Published in final edited form as:

Wiley Interdiscip Rev Membr Transp Signal. 2012 September 1; 1(5): 605–620. doi:10.1002/wmts.55.

Biophysical properties of the voltage gated proton channel H_V1

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Abstract

The biophysical properties of the voltage gated proton channel (H_V1) are the key elements of its physiological function. The voltage gated proton channel is a unique molecule that in contrast to all other ion channels is exclusively selective for protons. Alone among proton channels, it has voltage and time dependent gating like other “classical” ion channels. H_V1 is furthermore a sensor for the pH in the cell and the surrounding media. Its voltage dependence is strictly coupled to the pH gradient across the membrane. This regulation restricts opening of the channel to specific voltages at any given pH gradient, therefore allowing H_V1 to perform its physiological task in the tissue it is expressed in. For H_V1 there is no known blocker. The most potent channel inhibitor is zinc (Zn²⁺) which prevents channel opening. An additional characteristic of H_V1 is its strong temperature dependence of both gating and conductance. In contrast to single-file water filled pores like the gramicidin channel, H_V1 exhibits pronounced deuterium effects and temperature effects on conduction, consistent with a different conduction mechanism than other ion channels. These properties may be explained by the recent identification of an aspartate in the pore of H_V1 that is essential to its proton selectivity.

The voltage gated proton channel has a number of surprising characteristics, but the most prominent may be its ability to conduct protons (H⁺) through the cell membrane with perfect selectivity. Experimentally this can be determined by reversal potential measurements and ion substitution. For both native^{4–9} and expressed proton channels^{10, 11}, these experiments indicate high selectivity. It is astonishing that this is the only known voltage gated ion channel which conducts solely protons. There are other voltage gated channels which are selective to their “main ion” and in addition can conduct protons. Thus, voltage gated sodium channels have been reported to conduct protons in addition to sodium^{12–15}, with $P_H/P_{Na} \sim 250$. Sodium channels conduct only a proportionally small amount of protons depending on the solutions they are recorded in. Under the ionic conditions and ion concentrations in the body (150 mM Na⁺ and 60 nM H⁺, a 2,500,000 fold difference), for example, if the permeability for protons were the same as for sodium in a sodium channel, 1 H⁺ would be conducted with every 2.5×10^6 sodium ions. This ratio would change to 1.5 Na⁺ to 1 H⁺ at pH=1. Protons are the smallest ions. This characteristic makes it hard for molecular sieves (ion channels) to exclude them. Table 1 compares the mass and radius of protons with other ions and electrons. It emphasizes the difference between the next smallest cation Li⁺ and H⁺ with a radius difference of 39,000 times.

One large family of cation channels, the potassium channels, have not been reported to conduct any protons¹⁶. In the absence of experimental data it is not completely clear whether protons can pass the potassium channel pore with at least some conductivity.

Exclusive proton selectivity is a hallmark of the voltage gated proton channel. Protons are cations, but in contrast to all other cations, they lack the electron shell. When protons are hydrated they exist mainly as hydronium ions (H₃O⁺), but also as more complex forms such as Zundel (H⁺ + 2H₂O)¹⁷ or Eigen cation (H₃O⁺ + 3H₂O)¹⁸. Movement of protons through

water must be considerably fast since the lifetime for hydronium is estimated between 0.24–3 ps^{18–26}.

The Grotthuss mechanism

The general movement of ions with a hydration shell through water is defined by friction. The hydrated ion collides with water molecules slowing it down. Proton diffusion through water occurs by a different mechanism called “Grotthuss mechanism” (Fig. 1), with the result that H⁺ movement is 4.8 times faster than potassium ion in pure water at room temperature. During the Grotthuss mechanism a single proton binds to a water molecule and creates a hydronium. Any one of the three protons may leave the hydronium ion and move to the nearest water molecule orientated to accept the proton, producing a new hydronium. The former hydronium is transformed into a water molecule again by dispensing the proton.

The way a proton moves through a protein is envisioned as via a hydrogen bond chain (HBC). It can be imagined as proton hopping through a water file, where parts of an amino acid will be donor & acceptor for the proton instead of a water²⁷.

Neither carrier nor pump, but a channel

Despite being an individualist in the cation channel family, the voltage gated proton channel is unquestionably an ion channel²⁸. The first pages of several basic physiology text books^{16, 29–31} describe the essential distinction between pumps, carriers and ion channels. Table 2 summarizes how the proton channel fits into these three categories. The proton channel shows conformity with all but one of the characteristics for an ion channel. The proton channel does not consume energy. In excised and whole cell patches without GTP or ATP in the pipette or bath it still conducts protons. There is no co- or counterion for the proton channel. The reversal potential (E_H) of the proton channel follows almost perfectly the Nernst equation for protons. The voltage gated proton channel is electrogenic.

Returning back to the categories of Table 2, ion channels do have conformational changes. Gating is a conformational change of the proton channel, but it happens before the channel can conduct and gives valuable information about structure and function of the channel^{3, 32, 33}. After opening of the proton channel there are no further known conformational changes and therefore, it has no conformational changes during conduction.

The conductance of protons through the channel is considerably small, compared with all other ion channels. The turnover rate is among the smallest in the ion channel field except for some chloride proton antiporters³⁴ which have low turnover rates and the CRAC channels with a turnover rate of about 11,000 ions per second³⁵. The smallest turnover rate for a channel may be by the viral M₂ proton channel with estimates based on different measurements ranging from ~1–7 H⁺ per second³⁶ to 80 H⁺ s⁻¹^{37, 38}, to 62,500 H⁺ s⁻¹³⁹. The turnover rate of ~6,000 ions per second⁴⁰ is clearly more in the range of carriers than ion channels. Here again the low concentration of protons, ~60 nM in cytoplasm, might be responsible for this lower turnover rate. It would be intriguing to measure experimentally whether the proton channel turnover rate is more in the ion channel range at a pH 1 (100 mM H⁺) or 2 (10 mM H⁺). Based on these classifications the proton channel is an ion channel that conducts a rather rare cation.

Measurable properties of the voltage gated proton channel

To introduce the description of the biophysics of the proton channel it might be helpful to describe the properties most commonly measured during electrophysiological experiments. There are six basic properties, which can be determined from patch clamp measurements.

Max I_H

The **maximal proton current** measured at steady state. This is surprisingly difficult to determine experimentally, because all proton currents are subject to proton depletion^{41, 42}. Even with proton buffers as high as 100 – 200 mM in the pipette solution, proton channels exhibit depletion. Depletion can be explained as protons leaving the cell through proton channels faster than buffer molecules can diffuse from the pipette into the cell to restore the pH_i . The result is droop in currents, wrong maximal amplitudes, changed kinetics of activation, and shifts of the reversal potential. Numerous studies in which proton current and internal pH were measured simultaneously have shown that the droop is a result of increasing pH_i ^{41–45}. Only small amplitude currents close to threshold can be assumed not to be seriously affected by proton depletion. Maximal currents can differ substantially over the time course of a long experiment⁴⁰.

One way to reduce the influence of depletion is to estimate the maximal current by fitting the current traces with a single exponential function plus a short delay and taking the extrapolated maximal value as the maximal proton current (Fig. 2). Thus finding the correct fit for proton current might not be a trivial task. Often unmentioned is that determining the maximal current amplitude deducted from single exponential fits has some complications, too. In some cells a steady turn on of proton current is measured over several minutes. Maximal currents can differ substantially over the time course of a long experiment⁴⁰.

g_H -V

The numerical value of the conductance is not directly visible during the recording. The conductance must be calculated and then can be plotted as function of voltage. These diagrams are called conductance voltage plots (g_H -V). Comparing conductance voltage curves conveniently reveals changes in conductance and threshold. Furthermore they can be used to determine gating charges. We will return to these later in the review.

It is necessary to determine the proton current correctly to deduce the conductance. To avoid depletion problems in g_H -V curves Musset et al.¹ used an elaborate “end of pulse & tail current” approach to minimize some of the problems with conductance voltage curves. The following equation was used: $(I_{\text{end}} - I_{\text{tail}})/(V_{\text{test}} - V_{\text{hold}})$ (Fig 3).

At the endpoint of a pulse a certain number of channels are open giving rise to current (I_{end}). When the tail (I_{tail}) current is measured milliseconds later, approximately the same number of channels are open. Constructing a line through these two points on the current voltage graph, allows one to directly identify the reversal potential at the intersection with the abscissa⁴⁶. The result maybe slightly offset, if there is leak current or the instantaneous current voltage (I -V) curve is not linear. The slope of the constructed line is also a direct indication of the conductance (the “slope” conductance).

The “end of pulse & tail current” method has the advantage that the conductance of each pulse can be defined without knowing the reversal potential, because the calculation gives the slope conductance, not the more usual chord conductance.

The g_H -V is strongly affected by changes in pH_i through depletion. Through the slope conductance it is possible to adjust the conductance according to the shift in reversal potential due to depletion. There is often little saturation of H^+ conductance at potentials very positive to the reversal potential, which makes a correct Boltzmann fit less applicable. Boltzmann fits are commonly used for classical voltage gated channels to show differences in conduction and voltage dependence. For classical voltage dependent ion channels depletion is almost unknown, since in most cases the physiological solutions have at least millimolar concentrations of the conducted ion.

The best way to describe the conductance of the voltage gated proton current is maybe to be as clear as possible about the way it has been determined. In general, using the equation $(I_{\text{end}} - I_{\text{tail}}) / (V_{\text{test}} - V_{\text{hold}})$ is a practicable way.

τ_{act}

The time course of the increase of current during a voltage pulse reflects the speed with which the average proton channel opens. This has been quantified in several ways. τ_{act} is determined as a time constant (τ) of a single exponential equation. For proton channels τ_{act} is a rather useful tool to compare the speed of opening from different tissues, mutants, species, etc. The activation kinetics were previously defined as maximal rate of current rise of activation but this parameter depended strongly on the g_{H} of the each cell, so it was less applicable for comparisons^{5, 47}.

Another method was used by Koch et al.⁴⁸ who defined the activation kinetics as the time to half peak during depolarization for 500 ms. This method might introduce error since not every current trace might reach maximal value in a 500 ms pulse. Taken together the exponential method is the most practicable used today.

Even though the time course of current activation is not a simple exponential curve, for convenience it can be fitted as single exponential with a delay (Fig. 2).

V_{thres}

V_{thres} is the **voltage threshold of activation**. The parameter gives the voltage where the proton channel first opens (Fig. 4). It is dependent on the pH gradient across the membrane⁴⁹. In most cells at symmetrical pH it is 20 mV positive to the reversal potential. It changes 40 mV per unit change in ΔpH ⁵. This regulation of the voltage threshold by pH allows the channel to conduct protons solely in the outward direction. This feature makes the channel comparable to a diode in allowing unidirectional flow. Due to closing of the channel at sub threshold voltages the tail currents exhibit inward currents negative to reversal. Thus inward current is possible, but does not normally occur in conditions present in most cells. Unexpectedly the expressed voltage gated proton channel does not share this property of having a threshold only positive to reversal¹. For unknown reasons the threshold of the expressed channel is somewhat more negative than in the endogenous channel; at symmetrical pH V_{thres} was -10 mV ¹ or $+7$ ⁵⁰. By careful screening of 95 human genomic DNA samples, the Fischer group found a natural occurring mutation in the human proton channel at position M91T, which had an influence on V_{thres} ⁵¹. Threshold changes will be discussed in more detail when talking about the “enhanced gating mode” of the voltage gated proton channel.

τ_{tail}

The kinetics of channel closing can be determined by the time constant of the deactivating current (the “tail current”). Deactivation is not to be confused with inactivation. Inactivation typically occurs during a depolarization step, when the channel closes and therefore the current diminishes. After repolarization recovery from inactivation must occur before the channels can be re-opened. Deactivation is the closing of the channel after the voltage pulse. The proton channel does not close during sustained depolarizing pulses! Many voltage gated ion channels (Na^+ , K^+ , Ca^{2+} , Cl^-) do inactivate. The first experimental evidence to provide a reasonable physical mechanism for inactivation was by Armstrong⁵². In contrast to other channels, proton channels do not inactivate. Sometimes change of reversal potential during pulses can result in decaying currents during the pulse. This phenomenon is not connected with inactivation; it is caused by depletion.

In all proton channels measured so far the tail currents could be fitted with a single exponential function (Fig. 2). One exception is in rat alveolar epithelial cells near V_{thres} , where a second slower component appears⁵. The tail currents are used for reversal potential measurements (Fig. 5) and also describe the average time constant for single channel closing.

In stationary noise measurements and their resulting power spectra, Lorentzian fits exhibited a corner frequency ($\tau_{\text{Lorentzian}} = [2\pi f_c]^{-1}$) that reflected the kinetics of the tail current⁵³. Since the power spectrum is taken from the stationary noise measurement during depolarization, it may not be obvious why a channel closing rate can be seen. Channel noise is generated from the stochastic opening and closing of multiple channels. Thus even at a depolarizing voltage a fraction of channels closes, some open or re-open.

In addition, analysis of single channel data⁵³ showed that the mean open time of the single channel current was similar to the tail current kinetics.

E_{rev}

The reversal potential is maybe the most important parameter of an ion channel. It directly indicates selectivity. Experimentally in proton channel voltage clamp studies it can be determined by tail currents⁴¹ (Fig. 5), or tail ramps^{3, 42, 54} and sometimes it can be directly seen during a family of pulses if the channel opens before reaching the reversal potential¹. Because of its perfect selectivity, theoretically and practically a proton channel can be used as a pH meter. The Nernst Equation predicts a change of roughly 59 mV per unit pH at 25°C. A shift of half a unit pH higher outside would produce a 29.5 mV shift negative in the reversal potential. By measuring V_{rev} and knowing pH_o one can calculate pH_i . The reader might think about other uses.

The six criteria are the basis of the further topics of this review.

One gene codes for the proton channel in mammals but H_V1 exhibits varying biophysical properties

Proton currents have been measured in various human and mammalian tissues as well as in more distant species as snails, sea urchin, and unicellular marine life forms. It is possible to generate a reasonable list of tissues still to be tested and furthermore animals and even plants to be screened for proton channels⁵⁵.

Only one gene has been found to code for the voltage gated proton channel in mammals. Lee et al.⁵⁶ expressed the human channel and purified it before reconstituting it into liposomes. They showed that the H_V1 gene product alone and no other protein is needed to generate voltage gated proton currents.

Interestingly there are profound variations in the properties of proton currents in phagocytes. Phagocytes are white blood cells which are able to ingest harmful and foreign particles (phagocytosis) e.g. bacteria, dead cells and proteins. As discussed below, this pleiotropic behavior results from a single gene product.

Enhanced gating mode

The proton channel in phagocytes, has slow (>5s) activation kinetics and the tail currents exhibit kinetics with a time constant of about 200 ms. The activating current is clearly sigmoid and the deactivating current is exponential. Proton channels of the phagocyte type are detectable in neutrophils, eosinophils, microglia, macrophages, mast cells, basophils

etc.^{40, 42, 43, 54, 57–61}. One unique feature of the phagocyte proton channel is its ability to switch into a more active state. The active state was first described by Bánfi et al.⁶⁰, and was defined as “enhanced gating mode” by DeCoursey²⁸. In neutrophils or eosinophils, enhanced gating means that less depolarization will occur before H⁺ efflux balances electron (e⁻) efflux, which in turn means reactive oxygen species (ROS) production will be 15–20% greater⁶².

Agonists that activate the phagocyte “respiratory burst” (e.g. PMA, AA, fMLF) enhance proton channel gating. The respiratory burst is a drastic increase (up to over 50 times) in oxygen uptake by phagocytes⁶³. As a result of electron flux through NADPH oxidase, the oxygen is converted into superoxide anion (O₂⁻). O₂⁻ is the precursor for ROS responsible for killing bacteria. The activity of the NADPH oxidase results in a depolarization of the phagocyte. Furthermore the cytosol of the phagocyte becomes acidic⁶⁴. The proton channel in phagocytes is responsible for charge compensation, by balancing the e⁻ efflux, and pH regulation, by conducting protons out of the cell.

The “enhanced gating mode” changes the following biophysical properties. The conductance of the proton channel in enhanced gating mode is increased 1.9–2.9 times⁶⁵. The V_{thres} is more negative (Fig. 6) and fascinatingly inward proton conduction is possible. The activation kinetics are up to 5 times faster than in the unmodified channel^{59, 60, 66}. The tail current kinetics are in some way special. Tail current kinetics are strongly slowed in cells which have an active NADPH oxidase. However cells which do not have an active NADPH oxidase exhibit the same increase in activation kinetics but show no slowing of the tail current kinetics at all. There is evidently a link between NADPH oxidase activity and voltage gated proton channel properties, whose mechanism is still unknown. Experiments with different isoforms of the NADPH oxidase or mutated forms of Hv1 may illuminate this riddle.

Enhanced gating could be explained in part in terms of channel phosphorylation^{67, 68}. Morgan et al.⁶⁹ showed that the enhanced gating mode is induced mainly by a phosphorylation step. Even the strong enhancing effects of arachidonic acid^{40, 70} could be partially inhibited by the PKC (Protein Kinase C) inhibitor GFX (GF109203X). These findings led to the conclusion that mainly PKC is modifying the channel. Musset et al.⁶⁸ showed that the expressed proton channel could be phosphorylated in the LK35.2 cell line. During this phosphorylation the activation kinetics got faster, threshold became more negative and maximal current increased. All these changes were comparable to the changes in human basophils⁵⁹. Human basophils do not express a working NADPH-oxidase⁷¹. LK 35.2 cells have low level oxidase activity detectable by luminescence⁷², but oxidase activity was below resolution of patch clamp experiments. Taken together, phosphorylation transforms the proton channel into “enhanced gating mode” but does not reproduce all the changes that occur during the respiratory burst in cells with a working NADPH oxidase.

Oxidase activity manifests itself as an inward current (electron current) with a reversal potential independent of all ion concentrations⁷³. This inward current is inhibitable by diphenylene iodonium (DPI). DPI inhibits NADPH oxidases in white blood cells⁷⁴. NADPH oxidase activity can further be measured as the increase of superoxide radicals or hydrogen peroxide outside of the cell.

To specify the location of phosphorylation on the proton channel Musset et al.⁶⁸ inserted point mutations into two high probability phosphorylation sites in the N-terminus to ablate their function. Only one point mutation at one phosphorylation site (Thr²⁹) prevented the “enhanced gating mode” in the expressed channel. Charge exchanges on this position to a negatively charged phosphorylation mimic (T29D) had no effect on the currents. Thus the

modulation of proton current through phosphorylation seemed not to be based on charge alone.

Since the phosphorylation site is part of the channel, modulation of the expressed channel is possible without any accessory proteins. On the other hand the “enhanced gating mode” in LK35.2 cells was less pronounced than the enhanced gating in phagocytes, perhaps indicating other factors in enhancement.

Refocusing that in each species only one gene of the proton channel is known; it is reasonable to speculate that expression of different PKCs in the cells may explain why the proton channel in phagocytes, LK35.2 and basophils can convert into “enhanced gating mode”, but in alveolar epithelial cells it cannot.

Additional support for the PKC as origin of the “enhanced gating mode” (rather than a novel variant of proton channel, as originally envisaged⁶⁰) is that in the H_V1 knock out mouse, proton channel currents could not be found in alveolar epithelial cells, B lymphocytes, neutrophils and monocytes^{64, 72, 75–78}, indicating that the knock out of the *HVCN1* gene inhibits all functional H_V1 expression. Thus it may be that the key difference between alveolar epithelial cells and phagocytes is the availability of PKC. Alternatively it is possible that different tissues have slightly different proton channel properties through splice variants or posttranslational modification. A short form of H_V1 is detectable in B lymphocytes and related cell lines^{72, 79}.

Before the phosphorylation site was discovered, multiple suggestions were made as to the cause of the “enhanced gating mode”. Bánfi et al.⁶⁰ attributed the changes in V_{thres} , kinetics and zinc (Zn^{2+}) sensitivity to a new proton channel which is connected to the NADPH oxidase. Later DeCoursey and colleagues concluded that the same proton channel is modified to show these new characteristics: first the electron current and the proton current amplitude were not correlated⁶⁶, additionally the apparent difference in zinc sensitivity could be explained due to the conductance shift to more negative potentials in “enhanced gating mode”⁸⁰.

Another possibility to explain the changes of kinetics in the “enhanced gating mode” was introduced by Koch et al.⁴⁸. Koch et al. found that the monomerized form of the proton channel exhibits faster kinetics than the dimer and suggested monomerization as a possible mechanism for the “enhanced gating mode”. However several observations speak against the hypothesis: a) monomeric H_V1 have weaker zinc sensitivity³² than the dimeric form. The zinc sensitivity of proton channels in “enhanced gating mode” is not different than in resting channels⁸¹; b) the monomerized channel has a V_{thres} which is somewhat more positive than the V_{thres} of the dimer⁸¹, in contrast with the 30–40 mV negative shift in V_{thres} seen in the enhanced gating mode; c) activation kinetics in enhanced gating mode is sigmoid in contrast to the exponential activation kinetics in the monomer. It can be concluded that the main reason for “enhanced gating mode” is phosphorylation at Threonine²⁹ and not monomerization of the channel. It would be intriguing to test the response of monomeric channel to phosphorylation.

Brief overview of the voltage gated proton channel structure

The human voltage gated proton channel is composed of 273 AA (Amino acids)¹¹. Based on the primary sequence the channel is predicted to consist of 4 transmembrane domains with a long N-terminus about 100 AA and a shorter C terminus about 52 AA. Between species there is a high consistency of sequence and predicted secondary structure⁸². The transmembrane domains S1–S4 show several consistencies with the S1–S4 of cation

channels, but in contrast to the cation channels, S5–S6 domains known as the pore region of many voltage gated channels are missing (Fig 7).

Voltage gated cation channels are built out of 4 subunits arranged as a tetramer. For the voltage gated proton channel three independent research groups, Tombola, Koch and Lee, produced evidence that Hv1 in expression systems is composed out of two subunits assembling a dimer^{33, 48, 83}. Petheo further showed with Western blots that Hv1 is a dimer in the membrane of human granulocytes⁸⁴.

In stark contrast to the voltage gated cation channels, each subunit of Hv1 by itself is able to conduct protons. Chloride channels share this form of organisation; they are also double barrelled channels composed of two subunits^{85–88}.

Monomerized forms of the proton channel showed proton conductivity (Table 3) indicating that each subunit of the dimer has its own pore. Monomerization could be achieved either by removing the C-terminus of the channel close to the S4 segment^{48, 89}, or by exchanging the N-Terminus and the C terminus for the N- and C-Terminus of the voltage gated phosphatase Ci-VSP³³. Ci-VSP⁹⁰ is thought to be a monomer⁹¹. The monomeric channel is biophysically slightly different from the dimer. Table 3 compares the biophysical properties of the monomer and the dimer with the channel classifications made by DeCoursey⁹². Monomers exhibit faster gating than the dimer, both tail kinetics and activation kinetics are sped up. The activation energy needed to open the monomer is half the energy needed for the dimer, also closing of the channel is less energy consuming. The Q_{10} of conductance is nearly the same, implying that there is no shared conduction pathway in the dimer. The current shape is sigmoid for the dimer but exponential for the monomer. Activation kinetics of sodium and potassium currents were analyzed and modelled by Hodgkin and Huxley in (1952)⁹³. The mathematical equations showed that if multiple subunits participate in the opening of an ion channel, pronounced sigmoicity of the activation kinetics may result. This is consistent with cooperative gating of the proton channel dimer. The monomer follows the prediction further in exhibiting exponential kinetics, due to the lack of interaction partner. Enhanced gating has been described for the dimer but it is not clear whether the monomer responds to phosphorylation in the same way, or if the “enhanced gating mode” involves both subunits interacting with each other.

Lee et al.⁸³ proposed an orientation of the dimer interface. Biochemical crosslinking experiments led to the conclusion that cysteine²⁴⁹ in the C terminus and isoleucine¹²⁷ in S1 are the main connection sites. Interaction between both S1 segments and a coiled coil interaction at the C terminus link the subunits together as a dimer. A variety of evidence revealed that there is cooperativity between the subunits during the opening of the channel. Tombola et al.⁹⁴ based his approach on the E153C mutation shifting the g_H - V curve 50 mV more negative compared to the wild type. The g_H - V shift occurred only in homodimers. In heterodimers (WT-E153C) the g_H - V shift was not observed. They concluded that one monomer influences the other, because independent movement of monomers would predict a biphasic g_H - V curve between the homodimer curves. With a further set of mutations including the g_H - V shifted mutant (I218S) and exploiting the zinc insensitivity of the double histidine mutations (H140A-H193A) they further strengthened their hypothesis. Tombola et al. also used the N214C mutant to block the proton channel with an MTSET reagent to make one of the monomers in the dimer non-conducting. The results confirmed that the opening of the two pores was not independent.

Gonzales et al.³ used voltage clamp fluorometry to simultaneously measure the changes of a fluorophore at position S242C on top of the S4 domain in the *Ciona intestinalis* proton channel while recording the current in oocytes with two electrode voltage clamp. For the

activation kinetics in the dimer they observed the current activating with a short delay after the fluorescence signal from S4 had changed. When the time course of the fluorescence was raised to the power of 2 both traces overlapped. In the monomer no correction was needed since the current and the fluorescence exhibit the same kinetics. Gonzales et al.³ concluded that the delay in the dimer is explainable by strong cooperativity, thus current flows through each subunit only if both S4 have moved into open position.

In a purely electrophysiological approach they identified the gating charges of monomer and dimer by Boltzmann fit of the g_H - V curve and by limiting slope^{95,96}. Both methods revealed that the gating charge of the dimer was twice the charge of the monomer (Table 3).

Musset et al.³² used two methods to show cooperativity. The first was to test whether zinc sensitivity of the proton channel is changed by monomerization. The second was to determine temperature dependence of gating kinetics of monomer and dimer (Table 3).

Zinc sensitivity of voltage gated proton channels

Proton channels are inhibited by extracellular polyvalent cations. Zinc (Zn^{2+}), Cadmium (Cd^{2+}), Copper (Cu^{2+}), Nickel (Ni^{2+}), Cobalt (Co^{2+}), Mercury (Hg^{2+}), Beryllium (Be^{2+}), Manganese (Mn^{2+}), Aluminium (Al^{3+}), and Lanthanum (La^{3+}) have been used as inhibitors^{4, 6, 40–46, 49, 54, 97–99}. Zinc has been the most characterized metal for blocking proton channels. It could discriminate proton channels from potassium channels in *Helix aspersa* neurons, because the potassium channels are 80 fold less sensitive to zinc⁹⁷. The inhibition by zinc is dependent on pH_o . The concentration required to slow τ_{act} twofold is (μM) 0.22 at pH_o 8, 0.46 at pH_o 7, 5.4 at pH_o 6, 89 at pH_o 5.5 and 1,000 at pH_o 5 in rat alveolar epithelial cells¹⁰⁰. A careful analysis of the effects of Zn^{2+} on the native proton channel resulted in the conclusion that two to three histidines on the outside of the channel are responsible for the pH dependent Zn^{2+} effects on gating and conductance¹⁰⁰. After the discovery of the human voltage gated proton channel,¹¹ mutation of two histidines to alanines facing the extracellular milieu abolished most of the Zn^{2+} sensitivity. Single histidine mutations resulted in a higher affinity to zinc than both of the histidines mutated together. Thus both histidines contribute to Zn^{2+} effects.

Musset et al.³² investigated the Zn^{2+} sensitivity of wild type (WT) and C-terminal truncated human proton channels, which express as monomer. Two main questions could be addressed in this study: **first** if the Zn^{2+} sensitivity of expressed proton channels is comparable to that of the native channel; **second** if Zn^{2+} binds between the subunits of the dimer or in each subunit alone.

The monomer is less sensitive to zinc than the dimer³². This result could be explained if Zn^{2+} binding occurred at the interface between monomers. This result led further to a model of the dimer in which the S2 domain (H140) and the S3 domain (H193) of each subunit face each other in contrast to the Lee model where S1 domain is the main interface. It is possible that the dimer does not have a unique interface, but instead can be joined in different orientations that vary in time. The Zn^{2+} sensitivity data was additionally supported by tandem (concatemer) constructs where all other possible Zn^{2+} binding configurations between the monomers in the dimer were tested. Only constructs in which at least one histidine was present in each monomer were slowed by Zn^{2+} . It was concluded that the slowing of H^+ channel opening occurs when Zn^{2+} binds at the interface.

Temperature dependence of the voltage gated proton channel

The temperature coefficient (Q_{10}) is defined as the change of any property of a biological or chemical process due to the increase of temperature of exactly 10°C. It is a measure of the

activation energy of the process. Systems with low temperature dependence convert more easily from one state to another than those with high temperature dependence. Activation energy (E_a) was introduced in 1889 by Svante Arrhenius and its given unit is kJ/mol. Thus it is possible to convert activation energy into Q_{10} and vice versa, but the E_a is dependent on the temperature range it was measured in.

One of the unusual characteristics of the voltage gated proton channel is its high temperature dependence. There is a high value for the Q_{10} for four of the six basic parameters (activation kinetics, tail kinetics, maximal current and conductance). The Q_{10} for the kinetic parameters, tail and activation kinetic have extraordinarily high values that exceed the values for sodium and potassium channels ($Q_{10} \sim 3$) by 2–3 times. In fact the temperature dependence of proton channels seems to be one of the most pronounced in the ion channel field. CLC-0 takes the lead with a $Q_{10} = 40$ for its slow inactivating component¹⁰¹, followed by channels TRPV1 $Q_{10} = 26$ and TRPM8 $Q_{10} = 24$ for gating¹⁰². TRPV1 and TRPM8 are channels that ought to be temperature sensitive because their function is sensing temperature. The high Q_{10} of gating properties of proton channels might give an insight into structural rearrangements of the channel during opening and closing. The high E_a value in H_V1 might reflect major structural changes in this channel. Maybe the high Q_{10} could point to a different mechanism of voltage gating of H_V1 compared to the voltage gating found in potassium and sodium channels. For sodium and potassium channels a movement of S4 has been postulated. The nature of the movement is discussed in detail by others and should not be part of this review. Thus, despite all the similarities in S4 between K_V-channels and H_V1: Is the movement of the S4 domain in both channels similar or qualitatively different? Voltage clamp fluorometry (VCF) was used to illuminate multimerization status of H_V1 and the properties of gating. Gonzales and co-workers³ marked one amino acid (S242C) at the external part of the S3–S4 linker above the three arginines in CiH_V1. These three arginines are comparable to the four arginines in potassium channel S4 domains, which are thought to be voltage sensing basic residues that move through the electric field¹⁰³.

Gonzales et al.³ recorded changes in the environment of the fluorophore which were synchronous to the current kinetics of opening in the monomerized channel. Assuming that the fluorophore's light intensity decreases with more water than lipid surrounding it, the result suggests that fluorophore coupled to S4 moves away from the lipids during channel opening. Comparable movements of the S4 have been widely characterized in potassium channels. Thus the S4 segment of H_V1 responds to voltage changes in a "classical" way and is part of the gating process, it seems that the differences in Q_{10} of activation are not explained by a revolutionary new voltage sensing concept. However H_V1 differs from K_V in the way S4 movement is coupled to channel opening.

Musset et al.³² compared the monomeric form of the proton channel and the WT dimer in inside out patches. The monomer on its own has a faster activation kinetic than the dimer. Furthermore the Q_{10} of the human H_V1 dimer was double that of the monomer (Table 3). Musset et al.³² found that the sigmoidity of the current in the dimer is lost in the monomer. The current in the monomer is perfectly exponential.

Both the temperature dependence and the shape of the current are signs of strong cooperativity of the subunits in the dimer. To conclude: to open two gating subunits cooperatively more energy is needed and the structural changes are more complex than in the monomer. Notably, the Q_{10} of gating of the monomer is in the same range as the Q_{10} in K_V channels. Perhaps S4 movement results in a Q_{10} near 3, but the additional requirement of cooperative gating in H_V1 is more demanding than the transmission of VSD movement to K⁺ channel opening.

What does the Q_{10} of conductance imply?

The Q_{10} of conductance may hold some information about how a proton moves through the proton channel. The channel has a closed and an open configuration. In the open configuration protons traverse the channel at a certain rate which is equatable to conductance. The average Q_{10} for the conductance is between 2.8 at high temperatures (20–35°C) and Q_{10} of 5.3 at low temperatures (<20°C)¹⁰⁴. No voltage gated potassium nor sodium channels exhibit comparably high values of the Q_{10} . The Q_{10} for potassium channels and sodium channels range between 1.18 and 1.7. DeCoursey and Cherny¹⁰⁴ after measuring the temperature dependence discussed at length whether temperature dependence on its own could explain the conduction mechanism of voltage gated proton channels. They suggested one highly probable and one less likely scenario.

Highly probable scenario

The conduction pathway includes at least one protonation site, most likely the side chain of an amino acid. The amino acid is part of an HBC through the channel. The process of protonation and deprotonation of an amino acid is more temperature dependent than the movement of protons through water. This does not exclude that the proton reaches the protonation site by moving through waters which are in crevices of the proton channel. The protonation site by itself will be a bottleneck that every conducted proton has to pass. Here the activation energy for the process of protonation and deprotonation will be higher than for a proton transfer by a water molecule. This will result in an overall higher Q_{10} for conduction.

Less likely scenario

The voltage gated proton channel has constricted water as part of a water wire. This constriction would morph the water molecule into an ice like state due to its inability to move freely. In ice the water molecules have tetrahedral bonding to each other, which prevents the individual molecule from moving. Reduction in movement might hinder the turning step of the water molecules after a proton has passed. The turning of the water molecule still might be possible but it demands more energy than just the simple movement of protons through liquid water. Thus the turn of the constricted water might have steeper temperature dependence and a higher Q_{10} for this mechanism might result^{105, 106}.

Overall the voltage gated proton channel has stronger temperature dependence in four of the six basic parameters than other ion channel. The Q_{10} of conduction suggests a different conduction mechanism than in other ion channels. The Q_{10} of gating suggest a more complex gating mechanism than most other ion channels. Only the voltage dependence of the activation threshold is an exception. The V_{thres} is unaltered by temperature^{104, 107}. The reversal potential is only very slightly altered by temperature. For the temperature range from 4°C to 40°C a ~2 mV positive change for every 10°C is predicted by the Nernst equation.

Conduction mechanism coupled to selectivity

In all ion channels known, the permeation pathway contains the selectivity filter. This is logical and intuitive. For the voltage gated proton channel the selectivity mechanism is not completely understood. Ramsey et al.⁵⁰ mutated charged amino acids in or near the membrane spanning domains in the human proton channel. The mutated channels were expressed for electrophysiological measurements, but no single amino acid was required for conduction. However they found in every mutant a roughly 40 mV threshold shift of the $g_{\text{H}}-V$ relationship per unit pH shift. Ramsey et al.⁵⁰ mutated up to three candidate residues simultaneously in the same construct to exclude a conduction mechanism, in which the

selectivity is dependent on the concerted action of these amino acids. The multiple mutants showed no clear deviation from the fixed (40 mV) threshold to pH ratio.

Tombola et al.³³ reported that mutation of the asparagine at position 214 to Arg abolished proton current, and that in the open channel Asn²¹⁴ likely formed the selectivity filter at a constriction off the pore. Ramsey et al mutated this Asn to Arg or Lys and found that the mutants exhibited robust H⁺ current. Sakata et al.⁸⁹ observed proton currents with the analogous mutation in the mouse Hv1. Musset et al.¹⁰⁸ observed robust H⁺ current in N214D mutants. Ramsey et al.⁵⁰ concluded from experimental data and MD simulations that a water wire is the conduction mechanism. This water wire mechanism is described as coordinating a water network at a constriction of the proton channel; most likely in the form of an asymmetric Eigen cation (H₉O₄⁺). This water network has access to the outside and the inside of the plasma membrane, allowing Hv1 to have a fixed threshold to reversal potential ratio. Ramsey et al.⁵⁰ explained how this water network achieved selectivity by the “frozen water” scenario discussed above.

Any selectivity mechanism must also account for the strong deuterium isotope and temperature effects on proton currents. Ramsey et al compared deuterium effects in the proton channel (1.9 H/D) reported by DeCoursey and Cherny², with that between proton and deuterium mobility between water (1.5 H/D¹⁰⁹) and ice (numbers below).

The isotope effects for proton and deuteron mobility in ice is variable; Eigen¹¹⁰ reports 8 for the relative mobility of protons and deuterons (H/D) in ice. Kunst and Warman¹¹¹ report an effective drift of 4 (H/D) and a virtual mobility quotient of 2.7 in ice. Cowin et al.¹¹² measures no proton or deuteron movement in ice below 190 K, which resolves into H/D = 0. Devlin reported H/D exchange in ice nanocrystals below 145 K as Bjerrum defect movement following proton transfer^{113–115}. Park et al.¹¹⁶ measured lateral surface H/D exchange but a near absence of proton movement into the ice film at 90–140K, H/D = 0. Finally Kang and co-workers¹¹⁷ concluded that “the proton mobility issue is considered yet unresolved”. They report that vertical movement of protons in an ice film is possible below and above 140 K and the differences between lateral and vertical transport in ice might be explained through the thermodynamic affinity from protons to the ice surface^{118, 119}. To summarize, there is no consensus value for the mobility of protons and deuterium in ice.

Recently the DeCoursey laboratory compared several voltage sensors of voltage-sensing phosphatases, voltage gated cation channels, voltage gated proton channels of different species, and a protein of unknown function C15orf27¹⁰⁸. In an alignment of voltage sensors, five residues were picked which are conserved in all voltage gated proton channels but not in all other VSDs. Since C15orf27 did not exhibit proton currents, single mutations were introduced into Hv1 based on corresponding residues in the non conducting C15orf27. Only one of those mutations (D112V) abolished proton current. This position was mutated further into basic, acidic, and neutral residues. The reversal potential was measured in seven mutations of the aspartate residue. All of the mutations except the conservative aspartate to glutamate exchange revealed an anion conductance instead of the WT proton conductance. Even a mutation to histidine exhibited anion permeation. The results suggest that D112 is part of the selectivity mechanism of the proton channel. The results further suggest that in the non conducting D112V mutant the conduction pathway is occluded therefore D112 is also part of a narrow constriction in the conduction pathway of the channel. The change to anion but not cation permeation may show that cations are excluded and cannot permeate. It might be reasonable to speculate which other amino acids may also be involved into selectivity mechanism, for example which residues are responsible for the anion permeation. Further measurements would be helpful to shed light on this mystery.

In a dinoflagellate proton channel (kH_V1) discovered recently by Susan Smith¹²⁰, a comparable aspartate was found. This aspartate 51 is in a position in the S1 domain similar to Asp¹¹² in hH_V1. Mutations of this Asp⁵¹ to His, Ala and Ser turned the perfectly proton selective kH_V1 into an anion selective channel. Reversal potential measurements with chloride solutions gave almost identical reversal potential shifts as had been recorded with the human proton channel (hH_V1). The mutation to glutamate retained the perfect proton selectivity similar to hH_V1. The discovery of this proton channel which shares only 15% sequence identity with the human proton channel indicates the importance of an acidic residue at this crucial position. The selectivity mechanism appears to be identical in unicellular dinoflagellates and humans.

$$\frac{R \ln(Q_{10})}{\left(\frac{1}{T_1} - \frac{1}{T_2}\right)} = E_a$$

Equation to convert Q₁₀ into E_a

This equation allows to convert Q₁₀ into activation energy. Temperature in Kelvin. R= Gas constant 8.314 kJ mol⁻¹

$$Q_{10} = \left(\frac{X_2}{X_1}\right)^{\frac{10}{(T_2 - T_1)}}$$

Q₁₀ equation

Q₁₀ equation with X₁ and X₂ are the measured values and T₁ and T₂ their temperature. The Q₁₀ value gives the change through a 10 degrees temperature shift.

$$E_a = \frac{RT_1 T_2}{T_2 - T_1} \ln \frac{X_2}{X_1}$$

Activation Energy Equation

Q₁₀ is a expression of activation energy. The above equation allows to calculate out of the data (X = data/T = temperature) the activation energy directly. Temperature in Kelvin. R= Gas constant 8.314 kJ mol⁻¹

Acknowledgments

This work was supported by the Iacocca Family Foundation (B.M.), by NIH R01GM087507 (T.E.D) and by NSF MCB-0943362 (T.E.D).

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Fig. 1. Proton movement in a single water file pore

The proton hops on the first water molecule and each intermediate hydronium releases a proton to the nearest oxygen in the next water molecule. The picture below shows the Newton cradle as a mnemonic device for proton movement through a single file of water.

Fig. 2. Kinetic of outward- and tail currents

The figure shows the outward current fitted by a single exponential (red). There is a short delay before the exponential starts. The maximal value for the exponential gives the value for the maximal proton current at this voltage. Here the end of the pulse and the maximal current are almost the same. The tail current is perfectly describable with a single exponential (red).

Fig. 3. End of pulse

The figure shows a current trace during a pulse protocol. The amplitude of the current at the end of the pulse (I_{end}) is depicted together with the amplitude of the maximal tail current (I_{tail}) minus the leak current. The reversal potential (V_{rev}) is calculated with the equation in the center of the current trace. The reversal potential thereby is able to reflect changes in pH_i .

Fig. 4. Threshold of activation in voltage gated proton channels

Current family shows in red the first appearance of proton channels in outward and tail current. On the right the voltage pulses are displayed exhibiting in red the threshold voltage.

Fig. 5. Tail currents to determine the reversal potential

A prepulse to 50 mV is given followed by pulses to -30 through 20 mV. At 0 mV the tail current is nearly a straight line indicating the reversal potential. (pHi = pHo = 7).

Fig. 6. enhanced gating mode g-V shift

The figure shows the conductance voltage plot (g-V) of a human monocyte before and after PMA stimulation. The “enhanced gating mode” is detectable as a left shift in the g-V curve. The pHo 7 is pHi 7 in perforated patch mode.

Fig. 7. secondary structure of HV1 and voltage gated cation channels

S1–S3 are depicted in yellow, S4 orange, S5–S6 in red. The general concept of S1–S4 in the proton channel and voltage gated cation channels is the same with S4 as voltage sensor. The proton channel lacks S5–S6

Table 1
Ionic radius and mass of electrons protons and other ions

Mass of the proton is compared to all other ions. The electron radius is considered as the classical radius ¹. The classical radius is derived from the assumption that the electron is a charged shell.

	Radius ($\times 10^{-15}$ m)	Mass relative to H^+
e^-	1.42	1/1836
H^+	2	1
Li^+	78000	7
H_3O^+	99000	19
Na^+	102000	23
Cl^-	181000	35
K^+	138000	39

Table 2
Comparison between channel, carrier, and pumps to the voltage gated proton channel

The proton channel is in all but one property similar to an ion channel (green). Note the turnover rate might be connected to the concentration of the conducted ion.

	Pumps	Carriers	Channels	H_v1
consumes ATP	yes	indirectly	no	no
Co/Counterions	sometimes	sometimes	no	no
electrogenic	sometimes	sometimes	yes	yes
conformational changes during transport	several	yes	no	no
Turnover rate (Ions/sec)	10 ²	10 ⁴	10 ⁶	10 ⁴

Table 3
Comparison of the biophysical properties of mammalian H_v1

The Values show the biophysical properties measured mostly in human white blood cells, but also mice and rat phagocytes are included. Only the human H_v1 is shown in the table. The gating charge for dimers was measured by Musset et al.², DeCoursey et al.³, and dimers and monomers by Gonzales et al.⁴ Patch clamp configuration are abbreviated with pp = perforated patch (unpublished) and wc = whole cell configuration. Enhanced gating mode could only be detected in LK.35.2 cells in an expression system.

	MAMMALIAN CELLS			
Type	Phagocyte & Basophils		Human H _v 1	Human H _v 1
	Resting state	Phosphorylated “enhanced gating mode”	Expressed	C-terminus truncated
			Dimer	Monomerized
Gated by	V, ΔpH	V, ΔpH, NADPH oxidase activity	V, ΔpH	V, ΔpH
τ _{act} (at +60 mV)	Slower (5 s)	Slow (1.6s)	Slow (2.5 s)	Medium (0.4s)
Sigmoid activation?	Yes	Yes	Yes	No (exponential)
τ _{tail} (at −40 mV)	Slow (200 ms)	Very slow (1s)	Slow (370ms)	Medium (60 ms)
τ _{tail} components	1	1	1	1
Q ₁₀ τ _{act}	6.1 wc 4.4 pp	3.3 pp	7.2	3.6
Q ₁₀ τ _{tail}	7.1 wc 3.9 pp	4.5 pp	7.5	2.3
Q ₁₀ g _H	2.8	2.8	3.3	2.8
Enhanced gating	Yes	Enhanced	Yes dependent on expression system	Not tested
Gating charges	~6.0	Not known	~6.0	~3.0
Cells expressing	Microglia, Neutrophils, Eosinophils, Mast cells, Macrophages, Basophils, HL-60, PLB			
Cells expressed in			HEK-293, HEK 293T, COS cells, LK-35.2 (only the dimer)	