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Review

Voltage-gated proton channels

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Abstract. The history of research on voltage-gated proton channels is recounted, from their proposed existence in dinoflagellates by Hastings in 1972 and their demonstration in snail neurons by Thomas and Meech in 1982 to the discovery in 2006 (after a decade of controversy) of genes that unequivocally code for proton channels. Voltage-gated proton channels are perfectly selective for protons, conduct deuterons half as well, and the conductance is strongly temperature dependent. These properties are consistent with a conduction mechanism involving hydrogen-bonded-chain transfer, in which the selectivity filter is a titratable amino acid residue. Channel opening is regulated stringently by pH such that only outward current is normally activated. Main functions of proton channels include acid extrusion from cells and charge compensation for the electrogenic activity of the phagocyte NADPH oxidase. Genetic approaches hold the promise of rapid progress in the near future.

Keywords. pH, proton channel, voltage-dependent gating, ion channel, gating, phagocyte.

This review summarizes the properties, physiological regulation, and functions of voltage-gated proton channels. Although several reviews have appeared in the past few years [1–6], events in this area have moved forward rapidly, so that another review may be justified.

History

Voltage-gated proton channels were first proposed to exist in 1972 in dinoflagellates (e.g., *Noctiluca milaris*, *Gonyaulax polyhedra*) by J. Woodland Hastings [7]. Remarkably, the bioluminescence response to mechanical stimulation is evoked by an action potential [8, 9] that is evidently mediated by a proton conductance [10] in the tonoplast (the membrane of the large central vacuole). Protons flow from the vacuole interior at pH 3.5 [11] into small evaginations of the membrane called scintillons, which contain luciferase and luciferin. It appears that proton influx during the action potential directly lowers the pH in the scintillons, which releases luciferin from a binding protein and also activates luciferase [12], permitting the reaction with luciferase that produces a 100-ms flash [13]. Although more direct evidence of this scenario would be helpful, existing data suggest that an action potential mediated by voltage-gated proton channels directly activates the bioluminescence response [13]. The first voltage-clamp study of proton channels was by Roger Thomas and Robert Meech in snail neurons [14]. In a *tour de force*, they impaled *Helix aspersa* neurons with four electrodes to measure membrane potential, membrane current, and pH_i while injecting HCl. The formal electrophysiological properties of proton currents were further elucidated by Byerly, Meech, and Moody [15] at UCLA. In Susumu Hagiwara's laboratory next door, Christine Baud and Michael Barish found similar voltage-gated proton currents in *Ambystoma mexicanum* (salamander

or axolotl) oocytes [16, 17]. Martyn Mahaut-Smith explored the sensitivity of proton current to inhibition by Zn^{2+} [18]. Byerly and Suen observed no excess current fluctuations when proton current was activated and concluded that the single-channel current (at +10 mV at pH_o 7.4, pH_i 5.9) was at most 4 fA, about 1000 times smaller than ordinary ion channels [19]. Early themes of these studies included attempts to demonstrate that proton channels were molecular entities distinct from other ion channels (i.e., that protons had their own channels and did not simply permeate other ion channels adventitiously), that proton channels were not artifacts of the unusual recording solutions used to isolate them from other conductances, and that proton channels were actual proteins, rather than strange leaks through the phospholipid bilayer [discussed in ref. 20]. These kinds of doubts lingered in some minds until the recent identification of proton channel genes in human [21], mouse, and the sea squirt, *Ciona intestinalis* [22]. Nearly a decade after their discovery in snail neurons [14], proton channels were found in mammalian (rat alveolar epithelial) cells [23] and then human cells [24–26]. A number of fundamental measurements were made, including pH dependence [27, 28], deuterium isotope effects [29], temperature dependence [30, 31], quantification of Zn^{2+} effects on proton current [32], and eventually, single-channel conductance [33]. Gradually interest shifted toward determining the functional relevance of proton channels. Their uniquely pH-regulated voltage dependence results in activation of only outward proton current, leading to universal agreement that their primary function is proton extrusion. A more specific function was proposed in phagocytes, namely charge compensation for the electrogenic activity of NADPH oxidase [34]. A key observation by Bánfi and colleagues was that when NADPH oxidase was activated in phagocytes, proton currents exhibited radically altered gating properties, the most remarkable of which was the appearance of inward currents [35]. Using the perforated-patch recording configuration, we found that the gating kinetics of proton channels could be transformed from normal or 'resting' into 'enhanced gating mode' behavior by stimulation with activators of NADPH oxidase, such as the phorbol ester, PMA [36, 37], arachidonic acid [38], or the chemotactic peptide, fMLF [39]. Recent studies have focused on delineating the signaling pathways that govern proton channel 'activation.'

The pathway toward identifying the proton channel gene has been rocky. After Henderson and colleagues proposed the existence of proton channels in human neutrophils [34], Grinstein and colleagues reported that conductive proton efflux seen in neutrophils upon PMA stimulation was defective in patients with chronic granulomatous disease (CGD), although both Na^+/H^+-antiport and H-ATPase activity were normal [40]. CGD occurs when a mutation (several hundred have been indentified) prevents the function of any of several essential components of NADPH oxidase. One logical interpretation was that the oxidase itself mediated proton extrusion [40]. However, this group subsequently demonstrated that monocytes from CGD patients lacking expression of $gp91^{phox}$, the largest membrane-spanning subunit, had normal voltage-gated proton currents, apparently ruling out direct participation of $gp91^{phox}$ in proton conduction [41]. Nevertheless, Henderson and colleagues reported in 1995 that expression of $gp91^{phox}$ resulted in proton currents in CHO cells, and concluded that $gp91^{phox}$ was the phagocyte proton channel [42]. This idea was later expanded to include not only $gp91^{phox}$, but also other homologs in the NOX family of proteins, which were reported to function as proton channels [43–45]. To explain the presence of normal proton currents in CGD cells lacking $gp91^{phox}$, Bánfi and colleagues proposed that there were two types of proton channels in phagocytes, one of unknown identity in resting cells that was distinct from NADPH oxidase, and that $gp91^{phox}$ functioned as a second type of proton channel when NADPH oxidase was active [35]. This proposal was particularly seductive because of the discovery that in eosinophils with active NADPH oxidase, the proton conductance increased substantially, and its gating kinetics and voltage dependence were drastically altered [35]. However, a variety of studies failed to support these proposals. (1) In perforated-patch studies of human neutrophils, we found that H^+ channels with normal 'resting' properties were apparently converted to the 'activated' state upon stimulation with PMA, a phorbol ester that activates NADPH oxidase. All of our data were consistent with the presence of a single type of proton channel in unstimulated cells whose gating properties were modified uniformly by activators of NADPH oxidase, rather than two populations of channels with distinct gating properties [36]. (2) The absence of $gp91^{phox}$ in granulocytes from CGD patients with mutations that prevented $gp91^{phox}$ expression [41] or in PLB-985 cells with the gene knocked out did not affect the amplitude or properties of proton currents in unstimulated cells [46], nor did it prevent the increase in proton current seen in perforated-patch studies upon stimulation with PMA [1, 46]. (3) The converse study revealed that expression of $gp91^{phox}$ in COS-7 cells that lack endogenous proton currents failed to produce detectable proton currents [47]. (4) Several studies have presented evidence that other NOX family proteins,

including Nox1, Duox-1, and Duox-2, similarly appear to lack proton channel function [48–50]. Numerous other studies [43, 45, 51, 52] and reviews [1, 3, 53–56] have addressed this question, without achieving consensus. My opinion is that neither gp91^{phox} nor any of its NOX homologues function as proton channels. Recent identification of genes for a voltage-gated proton channel in humans [21], mice, and *C. intestinalis* [22] only partially resolves the debate, because of the possibility that phagocytes have two types of proton channel [35], although some of its proponents have abandoned this idea [57]. It is clear that the newly identified gene products are voltage-gated proton channels that closely resemble native proton currents, but with one intriguing difference: the voltage at which heterologously expressed proton channels open appears to be ~30 mV more negative than that of native proton channels [58]. The human, H_V1, or mouse, mVSOP, gene products are structurally unrelated to gp91^{phox} or other NOX proteins, and they are present in leukocytes [21, 22]. However, they activate at sufficiently negative voltages such that inward currents can be detected [58], reminiscent of the putative gp91^{phox}-related channel, according to the two-channel viewpoint. Consequently, because they are distinct from gp91^{phox}, H_V1 and mVSOP must be considered to be the 'resting' channel despite their negative voltage dependence. It is hoped that this question can be resolved in the near future by studies of mVSOP knockout mice. Demonstration of a complete absence of proton currents in granulocytes from knockout mice before and after stimulation would provide virtually conclusive evidence that gp91^{phox} does not function as a proton channel.

Attempts to determine whether gp91^{phox} is a proton channel were greatly complicated by several paradoxical observations that indicate profound interactions between NADPH oxidase and proton channels. (1) Proton channels exhibit a full PMA response only in cells that have an active NADPH oxidase, including neutrophils [36], eosinophils [37], and PLB-985 cells [46]. The 'standard' PMA response in human phagocytes is characterized by increased proton conductance (the maximum H^+ conductance, $g_{H,max}$ increases two- to fourfold), faster turn-on of H^+ current with depolarization (τ_{act} decreases three- to fivefold fold), slower closing of H^+ channels upon repolarization (τ_{tail} increases three- to sixfold), and the entire g_H-V relationship is shifted negatively by 40 mV [36, 37, 39, 46]. The PMA response is qualitatively identical, but weaker in murine granulocytes or osteoclasts [39, 59]. In non-phagocytes under identical perforated-patch recording conditions, no clear response has been detected in rat alveolar epithelial cells [36] or in HEK-293 cells or COS-7 cells expressing H_V1 or mVSOP [58]. (2) In phagocytes from CGD patients whose NADPH oxidase is dysfunctional, the proton channel response is only partial. In response to PMA, $g_{H,max}$ increases and τ_{act} decreases (the current turns on faster) identically to normal cells; however, the hyperpolarizing voltage shift of the g_H-V relationship is only half as large and there is no slowing of τ_{tail} [1, 46]. (3) In human basophils, which are closely related to phagocytes but which lack NADPH oxidase [60], the proton channel response to PMA is indistinguishable from that of a CGD phagocyte [61]. (4) In activated phagocytes, inhibition of NADPH oxidase activity with diphenylene iodonium (DPI) reverses the slowing of tail current decay without affecting any other property of proton currents [36]. DPI has no effect on resting proton current kinetics; evidently, proton channels can sense whether NADPH oxidase is active. We speculated that the link might be locally released H^+ which would be expected to slow deactivation if pH near the channel decreased [36].

The proton channel molecules whose genes were discovered in 2006 [21, 22] have remarkable and surprising properties. The protein closely resembles the first four membrane-spanning regions of other voltage-gated ion channels, S1–S4, known as the voltage-sensing domain (VSD), but lacks the other two regions, S5–S6, that form the pore where conduction takes place (Fig. 1). At this time, we do not know whether the proton channel functions as a monomer or a tetramer or something else, but recent evidence suggests that the functional unit is a dimer [62–64]. The Okamura group identified the gene during a search for homolog of another protein in *Ciona* called Ci-VSP, which also resembles a voltage sensor, but has a phosphatase moiety attached whose activity, remarkably, is regulated by membrane potential [65, 66]. The possibility exists that the proton channel is a representative of a group of 'modular' proteins that might assemble with other membrane proteins to confer voltage sensing to their function [67]. The S4 region of the proton channel includes four of the charged amino acids that characterize this region in *Shaker* K^+ channels (Table 1) and presumably mediate its sensitivity to membrane potential [68–70]. Now the story becomes more bizarre. Bezanilla and colleagues found that single Arg-to-His mutations at key points in the S4 region of a K^+ channel (R365H or R368H) resulted in proton transport through the voltage sensor. These Arg residues evidently traverse the entire membrane potential field during gating, and are thus alternately accessible to the internal and external solutions [71]. More striking still, the R371H mutation transformed the voltage sensor into a proton-selective channel that was opened by membrane depolarization [72]. Finally,

Table 1. S4 regions of voltage-gated K^+ and H^+ channels.

Shaker	L	**R** 362	V	I	**R** 365	L	V	**R** 368	V	F	**R** 371	I	F	***K*** *374*	L	S	***R*** *377*	H	S	***K*** *380*	G	**13 e_0**
mSlo1	W	L	G	L	***R*** *207*	F	L	***R*** *210*	A	L	**R** 213	L	-	I	Q	F	S	***E*** *219*	I	L	Q	**2.3 e_0**
H_V1	L	I	L	L	**R** 205	L	W	***R*** *208*	V	A	***R*** *211*	I	I	N	G	I	I	I	S	V	***K***	**6 e_0**
mVSOP	L	I	L	L	***R*** *201*	L	W	***R*** *204*	V	A	***R*** *207*	I	I	N	G	I	I	I	S	V	***K***	**6 e_0**

The sequence of 21 amino acids in the putative S4 regions of the *Shaker* K^+ channel, the maxi-K calcium-activated K^+ channel (mSlo), the human proton channel (H_v1), and the mouse proton channel (mVSOP). Potentially charged amino acids are indicated in bold. Single neutralization mutations at the numbered positions in boxes reduce the gating charge; those in italics do not affect gating charge or were inconclusive [21, 22, 58, 68–70, 129]. Total gating charge (e_0) estimates are listed in the final column. The mouse R204Q mutant did not express well; R207Q was identical to wild type, and for R201Q activation was faster and the g_H-V relationship was shifted by -50 mV and had a slightly steeper slope ($z = 1.9$ vs 1.4 for wild type) [22]. In human proton channels, gating was faster for all three Arg mutants, and for R205A, the midpoint was shifted positively, and the slope was less steep by 1/3 ($z\delta = 0.57$ vs 0.90 for wild type) [21]. The VSD of the *Shaker* K^+ channel can be transformed into a proton channel by the R362H mutation [73], and R362X where X = Cys, Ala, Ser, or Val produces non-selective cationic 'omega' current through the voltage sensor [75].

the R362H mutant is also a proton-selective channel, but it is opened by membrane hyperpolarization [73]. Isacoff and colleagues have shown that the latter discovery can be exploited to reveal details of the K^+ channel gating process [74]. They found that other mutations at the same location (R362X) produce non-selective cation current that is activated by hyperpolarization and hence reflects the channel in the closed state with respect to the central 'alpha' pore [75]. Their elegant studies demonstrate clearly that there are four 'omega' pores, one in each of the four voltage sensors, for every 'alpha' channel, which is the normal K^+ pathway through the center of the tetramer [75]. The possibility that the voltage sensor of K^+ channels and the voltage-gated proton channel will reveal clues to how each channel functions makes this an exciting area of research. An assortment of chimeras that combine structural elements of both channels have already been shown to result in functional K^+ or H^+ channels [76].

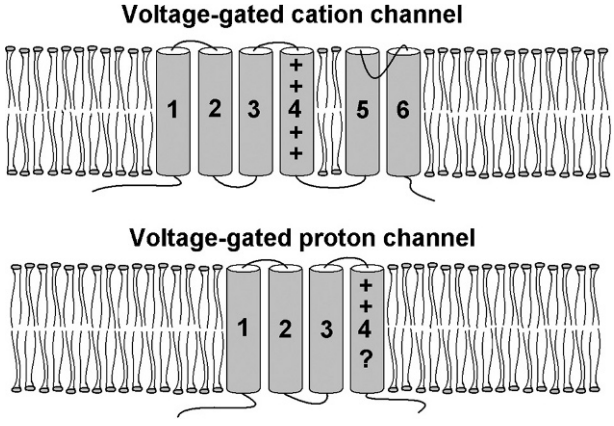

Figure 1. The molecular architecture of voltage-gated proton channels and ordinary voltage-gated cation channels. Most voltage-gated K^+, Na^+, and Ca^{2+} channels are tetramers formed from four copies of the illustrated subunit with its six membrane-spanning regions. Regions S1–S4 comprise the 'voltage-sensing domain' or VSD, within which S4 in particular has a number of charged Arg residues that appear to sense the membrane potential (cf. Table 1). The S5–S6 regions from all four copies of the illustrated subunits form a single central pore that opens and closes during gating. The voltage-gated proton channel has a generally similar sequence to S1–S4, but the S5–S6 regions are absent. Although three Arg resides are present in the proton channel S4 region (cf. Table 1), it is not clear that they contribute to voltage gating. Because the S5–S6 regions that comprise the pore of ordinary voltage-gated channels are absent, the conduction pathway is not obvious. Recent studies suggest that the proton channel functions as a dimer, each subunit with its own pore [62–64]. It is conceivable that the proton channel gene product associates with some other molecule that forms a more traditional pore, but expression of the gene alone produces voltage-gated proton currents.

Proton selectivity

A characteristic property of voltage-gated proton channels is perfect proton selectivity. When measured in a variety of ionic conditions and liquid junction potentials are corrected [77], reversal potentials change only with changes in pH [1]. Thus, although measured reversal potentials almost invariably deviate somewhat from the Nernst potential, the reason for the deviation is most likely experimental inability to control pH perfectly on both sides of the membrane. Even assuming that deviation from Nernst reflects permeation by other ions, the calculated proton permeability is still 10^6–10^8 greater than that for other ions [20, 26, 27, 29, 46, 78–83].

How can a channel be perfectly proton selective? Following the proposal by Nagle and Morowitz [84], the perfect selectivity of voltage-gated proton channels has been interpreted to indicate that the conduction pathway is not a simple water-filled pore but, instead, includes at least one titratable amino acid side

group [1, 3, 20, 45, 51, 82, 85]. Conventional ion channels have aqueous pores that traverse the membrane, usually with a single-file region. The archetypal, highly studied gramicidin channel, for example, contains 8–12 water molecules in single file [86], and is non-selective among cations, but conducts protons much better than any other ion [87]. This high proton conductance reflects the ability of protons to hop across the water molecules in the pore without displacing them, as must occur when other cations permeate. A titratable group that occludes the conduction pathway would comprise a selectivity filter that would limit permeation to protons [1].

A large number of proton pathways through other molecules appear to make use of this mechanism [1]. However, for some pathways, proton selectivity may be achieved by other mechanisms. For example, the M_2 viral proton channel is perfectly selective for protons [88–91]. The pore appears to be occluded at one point by four His^{37} residues (one from each subunit of the homotetramer). One school of thought is that protonation of one or more His^{37} leads to both activation of the conductance and proton selectivity, with protons 'shuttled' via one of the His^{37} residues [92–95]. Alternatively, proton conduction, and presumably proton selectivity, is achieved by the pore narrowing at this point so that the waters on either side can transfer a proton, but other ions cannot pass [96–101]. Similar proposals have been made for the voltage-gated proton channel. Molecular dynamics simulations based on the amino acid sequence of the voltage-gated proton channel, mapped onto the crystal structure of a K^+ channel voltage sensor, led to suggestions of a water-wire that narrows at one point and presumably achieves proton selectivity without benefit of a titratable group in the pathway [102, 103]. Molecular dynamics simulations suggest that a sufficiently narrow pore can effectively 'freeze' the waters and inhibit water or other ion flux, while still permitting protons to hop across the waters [104]. Regardless of the mechanism of proton selectivity in M_2, several other molecules include a titratable group in the proton conduction pathway that is almost certainly protonated and deprotonated during each proton conduction event, which results in proton selectivity or other crucial activities. Introduction of His by mutation at different locations in a K^+ channel voltage sensor produced voltage-gated proton channels or proton carriers, depending on the precise location [71–73], as discussed above. It is clear that protons exit carbonic anhydrase II by direct proton transfer from the His^{64} residue near the mouth of the channel to buffer in the external solution [105–107]. The Asp^{61} in H^+-ATPase is unequivocally protonated during operation of the pump [108]. Glu^{286} almost certainly transfers protons at a critical point in the D channel of cytochrome *c* oxidase [109–112]. Proton selectivity in a Cl^-/H^+ exchanger may result from two conserved Glu residues, E^{148} and E^{203}, that are presumably protonated during conduction [113], but in addition, E^{148} may act as a gate for the Cl^- pathway [114]. In these examples, proton selectivity is likely achieved by a titratable residue, but such conclusions are not set in stone. It is difficult to conceive experimental evidence that would 'prove' unambiguously that a titratable residue is the selectivity filter. For example, protons can exit carbonic anhydrases III or V or carbonic anhydrase II mutants that lack the titratable His^{64} residue, but the transfer rate is reduced drastically [115–117]. Similarly, Asp^{132}, the namesake of the D channel of cytochrome *c* oxidase, is almost certainly protonated during normal enzyme operation, but function remains in its absence, albeit proton transfer is slowed by up to 1000-fold in the D132N mutant [112, 118, 119]. Activity above wild type is restored upon introduction of another Asp in the vicinity [120]. Similarly, the D135N mutant of cytochrome bo_3 ubiquinol oxidase has reduced function [121] that is fully or partly restored by introduction of an acidic residue at nearby positions 139 or 142 [122]. These results suggest that the presence of an acidic residue is critical, but its precise location is not.

Proton conductance

Several early attempts to measure the single proton channel conductance were compromised by poor signal-to-noise (S/N) ratios of <1, meaning that the background noise was larger than the excess fluctuations that could be attributed to proton channel gating [19, 24, 25]. Cherny and colleagues [33] found that in human eosinophils where no other ionic conductances were present, by using excised patches with high seal resistance (>1 T$\Omega = 10^{12}$ Ohms) and appropriate filtering, stochastic gating (opening and closing) of proton channels produced fluctuations whose variance was >100 times larger than background (S/N >100). At low pH_i near $V_{threshold}$, single-channel-like events could be observed with amplitudes of ~10 fA, which is the smallest directly-recorded unitary current of which we are aware. The unitary current estimated from the variance was about half as large, which might indicate that the resolved events reflected occasional coupled gating of two-pore dimers, like some Cl^- channels [123], or might simply reflect the difficulty of resolving these tiny currents.

At low pH_i (5.0 or 5.5), the variance was maximal near the midpoint of the g_H-V relationship, which is predictable for a simple two-state open-closed gating

process. The unitary conductance was 38 fS at pH_i 6.5 and 140 fS at pH_i 5.5, and was independent of pH_o. The maximal open probability of the proton channel was 75 % at pH_i 6.5 and increased to 95 % at pH_i 5.0–5.5. Two results were surprising. First, the unitary conductance increased 3.7-fold from pH_i 6.5 to 5.5 [33], in contrast with the weaker pH dependence of the g_H reported in a large number of macroscopic studies; typically a 2-fold increase per unit decrease in pH_i [124]. Because the unitary conductance increased at lower pH_i more than the macroscopic g_H, the apparent number of channels available to open was reduced at low pH_i [33]. The mechanism of this inhibition by low pH is not known.

Extrapolated to physiological pH_i, the unitary proton conductance is ~15 fS at room temperature (78 fS at 37 °C) [33], which reflects roughly 10^4 H^+/s for a 100 mV driving force. Although this turnover rate is more in the range of carriers than most ion channels, in all other respects proton channels behave as channels. Their conductance is simply low due to the paucity of permeant ions [Fig. 13 in ref. 1]. Proton channels do not require ATP, co- or counter-ions, they exhibit voltage- and time-dependent gating, and they appear to provide a passive, selective pathway for protons to cross the membrane. They are therefore most reasonably considered to be ion channels.

The single-channel conductance increased at lower pH_i less than the 10-fold/unit one might expect for simple diffusion-limited entry into the channel [33]. However, this observation provides support for the idea that H^+ is the conducted ionic species rather than OH^- (in the opposite direction). The outward g_H is relatively insensitive to pH_o over a wide range (5.5–10) which argues against OH^- being the conducted species [23]. The conductance of deuterons through proton channels is only about half that of protons [29], which strongly supports the idea that protons, rather than OH^- or H_3O^+, carry current. The open-channel conductance is much more strongly dependent on temperature (Q_{10} values 2–5) [31] than is H^+ conductance in bulk water [125] or H^+ permeation through the water-wire that forms the conduction pathway through gramicidin channels [126, 127]. Proton pathways that include titratable amino acid residues have mainly larger Q_{10} values, in the range of proton channels [31]. Finally, perfect selectivity can be explained by protons hopping across a hydrogen-bonded-chain, but if H_3O^+ were the species carrying current, excluding other ions would be difficult. If selectivity is achieved by a mechanism other than HBC conduction, then this mechanism must also explain the perfect selectivity, temperature dependence, and isotope effects just discussed.

Regulation of gating by pH and by voltage

A unique property of voltage-gated proton channels is their exquisite sensitivity to both pH_o and pH_i. Increasing pH_o or lowering pH_i shifts the voltage-activation curve (g_H-V relationship) by 40 mV/unit pH toward more negative voltages [27]. This property appears to be shared by proton currents in all cells studied thus far [1]. Empirically, the relationship between the threshold voltage at which the g_H is first activated, $V_{threshold}$, and the pH gradient as determined from the reversal potential, V_{rev}, can be described as:

$$V_{threshold} = k\, V_{rev} + V_{offset}$$

where k was 0.76 in alveolar epithelial cells [27] and 0.79 in a fit to all published data as of 2003, representing 15 cell types [1], and V_{offset} was 18 mV in alveolar epithelium and 23 mV for all cells. The slope of the relationship $k < 1.0$ indicates that $V_{threshold}$ does not change as fast as V_{rev} when ΔpH is varied. A positive V_{offset} ensures that over a wide range of pH, only outward H^+ currents can be activated. The practical consequence of this regulation by the pH gradient, ΔpH, is that H^+ channels only open when there is an outward electrochemical gradient, i.e., when opening will permit protons to leave the cell. The general function of voltage-gated proton channels is therefore acid extrusion. Other, more specific functions will be described later.

When expressed in HEK-293 or COS-7 cells, human (H_V1) and mouse (mVSOP) proton channels display sensitivity to pH_o and pH_i generally like that of native proton channels [21, 22]. Surprisingly, however, $V_{threshold}$ is ~30 mV more negative for both than for native proton currents [58]. As a result, distinct inward currents can be elicited, especially when ΔpH is inward ($pH_o < pH_i$). At present this anomaly has no explanation.

The S4 region of voltage-gated K^+ and Na^+ channels contains seven potentially charged amino acid residues at every third position, mainly Arg residues (Table 1). Mutations that neutralize certain of these charges decrease the steepness of the voltage dependence [69, 128], consistent with the idea that these charged residues sense the membrane potential, and that they move relative to the electric field during gating. Specifically, each of four Arg residues in S4, when individually substituted by neutral amino acids, reduce the effective gating charge moved during channel opening [68–70]. In contrast, neutralization of only one of the three Arg residues in S4 of the human voltage-gated proton channel reduced the apparent gating charge [21] and none of the three Arg residues in S4 of the mouse H^+ channel affected the

steepness of the g_H-V relationship when neutralized [22]. Thus, despite the structural similarity between voltage-gated proton channels and the voltage sensor of voltage-gated K^+ channels, their voltage-sensing mechanism appears to be different. A similar conclusion was reached for the BK channel, in which only one Arg in S4 contributes to the gating charge [129]. On the other hand, despite minimal effects on the effective gating charge, neutralization of each of the three Arg altered the gating kinetics in the human channel [21], supporting the idea that S4 does participate in gating.

Three features of the voltage-sensitive gating mechanism of proton channels appear to distinguish them from voltage-gated K^+ channels. First, the K^+ channel VSD transmits its response to voltage to a physically distinct 'gate' that opens a pore in S5–S6. The conduction pathway through proton channels may be near or at the same location as the Arg residues in S4, the equivalent of the omega current discussed above. Second, the voltage dependence of K^+ channels is essentially absolute. Although both permeant ions and pH affect K^+ channel gating, the effects are negligible compared with the profound effects of protons, the permeating species, on voltage-gated proton channel gating. Any proposed gating mechanism must account for the ΔpH dependence of gating. Third, the effective gating charge is quite large, 12–14 elementary charges (e_0) in voltage-gated sodium [130] and potassium channels [68, 69, 131]. In contrast, estimates of the gating charge movement in proton channels are half as large, 6–8 e_0 [132] or 5.4 e_0 for the native H^+ channel in rat alveolar epithelial cells [29] and 6.0 e_0 for H_V1 or mVSOP expressed in HEK-293 cells [58]. The values for proton channels were estimated by the limiting-slope method [133] from data spanning a narrow range of open probability values, due to the impracticability of recording single-channel currents, and thus likely underestimate the true gating charge. Intriguingly, BK channels also have three of the four key Arg residues in S4, yet they have weak voltage dependence, with $e_0 = 2.3$ [129]. Part of the explanation is that over half the gating charge arises from charge movement in S1–S3, and part is that for the BK channel, gating is not strictly coupled to voltage sensor movement [129]. Measuring proton channel gating current is conceivable only at the reversal potential, because 'blockers' such as Zn^{2+} act by altering gating rather than by pore occlusion [32] and the permeant ion cannot be removed. Removing intracellular protons or adding extracellular Zn^{2+} simply shifts gating to more positive voltages, but does not abolish ionic current. Attempts to detect gating current have failed, probably because the slow activation process smears the gating current temporally over several seconds. Although the effective gating charge estimates for proton channels are only half as large as those for other channels, the proton channel may not function as a tetramer. Recent studies suggest that the proton channel functions as a dimer, each subunit with its own pore [62–64]. If gating is cooperative, the gating charge per subunit is comparable to that of the K^+ channel, with 3.2 e_0 for each of the four VSDs of K^+ channels. On the other hand, there is no reason beyond evolutionary inertia to believe that the charge movement that accompanies voltage sensing in proton channels should resemble that which opens K^+ channels, especially since the latter involves transmitting the gating process to a separate gate that is absent in the proton channel. As in the BK channel [129], the key charges that move during proton channel gating may not be concentrated in S4.

Pharmacology

There are no blockers of proton channels, in the strict sense of a drug that sterically occludes the conduction pathway. Most organic inhibitors alter gating, generally by shifting the g_H-V relationship in the positive direction [1]. Recently, hanatoxin, from tarantula venom, has been shown to inhibit in this manner, apparently by interacting with the residues in the proton channel corresponding with the 'paddle region' of voltage-gated K^+ channels [134]. Classical inhibitors of proton currents are inorganic, namely polyvalent cations, most frequently Zn^{2+} and Cd^{2+}. The potency sequence for divalent metal cations is:

$$Zn^{2+} \approx Cu^{2+} > Ni^{2+} > Cd^{2+} > Co^{2+} > Mn^{2+} > Ba^{2+}, Ca^{2+}, Mg^{2+} \approx 0$$

[124]. In addition to divalent cations, La^{3+}, Gd^{3+}, and Al^{3+} are also effective. All of these metal cations slow the activation of proton currents during depolarizing pulses and shift the g_H-V relationship positively. The maximum conductance, $g_{H,max}$, may also be reduced at high concentrations. The effects on gating resemble the effects of Ca^{2+} and other polyvalent cations on most voltage-gated ion channels. They are roughly what one would expect if the metal cation bound near the outside of the channel and altered the membrane potential sensed by the voltage sensor of the channel, as proposed by Huxley, Frankenhaeuser, and Hodgkin for voltage-gated Na^+ and K^+ channels in squid axon [135]. In snail neurons, Cd^{2+} appears to slow channel opening more than predicted from the shift of the g_H-V relationship [15]. In alveolar epithelium, Zn^{2+} slows activation more than predicted, but Cd^{2+} does not [32].

A striking feature of metal effects on proton currents is their exquisite sensitivity to pH_o. Considering the slowing of τ_{act}, Zn^{2+} was ~10 times less potent at pH_o 6 than pH_o 7, and ~100 times less potent at pH_o 5 than at pH_o 6. This result could be explained if Zn^{2+} bound competitively with protons at a site comprising two or three titratable groups with a pK_a of 6.2–6.6, strongly suggesting His residues [32]. Mutagenesis of the human channel, H_V1, confirmed this prediction. Two His residues, His^{140} and His^{193}, face the external solution, and neutralizing mutations eliminate Zn^{2+} sensitivity [21].

Zn^{2+} applied to the intracellular solution has more subtle effects, slowing τ_{tail}, reducing $g_{H,max}$ by about 50 % at 170 μM Zn^{2+} at pH_i 6.5, and possibly shifting the g_H-V relationship toward more negative voltages (in the direction expected for surface charge screening effects) [32]. Intracellular Zn^{2+} may also compete with H^+, because 45 μM Zn^{2+} applied at pH_i 7.5 reduced H^+ currents by 70 % [57], suggesting higher apparent potency at higher pH_i. More detailed discussion of Zn^{2+} effects can be found in a recent review of the pharmacology of proton channels [6].

Distribution of proton channels

Table 2 lists the species and cells in which proton currents have been identified by voltage clamp or in which the gene has been identified. Each year a few cells are added to this list. The only cells that have been found not to express proton channels are the COS-7 monkey kidney cell line [45, 47], the HM1 derivative of the HEK-293 cell line [21], and the human hepatoma cell line HUH7.5 [V.V. Cherny, unpublished data]. The gene is expressed in at least two protozoa, indicating that it has been around for some time, evolutionarily. It exists in sea urchins, sea squirts, birds, and numerous mammals, including of course, humans. In humans, proton channels are present in various epithelia and are ubiquitous in leukocytes where they serve several specific functions [4, 34].

A number of cells that may have proton channels are omitted from Table 2. Suggestive evidence supports the expression of voltage-gated proton channels in the human bronchial epithelial cell line 16HBE14o- [136], rat hippocampal CA1 neurons [137, 138], and human synovial cells [139]. An intriguing observation is the nearly parallel expression of Nox2 and proton channels. Proton channel homologs are present in at least 10 of 12 species that express Nox2 [140]. This coincidence may reflect that evolutionary forces find proton channels useful in compensating the electrogenic activity of NADPH oxidases. On the other hand, it may simply reflect the relatively small number of species for which extensive genetic data exist.

In the cells listed in Table 2, proton channels are present in the plasma membrane. It is generally assumed that proton channels in phagocytes are present in the phagosome membrane, which is derived from plasma membrane that clearly contains proton channels. Indirect evidence suggests that proton channels also exist in the membrane of the Golgi complex where they help regulate the pH [141].

Biochemical regulation of proton channels

Intracellular Ca^{2+}, $[Ca^{2+}]_i$

Although increasing $[Ca^{2+}]_i$ may enhance proton currents, this effect seems to be indirect. It is clear that voltage-gated proton channels are not activated by increases in intracellular Ca^{2+} [1]. However, in whole-cell studies of human eosinophils, when $[Ca^{2+}]_i$ in the pipette solution was increased from ≤ 100 nM to 1 μM, proton currents were 2.2-fold larger at pH_i 7.2 [142], 2.4-fold larger at pH_i 7.0, and 1.4-fold larger at pH_i 6 [78]. However, increased $[Ca^{2+}]_i$ did not detectably enhance proton currents in snail neurons [15], HEK-293 cells [44], or human neutrophils [143], or in inside-out patches from human lymphocytes [80], rat alveolar epithelial cells [6], or human eosinophils [57]. The results from excised inside-out patches of membrane are more convincing because the $[Ca^{2+}]_i$ can be changed in the same experiment. These observations may be reconciled by assuming that Ca^{2+} has no direct effect on the channel itself. Changes in $[Ca^{2+}]_i$ in intact cells might enhance proton currents indirectly [1], for example, by increasing PKC activity, which is known to 'activate' proton channels [39].

Arachidonic acid

There is no question that arachidonic acid (AA) has pharmacological effects that enhance proton currents. However, it remains controversial whether AA achieves effective concentrations *in vivo*. In whole-cell studies, AA increases H^+ currents, shifts the g_H-V relationship toward more negative voltages and hastens channel gating: both opening during depolarizing pulses (smaller τ_{act}) and closing upon hyperpolarization (smaller τ_{tail}) become more rapid [25, 78, 142, 144]. Other unsaturated fatty acids had similar effects with a potency sequence: AA > palmitoleate ≈ palmitelaidate > linoleate > oleate > elaidate; saturated fatty acids were ineffective [144]. Similar responses are observed in excised membrane patches [V.V. Cherny and T.E. DeCoursey, unpublished data], suggesting a fairly direct effect on the channel.

Table 2. I_H Density in Species and Cells Reported to Have H^+ channels.

Group	Species	Tissue	Cell type	$I_{H,max}$ (pA/pF) (pH_i)	Reference
Protozoan	*Trypanosoma brucei*		kinetoplastid	gene	GenBank
	Tetrahymena thermophila		ciliate	gene	GenBank
Echinoderm	*Strongylocentrotus purpuratus*		purple sea urchin	gene	GenBank
Mollusc	*Helix aspersa*	neuron	neuron	4.5 (~6.8)	213
	Lynmaea stagnalis	neuron	neuron	14.6 (5.9)	19
	Tritonia diomedea	epithelium	ciliated epithelium	91.1 (7.3)[§]	174
Tunicate	*Ciona intestinalis*		sea squirt	gene, X	22
Fish	*Danio rerio*	–	–	gene	GenBank
	Tetraodon nigroviridis	–	–	gene	GenBank
Amphibia	*Ambystoma*	oocyte	oocyte	8.4 (~7.2)	16
	Rana esculenta	oocyte	oocyte	~32	160
	Rana pipiens	epithelium	renal proximal tubule	<75 (6.5)	214
	Xenopus laevis	–	–	gene	GenBank
	X. tropicalis	–	–	gene	GenBank
Birds	*Gallus gallus*	–	–	gene	GenBank
	chicken	macrophage	osteoclast	? (7.3)[a]	215
Mammals	hamster	epithelium	ovary CHO	1.6 (5.5)	216
	mouse	connective tissue	fibroblast 3T3	2.5 (5.3)	50
	mouse	macrophage	peritoneal	~30 (6.0)	79
	mouse	microglia	microglia	42 (6.0)	217
	mouse	microglia	BV-2	~20 (5.5)	218
	mouse	granulocyte	mast cell	9.6 (5.5)	30
	mouse	macrophage	osteoclast		219
	mouse	granulocyte	granulocyte	~20 (7.0)	39
	mouse	granulocyte	neutrophil	~50 (7.0)	220
	Rattus norvegicus (rat)	–	–	gene	GenBank
	rat	neuron	hippocampal CA1	0.9 (6.0)	173
	rat	epithelium	alveolar	27.3 (5.5)	23
	rat	macrophage	alveolar	~2 (7.5)	218
	rat	microglia	microglia	~66 (7.2)[a]	221
	rat	microglia	microglia	20 (5.5)	148
	rat	microglia	GM1-R1	26 (5.5)	222
	rat	microglia	MLS-9	0.22 (5.5)	218
	Rabbit (bunny)	macrophage	osteoclast	6.7 (6.0)	154
	Canis familiaris (dog)	–	–	gene	GenBank
	Bos taurus (cow)	–	–	gene	GenBank
	cow	chondrocyte	articular	17 (6.0)	223
	Pan troglodytes (chimp)	–	–	gene	GenBank
Human		epithelium	lung A549	~2 (5.5)	20
		epithelium	prostate PC-3	~4 (5.5)	20
		epithelium	kidney HEK-293	~1 (6.5)	218
		epithelium	airway JME	1.38 (5.3)*	168
		epithelium	airway gland Calu-3	1.8 (5.3)**	49
		epithelium	tracheal	2.9 (5.3)**	49
		epithelium	cervical Hela	1.38 (5.3)	1
		fibroblast	cardiac	~2.6 (6.2)	224
		skeletal muscle	myocyte	~10 (5.5)	24
		lymphocyte	T lymphocyte	0.9 (6.0)	80
		lymphocyte	B lymphocyte	94.7 (6.0)	80
		lymphocyte	Jurkat E6–1	36.3 (6.0)	80
		macrophage	monocyte	28 (6.0)	41
		macrophage	THP-1 monocyte	22 (5.5)	83
		microglia	microglia	? (7.3)[a]	225
		granulocyte	neutrophil	17 (6.0)	25
		granulocyte	eosinophil	~200 (6.0)	78
		granulocyte	basophil	~100 (5.5)	81
		granulocyte	HL-60	133 (5.5)	26
		granulocyte	K-562	~5 (6.0)	20

This table includes only cells in which the existence of H^+ channels was established by direct voltage-clamp studies, and species in which homologs of the proton channel exist. X = proton currents demonstrated by heterologous gene expression. $I_{H,max}$ is the largest H^+ current measured in a given cell (normalized to capacitance, which reflects surface area), usually at ~150 mV positive to V_{rev}; $g_{H,max}$ values were converted to current at V_{rev} + 150 mV. In studies where typical values or cell size was not specified, estimates were based on data in e.g. figures and are preceded by a tilde (~), as are values from surveys including a small number of cells. All values are at room temperature (20–25 °C), except where noted (*) at 37 °C, (**) at 26 °C, or ([§]) at 10 °C. Table updated from DeCoursey [1].

[a] Identity of the conductance not established with certainty.

However, when AA was applied in perforated-patch (rather than whole-cell) configuration, its enhancement of proton currents in human eosinophils was distinctly more profound [38]. Furthermore, its effects were partially reversed by protein kinase C (PKC) inhibitors in the continued presence of AA [39]. The implication is that AA can enhance proton channel gating directly, but also indirectly through activation of PKC. A further distinction is seen with the effects of oleic acid, which was as potent as AA in the perforated-patch study [38], but much weaker than AA in the whole-cell study [144]. Evidently, oleate and AA activate PKC with similarly efficacy, but differ in their direct effects on proton channels. The enhancement of proton currents by AA occurs over a narrow concentration range, with distinctly increased H^+ current at 1 μM AA, large effects at 5 μM, while at >5 μM AA, membrane damage usually occurred within a few minutes [38]. The proximity of the effective concentration of AA to the critical micelle concentration has been noted [145, 146]; however, novel delivery methods such as platelet microparticles might enable high concentrations locally [147]. The concept of concentration for an extremely lipophilic molecule like AA is somewhat ill-defined, and thus the physiological relevance of AA may remain obscure. However, it has been shown that the generation of AA by cPLA$_2\alpha$ is not required for normal activation of proton currents in human eosinophils or in murine granulocytes, by pharmacological inhibition and knockout, respectively [39].

Cytoskeletal interactions: response to swelling

Cell swelling induced by lactic acid exposure has been reported to double $g_{H,max}$ in rat microglial cells [148]. This weak acid enters the cell in neutral form and decreases pH_i, which accounts for much of this effect. However, swelling induced by hypotonic stress also reportedly increased H^+ currents [148]. The actin stabilizer phalloidin inhibited the acidosis-induced swelling [148]. PKC inhibitors also prevented the slow increase in H^+ current [149].

Longer-term cytoskeletal interactions were observed in murine microglia. Acute exposure (30 min) to intracellular cytochalasin D or colchicine had no discernable effect. However, 24-h exposure to the cytoskeletal disrupters cytochalasin D or colchicine reduced proton currents, whereas cytoskeletal stabilizers phalloidin or taxol had no effect [150].

Phospholipids/lipoprotein

It was reported that low-density lipoprotein (LDL) 'activated' a voltage-gated non-selective cation current in mouse macrophages by shifting the voltage-activation curve negatively by ~20 mV [151]. The currents reported closely resemble proton currents and the experiments did not rule out this possibility. However, commercial preparations of LDL often contain EDTA, which produces very similar enhancement of voltage-gated proton currents [27], presumably by chelating contaminant metals in the water or salts used. A similar LDL effect in rat alveolar macrophages was abolished by including EGTA in all solutions [V.V. Cherny and T.E. DeCoursey, unpublished observations].

The 'activated' state of proton currents, which is discussed below, was reported to exhibit rapid spontaneous rundown (i.e., reversion to the 'resting' state) in excised inside-out membrane patches from eosinophils [57, 152]. The enhanced gating mode was preserved only by a combination of 5 mM ATP, 50 μM ATPγ-s, and 25 mM phosphoinositide 3,4-bisphosphate [152]. Under similar conditions, we do not consistently observe rundown; however, it may be that our excised patches include submembrane components that preserve function.

Functions of proton channels

Acid extrusion

Because the pH and voltage dependence of g_H activation results in the conductance being activated only when there is an outward electrochemical gradient for H^+, it is universally agreed that a fundamental function of voltage-gated proton channels is to extrude acid from cells, especially under conditions of acute acid load. Thomas and Meech [14] showed that proton currents contributed to pH_i recovery after HCl injection in snail neurons. Proton channel involvement of pH_i recovery from an acid load due to an NH_4^+ prepulse [153] has been reported in rabbit osteoclasts [154, 155], murine mast cells [30], rat microglia [148], human neutrophils [156, 157], rat alveolar epithelial cells [158], and rat hippocampal neurons [138].

Cells can choose from a number of different transporters that move H^+ equivalents across membranes. Proton channels are an extremely efficient means of extruding acid. First, they 'cost' the cell nothing. Other proton transporters consume ATP either directly, as the H-ATPase, or indirectly, such as the Na^+/H^+-antiporter, which uses and consequently dissipates the Na^+ gradient to drive H^+ transport. Second, proton channels have a prodigious capacity to transport protons and hence to change pH_i. In murine macrophages at room temperature, the proton conductance can increase pH_i at a rate of 1.8 units/min even when the cell is dialyzed with 100 mM buffer [79]. In contrast, in human

neutrophils even at 37 °C, the peak rate of pH change due to Na^+/H^+-antiport is only 0.34 units/min [156], 0.077 units/min due to Cl^-/HCO_3^- exchange [159], and a meager 0.058 units/min due to H-ATPase [156]. Another consideration in deciding which transporter is most useful is electrogenicity; proton channels and H-ATPase are electrogenic, whereas Na^+/H^+-antiport and Cl^-/HCO_3^- exchange are electroneutral. If the goal is to move charge across the membrane, then an electrogenic transporter is required. If pH change is the goal, then electrogenicity may be a disadvantage. As will be discussed below, during the respiratory burst, the phagocyte needs both charge compensation and pH regulation, and the voltage-gated proton channel performs both tasks admirably.

Amphibian oocytes

Proton channel expression changes during maturation of salamander (*Ambystoma*) oocytes [17]. Proton currents in *Rana esculenta* (frog) oocytes [160] have been suggested to regulate calcium oscillations [161].

Alveolar epithelium

Proton channels are highly expressed in rat alveolar type II cells and persist after differentiation in culture into type-I-like cells [23]. Other than the ubiquitous function of acid extrusion [158], no specific function has been demonstrated. One hypothesis was that proton channels might contribute to CO_2 elimination by the lung by supporting facilitated diffusion. Carbonic anhydrase in alveolar epithelial cells converts CO_2 to H^+ and HCO_3^-, amplifying the total concentration of diffusible species 10- to 20-fold, and thereby facilitating their diffusion [162]. The main drawback of this mechanism is that it requires spontaneous recombination of H^+ and HCO_3^- in the alveolar subphase (liquid lining the alveolar air space) and because this fluid lacks carbonic anhydrase [163, 164], the reformation of CO_2 would occur slowly. Although indirect evidence opposing this mechanism has been published [165–167], a definitive test has not been reported.

Airway epithelium

Stimulation of human airway epithelial cells by histamine or ATP results in proton secretion, which helps acidify the mucosal surface. This acid secretion is largely mediated by voltage-gated proton channels, and is inhibited by apical Zn^{2+} application [168]. An expansion of this role came with the discovery that both human tracheal epithelial cells [49, 169] and fetal human alveolar type II epithelial cells express homologs of NADPH oxidase, including DUOX-1 [170]. In both types of lung cell, the epithelial cells expressing DUOX-1 release H_2O_2 (possibly for its antibacterial effect) in addition to secreting acid. As shown in Figure 2, the NADPH oxidase activity generates intracellular protons that are extruded via proton channels, just as in phagocytes (see below). Because the apical membrane potential of airway cells is relatively stable [171], it has been proposed that the outwardly directed H^+ gradient is the key regulator of H^+ channel activity. The H^+ gradient may be maintained by HCO_3^- secretion across the apical membrane through CFTR channels [172].

The human bronchial epithelial cell line, 16HBE14o-, acidifies upon exposure to ATP, and this response is inhibited by Zn^{2+} [136]. However, it would be unusual for a voltage-gated proton channel to permit H^+ influx, unless its gating were enhanced, as occurs in activated phagocytes. Stimulation of rat alveolar epithelial cells with PMA in perforated-patch configuration does not alter the gating of proton channels [36]. Thus it is not clear whether proton channels are involved in the reported proton influx.

Rat hippocampal neurons

Rat hippocampal neurons exposed to anoxic conditions exhibit a transient drop in pH_i followed by depolarization and recovery of pH_i which is inhibited by Zn^{2+}, and appears to be mediated by voltage-gated proton channels [137, 138]. Direct recording of proton currents was complicated by their small amplitude and their being masked by a larger, non-selective conductance [173].

Tritonia diomedea

The ciliated epithelium of nudibranch molluscs expresses large proton currents of uncertain function [174].

Snail neurons, human skeletal myotubes

A proposed function of proton channels in snail neurons is to extrude acid that accumulates during trains of action potentials [14, 15]. Consistent with this function, snail proton currents activate very rapidly during depolarizing pulses, in a few milliseconds, compared with mammalian proton channels that open with time constants of the order of seconds. A similar function has been proposed in human skeletal muscle [24].

The phagocyte respiratory burst

Well before the presence of voltage-gated proton channels had been demonstrated in human neutrophils by voltage-clamp [25], their role in charge compensation during the 'respiratory burst' had already been proposed by Henderson and colleagues [34]. Although this remains the most distinctive

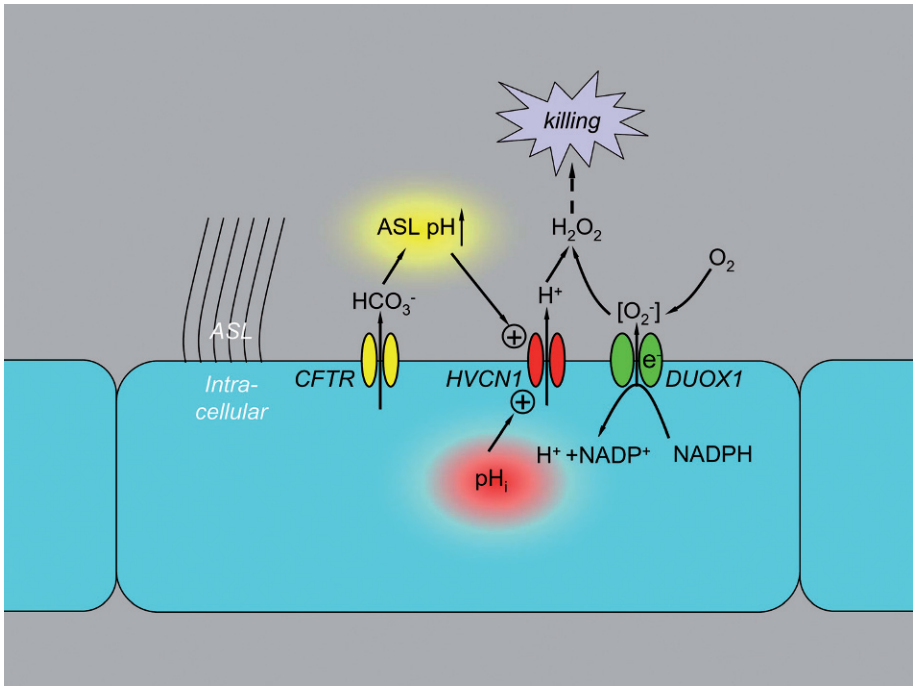

Figure 2. The NADPH oxidase isoform in the airways is DUOX1. DUOX1 is highly expressed in the apical membrane of lung epithelial cells [170]. Like other NADPH oxidases (Fig. 3), DUOX1 generates intracellular acid as it transports electrons (e^-) across the membrane to form extracellular superoxide. H^+ extrusion through proton channels prevents excessive intracellular acidification and acts as a substrate to form H_2O_2. Release of H_2O_2 into the airway surface liquid (ASL) may support bacterial defense. Apical CFTR channels are permeable to HCO_3^-, which tends to alkalinize the subphase liquid and maintain the H^+ gradient that is necessary to keep the H^+ channel open [adapted from ref. 172].

function of proton channels in phagocytes, several additional consequences of proton flux may be equally important [4]. Interested readers may consult more focused reviews of the physiological roles of proton channels in phagocytes [1, 3, 4, 175, 176]. Figure 3 illustrates the main features of the respiratory burst with respect to proton husbandry. When neutrophils are challenged by bacteria or other stimuli, NADPH oxidase assembles and begins to consume O_2 and produce $O_2^{\cdot-}$ at rates drastically greater than in resting cells [177]. This process is called the 'respiratory burst' [178, 179], despite the more conventional nomenclature in which cellular respiration reflects mitochondrial O_2 consumption. The $O_2^{\cdot-}$ produced is a precursor for a variety of other reactive oxygen species, including many with greater bactericidal activity. Because many reactions involving $O_2^{\cdot-}$ produce O_2 as a product, there is substantial recycling [177]; consequently O_2 consumption substantially underestimates the actual turnover rate of NADPH oxidase. In eosinophils, which attack helminths too large to engulf, NADPH oxidase assembles at the plasma membrane and extrudes electrons to produce extracellular $O_2^{\cdot-}$, whereas in neutrophils and macrophages that phagocytose bacteria, the oxidase assembles in the phagosome membrane and electrons are injected into the phagosome. Each electron that exits the cytoplasm via NADPH oxidase effectively 'leaves behind' at least one proton, producing charge separation. Consequently, electron translocation across the membrane produces immediate and profound depolarization. Because NADPH oxidase works by translocating electrons, its activity can be measured directly in single voltage-clamped cells as an electron current [180]. In contrast with O_2 consumption, this electron current is a direct, real-time reflection of NADPH oxidase activity. PMA-stimulated electron current is 2–3 pA in human and mouse neutrophils [36, 39], and 5–12 pA at room temperature at pH 7.0 in human eosinophils [37, 39, 181, 182], paralleling the threefold higher $O_2^{\cdot-}$ production rate by eosinophils, measured biochemically [181, 183–188].

Proton channels are activated during the respiratory burst by a combination of factors. (1) NADPH oxidase is electrogenic and produces a rapid and profound depolarization [34, 176, 189–192]. Because translocation of only a few charges is required to produce depolarization, substantial depolarization occurs as soon as a small fraction of the NADPH oxidase complexes in the cell have been activated [4]. In fact, several early studies concluded (understandably but erroneously) that depolarization preceded detectable NADPH oxidase activity [193–196], and might in fact be the trigger for the respiratory burst [197–199]. That the g_H is activated and its activity limits the extent of the depolarization during the respiratory burst is confirmed by experiments in which depolarization was increased by Zn^{2+} or Cd^{2+} [3, 34, 35, 192, 200]. (2) NADPH oxidase activity also generates intracellular protons, tending to produce cytosolic acidification, which would promote H^+ channel activation. Prevailing wisdom holds that the Na^+/H^+-antiport not only prevents significant acidification during the respiratory burst, but increases pH_i by changing the set point

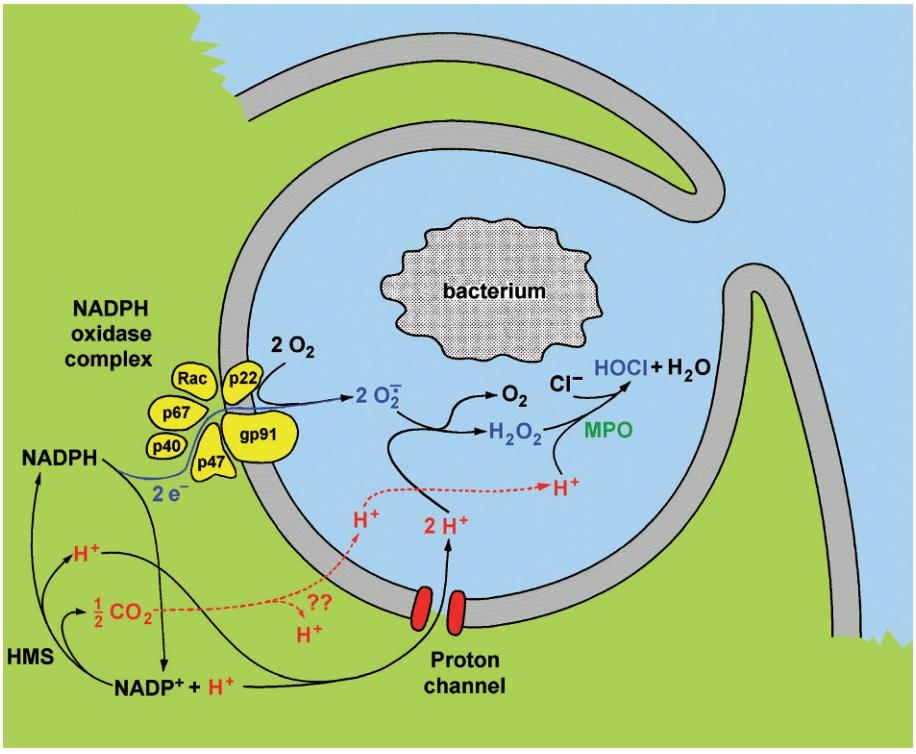

Figure 3. The main reactions that influence phagosome membrane potential and $pH_{phagosome}$ during the respiratory burst are summarized. The activity of NADPH oxidase is electrogenic [34], and most of the charge movement is compensated by proton efflux through voltage-gated proton channels. Each NADPH molecule consumed by NADPH oxidase activity and regenerated by the hexose monophosphate shunt (HMS) produces two H^+ in the cytoplasm. Two electrons from each NADPH cross the membrane through an electron pathway within cytochrome b_{558} (the combination of $p22^{phox}$ and $gp91^{phox}$), generating two $O_2^{\cdot -}$ molecules, which dismutate spontaneously, consuming two H^+ to form H_2O_2. Myeloperoxidase (MPO) converts roughly half of the H_2O_2 into HOCl. In addition, the HMS produces one CO_2 for every two NADPH regenerated. The CO_2 may dissociate in the cytoplasm or cross the phagosome or plasma membrane before dissociating, with various consequences [from ref. 4].

of the antiporter [201, 202]. However, recent studies on human neutrophils indicate that upon phagocytosis, pH_i decreases significantly and proton efflux is rapidly activated [203]. (3) Finally, proton channel opening is greatly enhanced by respiratory burst agonists, due to phosphorylation by PKC of either the channel itself or a regulatory molecule [39]. All four of the changes in gating discussed previously serve to increase the probability of proton channel opening. Modeling indicates that if proton channel gating were not enhanced, the membrane potential would depolarize 25–30 mV more than occurs, which would attenuate the respiratory burst by ~18% [4] due to the inhibition of NADPH oxidase activity by depolarization [204].

The respiratory burst provides an extreme challenge to the cell. Its magnitude can be realized by expressing the cumulative electron translocation into the phagosome as a concentration; estimates range from 2 to 4 M or more [4, 205, 206]. This value reflects the tiny volume of the phagosome, which changes rapidly as granules fuse and add their contents [4, 207, 208]. Of course, the electrons do not accumulate – they reduce O_2 to $O_2^{\cdot -}$ which dismutates rapidly to form H_2O_2 that in turn is converted by myeloperoxidase to HOCl, which increases at a rate estimated at ~2 mM/s [209]. The respiratory burst requires continual turnover of cytoplasmic NADPH, estimated to occur 4–8 times per second [182], which is reconstituted by the hexose monophosphate shunt. As shown in Figure 3, each electron leaves a proton in the cytoplasm, which tends to lower pH_i and depolarize the membrane. Depolarization inhibits NADPH oxidase directly, by preventing electrons from crossing the membrane [204, 210]. Low pH_i also inhibits NADPH oxidase [182]. Both effects can be compensated by proton flux that precisely balances electron flux [4, 206]. If the electronic charge were compensated by other cations, the introduction of 2–4 M cation into the phagosome would cause extreme swelling [4], which does not occur, and the pH would change drastically, which does not happen, at least in neutrophils [211, 212]. An additional benefit of compensating charge with protons is that many products of $O_2^{\cdot -}$ and H^+ reactions in the phagosome, such as O_2, H_2O_2, H_2O, and HOCl, are membrane permeant and would minimize osmotic

perturbations [4]. Finally, both H^+ and Cl^- are required in large quantities in the phagosome as substrate to produce reactive oxygen species. Taking into account these ramifications of compensating charge with various ions, we concluded that no more than about 5% of charge compensation could be ascribed to ions other than H^+ [4]. In addition to charge compensation, proton currents help maintain both cytoplasmic and phagosomal pH, minimize osmotic changes and provide substrate for producing reactive oxygen species.

Conclusion

Voltage-gated proton channels originated as phenomenological observations on dinoflagellates and snail neurons. Perhaps because of the novelty of a proton-conducting ion channel, for over a decade it was hard to convince skeptics that proton channels existed as genuine membrane proteins, as opposed to being a peculiar leak through the membrane or an adventitious conductance through another channel. Nevertheless, much progress was made in defining the fundamental properties of these channels. Now that the gene has been identified, a whole host of questions can be addressed, including the whole gamut of structure-function questions. Is the channel really a dimer? Where is the permeation pathway? How does the channel achieve perfect proton selectivity? How does the channel gate? How does the gating machinery sense pH_o and pH_i? Although substantial evidence supports a variety of functions for proton channels, most of the evidence is based on pharmacological lesion experiments using the non-selective inhibitor Zn^{2+}. It is now possible to supplement this approach using small interfering RNA (siRNA), antibodies to the channel protein, and the creation of knockout mice. The remarkable topological similarity of the proton channel to the VSD of other voltage-gated channels may lead to insights into the mechanisms involved in both types of channel. We are thus in the happy position that rapid progress in the next few years seems inevitable.

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