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The Voltage-Gated Proton Channel: A Riddle, Wrapped in a Mystery, inside an Enigma

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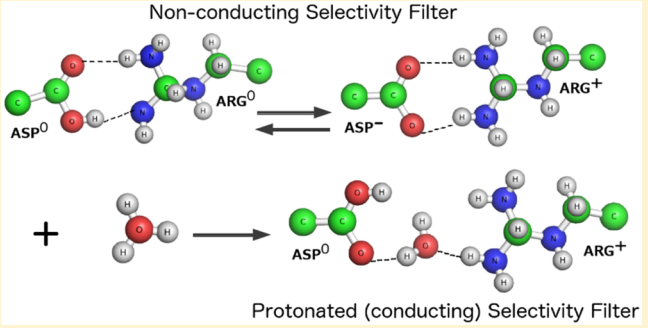
ABSTRACT: The main properties of the voltage-gated proton channel (H_V1) are described in this review, along with what is known about how the channel protein structure accomplishes its functions. Just as protons are unique among ions, proton channels are unique among ion channels. Their four transmembrane helices sense voltage and the pH gradient and conduct protons exclusively. Selectivity is achieved by the unique ability of H_3O^+ to protonate an Asp−Arg salt bridge. Pathognomonic sensitivity of gating to the pH gradient ensures H_V1 channel opening only when acid extrusion will result, which is crucial to most of its biological functions. An exception occurs in dinoflagellates in which influx of H^+ through H_V1 triggers the bioluminescent flash. Pharmacological interventions that promise to ameliorate cancer, asthma, brain damage in ischemic stroke, Alzheimer's disease, autoimmune diseases, and numerous other conditions await future progress.



This review will highlight some of the distinctive features of the voltage-gated proton channel (HVCN1 or H_V1). The title borrows from Winston Churchill's observation about the unpredictability of Russian behavior and intends to evoke the elusiveness of the proton channel. H_V1 prehistory begins in 1806 with a classical paper "Sur la décomposition de l'eau et des corps qu'elle tient en dissolution à l'aide de l'électricité galvanique," by C. J. T. de Grotthuss (also known as Freiherr Christian J. Theodor von Grotthuss),[1] the namesake of the "Grotthuss mechanism". Reading a translation of this paper,[2] one realizes that the mechanism proposed differs substantially from the current view,[3] not least because Grotthuss described the water molecule as OH. Details aside, the contribution of Grotthuss was acknowledged in 1905 in "Notiz über Ionengeschwindigkeiten",[4] in which Danneel provided a lucid explanation of the anomalously rapid conductance of H^+ (which has a mobility 5-fold higher than those of other ions) and, to a lesser extent, OH^-. Danneel recognized that the proton, alone among cations, could bind reversibly to water and further that in protonated water, H_3O^+, the three protons are equivalent and interchangeable. One proton binds to water, and another proton leaves on the far side, saving distance to be traveled.

It was perhaps inevitable that an ion with such a unique conduction mechanism in water would follow different rules in its interaction with proteins. Indeed, both groups who identified HVCN1 channel genes in 2006[5,6] noted the presence of a voltage-sensing domain resembling that found in other voltage-gated ion channels, but the striking absence of any traditional pore domain (Figure 1). In light of the fact that ion channels exist to create regulated passive flow of specific ions across cell membranes, the lack of a permeation pathway seems a deplorable omission, almost a fatal flaw. Nevertheless, the gene product alone, when incorporated into liposomes, functions as a proton conductor (Figure 2), confirming that no accessory protein, such as a separate pore domain, is required.[7]

Traditional ion channels[16] are thought to contain a narrow pore through which a column of water molecules[17] interspersed with ions is obliged to march in single file.[18] Exceptions exist, such as wide-pore nonselective channels.[19] The single-file region usually comprises a small part of the distance across the membrane, with relatively wide aqueous vestibules at either end.[20] Protons can hop through the single-file region of a water-filled pore without displacing the water molecules,[21] whereas ions must wait for the waters in front to pass through.[17,22] Protons do not need to travel with an entourage of waters of hydration and can in fact move quite well as a bare proton, hopping from one ligand to another through hydrogen bonds.[23−26] As will be discussed later, this ability underlies the mechanism by which H_V1 channels are able to select for protons. Consequently, it is appropriate that these channels be termed "proton channels" as opposed to "hydrogen ion channels", because the permeating species is the proton, not the hydronium ion, H_3O^+[27−30] or the other common forms of hydrated hydrogen ion, $H_5O_2^+$, recently named the "Zundel cation"[31] but described by Huggins in 1936,[32] and $H_9O_4^+$, the "Eigen cation".[33,34] For the same reason, the official (HUGO Gene Nomenclature Committee) gene product name HVCN1 (hydrogen voltage-gated channel 1) is a misnomer. Even in

Received: April 3, 2015
Revised: May 7, 2015
Published: May 12, 2015

ACS Publications © 2015 American Chemical Society

DOI: 10.1021/acs.biochem.5b00353
Biochemistry 2015, 54, 3250−3268

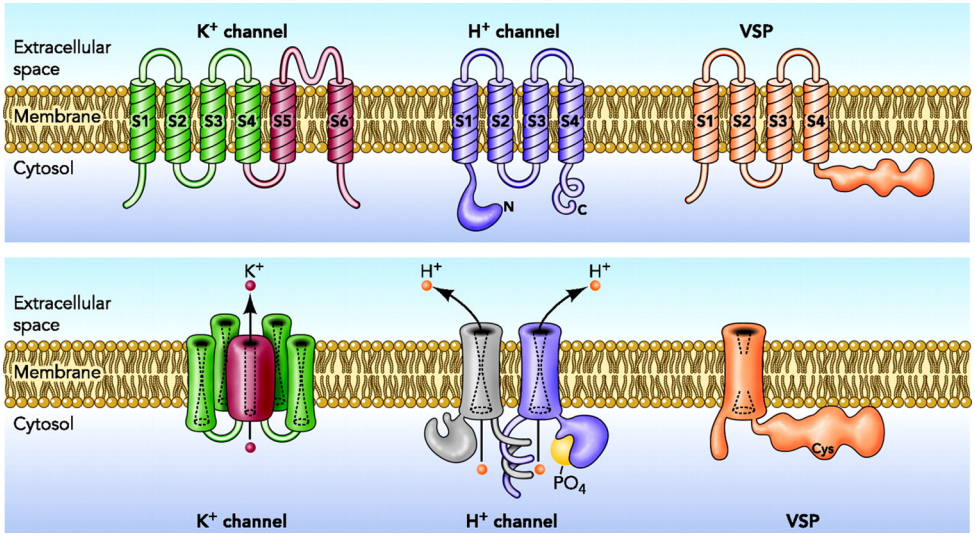


Figure 1. Topology of three molecules that contain voltage-sensing domains (VSDs): a generic K^+ channel, the proton channel, and a voltage-sensitive phosphatase (VSP). The top row shows the orientation of a monomer in the membrane, with the transmembrane helical segments labeled S1, S2, etc., beginning at the amino terminus. The bottom row shows the assembled tetramer, dimer, or monomer, respectively. Ion conduction in the K^+ channel occurs through the single central pore (red) that is comprised of four S5–S6 pairs. H_V1 is a dimer in mammals,[8−10] but each monomer has an intrinsic conduction pathway; monomeric constructs retain the major functional properties of the dimer.[8,10−14] S1–S4 form the VSDs that sense membrane potential, which is transduced into channel opening or closing (gating) in "voltage-gated" ion channels, or into changes in the rate of phosphatase activity in the voltage-sensing phosphatase. Reprinted with permission from ref 15. Copyright 2010 American Physiological Society.

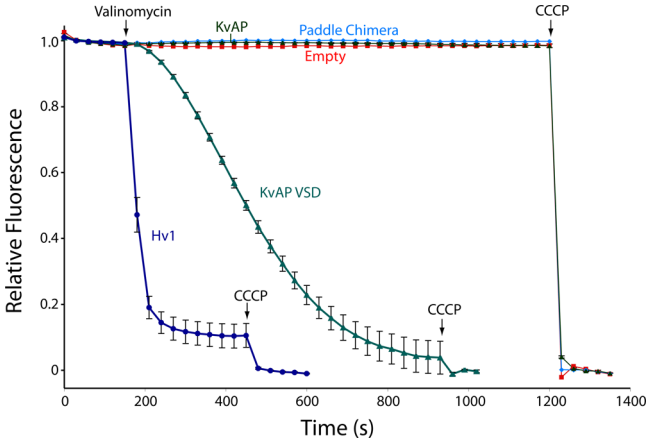


Figure 2. Fluorescence-based H^+ flux assay for vesicles containing hH_V1 (dark blue), the VSD of KvAP channels (green), KvAP (dark green), the paddle chimera (cyan), and empty vesicles (red). Valinomycin and protonophore CCCP were added at the indicated time points. Vesicles loaded with K^+ were placed in a low-K^+ buffer. Valinomycin allows K^+ efflux, which drives H^+ influx, detected by a fluorescent dye. Reprinted with permission from ref 40. Copyright 2014 James Anthony Letts.

water, bodily diffusion of H_3O^+ is negligible.[35,36] If we know one thing about proton movement through channels, it is that H_3O^+ is not the permeating species.[17,21,23,29,37−39]

■ PROTON CHANNELS ARE WIDELY DISTRIBUTED

Figure 3 shows the phylogenetic relationships among 8 confirmed and 29 suspected H_V1s. Proton channels are found in several single-cell marine creatures, invertebrates, and mammals, including humans. The short branch lengths in Figure 3 for mammals reflect strong sequence similarity. In contrast, H_V1 in lower life forms have more divergent sequences. The expectation that sequence differences presage functional differences was borne out in the dinoflagellate *Karlodinium veneficum*, whose H_V1 is unique in generating inward current.[41] H_V1 in coccolithophores are involved in calcium carbonate formation, an important part of the global carbon cycle[42] that is sensitive to atmospheric CO_2 levels.[43] Taylor and colleagues suggested that higher plants might not find H_V1 useful, because plant cells normally exploit a large inward proton gradient that drives nutrient uptake and solute exchange.[44] As will be shown later, H_V1 is exquisitely adapted to extrude protons down their electrochemical gradient; consequently, in plants, the channel might never open.

Thus far, only one H_V1 gene has been identified in any species; no paralogs exist. An analysis of 608 SNPs (single-nucleotide polymorphisms) in human H_V1 (hH_V1) identified seven nonsynonymous SNPs (i.e., which change the amino acid sequence), two of which occur more frequently than 1%.[45] More recently, missense variants of 41 of the amino acids in hH_V1 have been identified (http://useast.ensembl.org/Homo_sapiens/Transcript/ProtVariations?db=core;g=ENSG00000122986;r=12:110627841-110704950;t=ENST00000439744). One mutation identified in a human subject and characterized electrophysiologically is M91T, which requires a larger-than-normal stimulus to open the channel.[46] No human deficient in H_V1 is known; the HVCN1 knockout mouse exhibits altered immune cell responses[47−51] and indications of an autoimmune-like phenotype.[52]

Human B lymphocytes express two isoforms of H_V1.[47] Normal B cells have mainly the full-length protein, which contains 273 amino acids. They also express small amounts of a shorter isoform, as a result of alternative mRNA splicing that produces a protein identical to the long isoform but lacking the first 20 amino acids.[53] The short isoform is present at much higher levels in malignant B cells, such as chronic lymphocytic leukemia, where its higher activity and reactivity (see Figure 14) may contribute to the pathology.[53] T lymphocytes have only

DOI: 10.1021/acs.biochem.5b00353
Biochemistry 2015, 54, 3250−3268

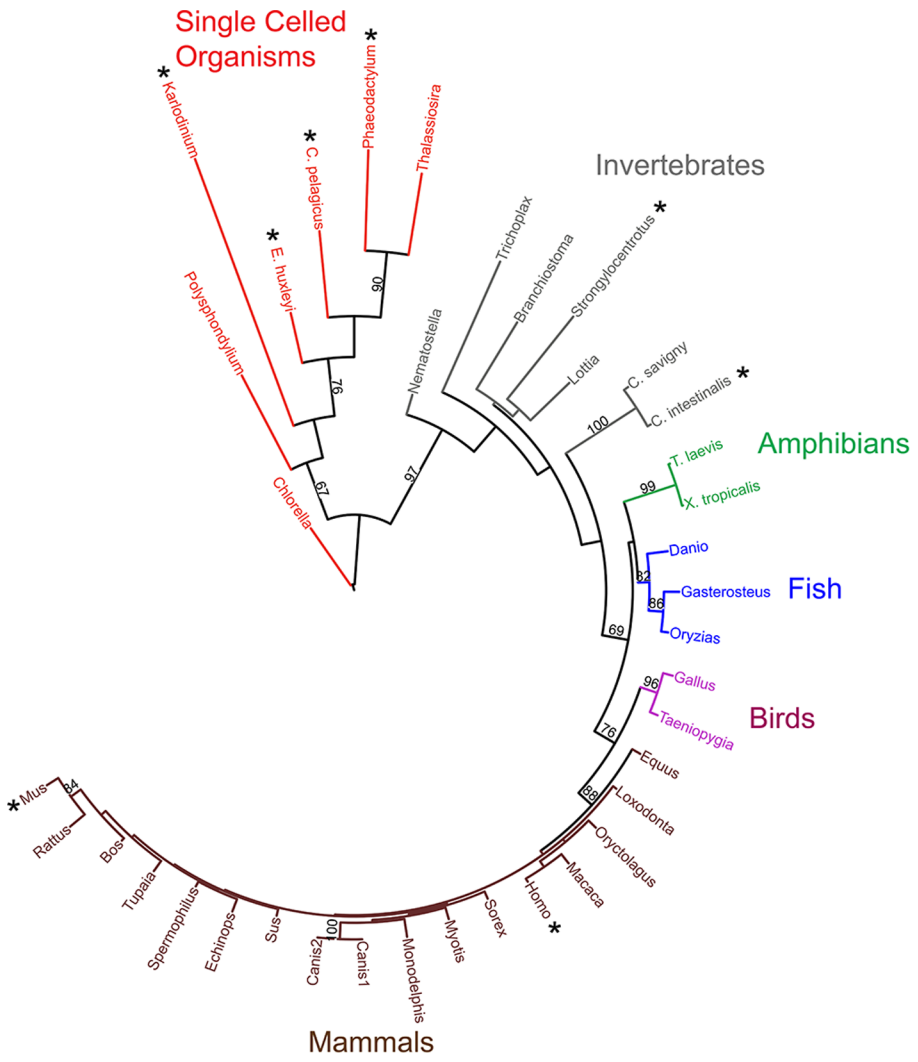


Figure 3. Maximum likelihood phylogenetic tree from a multiple-sequence alignment of the VSD portion of 37 H_V1s. Branch lengths are proportional to the evolutionary distance between sequences. Eight genes confirmed to be H_V1 by direct electrophysiological measurement are starred; the rest are predicted to be H_V1. Two other genes not shown have been confirmed recently by voltage clamp, from a bioluminescent dinoflagellate, *Lingulodinium polyedrum*,[89] and a parthenogenetic insect, *Nicoletia phytophila* (personal communication from G. Chaves, C. Derst, and B. Musset). Reprinted with permission from ref 41. Copyright 2011 The National Academy of Sciences.

~1% of the proton current of B cells,[54] but T cells express more short than long isoform.[52]

H_V1 have been identified directly by voltage-clamp recordings in dozens of primary tissue cell types, including snail neurons,[55−58] amphibian oocytes,[59,60] rat alveolar epithelial cells,[61] murine macrophages,[62] human[63] and mouse neutrophils, murine microglia,[64] cultured human myotubes,[65] rabbit osteoclasts,[66] human basophils,[67,68] human tracheal epithelium,[69] human[70,71] and mouse eosinophils,[72] human cardiac fibroblasts,[73] murine dendritic cells,[74] and human monocytes,[75] but are likely present in many cells that have not yet been studied. Their identified functions differ according to the cell. Functions proposed in several cells and tissues are discussed below. In most cells, H_V1 exist in plasma membranes. Indirect evidence hints at their presence in Golgi membranes.[76] A major role of H_V1 has been established in the phagosome,[48,50,77,78] an intracellular organelle in white blood cells where pathogens are engulfed and destroyed. Finally, H_V1 is thought to mediate the action potential in the vacuolar membrane of bioluminescent dinoflagellates[41,79−83] and trigger the bioluminescent flash in smaller organelles called scintillons.[41,84−88]

H_V1 Structure: An Elusive Pimpernel. H_V1 (the entire protein or even just the membrane-spanning S1−S4 domain) has so far eluded valiant efforts to determine its crystal structure.[40] The structure of a C-terminal fragment alone was reported, which confirmed predictions from protein sequence analysis programs of coiled-coil architecture.[90] A structure of the mouse H_V1 presumed to be closed (nonconducting) was a chimera of three proteins from three species.[91] The C-terminus was replaced with a leucine zipper motif from *Saccharomyces cerevisiae*; the portion from the middle of S2 to the middle of S3 was replaced by the corresponding part of the *Ciona intestinalis* voltage-sensing phosphatase (cf. Figure 1), and the N-terminus was truncated. Nevertheless, the resulting protein exhibited the main electrophysiological characteristics of voltage-gated proton channels. Gating appeared to be faster than in the WT channel; however, replacing the C-terminus may account for this subtlety. In species where H_V1 is a dimer (Figure 1), forcing expression as a monomer speeds channel opening (activation) several-fold.[8,10,12,92] No open structure exists.

DOI: 10.1021/acs.biochem.5b00353
Biochemistry 2015, 54, 3250−3268

WHY DOES H_V1 NEED SPECTACULAR PROTON SELECTIVITY?

Many ion channels are selective; they allow certain ions to permeate but exclude other ions. Usually, this selectivity is not perfect: a Na^+-selective channel may allow K^+ to permeate every so often, with every third to 20th ion being K^+.[93−97] Potassium-selective channels tend to be more discriminating, with an error rate as low as 1 in 1000.[98] Evolutionary forces evidently did not consider these error rates to be problematic, so why should the proton channel be any different? In fact, H_V1 is quite different indeed. As nearly as can be determined experimentally, H_V1 is perfectly selective for protons.[99] No other ion detectably permeates. The requirement for exquisite selectivity is a consequence of biological ion concentrations. Protons inside mammalian cells or in bodily fluids exist at nanomolar concentrations (40−70 nM). This concentration is literally more than 1 million times lower than that of the major cations, Na^+ and K^+. Selectivity can be quantified in terms of relative permeability, roughly how often a particular ion present at the same concentration would permeate in competition with the intended ion. Because of the low concentration of protons, a channel with a million-fold preference for protons would still end up allowing other ions to permeate more than half the time. Such behavior would defeat or at least compromise the purpose of the channel in most situations. To do its job at even a rudimentary level, the proton channel needs a relative permeability $>10^6$ higher for protons than for any other ion. In practice, it is difficult to measure selectivity much greater than this, but estimates of $>10^7$,[100,101] $>10^8$,[27] and even perfect selectivity[41] have been reported. The other side of the coin is that no evidence that other ions can permeate has been produced. We therefore consider H_V1 to be proton specific, which makes H_V1 unique; no other perfectly selective ion channel is known.[20]

HOW IS PERFECT PROTON SELECTIVITY ACHIEVED?

H_V1 must be and is perfectly selective for protons, but how is this accomplished? Some insight comes from mutation studies that have identified certain amino acids that appear to be essential. Of central importance is Asp112 in hH_V1 (Asp51 in kH_V1), located in the middle of the S1 transmembrane helix (Figure 4). Mutating this Asp to any other amino acid except Glu (another acidic amino acid) eliminates proton specific conduction and converts the channel to anion selectivity.[41,102] This result was astonishing at the time, because the expectation was that mutants with compromised selectivity might be nonselective among cations. The D112H mutant (we use the standard convention for point mutations, the one-letter abbreviation for the native amino acid, its numerical position, and its replacement), in particular, was expected (by this author) to retain proton selectivity. Starace and Bezanilla[103−105] had shown that introducing a His into the central narrow section of a K^+ channel VSD (Figure 1) resulted in a proton-selective conductance. The K^+ channel VSD normally does not conduct at all; it simply moves in response to voltage. These studies showed that a His residue at a constriction, given access to aqueous crevices on either end, could transfer protons selectively across the membrane. Another precedent was the viral M2 channel, whose proton selectivity is due to the presence of a tetrad of His resides at a constriction.[106,107]

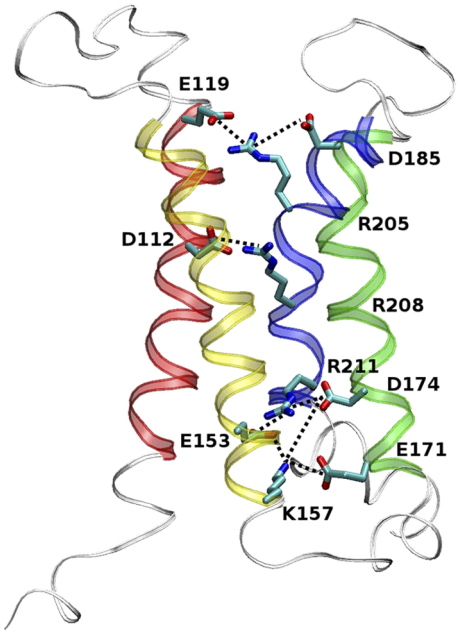


Figure 4. Transmembrane domain of the human voltage-gated proton channel, hH_V1. The four helical segments are color-coded (S1, red; S2, yellow; S3, green; S4, blue); the extracellular end is at the top. Acidic and basic residues are labeled; dashed lines indicate salt bridges predicted by molecular dynamics simulations of a homology model to stabilize the open channel.[108] D112 is crucial to proton selectivity.[102] Reprinted from ref 108. Copyright 2013 The Rockefeller University Press.

In retrospect, the anion selectivity of Asp112 mutants seems reasonable, based on structural considerations. As mentioned above, a crystal structure of a closed mouse H_V1 channel was reported recently,[91] but no open channel structure exists. The best estimates are homology models that are based on structures of the VSDs of other channels. Because of a greater degree of sequence similarity, the VSDs of Na^+ and Ca^{2+} channels are preferable to K^+ channels as templates (Figure 5), but crystal structure resolution is also a factor. Pupo and colleagues[109] analyzed eight homology models proposed to date. In most open state models (Figure 4), Asp112 (or its equivalent in a CiH_V1 model)[110] interacts with Arg residues in the S4 segment via salt bridges.[13,108,111,112] This is reasonable enough; it is *a priori* unlikely that a negatively charged Asp residue could exist in a narrow region of H_V1 without its charge being compensated in some way. When Asp is replaced with an uncharged amino acid, this leaves the cationic Arg without its usual partner, and the now uncompensated positive charge produces anion selectivity. The permeation rate of anions might be low if they are trapped by the cationic group, as appeared to be the case with some of the mutants;[102] a native anion channel might evolve a more refined and efficient mechanism.[135] Nevertheless, this phenomenon illustrates one of the simplest charge selectivity mechanisms: mutations that change the net charge at a constriction in a channel can switch the selectivity between anion and cation.[113−115]

Asp Produces H^+ Selectivity Only within a Certain Molecular Context. Clearly, the presence of a single Asp residue is not sufficient to produce proton selectivity. To understand what molecular context was required, we moved the Asp from position 112 to all positions on the S1 helix from position 108 to 118. At most positions, no current was

DOI: 10.1021/acs.biochem.5b00353
Biochemistry 2015, 54, 3250−3268

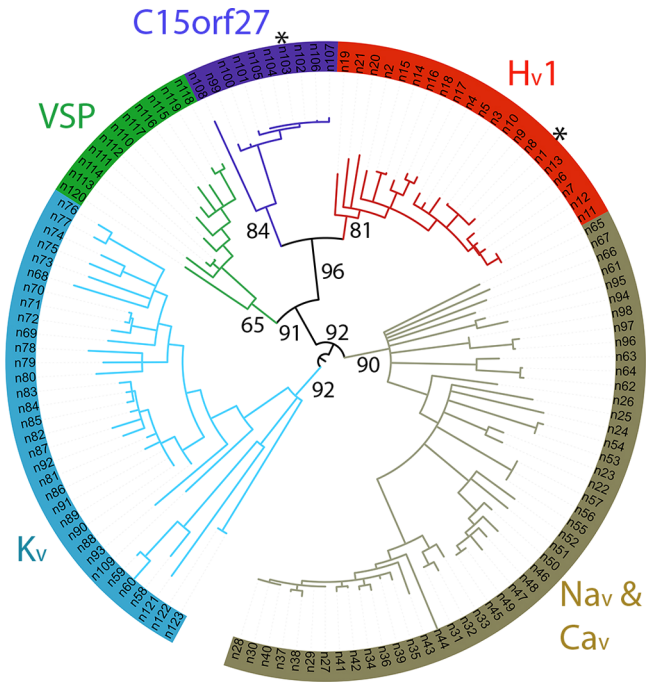


Figure 5. Unrooted phylogram of 122 VSDs from several classes of VSD-containing molecules. The branch length shows the degree of difference from neighbors. Despite the inclusion of only the VSD (S1−S4) and excluding the pore-forming S5−S6 domain, all K^+ channels separated onto one branch and Na^+ and Ca^{2+} channels onto another branch. A third branch includes voltage-sensing phosphatases, H_V1, and c15orf27, molecules of unknown function. Reprinted with permission from ref 102. Copyright 2011 Nature Publishing Group.

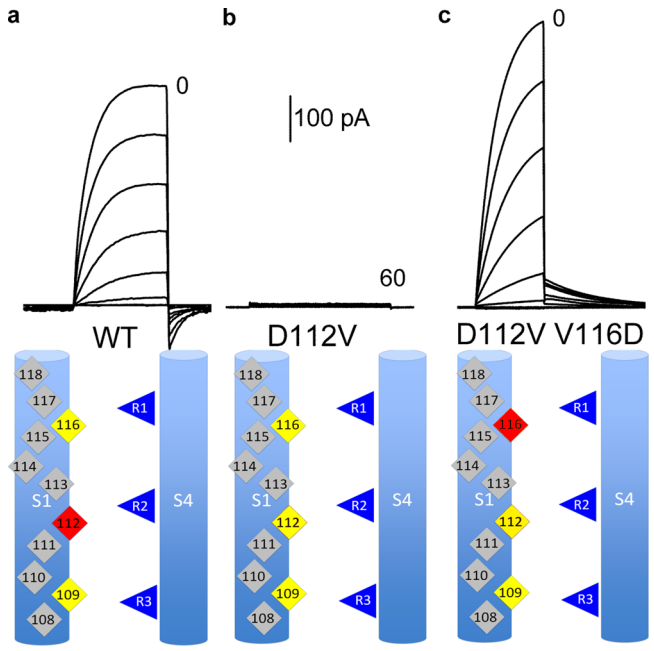


Figure 6. Asp residue that is crucial to proton selectivity in hH_V1 can be shifted from position 112 to 116 in the S1 helix. The diagrams show S1 and S4 segments schematically in the open hH_V1 channel. The three S4 Arg residues (R1−R3 colored blue) are numbered from the outside (Arg205, Arg208, and Arg211). Yellow indicates Val, red Asp, and gray all positions where Asp did not produce functioning channels. The families of voltage-clamp currents (in 10 mV increments up to the voltage indicated) above each diagram illustrate H^+-selective currents in WT, no current in D112V, and H^+-selective currents in the D112V/V116D double mutant, all at pH_o 7.0 and pH_i 5.5. Reprinted from ref 111. Copyright 2013 The Rockefeller University Press.

observed (gray diamonds in Figure 6). Remarkably, at position 116, which is one turn of the helix above (toward the external side of the membrane) the normal position, 112, in the WT (wild-type) channel, Asp restored proton-selective currents. A similar situation exists in the proton channel leading into the H^+-ATPase. In this molecule, Asp61 is essential but can be moved with its function preserved to one other location.[116] In both cases, the fact that the key Asp can be relocated shows that not every detail of the normal WT location is essential. On the other hand, the fact that Asp restored function at only one other location means that there are fairly strict constraints. Logically, one should examine the similarities and differences between the two locations to deduce what is truly essential.

Tentative conclusions drawn from shifting Asp along the S1 segment include the following. Proton selectivity of H_V1 requires a carboxyl group (Asp or Glu worked at either location) that faces the pore directly (at other positions there was no function)[111] at a fairly narrow location (Asp185 and Asp123 in the WT channel do not contribute to selectivity but are located at a higher position where the pore is wider).[102] Evidently, Asp must be on the S1 helix, because upon insertion of Asp at several pore-facing locations in S2 or S3, proton-selective conduction was not observed.[111] Surprisingly, the mean hydration profile generated by molecular dynamics (MD) simulations did not differ in H^+-selective, anion-selective, or nonconducting mutants; hence, this frequently reported property is not useful as a predictor of H^+ selectivity.[111] Finally, charge compensation is essential, as discussed next.

A "Salt-Bridged" Selectivity Filter Appears To Be Necessary for H^+ Selectivity. In another parallel with the ATP synthase, the crucial Asp in H_V1 needs to be within interaction distance of an Arg residue. The only absolutely essential amino acid in ATP synthase besides Asp61 is Arg210.[117−119] We assume that Arg208 is critical in WT hH_V1, but either Arg205 or Arg208 can perform the same function in the D112V/V116D double mutant. When Asp was moved to position 116, MD simulations produced two types of configurations: in the prevalent form there were hydrogen bonds between Asp116 and Arg205, Arg208, or both. Less often, the hydrogen bonds were broken. In the latter configuration, dragging a point charge through the pore revealed a large (~10 kcal/mol) barrier to cation permeation, comparable to that seen in anion-permeable D112x mutants. When the salt bridge was intact, this barrier disappeared,[111] suggesting that proton selectivity requires an intact linkage.

In the WT hH_V1 channel, MD simulations show that the Asp112−Arg208 interaction is present 90% of the time, while the rest of the time a continuous hydrogen-bonded chain of water molecules (a "water wire") exists, with a lifetime of usually <1 ns.[108] Water wires are demonstrably extremely efficient proton pathways,[23,120−125] although they are not intrinsically proton-selective. MD simulations cannot resolve whether protons permeate H_V1 via a water wire that is only fleetingly patent,[108,110] or if proton-selective permeation requires a salt linkage. To answer this question, Todor Dudev, Carmay Lim, and colleagues produced a reduced quantum model of the selectivity filter, by positioning the side chains of Asp and Arg on a scaffold. In the initial bidentate salt bridge, there are two hydrogen bonds between Asp and Arg (Figure 7, top). Surprisingly, the proton that might be expected

DOI: 10.1021/acs.biochem.5b00353
Biochemistry 2015, 54, 3250−3268

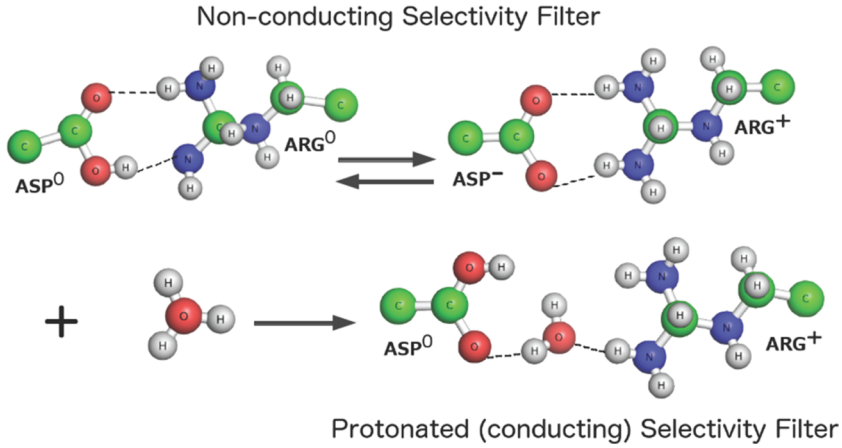


Figure 7. Proton selectivity occurs because H_3O^+ is uniquely able to break the Asp−Arg connection in the selectivity filter, opening its own conduction pathway. Quantum calculations reveal that Asp and Arg interact in the hH_V1 selectivity filter via two hydrogen bonds (dashed lines), in stable optimized configurations with Asp (top left) or Arg (top right) protonated. Introducing a hydronium ion, H_3O^+, into either (bottom row) results in protonation of Asp, yielding a neutral water molecule that mediates interactions between side chains. Computed ΔG values are negative, indicating a favorable forward reaction, assuming dielectric constants of 4 or 30 to reflect low or high solvent accessibility, respectively.[128] Figure generously provided by Karine Mazmanian.

to remain covalently bound to Arg due to its extremely high pK_a (12.5 in solution) can shift to Asp in a hydrophobic environment. The overall net charge is neutral for either Asp^-−Arg^+ or $AspH^0$−Arg^0. An Arg−carboxylate structural motif that has been identified in several other enzymes is thought to ensure equilibrium between protonated and deprotonated Arg.[126] When a proton in the guise of H_3O^+ approaches the selectivity filter, Asp becomes protonated and the hydrogen bonds break, resulting in $AspH^0$−H_2O^0−Arg^+ (Figure 7, bottom). The positioning of H_3O^+ between a deprotonated acid and a base has been observed spectroscopically.[127] In a sense, H_3O^+ "opens" the pore to allow its own permeation. From the protonated selectivity filter configuration depicted in Figure 7 (bottom), reprotonation of a water would result in net H^+ permeation. If any ion besides H_3O^+ approaches the selectivity filter, the result is quite different. The Asp−Arg linkage remains intact, and the ion becomes trapped by the side chain of opposite charge. In this ingenious mechanism, the unique abilities of protons to travel by riding a water molecule and to transfer readily and reversibly to other groups are exploited by H_V1 in achieving proton selectivity.

In WT hH_V1, Asp112 and Arg208 function as the selectivity filter. Experimentally, proton selectivity is preserved when Asp is replaced with Glu,[102] or when Arg is replaced with Lys,[128] indicating an angstrom of leeway in side chain length. In the quantum model, Lys protonates Asp, because its pK_a is lower than that of Arg, producing $AspH^0$−Lys^0, but a stable Asp^-−Lys^+ ion pair minimum was not observed.[128] Thus, proton selectivity can occur without a formal salt bridge (which by definition requires both hydrogen bonding and electrostatic interaction) but still requires intimate interaction between a carboxyl group and a basic amino acid.

As mentioned, an essential Asp−Arg pair also occurs in ATP synthase; indeed, superposition of Asp in the two molecules reveals that the geometries of the pairs are strikingly similar. Upon examining several other molecules that contain Asp−Arg/Lys pairs thought to be important in proton pathways,[128] we found similar distances between the charge centers in crystal structures of Na^+ pyrophosphatase,[129] H^+ pyrophosphatase,[130] and the glucose symporter Xy1E.[131] Conversely, a search of 60 non-proton-selective ion channels and transporters failed to identify Asp−Arg pairs meeting the criteria for a H^+ selectivity filter.[128] The Asp−Arg motif may be one of Nature's tools for designing proton-selective pathways.

MD simulations predict two hydrophobic regions in the H_V1 pore, one at the selectivity filter and the other at a highly conserved Phe150 (in hH_V1).[108,110] How do protons get through the latter obstacle? One possibility arises from the remarkable ability of the proton (but not K^+) to promote solvation of hydrophobic nanotubes simply by entering one end.[132] The proton in essence injects its own water wire through the hydrophobic region. Perhaps the high activation energy for permeation[28,133] is a reflection of the work protons must do to permeate; they are uniquely able to open the selectivity filter and to hydrate dry regions of the pore.

WHAT IS THE DIFFERENCE BETWEEN AN OPEN AND CLOSED H_V1 CHANNEL?

All biologically useful ion channels open and close, which means they have two distinct conformations, one of which permits conduction and the other of which does not. The open channel typically contains at least a short section through which ions and water molecules are thought to pass in single file.[22] These expectations have been confirmed for several channels whose crystal structure has been determined.[134−137] A number of homology models have been produced for open H_V1 channels,[109] but only limited direct structural information exists. Earlier models[13,110,138] tended to assume that H_V1 would move during opening the way the voltage-sensing domain of other voltage-gated ion channels moves, namely by a large outward excursion of the S4 transmembrane helix, resulting in all three positively charged residues (Arg) moving past a central constriction delimited by a highly conserved Phe (Phe150 in hH_V1). More recently described evidence exists: (a) the lower gating charge in mouse than human H_V1 despite the same three S4 Arg residues,[92] (b) the accessibility of Arg → His mutants to Zn^{2+} applied from either side of the membrane,[108] (c) the crystal structures of the closely related voltage-sensing phosphatase in both "up" and "down" configurations,[139] and (d) the closed crystal structure of H_V1,[91] which all point to a

DOI: 10.1021/acs.biochem.5b00353
Biochemistry 2015, 54, 3250−3268

much smaller S4 movement in H_V1.[108,111] This difference is evolutionarily reasonable. The K^+ channel VSD must force the physically distinct pore domain to open. In contrast, the proton channel, in principle, requires only a subtle rearrangement that allows the critical Asp−Arg interaction to occur.

As discussed above, a crystal structure of what is presumed to be a closed H_V1 channel was reported recently.[91] Whether a crystallized channel is considered open or closed is based on whether it is normally open or closed at 0 mV, because there is no membrane potential in a crystal. Given that H_V1 at symmetrical pH opens just positive to 0 mV,[100] and because multiple closed states exist,[29] it is possible that the crystal structure depicts a shallow closed state. As illustrated in Figure 8, the closed structure exhibits two hydrophobic layers,[91] at

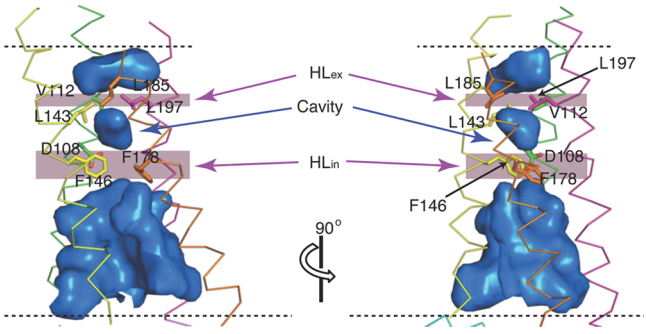


Figure 8. Predicted aqueous accessibility (blue surface) of the pore of the closed mouse H_V1 channel, based on its crystal structure. Dashed lines show the membrane surface; the top is the extracellular end. Reprinted from ref 91. Copyright 2014 Nature Publishing Group.

least one of which presumably creates a proton-impermeable barrier. Models of the open H_V1 channel also include two hydrophobic zones;[108,111] the hydrophobic region was proposed to be wider in the closed state.[110] In the closed structure, the Asp (Asp108 in mH_V1) that is critical to proton-selective conductance is directly in the middle of the larger hydrophobic zone.[91] Figure 9 presents evidence that a sufficiently hydrophobic region can occlude conduction in hH_V1.

REGULATION OF H_V1 BY ΔpH IS THE KEY TO ALL ITS FUNCTIONS

Like all ion channels, H_V1 must open and close at the appropriate times to serve a useful purpose in cells. Like many ion channels, H_V1 opens at relatively positive transmembrane voltages, making the voltage-clamp approach[141] a particularly attractive way to study its behavior. H_V1 is called a "voltage-gated" ion channel, because the transmembrane voltage strongly influences the probability that it opens or closes, opening and closing being collectively called "gating". Gating, in turn, refers to the fact that no ions pass through a closed channel, but when a "gate" in the channel opens, ions flow continuously until the gate closes again. In contrast to nearly all other ion channels, however, the voltage dependence of H_V1 opening is not absolute but is strongly modulated by the concentration of permeating ions, namely by pH. Admittedly, some other ion channels share this propensity. Some voltage-gated K^+ channels close more slowly when they are occupied by a permeating K^+ or Rb^+ ion.[142,143] The voltage dependence of the open probability of inwardly rectifying K^+ channels is more strongly influenced by the external than internal K^+

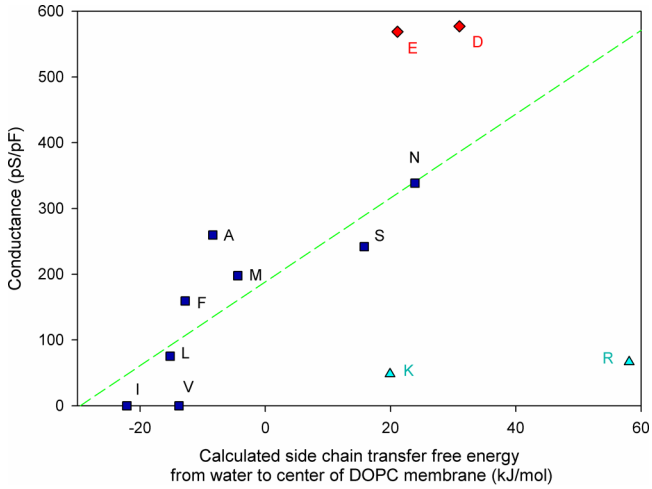


Figure 9. Maximal conductance of hH_V1 mutants in which Asp112 has been replaced with the indicated amino acid. Acidic, basic, or neutral side chains are colored red, aqua, or blue, respectively. The more hydrophobic the side chain, the smaller the conductance. Hydrophobicity, as defined in ref 140, increases to the left. Data are from ref 102 or from the same authors if the data are unpublished.

concentration,[144,145] as is that of Cl^- channels by the external than internal Cl^- concentration.[146,147]

H_V1 is unique in being equally sensitive to the permeant ion concentration on both sides of the membrane. Early studies identified this property qualitatively in snail neurons from *Lymnaea stagnalis*[55] and *Helix pomatia*.[56] Cherny and colleagues systematically studied the pH dependence of H_V1 in rat alveolar epithelial cells[100] and found that changes in pH_o and pH_i were equally effective in shifting the g_H−V (H^+ conductance−voltage) relationship along the voltage axis. As is the case for most voltage-gated ion channels, the probability that the channel will open is a sigmoid function of voltage, which can be determined by calculating the H^+ conductance, g_H, from currents measured over a range of voltages. Remarkably, the pH gradient, or ΔpH (defined as pH_o−pH_i), rather than the absolute pH on either side of the membrane, was the sole determinant of the position of the g_H−V relationship. As is evident in Figure 10B, whether pH_o and pH_i were 8.0 and 7.5, 7.0 and 6.5, or 6.0 and 5.5, respectively, channels began to open near 0 mV.

The biological significance of ΔpH-dependent gating is that over a very wide range of conditions encompassing all pH and membrane potentials likely to be encountered in living cells, H_V1 opens only when the electrochemical driving force is outward. In other words, the channel is regulated so that it can open only when doing so will result in outward H^+ current, that is to say, acid extrusion. This regulation is as perfect as could be imagined for a channel whose purpose is to eliminate excess acid from metabolically active cells.

Before going further, a glaring exception to this rule must be mentioned, to "prove the rule". Proton channels in dinoflagellates serve functions very different from those described to date in other species (Figure 11). Bioluminescent dinoflagellates need H_V1 that can conduct inward H^+ current (Figure 11A,B), and this is evidently what evolution provided. Although ΔpH-dependent gating occurs, the entire relationship is shifted by −60 mV, so that when kH_V1 opens, it allows H^+ influx.[41]

DOI: 10.1021/acs.biochem.5b00353
Biochemistry 2015, 54, 3250−3268

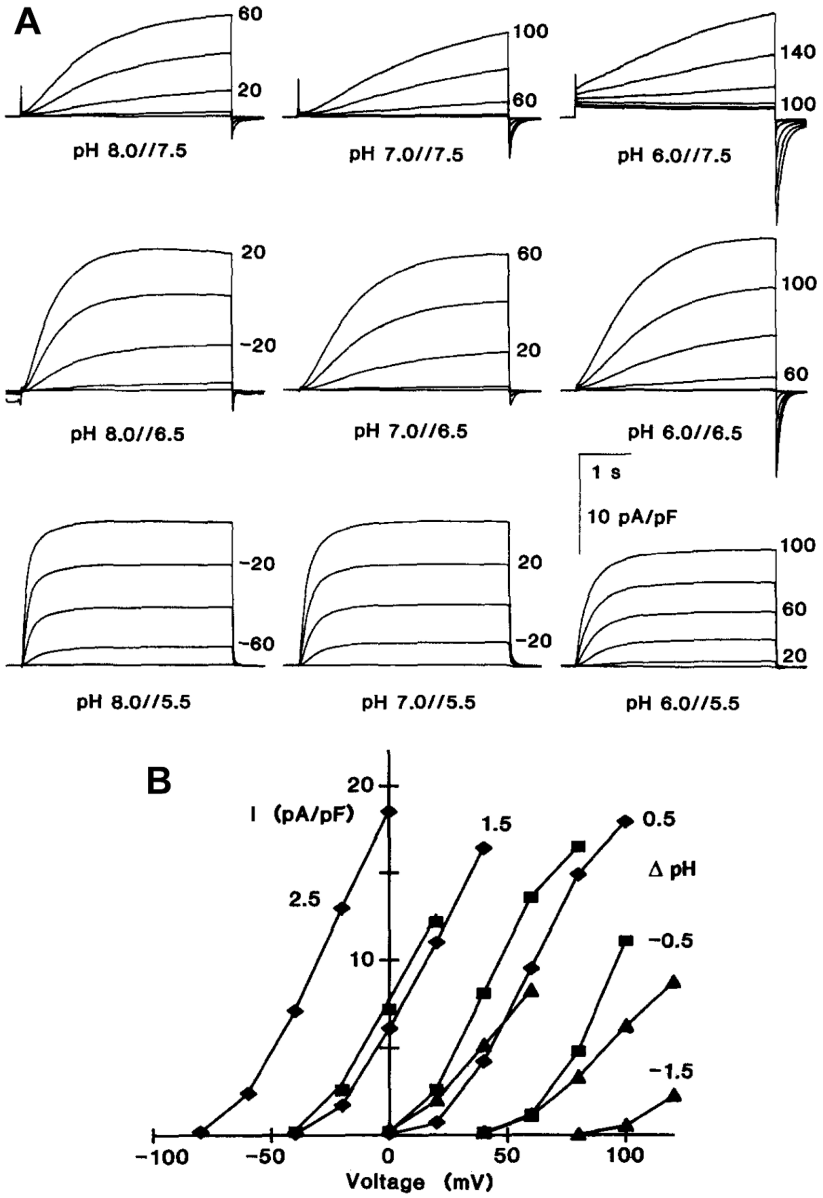


Figure 10. Voltage at which H_V1 channels open determined by the pH gradient, ΔpH (pH_o−pH_i). (A) Families of proton currents recorded in rat alveolar epithelial cells at several pHs, indicated as $pH_o//pH_i$, during depolarizing voltage pulses shown in 20 mV increments. The peak currents from these families are plotted in panel B, with diamonds for pH_i 5.5, squares for pH_i 6.5, and triangles for pH_i 7.5. Reprinted from ref 100. Copyright 1995 The Rockefeller University Press.

How Does ΔpH-Dependent Gating Work? Among those questions that remain completely unanswered, this is the most important and the least understood. Little progress has been made beyond the original model proposed by V. S. Markin and colleagues.[100] An attempt to identify one or more titratable groups that might contribute to ΔpH-dependent gating arrived at the remarkable result that the several dozen mutants tested all retained a very nearly 40 mV/unit pH shift in the g_H−V relationship.[138] In fact, there has been no report of a mutation that abrogates this function. Clearly, the mechanism is robust. Recently, Villalba-Gallea[148] attempted to separate the voltage and pH dependence of several steps in H_V1 gating, a potentially useful approach.

REGULATION OF H_V1 BY PHOSPHORYLATION: ENHANCED GATING MODE

Under normal conditions, the question of whether H_V1 will open can be predicted from ΔpH and membrane potential (Figure 10). In some situations, the cell may need to open more channels more quickly. Just such an enhanced responsiveness mode can be produced by phosphorylating the channel. Enhanced gating has been studied intensely in human phagocytes, including neutrophils,[154] eosinophils,[155,156] and monocytes,[75] but also in human B lymphocytes[53] and basophils,[68] and in mouse osteoclasts,[157] granulocytes,[158] and dendritic cells.[74] Confronted with a pathogenic stimulus such as a bacterium (Figure 12), phagocytes undergo a "respiratory burst", an enormous increase in their level of oxygen consumption that occurs during phagocytosis.[159] This term is a misnomer because most of the O_2 is consumed as substrate to

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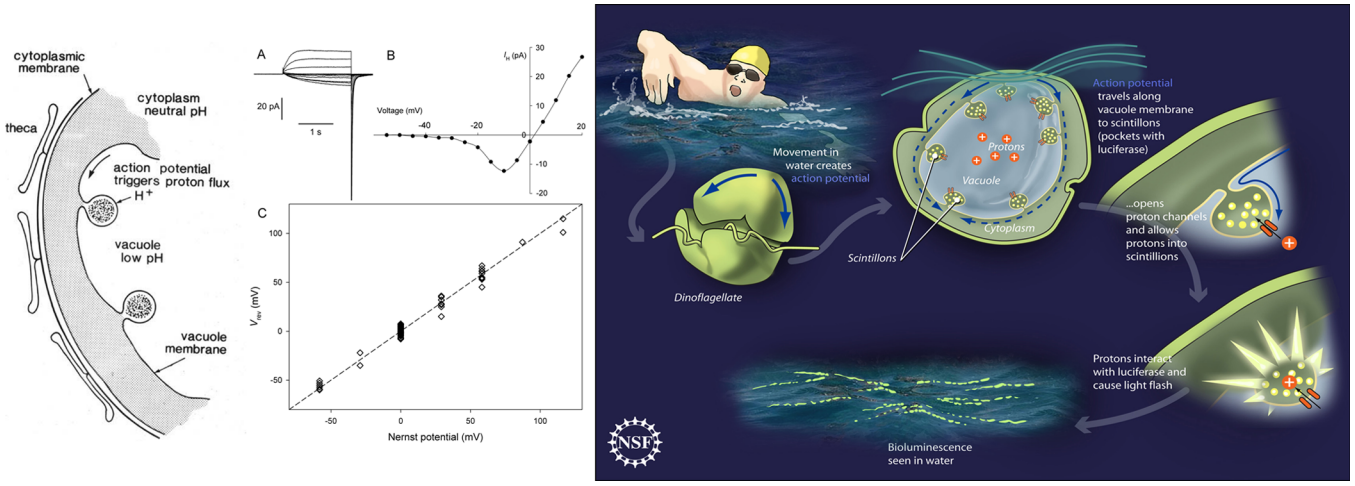

Figure 11. Proton channels trigger the flash in bioluminescent dinoflagellates. The diagram on the left and the cartoon on the right illustrate J. W. Hastings' original 1972 proposal,[87] which was the first conception of a voltage-gated proton channel. Movement of water initiates an action potential that travels along the vacuolar membrane (the tonoplast) and invades the scintillon[88] membrane where it opens voltage-gated proton channels. H^+ readily enters the luciferase-containing scintillon because of the enormous driving force; the vacuole pH is 3.5−4.5.[83,149] The resulting influx of H^+ activates the acid-activated luciferase[150,151] contained in the scintillon, as well as releasing luciferin from its binding protein.[152] Both events allow the enzyme luciferase to catalyze the oxidation of its substrate luciferin to an excited species that releases a photon, resulting in the flash. Reprinted with permission from ref 153. Copyright 2001 Elsevier. The first gene for a dinoflagellate proton channel (or ion channel of any kind) was obtained from a cDNA library from *Karlodinium veneficum*. When expressed in mammalian cells, the gene product (kH_V1) produced depolarization-activated currents (A) that reversed near the Nernst potential for H^+ (E_H) and were thus proton-selective (C). Unlike any other proton channel identified to date, kH_V1 opens well below E_H and thus conducts inward current, as is evident in the current−voltage curve in panel B. This property is ideally suited to the proposed purpose, because in the tonoplast, H_V1 would orient with the vacuole being topologically extracellular. H_V1 from any other species would prefer to extrude acid. To trigger the flash, H_V1 must open and allow inward current, precisely as observed. In addition to allowing the flux of H^+ into the scintillon, these proton channels may also mediate the action potential that triggers the flash. Reprinted with permission from ref 41. Copyright 2011 The National Academy of Sciences. Artwork by Zina Deretsky, National Science Foundation.

produce reactive oxygen species (ROS), not by mitochondrial respiration.[160] ROS are produced by NADPH oxidase, which is activated largely by phosphorylation[161−164] mainly by protein kinase C.[162,165−167]

Essentially all stimuli that activate NADPH oxidase (NOX2) also "activate" H_V1, producing a constellation of changes in channel properties that all increase H_V1 activity in what is called "enhanced gating mode" (Figure 13). Enhanced gating of H_V1 is produced by a number of physiological stimuli that also activate NOX2, including chemotactic peptides such as fMLF in neutrophils,[158] lipopolysaccharide (LPS) in dendritic cells,[74] IgE in basophils,[68] IL-5 in eosinophils,[178] and arachidonic acid in neutrophils and eosinophils.[63,179,180] Despite the diversity of stimuli, enhanced gating occurs when H_V1 is directly phosphorylated by PKC, at a single phosphorylation site (Thr29) located in the intracellular N-terminus.[53,181] Enhanced gating can be prevented by pretreatment with PKC inhibitors[74,157,158,178] and can be reversed after it is established, also by PKC inhibitors.[68,75,158,181] Even in the case of arachidonic acid, which has direct activating effects on both NADPH oxidase[179,182] and H_V1,[63,158,180,183] part of the enhanced gating response can be prevented by PKC inhibition.[158]

A number of questions about enhanced gating remain unanswered. First, H_V1 gating can be enhanced only in certain cells; some cells do not respond at all, such as rat alveolar epithelial cells[154] or HEK-293 or COS-7 cells that express hH_V1 heterologously.[184] A speculative explanation is that different PKC isoforms may be present in different cells. Even more puzzling is the association of the intensity of the enhanced gating response with the presence of an active NADPH oxidase complex.[185] Cells such as phagocytes with high NOX2 activity have the most pronounced enhanced gating, whereas closely related cells, such as basophils[186] or neutrophils from subjects with genetically impaired NADPH oxidase (i.e., with chronic granulomatous disease), exhibit a distinctly attenuated response.[68,187] Even more puzzling is that one and only one of the four manifestations of enhanced gating, namely slower tail current decay (channel closing), is temporally correlated with NADPH oxidase activity measured directly as electron current.[154,156,179] A plausible but not entirely satisfying explanation for these phenomena is that NOX2 produces intracellular protons at a sufficiently high rate to alter the local pH near H_V1, and a low pH_i promotes H_V1 opening.[154,185,188]

How Effective Is Enhanced Gating? It can be reasoned that enhanced gating makes H_V1 more likely to open or remain open under any particular circumstances. Therefore, a smaller stimulus will be required to activate the H^+ flux required for any purpose. In the case of the respiratory burst, the effect of enhanced gating on the ability of H_V1 to limit depolarization has been analyzed using a mathematical model based on directly measured properties of both NADPH oxidase and proton currents in human neutrophils and eosinophils. Because NOX2 activity is electrogenic[168,189] and is directly inhibited by depolarization,[171] the additional depolarization that would result if H_V1 gating were not enhanced would decrease the rate of ROS production by 15−20%.[77]

Similarly, when NOX2 is active, its activity tends to lower the pH_i (Figure 12). The extent to which the pH_i falls in a human neutrophil during phagocytosis when H_V1 is blocked by Zn^{2+} or in HVCN1 knockout mouse neutrophils[48,49] is sufficient by itself to inhibit NOX2 substantially.[190] A delayed and weaker H_V1 response due to a hypothetical absence of enhanced gating would compound the impairment of NOX2 activity. An

DOI: 10.1021/acs.biochem.5b00353
Biochemistry 2015, 54, 3250−3268

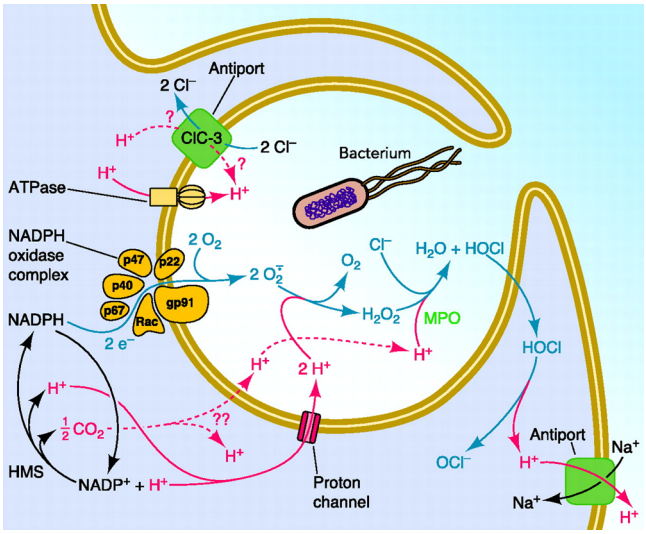

Figure 12. Symbiotic relationship that exists between H_V1 and NOX2, the phagocyte NADPH oxidase enzyme complex. When a bacterium is engulfed into the nascent phagosome, NOX2 rapidly assembles in the phagosome membrane (the membrane is depicted as a tan double line) and becomes extremely active, producing a "respiratory burst". This enzyme produces bactericidal reactive oxygen species (ROS) by extracting two electrons from NADPH inside the cell and transferring them sequentially across a redox chain to reduce extracellular oxygen (O_2) to superoxide anion ($O_2^{\bullet-}$), from which other ROS are produced. Because NOX2 moves electrons across the membrane, it is electrogenic and tends to depolarize the phagosome membrane,[168] while leaving behind a proton in the cell tends to acidify the cytoplasm.[169] H_V1 in phagocytes[50,62,63,170] acts as a countercharge, limiting depolarization.[48,155,171−175] At the same time, efflux of H^+ through H_V1 restores the cytoplasmic pH.[49] Two other vital consequences of H_V1 activity during the respiratory burst are limiting the osmotic consequences that would occur if other ions compensated charge and providing substrate (H^+) necessary for ROS production in the phagosomes.[77] Roughly 95% of the charge compensation required is provided by H_V1,[77] and ROS production is attenuated by up to 75% in H_V1 knockout mice[47,48,50−52,72,78,176] or rats.[177] Reprinted with permission from ref 15. Copyright 2010 American Physiological Society.

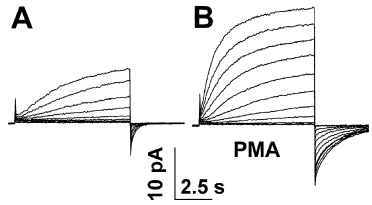

Figure 13. Enhanced gating mode of H_V1 induced in a voltage-clamped human neutrophil. Currents in 10 mV increments up to 80 mV (A) before and (B) after PMA (phorbol myristate acetate) stimulation. Phosphorylation of H_V1 by the PKC activator, PMA, results in faster activation, slower deactivation, larger currents, and activation at more negative voltages. All changes increase the activity and response of H_V1. Reprinted with permission from ref 154. Copyright 2008 National Academy of Sciences.

increased level of apoptosis in human eosinophils with HVCN1 knocked out was attributed to deleterious effects of both depolarization and low pH_i on cell viability.[72] Thus, H_V1 is needed not only to facilitate ROS production but also to prevent NOX2-induced apoptosis.

BIOLOGICAL FUNCTIONS OF PROTON CHANNELS

The Functions of H_V1 Differ Widely in Different Cells. When H_V1 channels open, several things change. Under normal circumstances, because of ΔpH-dependent gating, H^+ efflux occurs, which (a) increases the pH_i, (b) decreases the pH_o, and (c) hyperpolarizes the membrane potential. Each of these changes may have very different consequences in different cells. As an example of category a, human basophils are involved in immune responses and produce histamine. In these cells, IgE stimulates H_V1, producing enhanced gating, and inhibition of H_V1 by Zn^{2+} reduces the rate of histamine release, apparently by accumulation of excess intracellular acid.[68] By extruding acid, H_V1 keeps the pH_i within a range compatible with histamine secretion.

The first proton channels characterized by voltage clamp were in snail neurons.[58] The action potential in these cells occurs as a result of influx of Ca^{2+} through Ca^{2+} channels. Ca^{2+} is then rapidly extruded via Ca^{2+}/H^+ exchange at the expense of cytoplasmic acidification. The depolarization during the action potential is thought to open H_V1, which have very rapid activation kinetics in these cells,[55] resulting in H^+ efflux, thereby restoring pH_i[191] (category a).

In airway epithelium, H_V1 appears to regulate the pH_o in airway surface liquid,[69,192,193] an example of category b described above.

H_V1 plays multiple roles in human sperm, illustrating categories a and c. Inhibiting H_V1 reduces sperm motility, apparently by facilitating NOX5-dependent ROS production[194] (category c). Before a sperm can fertilize the egg, it must undergo capacitation, a kind of maturation process that is triggered by an increase in pH_i as well as ROS.[195−197] Because seminal fluid contains large amounts of zinc,[198,199] which in divalent cationic form (Zn^{2+}) potently inhibits H_V1,[200] it was proposed that the pH_i remains low until the sperm enters the female reproductive tract.[197] As Zn^{2+} inhibition is relieved, H_V1 extrudes acid, thereby increasing pH_i (category a) and triggering capacitation.

Two further examples of category c, in which the key effect of H_V1 activation is electrical, include compensating for the electrogenic activity of NADPH oxidase, discussed in Figure 12, and triggering the flash in bioluminescent dinoflagellates, as described in Figure 11.

Not Everything H_V1 Does Is Good: Used Incorrectly, H_V1 May Be Detrimental to Your Health. Under normal circumstances, the ability of H_V1 to facilitate ROS production in phagocytes is beneficial, in that pathogens are killed. However, when microglia (macrophages of the brain) produce too much ROS, this may cause local tissue damage. Thus, H_V1 in microglia[64] may enable the generation of excessive ROS in Alzheimer's disease.[201] Excessive ROS production occurs and is thought to contribute to myriad other pathological conditions, such as atherosclerosis, ischemic stroke, Parkinson's disease, ischemic liver disease, and aging.[166] In the case of ischemic stroke, a thorough study has confirmed the prediction that H_V1 can exacerbate brain damage precisely by this mechanism, namely by facilitating production of ROS by NADPH oxidase in microglia.[176] The involvement of H_V1 in B cell receptor signaling suggests that inhibitors might be useful in auto-immune diseases and B cell malignancies.[202,203]

Several genomic studies have identified the *HVCN1* gene as being relevant to disease. *HVCN1* was one of four genetic predictors of susceptibility to hyperoxia-induced lung injury

DOI: 10.1021/acs.biochem.5b00353
Biochemistry 2015, 54, 3250−3268

identified in mice.[204] *HVCN1* was one of five genes identified by microarray screening to correlate with Crohn's disease activity.[205] Intriguingly, *HVCN1* was among three genes with levels that were depressed in human cystic fibrosis but responded to therapy by returning to normal levels.[206]

Recent studies suggest that H_V1 may contribute to the malignancy of several cancers. Higher levels of H_V1 expression occur in breast cancer cell lines with greater metastatic tendencies.[207] Knockdown of H_V1 in breast cancer cell lines reduced the extent of proliferation and invasiveness.[208] H_V1 knockdown also decreased the rate of growth of tumors implanted in mice. Finally, in human patients, a high level of H_V1 expression was correlated with poor prognosis.[208] A very similar story was reported for colorectal cancer.[209] These studies were based entirely on biochemical and genetic measurements; however, it has been confirmed directly by the patch-clamp technique that human breast cancer cell lines do have hH_V1 currents.[210] Presumably, the mechanism by which H_V1 improves the lives of cancer cells is related to their abnormal metabolism. Cancer cells use glycolysis in preference to oxidative phosphorylation even in the presence of adequate oxygen. This phenomenon, named the Warburg effect after its discoverer,[211] creates a buildup of lactic acid that acidifies the cells, thus requiring enhanced activity of H^+ extrusion mechanisms to prevent cell death. Consistent with this view, inhibiting H_V1 with Zn^{2+} induced apoptosis of metastatic SHG-44 glioma cells.[212] As anthropocentric humans, we rejoice when H_V1 prevents eosinophil apoptosis,[72] but not when cancer cells are the beneficiaries!

In humans, hH_V1 is usually present as the full-length 273-amino acid protein. However, Melania Capasso, Martin Dyer, and colleagues identified a second translation initiation site 60 bp downstream from the normal ATG and showed that this generates a splice variant that lacks the first 20 amino acids.[47] This short isoform is barely detectable in immunoblots of normal B lymphocytes but is expressed strongly in several malignant B cell lines and comprises roughly one-third of the H_V1 protein in malignant B cells from patients with chronic lymphocytic leukemia.[53] In unstimulated cells, the electrophysiological properties of short form H_V1 were similar to those of the long form, but activation was slower. However, upon stimulation by phorbol ester (PMA), the enhanced gating response (Figure 14) was substantially more pronounced in short than in long form H_V1. The net result would be a higher level of H_V1 activity in malignant B cells. H_V1 colocalizes with the B cell receptor (BCR),[47] but the short isoform interacts more weakly. Consequently, internalization of the BCR-H_V1 complex occurs to a lesser extent in cells expressing the short isoform, which further increases the remaining H_V1 activity on the plasma membrane.[53] Proliferation and cell migration were also promoted by short form expression.[53] These properties of the short isoform are consistent with the idea that excessive H_V1 activity supports malignancy.

A study of HVCN1 knockout rats produced evidence that H_V1 in kidney thick ascending limb modulates NADPH oxidase activity according to intracellular sodium levels and thereby contributes to the development of hypertension and renal disease in rats fed a high-salt diet.[177]

WHY ARE MAMMALIAN H_V1 DIMERS?

Several lines of evidence support the conclusion that H_V1 from several multicellular species (human, mouse, and the sea squirt *Ciona intestinalis*) exist as dimers.[8−10,213] The main region of

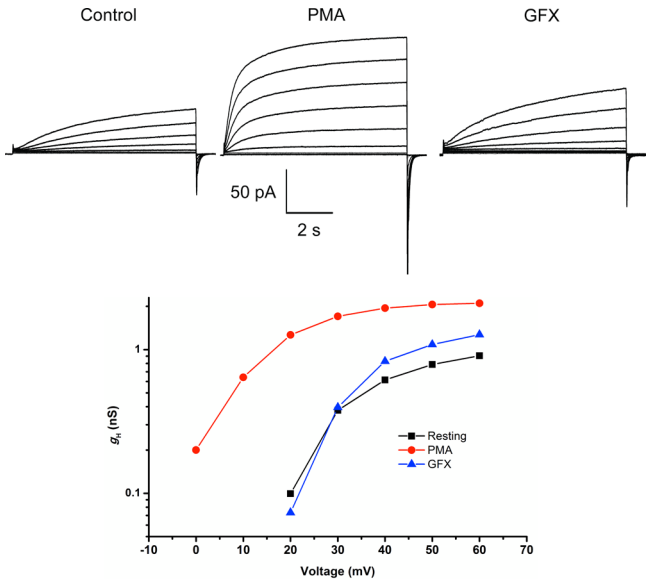

Figure 14. Enhanced gating response of hH_V1 in a B lymphocyte from a patient with chronic lymphocytic leukemia. The control is the g_H derived from a family of proton currents in a resting cell. After PMA stimulation, the current amplitude increased, activation (turn-on) became faster, and as is evident in the graph, the g_H turned on at voltages at least 20 mV more negative. The PKC inhibitor, GF109203X (GFX), reversed all of these changes, confirming they were due to phosphorylation of the channel. Reprinted with permission from ref 53. Copyright 2014 National Academy of Sciences.

attachment of the dimer is a coiled-coil region in the intracellular C-terminus.[8−10,13,78,90,214] H_V1 in several (but not all) unicellular species (*Karlodinium veneficum*, *Phaeodactylum tricornutum*, and *Thalassiosira*) lack predicted coiled-coil regions and therefore may exist as monomers.[213] When H_V1 from species with dimeric channels are forced to exist as monomers, they still function reasonably normally. Monomeric H_V1 open 3−7 times faster than their dimeric counterparts,[8,10,12,92] and their g_H−V relationship is somewhat (10−15 mV) more positive.[10,12] These are relatively subtle differences in the grand scheme of things, because if a cell really needed an H_V1 with these properties, one should have evolved.

A more probable explanation for the dimeric architecture of H_V1 arises from the observation that the protomers gate cooperatively, not independently. This kind of behavior is reasonable for tetrameric voltage-gated ion channels (Figure 1) whose four VSDs surround a single central pore and all contribute to prying it open. It *is* remarkable for H_V1, because each protomer has its own conduction pathway. Nevertheless, it appears that by some mysterious mechanism, both protomers must undergo a voltage-induced conformational change before either conduction pathway opens.[12−14,92,214,215] One consequence of this arrangement is that the probability that dimeric H_V1 will open is twice as steeply dependent on voltage as that of the monomeric form.[92,214,216] This property may be important enough to rationalize the existence of dimeric H_V1, as may become evident by considering H_V1 in phagocytes.

The best-studied function of H_V1 occurs in white blood cells that are phagocytosing bacteria or other alien life forms (Figure 12), where the primary role of H_V1 is to allow NOX2 (the NADPH oxidase enzyme complex) to produce large quantities

DOI: 10.1021/acs.biochem.5b00353
Biochemistry 2015, 54, 3250−3268

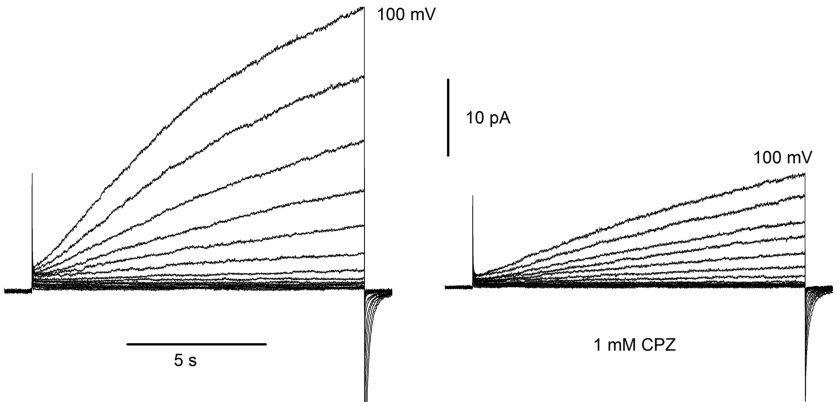

Figure 15. Weak base inhibitors are weak inhibitors of hH_V1. Families of currents during identical 12 s pulses in 10 mV increments up to +100 mV from an inside-out patch of membrane, in the absence or presence of 1 mM chlorpromazine (CPZ). The cationic form of CPZ was proposed to inhibit from the internal solution.[225] Unpublished data by V. V. Cherny and T. E. DeCoursey.

of reactive oxygen species (ROS) over a sustained time period. In this situation, H_V1 is activated mainly by the depolarization resulting from NOX2 activity,[77] which opens enough H_V1 channels to balance the efflux of electrons through the enzyme. If H_V1 were a monomer, greater depolarization would be needed because of the weaker voltage sensitivity of the monomer. Depolarization directly inhibits NOX2 activity, because it opposes the tendency of electrons to leave the cytoplasm.[171] In this context, a dimeric H_V1 has a decided advantage over the monomeric form.[23,213]

Phagocytes produce vast quantities of ROS, mainly to kill pathogens. Many other cells also produce ROS, but in much smaller amounts and for purposes of signaling rather than indiscriminately wreaking havoc. H_V1 knockout or inhibition impairs ROS production in several of these cells, presumably by the mechanism illustrated in Figure 12. Examples include human sperm, in which ROS is produced by NOX5,[194] human B cells,[47] and kidney thick ascending limb cell cells when the intracellular Na^+ concentration is low.[177] It is not obvious that cells with very low levels of ROS production, or cells that express abundant other channels, would have such severe problems with membrane depolarization as phagocytes do. In some cases, the role of H_V1 may involve direct interaction with other molecular complexes, as observed in B cells,[47,53] and as suggested by colocalization with NOX2 in human eosinophils.[78,217]

■ WHAT BLOCKS H_V1 CHANNELS?

There is substantial interest in identifying potent and selective inhibitors for H_V1.[45] Such an inhibitor would be useful in "pharmacological lesion experiments" to clarify biological functions of H_V1, and potentially for use as a drug to intervene in any of the numerous pathophysiological situations in which H_V1 are involved. For both purposes, a compound with an extracellular site of action is greatly preferred. There may also be applications in which one would like to increase H_V1 activity. Agents in the latter category thus far include mainly biological activators such as unsaturated long chain fatty acids like arachidonic acid,[63,70,158,179,180,183] as well as activators of enhanced gating (see Regulation of H_V1 by Phosphorylation: Enhanced Gating Mode). More comprehensive reviews of H_V1 pharmacology may be consulted for further details.[45,218] Here we present an overview.

Polyvalent Metal Cations. The first identified H_V1 inhibitors were polyvalent metal cations. The proton current in snail neurons was inhibited most potently by Zn^{2+}, Cu^{2+}, La^{3+}, Ni^{2+}, and Cd^{2+},[58,219−221] and H_V1 in amphibian[60,222] and mammalian cells share this sensitivity.[61,64,65,71] Obviously, these metal ions interact with many proteins, but Zn^{2+} inhibited H^+ currents 80 times more potently than Ca^{2+} currents, making it useful in separating these currents in *Helix* neurons.[220] During the *fin de siècle* era, as proton currents were identified in several new cells and species each year, demonstration of Zn^{2+} and Cd^{2+} inhibition was *de rigueur* in confirming a conductance to be proton current. Obviously, this was nonsensical, but only recently have confirmed H_V1 that are insensitive to polyvalent metal cations been identified.[42,99,223] A systematic study of interaction of Zn^{2+} with the rat alveolar epithelial proton channel revealed striking competition between Zn^{2+} and H^+ for an externally accessible inhibitory binding site.[200] Strongly pH_o dependent inhibition could be described by a site with at least two titratable groups with a pK_a of ∼7 that prevented channel opening when bound by Zn^{2+}. This prediction appeared to be confirmed seven years later when the gene was identified, along with two His residues that contributed to Zn^{2+} sensitivity.[5] Examination of an early homology model of the open hH_V1 suggested that His140 and His193 were too far apart to coordinate a Zn^{2+} ion, and it was proposed that Zn^{2+} binds at the interface between protomers in the dimer.[13] The recent crystal structure of mouse H_V1 in the closed state surprisingly revealed Zn^{2+} coordinated between these two His residues within each protomer.[91] In addition, two other residues (Glu119 and Asp123 in the human sequence) were found to contribute more subtly to Zn^{2+} binding.[91] Evidently, the homology model was imperfect, or the relative positions of the two His residues (which are on different helices) differ in closed and open channels. The recently published structural information does appear to reconfirm the original prediction that two His residues coordinate Zn^{2+} in the closed channel, and thereby prevent it from opening.[200]

Organic Inhibitors. In contrast to most ion channels, H_V1 has no high-affinity blockers that originate in venom or toxin. Tarantula toxins, including hanatoxin, do inhibit H_V1 at low micromolar concentrations.[224] However, the mechanism is not pore occlusion, but interaction with the "paddle motif" (helices S3b and S4), with the toxin approaching from the membrane interior and shifting the g_H−V relationship in the positive direction. At least two explanations for the lack of inhibitors

DOI: 10.1021/acs.biochem.5b00353
Biochemistry 2015, 54, 3250−3268

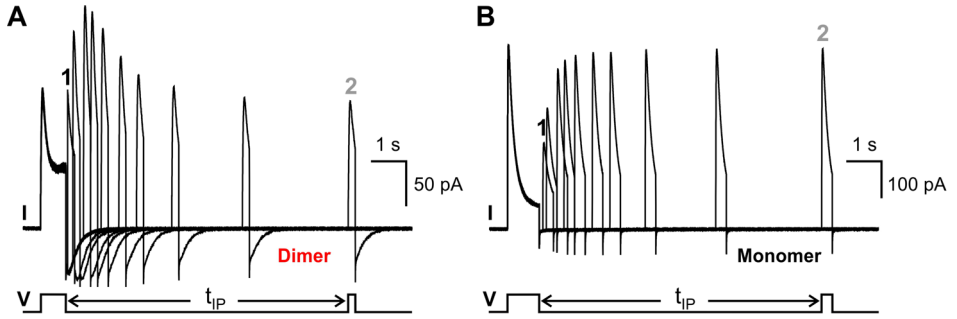

Figure 16. Complex interaction of 400 nM 2GBI with the F150A mutant of hH_V1 seen in dimer (A) but not in monomer (B). Pulse pairs to +140 mV were applied separated by a variable interval at −80 mV. Current decay during the pulses reflects block by internally applied drug, with the time course of recovery reflected in the peak current during the second pulse of the pair. Reprinted from ref 215. Copyright 2013 Elsevier.

may be postulated. The extracellular portions of the hH_V1 molecule are relatively small; both extracellular loops comprise fewer than a dozen amino acids. This property also limits the epitope possibilities for antibodies that might act externally, for example, on intact, living cells. The second explanation is perhaps more in keeping with the theme of this review: the challenge for an inhibitor to physically occlude a proton pathway is much greater than for a K^+ pathway, for example. Every proton-selective pathway reiterates the unique ability of protons to negotiate conduction through vanishingly small spaces. In fact, to create a proton-selective pathway, one key design principle is to invoke close protein packing, to prevent other ions from permeating.[23]

A large number of weak bases such as 4-aminopyridine,[55,56,60,64,221] amiloride,[226] verapamil or D600,[56,221] and others[188] inhibit proton currents to some extent. In several cases, it was suspected that the mechanism might be permeation of the weak base in neutral form into the cell where it would take up a proton and increase the pH_i, thus appearing to inhibit the current without directly interacting with the channel at all.[188,221] This mechanism was shown to explain the apparent inhibition of H_V1 by local anesthetics.[227] However, several other weak base inhibitors have been reported not to change the reversal potential or to shift the g_H-V relationship (both of which are highly sensitive to pH_i), suggesting a different mechanism. Drugs in this category include antidepressants (imipramine, amitriptyline, desipramine, and fluoxetine),[228] antipsychotics (chlorpromazine, haloperidol, and clozapine),[225,229] dextromethorphan,[230] and (−)-epigallocatechin 3-gallate.[231] These drugs were all studied with a large ΔpH (pH_o 7.3, pH_i 5.5), which would concentrate the charged form of a weak base 63-fold at the intracellular site of action;[232−234] hence, the reported IC_{50} values in the low micromolar range need to be multiplied by 63 to estimate the potency at a symmetrical pH of 7.3. For example, the K_D for chlorpromazine was reported to be 2.2 μM under the conditions described above.[225] Figure 15 shows the effect of 1000 μM chlorpromazine added to an inside-out patch at a symmetrical pH of 7. Given its high pK_a,[235] >99% of the drug is in the effective cationic form at the proposed internal site of block. The proton current is distinctly attenuated, but the required concentration is too high to be of pharmaceutical interest.

Guanidinium Derivatives. Guanidinium inhibits hH_V1 from the internal but not external side with a K_D of 1 mM.[10] Even weaker inhibition was evident in a later study reporting large hH_V1 currents in the presence of symmetrical 100 mM guanidinium chloride solutions.[99] Tombola's group has sought potent organic inhibitors by testing a number of compounds chemically related to guanidinium. Two potent inhibitors were identified, of which 2GBI [2-guanidinobenzimidazole (K_D = 38 μM)] was investigated in detail. It was found to bind only from the intracellular solution and only when the channel was open. As illustrated in Figure 16, the kinetics of recovery from 2-GBI block was simple when a monomeric hH_V1 construct was studied, but complex and nonmonotonic for the native dimer.[215] This interaction was interpreted to mean that binding of drug to one protomer affects the gating kinetics of the other protomer in the dimer. Analysis of additional related compounds revealed 5-chloro-2-guanidinobenzimidazole with a K_D of 1.6 μM and two others nearly as potent.[236] This potency approaches pharmaceutical interest, although the intracellular site of action remains an obstacle to be overcome.

■ FUTURE OUTLOOK FOR H_V1 CHANNELS

Reprising a review written in 2008 that listed outstanding questions about H_V1[237] reveals some genuine progress, some backsliding, and many incremental advances. The number of species with confirmed H_V1 genes is now ten instead of four. H_V1-like genes exist in dozens of species in which channel activity has not yet been confirmed by expression and voltage-clamp study. A short isoform of hH_V1 that exhibits functionally important differences compared to the full-length protein has been identified. The number and variety of biological functions of H_V1 have proliferated to an extent that both mimics and includes cancer. It would be surprising if H_V1 were not discovered in numerous additional cell types and tissues, where novel and important functions will doubtless be identified. Real progress has been made in understanding how H_V1 achieves proton selectivity. However, a number of central questions linger, and important properties of H_V1 remain mechanistically mysterious. Current models of closed and open H_V1 channels are comically diverse and mutable. Which part of the molecule moves during channel opening and by how much are other topics that are subject to creative interpretation of inadequate data. That H_V1 is a dimer whose protomers gate cooperatively has been established, but the mechanism of cooperative gating of dimeric H_V1 is poorly understood. Potent, selective, inhibitors that act through external application are elusive. Finally, the mechanism of the quintessential feature of H_V1, namely ΔpH-dependent gating, remains inscrutable. There is plenty to keep us occupied and entertained for some time.

DOI: 10.1021/acs.biochem.5b00353
Biochemistry 2015, 54, 3250−3268

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Funding

Supported by National Institutes of Health Grant GM102336 and National Science Foundation Grant MCB-1242985.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

ΔpH, pH gradient defined as $pH_o - pH_i$; E_H, Nernst potential for H^+; g_H, H^+ conductance; g_H–V, g_H versus voltage; H_V1, proton channel; HVCN1, proton channel gene product; K_D, dissociation constant; pH_i, intracellular pH; pH_o, extracellular pH; PKC, protein kinase C; ROS, reactive oxygen species; S1, transmembrane segment 1; VSD, voltage-sensing domain; WT, wild-type (native protein without mutations).

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DOI: 10.1021/acs.biochem.5b00353
Biochemistry 2015, 54, 3250−3268