three S4 arginine residues are dispensable, the R205A R208A R211A triple mutant failed to generate measurable H^+ current, possibly due to the complete loss of the crucial voltage-sensing residues¹⁻³. Consistent with histidine scanning studies in the Shaker Kv channel S4 segment¹⁴, our data reinforce the idea that S4 arginine residues are not sufficient to form a pathway for sustained transmembrane H^+ conduction in VSD-containing proteins. Nonconducting mutant Hv1 channels could also result from a dramatic decrease in cell-surface expression, and we observed prominent intracellular localization of GFP fluorescence and small current amplitudes in a variety of the Hv1 mutations tested here, particularly those showing negative V_{thr} values that will be open and therefore conduct inward H^+ currents at the cell's resting potential. Although we cannot definitively rule out the possibility that proton exchange between water and/or hydronium molecules and amide groups of the peptide backbone, measured hydrogen-deuterium exchange rates are typically several orders of magnitude slower than H^+ conduction through voltage-gated proton channels²⁸. Finally, although it is possible that proton conduction in Hv1 results from a complex mechanism of H^+ transfer among several residue side chains, functional redundancy of H^+ shuttle residues or rapid H^+ exchange with the protein backbone, the simplest explanation for the sum of our data is that protons are transferred through a water wire in Hv1.

Studies on chimeric Kv channels containing transplanted S3b-S4 segments from other VSD proteins, including Hv1, showed that distantly related VSD proteins are likely to share a common structural motif in the voltage sensor and therefore to operate by a similar overall mechanism²⁹. In the modular VSD scheme, mutation of conserved acidic and basic residues may perturb networks of electrostatic interactions that contribute to the energetics of voltage-dependent gating^{1,3}. Some acidic residues that participate in electrostatic interactions with basic residues in S4 to control protein stability and voltage dependent activation²⁵ are conserved in Hv1 (Supplementary Fig. 1), and the effects of conserved acidic residue mutations on the voltage-dependent activation of NaChBac³⁰ compare favorably with those reported here for Hv1 (Supplementary Table 1). Glu153 (S2) and Asp174 (S3a), which are conserved in all known Hv1 species orthologs, appear to be especially important for determining voltage-dependent activation. Even the conservative

mutations (such as E153D and D174E) caused large shifts in V_{thr} (Supplementary Table 1), indicating that these side chains may normally participate in precisely coordinated interactions with other groups. Other residues that are broadly conserved among VSD proteins may be either conserved (Glu119, Lys157) or divergent (Ser143, Lys157, Ser181, Asn214) in Hv1, but nonetheless seem to have only minor effects on Hv1 activity when mutated (Supplementary Table 1 and Supplementary Fig. 1). We also identified residues that appear to be uniquely conserved among Hv1 orthologs which dramatically affect V_{thr} when mutated (Asp112, Asp185). Although the presently available data suggest that Hv1 shares functional and structural homology with the VSDs of channels and phosphatases^{8,15,22}, important differences are likely to endow Hv1 with the apparently unique ability to mediate a H^+ -selective conductance and couple voltage sensing to pH-gradient sensing.

Modification of Hv1 N214C, which is typically occupied by a basic residue in VSD protein S4 helices, with the positively charged reactive thiol MTSET, renders Hv1 nonconducting, suggesting that this residue is in or near the H^+ conduction pathway⁸. Consistent with this result, Asn214 was found to lie near a constriction in the central aqueous crevice of Hv1 model structures (Fig. 3). However, introducing positive charge (by mutation to arginine or lysine) at position 214 is not structurally or mechanistically equivalent to MTSET modification because the mutant channels generate robust H^+ currents^{15,16} (Supplementary Fig. 2a). Our experimental data and structural models are consistent with recent S4 residue-accessibility studies and the notion that, although Asn214 lies near the central crevice through which protons move in the Hv1 open state, the nonionizable polar asparagine residue is not required for Grotthuss-type HBC proton transfer in Hv1 (refs. 15,22). The Asn214 equivalent in Shaker and the Kv1.2-2.1 chimera is a lysine that interacts with a conserved aromatic residue in S2 (Phe150 in Hv1) to form an occlusion site that is involved in the movement of voltage-sensing arginine residues in S4 (ref. 31). However, substitution of nonaromatic residues (alanine, cysteine, serine) in place of Phe150 does not markedly affect V_{thr} in Hv1 (I.S.R. and D.E.C., unpublished data), suggesting that Hv1 and Kv channels may show subtle but important differences in voltage-dependent gating.

Mutations of other polar residues that appear to line the aqueous crevice (such as Ser143 and Ser181) also did not abolish Hv1 function, suggesting that the water network is tolerant to structural changes resulting from point mutations. Delocalization of the excess charge in protonated water clusters (Eigen and Zundel cations)^{10,32} could endow the Hv1 water wire with enough conformational plasticity to remain functional despite perturbations in the surrounding protein structure that are caused by introduced mutations. Consistent with this hypothesis, water residency was found to be reduced, but not eliminated, in molecular dynamics simulations of mutant Hv1 proteins that remain conductive but show altered functional properties (Fig. 4 and Supplementary Fig. 5). The central VSD crevice in Hv1 therefore appears to be particularly suited, among VSD proteins, to harboring water molecules for H^+ permeation, and side chain hydrogen bonds may be important for restricting water mobility to produce a gel- or ice-like water structure. Notably, the effect of deuterium isotope substitution on native voltage-gated H^+ current (the measured conductance was 1.9 times smaller in D_2O compared to H_2O) is similar to the decreased D^+ conductivity in ice versus water. In contrast, D^+ conductance is 1.3 times smaller than that

of H^+ in bulk water, indicating that H^+ transport is not likely to be mediated by bulk water or hydroniumion (H_3O^+) flux through voltage-gated proton channels³³. The idea that H^+ permeation is independent of bulk H_3O^+ movement is intrinsic to our model of Hv1 mechanism (Fig. 5). An aqueous H^+ permeation pathway in Hv1 is also consistent with a recent study in which rapid temperature jumps were applied to native voltage-gated proton channels in microglia³⁴. Future studies will use *in silico* approaches to estimate the free-energy profiles for explicit H^+ residency in and flux through Hv1, as previously explored in other membrane proteins^{24,25,35}. A noteworthy functional parallel to Hv1 comes from the bacteriorhodopsin photocycle, in which a Grotthuss-type H^+ transfer in a water network mediates the extracellular H^+ release step²⁶. In both Hv1 and bacteriorhodopsin, the movement of arginine residues (S4 arginine residues in Hv1, Arg82 in bR) controls the opening of an aqueous proton-permeation pathway.

The existence of water deep within the central VSD crevice and in close proximity to voltage-sensing arginine residues in S4 suggests a mechanism for coupling voltage and pH gradients in Hv1. Indeed, an H^+ binding site within the dielectric was previously postulated; in a mathematical model, voltage sensing could be accomplished by H^+ binding, movement of a charged gating particle (such as S4) or both^{13,28}. The rate-limiting step in channel opening was previously predicted to be the deprotonation of an extracellularly accessible H^+ binding site (extracellular acidification shifts V_{thr} toward positive potentials)^{13,28}.

Our experimental and simulation data predict that the central proton binding site is in water rather than an ionizable Hv1 side chain, raising the possibility that protonated water directly interacts with voltage-sensing arginine residues at least in S4 (Fig. 5). In this scheme, open and closed channel states are differentiated by the position of S4 and proton accessibility to a central water network. At low pH_o , the probability of H^+ occupancy in the extracellularly accessible water network increases, thereby stabilizing the ‘down’ position of S4 by electrostatic repulsion of positively charged arginine residues. Conversely, an outward translation of S4 driven by membrane depolarization will tend to decrease H^+ occupancy of the water network, thus affecting the channel’s sensitivity to pH_o ^{13,28}. The control of channel opening by both voltage and pH_o is therefore the result of balancing the energetics of H^+ binding in the central water network and S4 arginine movement through this aqueous environment. Consistent with this model, we found that only a single conserved arginine in S4 (either Arg208 or Arg211) is required for voltage- and pH-dependent gating in Hv1.

At physiological voltages, Hv1 channel open probability and the voltage-gated H^+ conductance are miniscule ($P_{open} \sim 10^{-3}$)^{22,36}, but the probability of stochastic S4 movement between the up and down states of Hv1 channel opening and voltage-sensor movement nonetheless remains finite. The occasional spontaneous outward movement of S4 may allow water in the central crevice to receive a proton from the cytosolic side, thereby allowing both intra- and extracellular pH (that is, the pH gradient) to be sensed. Alternating membrane accessibility of an H^+ binding site is a central feature of a kinetic model of proton channel gating^{13,28}. However, our inability to identify Hv1 mutations that abolish coupling between the pH and voltage gradients indicates that water, rather than an ionizable side chain, functions as a ‘regulatory proton binding site’^{13,28}. A protonatable network of water molecules therefore serves not only as the pathway for the permeant ion but also as a crucial

element in a gating mechanism that couples transmembrane voltage and pH gradient sensing.

We hypothesize that in Hv1, protonated waters in the central crevice electrostatically interact with S4 arginine residues, thus explaining how information about local pH is communicated to the voltage-sensing apparatus to achieve coupling between V_{thr} and pH (Fig. 5). The proposed mechanism represents a variation on a common theme where hydrogen bonding and electrostatic interactions create coordinated networks of water molecules, through which an excess proton may be efficiently transferred. The existence of water networks harboring an excess proton (perhaps in the form of an asymmetric Eigen cation) was previously inferred from both experimental and theoretical studies of H^+ -selective transport pathways^{6,11,12,24,25,32,35,37}. Hv1 uniquely combines an H^+ -selective permeation pathway within an S4-type VSD, and the result is a proton channel in which membrane voltage and the concentration of the permeant ion, H^+ , coordinately determine the open probability of an aqueous proton wire.

In summary, we pursued two independent and complementary experimental strategies to investigate the molecular mechanism of H^+ permeation in Hv1. Neutralizing mutagenesis of candidate ionizable residues does not abrogate the expressed H^+ current, indicating that explicit side chain titration is not required to sustain the proton conductance. Strikingly, voltage and pH gradient sensing also remain coupled in the mutations tested here. Molecular dynamics simulations of Hv1 homology models revealed resident protein-associated water molecules within the central crevice of the VSD that are stabilized by hydrogen bond networks with specific residues. We conclude that conformational rearrangements in the Hv1 structure (gating), which result from changes in membrane potential or the pH gradient, permit the formation or function of a water wire for selective H^+ conduction.

ONLINE METHODS

Mutations and electrophysiology

HEK 293, HM1 or 293T cells were transfected with WT or mutant GFP-hHv1 as previously described⁴ and subjected to whole-cell voltage clamp 18–36 h later using TMA-MeSO₃ or NaCl-based recording solutions containing 100 mM pH buffer near its pK_a (sodium citrate, pH 3.5 and 4.5, MES, pH 5.5, bis-Tris, pH 6.5, HEPES, pH 7.5)⁴. Imposed pH gradients are indicated by $pH = pH_o - pH_i$, where pH_o is the pH of the extracellular bath solution and pH_i is the pH of the intracellular pipette solution. Site-directed mutagenesis of GFP-hHv1 in pQBI25-fC3 (Qbiogene) was performed by PCR (Phusion HF, Finnzymes) as described⁴ and verified by DNA sequencing (Mental Retardation/Developmental Disabilities Research Center Molecular Genetics Core Facility at Children's Hospital). V_{thr} was estimated by visual inspection of raw I_{tail} records elicited by voltage steps applied in increments of +10 mV from a holding potential where no time- and voltage-dependent current could be detected. Currents were filtered at 1–5 kHz and digitized at 2–10 kHz without series resistance compensation or correction for liquid junction potential. For S4 arginine mutations that showed rapid kinetics, data were digitized and filtered at 5–20 kHz and digitized at 20–100 kHz with 70–90% series resistance compensation, and V_{thr} was

estimated from I_{step} . Offline linear leak subtraction (Microcal Origin 6.0) was used to show $I_{\text{tail}}-V$ and $I_{\text{step}}-V$ relations.

Homology models

Hv1 (NP_115745) shares 15.2% and 11% sequence identity with KvAP¹⁷ (PDB 1ORS) and Kv chimera¹⁸ (PDB 2R9R) VSDs. We used the KvAP and Kv chimera VSD structures as templates to build two structural models (Hv1A and Hv1B, respectively) for Hv1. A structural profile (also known as a position-specific substitution matrix) was calculated for each X-ray structure aligned to homologous Kv sequences. A sequence-only profile was also created for Hv1 aligned to homologous sequences. Homologs were obtained from UniRef100 (<http://www.uniprot.org/>) using noniterative BLAST (e value $< 10^{-5}$). The query was aligned against the BLAST hits with MAFFT³⁸ based on BLOSUM62 substitution matrix³⁹, using the iterative option for best alignment quality. The profile-profile alignment method of FUGUE⁴⁰, which incorporates environment-specific substitution matrices, was used to align the structural profile for each template to the Hv1 sequence profile. Similar alignments were obtained using a modified version of the program (FUGUE_TM) developed for structure-sequence alignments specifically for membrane proteins (Mokrab *et al.*, unpublished data). The resulting structure-sequence alignments were manually adjusted in several ambiguous positions to ensure conservation of key residues and were used as input for MODELLER 8.0 (<http://www.salilab.org/modeller/>) to build ten three-dimensional models satisfying maximum restraints. Candidate models were evaluated based on the energy and violation values from MODELLER and the sequence-structure compatibility scores of pG, PROSA2003 (<https://prosa.services.came.sbg.ac.at/prosa.php>) and VERIFY3D (http://nihserver.mbi.ucla.edu/Verify_3D/). Unreliable regions in the models associated with ambiguous regions in the alignments were improved by altering the alignments manually using ViTO (<http://abcis.cbs.cnrs.fr/VITO/DOC/index.html>). Finally, the best model for each template was selected. An open-state homology model of the bacterial voltage-gated sodium channel NaChBac (Swiss-Prot Q9KCR8) was built by following a similar procedure.

Coarse-grained molecular dynamics simulations

Simplified coarse-grained models allow relatively longer processes such as bilayer self-assembly to be simulated in realistic time scales while capturing key physicochemical properties of the atomistic system^{18,41–43}. Coarse-grained molecular dynamics simulations were performed using GROMACS 3.3.3 (<http://www.gromacs.org/>) as previously described⁴⁴. Coarse-grained parameters were previously described for lipid molecules (dioleoyl-phosphatidylcholine and POPC), Na⁺, Cl[−], K⁺ ions and water⁴² and for residues⁴⁵. Coarse-grained protein models were generated from the corresponding atomistic structures and contain a chain of backbone particles and attached side chain particles. Details of protein bond and angle potential can be found in previous work⁴⁵. Lennard-Jones interactions were shifted to zero between 9 and 12 Å, and electrostatics were shifted to zero between 0 and 12 Å, with a relative dielectric constant of 20. The nonbonded neighbor list was updated every 10 steps. Simulations were performed at constant temperature, pressure and number of particles. The temperature of the protein, lipid and solvent were each coupled separately using the Berendsen algorithm⁴⁶ at 310 K, with a coupling constant $\tau_T = 40$ ps.

The system pressure was anisotropically coupled using the Berendsen algorithm at 1 bar with a coupling constant $\tau_p = 40$ ps and compressibility of 5×10^{-6} per bar. The time step for integration was 40 fs.

Coarse-grained models were generated for Hv1A, Hv1B and NaChBac homology models as well as the X-ray structures KvAP VSD (PDB 1ORS¹⁷); Kv1.2 (PDB 2A79²); Kv1.2-2.1 chimera (PDB 2R9R¹⁸); and Mlotik1 (PDB 3BEH⁴⁷). Tertiary structure was maintained using an elastic-network model^{48,49}. Harmonic restraints with a force constant of $10 \text{ kJ mol}^{-1} \text{ \AA}^{-2}$ and an equilibrium bond length equal to that in the starting structure were applied between all backbone particles within 7 Å of one another. Hv1A, Hv1B and KvAP VS were each placed in a box of $13 \times 13 \times 13$ Å; chimera, Kv1.2, NaChBac and MlotiK1 were each placed in a box of $15 \times 15 \times 13$ Å. Coarse-grained models were energy minimized using <1,000 steps of the steepest-descent method to relax steric conflicts in the protein. Subsequently, each system was combined with randomly positioned coarse-grained lipids (Supplementary Table 2) and solvated with coarse-grained water particles, and sodium or chloride counterions were added to maintain electrical neutrality. Each system was energy-minimized for <1,000 steps to relax steric conflicts between protein, lipid and solvent. Production simulations were run on Linux PCs.

Atomistic molecular dynamics simulation setup

Atomistic molecular dynamics simulations were prepared as described⁵⁰. Side chain ionization states were determined based on pK_a calculations performed using PROPKA (<http://propka.ki.ku.dk/>). Ionizable residues were predicted to be in the default states at pH 7 based on standard pK_a values for each residue. We adopted lipid parameters as used previously⁵¹. Atomistic lipid bilayers were reconstituted from the final configuration of the coarse-grained simulations by modeling atomistic fragments based on the coarse-grained particles and building them into entire lipid molecules, followed by energy minimization (P. Stansfield and M.S.P.S, unpublished data; Supplementary Table 2). The atomistic protein was superimposed on the coarse-grained protein by least-square fitting of the coordinates of the Ca atoms and particles. The resultant atomistic protein-bilayer systems were energy-minimized and solvated with simple point charge water molecules followed by removal of any water molecules that were too close to either protein or lipid molecules. The systems were then subjected to further energy minimization. Countercharge ions were added to both systems by replacing water molecules with ions at the most favorable electrostatic potential positions.

Prior to the production run, a 1-ns equilibration run was performed during which all of the heavy (that is, not H^+) protein atoms were harmonically restrained with an isotropic force constant of $1,000 \text{ kJ mol}^{-1} \text{ nm}^{-1}$. Restrained molecular dynamics runs were performed at 300 K for each protein-bilayer system. Finally, all positional restraints were removed, and 20-ns duration production run simulations were performed for each system. Simulations of KvAP were described previously⁵⁰. Simulations were performed using GROMACS 3.3 (ref. 52), implementing the GROMOS96 force field⁵³. The lipid parameters were based on GROMOS96, supplemented with additional bond, angle and dihedral terms⁵¹. All energy-minimization procedures used <1,000 steps of steepest-descent method to relax any steric

conflicts generated during system setup. Long-range electrostatic interactions were calculated using the particle mesh Ewald method, with a 12-Å cutoff for the real space calculation⁵⁴. A cutoff of 12 Å was used for the van der Waals interactions. The simulations were performed at constant temperature, pressure and number of particles. The temperatures of the protein, lipid and solvent (waters and ions) were separately coupled using the Nose-Hoover thermostat⁵⁵ at 310 K, with a coupling constant $\tau_T = 0.1$ ps. System pressures were semi-isotropically coupled using the Parrinello-Rahman barostat⁵⁶ at 1 bar with a coupling constant $\tau_p = 1$ ps and compressibility = 4.5×10^{-5} per bar. The LINCS algorithm⁵⁷ was used throughout to constrain bond lengths. The time step for integration in both simulations was 2 fs. All analyses used GROMACS tools and locally written code. Molecular graphics images were generated using VMD⁵⁸. The simulation parameters adopted here are comparable to those used previously^{21,50}. Structural features, including aqueous accessibility of the central crevice, were found to be similar in atomistic molecular dynamics—equilibrated Hv1A and Hv1B models (Supplementary Fig. 5). For simplicity, structural models shown in the figures refer to Hv1A unless otherwise stated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Charge-neutralizing mutations in Hv1 conduct H^+ current. **(a)** VSD sequence alignment of human, mouse and *Ciona* Hv1 orthologs showing ionizable residues (blue, histidine, arginine and lysine; red, aspartate and glutamate; orange, cysteine; green, serine and tyrosine). Conserved residues are shown in bold type. Approximate boundaries of transmembrane segments S1–S4 are indicated by solid lines. **(b)** Representative currents elicited by steps to the indicated voltages in cells expressing WT Hv1 or the indicated point mutant. Insets, magnified tail currents; diagrams, pH of bath and pipette solutions; red lines, current at the empirically determined V_{thr} . **(c)** Representative I_{tail} - V relations for D174A (red circles), E153A (orange squares), WT Hv1 (green triangles), K157A (cyan circles), D185A (indigo squares) and D112A (violet circles) from experiments that yielded the currents shown in b (pH = -1). Open symbols denote V_{thr} .

Figure 2.

Voltage- and pH-gradient sensitivity of Hv1 mutations. **(a)** V_{thr} -versus- ΔpH relations measured for D174A (red circles), E153A (orange squares), WT Hv1 (green triangles), K157wA (cyan circles), D185A (indigo squares) and D112A (violet circles). Solid lines, linear fits to the averaged data. **(b)** Effect of selected mutations on V_{thr} when $\Delta pH = 0$. Data in **a** and **b**, means $\pm$ s.e.m. from $n = 3-7$ experiments. Where not visible, error bars are smaller than symbols.

Figure 3.

An aqueous crevice in the molecular dynamics–equilibrated Hv1 open-state homology model. **(a)** Ribbon diagram of the open-state Hv1A homology model based on KvAP. Transmembrane segments are colored as follows: yellow, S1; green, S2; blue, S3; red, S4. **(b)** Atomistic system subject to molecular dynamics simulations, showing Hv1 VSD in solvated POPC bilayer. Lipids, green; lipid head groups, dark green; solvent, blue; protein, yellow mesh and tube; S4, red. **(c)** The final snapshot of an atomistic molecular dynamics simulation of Hv1A ($t = 20$ ns) showing water molecules along the central axis of the S1–S4 bundle. Inset figures, details of water coordination at the internal and external clusters of charged and polar residues. Residue side chains found to form extensive hydrogen bond interactions with water are highlighted in blue (basic), red (acidic) or green (polar uncharged). Water molecules, light blue spheres. For simplicity, only oxygen atoms of waters are shown. S1–S3 segments, yellow; S4, orange.

Figure 4.

Water density in molecular dynamics simulations of Hv1 and other VS domains. **(a–h)** Three-dimensional water-density profiles calculated over the last 10 ns of 20-ns simulations are shown as solid surfaces for Hv1A **(a)**, KvAP VSD **(b)**, Kv1.2-2.1 VSD **(c)**, Kv1.2 **(d)**, Mlotik1 VS **(e)**, NaChBac VSD **(f)**, Hv1 D112N **(g)** and Hv1 E153N **(h)**. The profiles in **c**, **d**, **e** and **f** were taken from simulation of the entire channel tetramers. Water accessibility shown in **g** and **h** correlates with robust H^+ currents measured in these mutations (Supplementary Table 1). S4 segment, orange. **(i,j)** Average number of waters as a function of position along the membrane normal over the last 10 ns of the simulations.

Figure 5.

H⁺ permeation in Hv1 channels. **(a)** Schematic representation of gating transitions and H⁺ permeation in Hv1. Gray shading, hydrophobic protein and lipid; white space, intraprotein water-accessible crevice. Changes in transmembrane voltage (V_M) and the pH gradient (ΔpH) cause conformational changes in the Hv1 protein. In the closed state (left), waters in the central crevice are accessible to H⁺ in the extracellular milieu. Protonation of the water network favors the closed state via electrostatic interactions with arginine residues in S4 (light blue circles). Acidic residues (light red circles) also participate in electrostatic networks that stabilize the closed state. Depolarization and/or extracellular alkalinization promote the transition to the open state, where H⁺ may simultaneously access water molecules in the central crevice for Grotthuss transfer in a water wire (arrows). Blue circles, basic residues; red circles, acidic residues; I, intracellular; O, extracellular. **(b)** Cutaway view of the aqueous H⁺ permeation pathway in the Hv1A model structure. Schematic Grotthuss-type proton transfer, dashed arrows. The side chains of selected hydrophilic residues are shaded according to character (top to bottom: Asp185, Arg211, Asp112, Glu153, Asp174; light red, acidic; light blue, basic); the remainder of the protein is gray, lipids are black and waters are red and white. Some residues whose side chains protrude into the aqueous crevice are omitted for clarity.