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Voltage-Gated Proton Channels Find Their Dream Job Managing the Respiratory Burst in Phagocytes

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The voltage-gated proton channel bears surprising resemblance to the voltage-sensing domain (S1–S4) of other voltage-gated ion channels but is a dimer with two conduction pathways. The proton channel seems designed for efficient proton extrusion from cells. In phagocytes, it facilitates the production of reactive oxygen species by NADPH oxidase.

In 1972, Hewlett-Packard introduced the HP-35, the first scientific pocket calculator, which cost $395 and used reverse Polish notation, which resembles the German language in putting the verb (or operator) at the end of the sentence. Democratic National Headquarters at the Watergate Hotel in Washington D.C. was burglarized. Ceylon was renamed Sri Lanka. Also in 1972, Woody Hastings and colleagues proposed the existence of voltage-gated proton channels in the dinoflagellate *Noctiluca miliaris* (70). One decade later, Roger Thomas and Bob Meech assailed a single snail neuron with a bewildering array of up to a half-dozen electrodes (to measure and control voltage, to measure current, pH_i, pH_o, and to inject HCl), resulting in the first voltage-clamp report of proton channels (211). Subsequent work, most notably the seminal study by Byerly, Meech, and Moody (22), confirmed that proton channels were genuine ion channels like the better known K^+, Na^+, and Ca^{2+} channels (23). Lydia Henderson, Brian Chappell, and Owen Jones provided strong indirect evidence that proton channels play a crucial role in compensating the electrogenic activity of NADPH oxidase in human neutrophils (84–86), which remains today their most clearly established specialized function. Voltage-clamp studies in the early 1990s confirmed the existence of proton channels in mammalian (43) and human cells (13, 49, 60). During the remainder of this *fin de siècle* era, fundamental properties of proton channels were determined, and the number of cells and species known to express proton channels grew to dozens (47). The discovery that proton channel gating was dramatically altered and enhanced in phagocytes during NADPH oxidase activity (9) combined with the application of the perforated-patch approach to study both proton and electron currents (the consequence of electrogenic NADPH oxidase activity) in living, responsive human neutrophils (56) advanced the understanding of the intricate interrelationship between proton channels and NADPH oxidase in phagocytes, one focus of this review. Genes coding for proton channels were discovered in 2006, ushering in a new era of unprecedented activity that is only just underway.

Properties of Voltage-Gated Proton Channels

Proton channel gene

After a decade of controversy and turmoil over the proposal that the $gp91^{phox}$ component of NADPH oxidase was a voltage-gated proton channel (8, 31, 45, 55, 56, 61, 76, 82, 83, 87, 88, 125, 127, 128, 139, 149, 161, 170, 188), genes coding for proton channels were finally identified in 2006 in human (173), mouse, and *Ciona intestinalis* (184). More recently, proton channel (*Hvcn1*) knockout (KO) mice have been shown to lack detectable proton current in neutrophils (65, 135, 168, 174), B lymphocytes (25), monocytes, and alveolar epithelial cells (unpublished observations with Ramsey IS, Cherny VV, Musset B, Morgan D, Clapham DE). The suggestion that $gp91^{phox}$ might be a second type of proton channel that is active only when NADPH oxidase is assembled and active (9) was finally ruled out conclusively by studies of KO mouse neutrophils in perforated-patch configuration, in which the existence of PMA-stimulated electron current directly demonstrated the presence of active NADPH oxidase complexes in the plasma membranes of cells that lacked detectable proton current (65, 135).

Thus far, only one proton channel gene has been identified in any species. The human *Hvcn1* gene codes for 273 amino acids (FIGURE 1), nominally a 32-kDa protein. In Western blots of human B cell lines, the *Hvcn1* gene product often appears as a doublet, which reflects the expression of both a full-length and a shorter protein that results from a second initiation site downstream from the initial ATG (Ref. 25; Capasso M, Dyer MJS, unpublished observations).

A naturally occurring missense mutation, M91T, has been identified that is estimated to occur in <1% of the human population (93). This mutation appears to shift the position of the proton conductance-voltage (g_H-V) relationship positively, decreasing the likelihood of channel opening and consequently reducing the H^+ efflux elicited by high pH_o in a heterologous expression system (93).

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Molecular structure

The proton channel gene products in human (178), mouse, and *Ciona intestinalis* (184) bear astonishing resemblance to the voltage-sensing domain of other voltage-gated ion channels (FIGURE 2, TOP), even sharing three of the four Arg residues in the S4 domain thought to comprise the main voltage sensors in K^+ channels (2, 193). It is nevertheless unclear whether these residues serve the same function of voltage sensing in the proton channel, because only one has been found to reduce voltage sensitivity on mutation (173, 184). In addition, truncating the COOH terminus of the mouse channel, mVSOP, between the second and third Arg residues in S4 does not eliminate function (181).

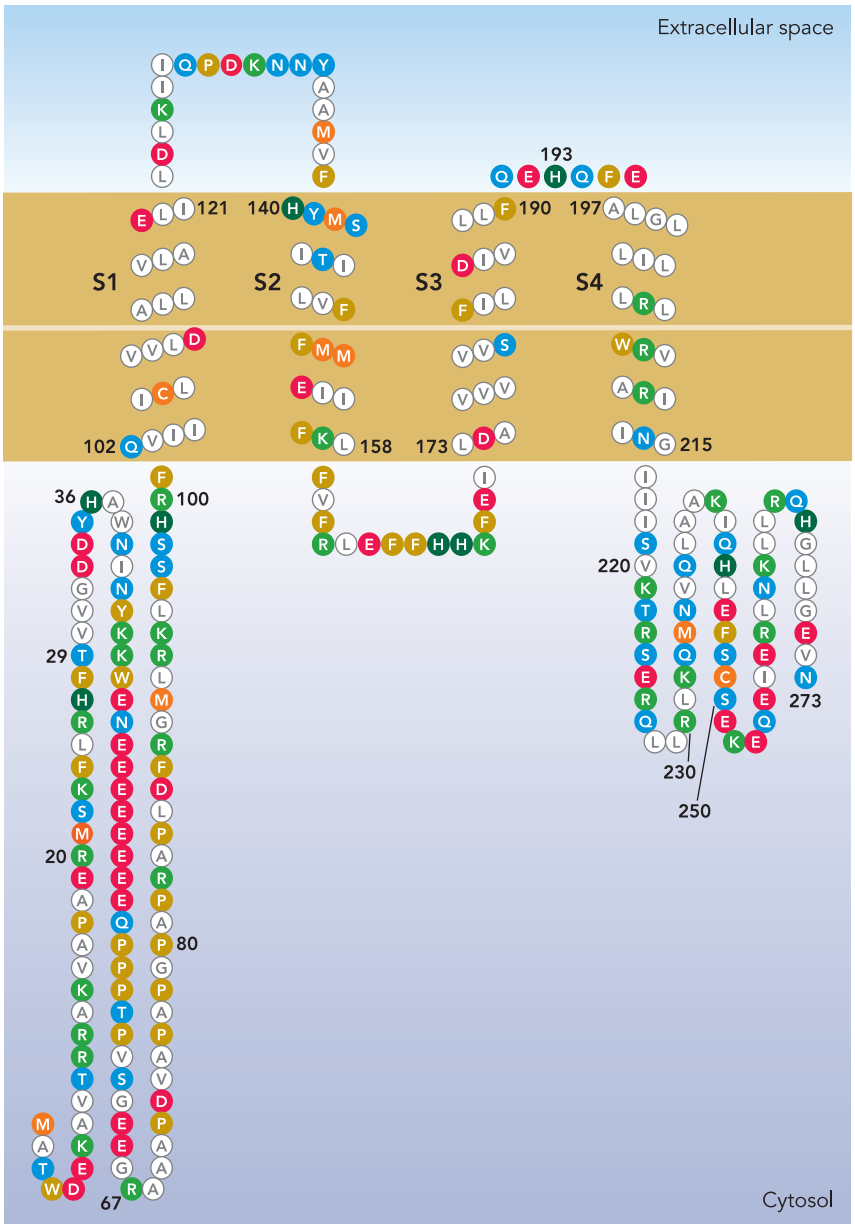

FIGURE 1. **Amino acid sequence of the human proton channel, H_V1 (*Hvcn1* gene product)**
The four membrane-spanning regions resemble S1–S4 of K^+ channels, including three of the four Arg residues in S4 that are thought to sense voltage (173). Membrane segments were determined by refined PHD prediction (179). Inhibition by Zn^{2+} reflects interactions at His^{140} and His^{193} (173). Phosphorylation of Thr^{29} produces enhanced gating shown in Fig. 3 (152).

The proton channel protein lacks the S5–S6 regions that form the pore of other channels. Evidently, a conduction pathway exists within the 273 amino acids that comprise S1–S4 in the human proton channel protein. This possibility seems entirely feasible, because the voltage-sensing domains (VSD) of K^+ channels can be converted into voltage-gated proton selective channels by single Arg-to-His mutations: R362H (203) or R371H (202, 204). All three classes of voltage-sensing molecules in FIGURE 2 appear to have several charged amino acid residues located in a narrow region within the membrane that has aqueous access on either end. The aqueous access regions serve to condense the membrane electrical field so that the charged resides can sense and respond to voltage without extensive physical translocation across the membrane (203). The aqueous access also provides a pathway for protons to cross most of the membrane; gating and selectivity may occur at the constriction, but neither function has been localized yet.

In 2008, three groups provided strong evidence that the proton channel exists as a dimer, with a conduction pathway in each monomer (107, 116, 212). It is not known whether the monomeric channels interact or gate independently or what benefit might derive from this arrangement. Indirect evidence suggests a cooperative gating mechanism in which both monomers must undergo an opening transition before either can conduct (157).

Perfect selectivity

A hallmark feature of voltage-gated proton channels is their extreme selectivity. In fact, there is no evidence that any other ion can permeate; evidently, their selectivity is perfect. The reversal potential does not change (after correction for liquid junction potentials) on substitution of the predominant cation or anion with a wide range of ions. Observed deviations of the reversal potential from the Nernst potential for protons, E_H, probably reflect imperfect control over pH. Even by assuming they reflect permeation of other ions, the relative permeability calculated with the Goldman-Hodgkin-Katz equation (78, 89, 91) is still $>10^6$–10^8 greater for H^+ than other cations (45). The identification of proton currents requires careful measurement of reversal potentials at different pH; other conductances may resemble proton currents but are not. For example, many cells have Ca^{2+}-activated Cl^- currents that turn on slowly during depolarizing pulses (6, 81, 126).

The perfect selectivity of proton channels suggests that permeation may occur by a hydrogen-bonded chain mechanism (HBC) including side chains of amino acids (50), as proposed originally by Nagle and Morowitz (159). Proton selectivity achieved by a titratable His residue in the permeation pathway was proposed for the M_2 viral proton channel (144, 171, 189) and occurs for Arg→His mutants of the K^+ channel VSD (202, 203). By comparison, protons permeate

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gramicidin channels by a Grotthuss-like mechanism, by hopping across a single-file row of a dozen water molecules (118). Gramicidin channels have a higher conductance to H^+ than to any other cation (normalized by permeant ion concentration), but they are not proton selective (90, 158). The high H^+ conductance reflects the ability of protons to hop across the water wire without the necessity of water diffusing through the pore, in contrast to other cations that must wait in single file for the waters to permeate.

Proton conduction via water molecules that form a discontinuous water wire has been proposed to account for proton selectivity in some channels. Lear and colleagues (3, 115) observed proton selectivity of the synthetic channel $(LSLLLSL)_3$ but nonselectivity of $(LSSLLLSL)_3$ by virtue of the former being a tetramer too narrow to contain a continuous row of water molecules and the latter being a water-filled pore. Similarly, some proposals regarding the high proton selectivity (permeability for H^+ vs. other cations $>1.5 \times 10^6$) of the M_2 viral proton channel (37, 120, 122, 133, 143) include narrow constrictions that allow proton transfer between waters but prevent free water flux (73, 183, 200, 205). Molecular dynamics calculations for constricted water wires in M_2 (28) and in $(LSLLLSL)_3$ (219) produced 6,000-fold and 100-fold selectivity for H^+ over other cations, thus falling short of reproducing the extreme selectivity reported experimentally. However, these models consider only the transmembrane domain and ignore entry and exit steps. Selectivity could be enhanced if protons preferentially approach the channel by rapid transfer in the plane of the membrane—the "antenna effect" (1, 28, 79, 145).

It is at present unclear how selectivity is accomplished in the voltage-gated proton channel. Two observations suggest that asparagine, Asn^{214}, may play a role. Along with the three Arg residues in S4 of proton channels that align with the first three of four Arg in S4 of K^+ channels, proton channels have a conserved Asn^{214} in the position of the fourth Arg. The point mutation N214R abolished proton current (212). By analogy with the movement of the four Arg in the K^+ channel VSD during opening, Asn^{214} was predicted to move to the constriction of the proton channel during opening, providing a polar environment compatible with proton permeation (212). Supporting the plausibility of such a mechanism, a recent study of the water channel, aquaporin, showed that extreme proton selectivity could be achieved by the double mutation H180A/R195V in the putative selectivity filter region (220), which is distinct from the canonical NPA region in the center of the pore. The NPA (Asn-Pro-Ala) region selects against cation permeation (12) and was thought to exclude protons (20, 146, 207) because it coincides with the free energy barrier opposing proton permeation (26, 58, 92). However, even with NPA intact, the H180A/R195V mutant permits selective proton permeation, leading the authors to propose that, at a constriction, Asn can act as a hydrogen bond donor compatible with H_3O^+ acting as an acceptor, while excluding other cations (220). The surprisingly high proton conductance through the NPA motif may reflect the unique advantage protons have over other cations, namely the capacity for charge delocalization (28). In light of the high proton selectivity of the H180A/R195V aquaporin mutant, which lacks any obvious titratable residues in the pathway, one might speculate that Asn^{214} may not only permit proton permeation but act as the selectivity filter in the voltage-gated proton channel. However, this attractive idea appears to be disproved by the persistence of proton-selective permeation after COOH-terminal truncation between the second and third Arg residues in S4 (181), ablating Asn^{214} altogether!

Permeation is nontrivial

Permeation of protons through voltage-gated proton channels almost certainly occurs by a Grotthuss-like mechanism of proton hopping, as opposed to hydrodynamic diffusion of hydronium ions, H_3O^+, or the flux of OH^- in the opposite direction (45). The macroscopic, and more importantly, single-channel conductance increases with proton concentration on the side of the membrane from which current flows but is unaffected by pH on the distal side of the membrane (34). The deuterium conductance is only about half that for protons (51), which is also strongly suggestive that H^+ (not H_3O^+ or OH^-) is the conducted species. In contrast, the deuterium isotope effect on proton conduction through gramicidin channels (4, 36) or on water permeation through aquaporin I channels (124) is not too different from corresponding values in bulk water. Finally, permeation has strong temperature dependence, with $Q_{10} > 2$ (23, 109) and as high as 5.1 in excised patches at <20°C, much higher than the Q_{10} for permeation of other ions through ordinary ion channels (52). By comparison, the temperature dependence of proton flux through water-filled gramicidin channels

Table 1. Effects of transporters on pH_i and $pH_{phagosome}$ at different voltages

Transporter	Hyperpolarized		Depolarized	
	pH_i	$pH_{phagosome}$	pH_i	$pH_{phagosome}$
NADPH oxidase	↓↓↓	↑↑↑	↓↓	↑↑
Proton channel			↑↑↑	↓↓↓
H^+-ATPase	↑	↓	↑	↓↓
Na^+/H^+ antiport	↑	↓	↑	↓
ClC-3	↓	↑	↑	↓↓
HOCl diffusion	↓	↑	↓	↑
CO_2 diffusion	↓	↓	↓	↓

Expected effects of several membrane transporters or processes on pH in the cytoplasm (pH_i) or phagosome ($pH_{phagosome}$) of a neutrophil. Number of arrows reflects both rate and extent of effects. For example, ClC-3 and Na^+/H^+ antiport in the phagosome will cease as soon as phagosomal Cl^- or Na^+, respectively, is exhausted. ClC-3 is unique in that it is expected to change pH in opposite directions at different voltages.

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(4, 35) is closer to that for protons in bulk water. In summary, protons permeate the water-filled gramicidin channel with more facility than they traverse voltage-gated proton channels.

Single-channel proton currents are only a few femtoamperes even at low pH, just on the edge of being directly measurable under optimal conditions of low noise and tight seals in the TΩ range (34), which is critical for minimizing low-frequency noise (117). However, proton channel gating introduces current fluctuations that can be used to determine the conductance. The unitary conductance is independent of pH_o but decreases as pH_i increases from 140 fS at pH_i 5.5 to 38 fS at pH_i 6.5 (34), and 15 fS extrapolated to physiological pH_i 7.2 (at 21°C). Although this conductance is ~1,000 times smaller than that of ordinary ion channels, the most probable explanation is that the permeant ion concentration is extremely low: cells contain $>10^{-1}$ M K^+ but $<10^{-7}$ M H^+, one million times lower. The largest recorded single channel current is that of protons through gramicidin, 350 pA (2×10^9 H^+/s) at 5 M HCl (42), but if one extrapolates the unitary proton conductance of gramicidin to physiological pH, the result is actually smaller than for proton channels (45). Despite their miniscule unitary conductance, proton channels are expressed in most cells at sufficiently high levels that the macroscopic proton current is comparable to or larger than other currents, including K^+ currents (23, 43, 60, 67, 138). Although this requires a proton channel density of up to 100–200 μm^2 (45, 49), two orders of magnitude greater density has been observed for other membrane proteins (89).

Gating is regulated by voltage and pH

Proton currents look remarkably like delayed rectifier K^+ currents, although their gating is generally slower. Both are activated by depolarization, activation becomes faster at more positive voltages, the currents turn on with a sigmoid time course whose sigmoidicity is increased by preceding hyperpolarization (the "Cole-Moore effect") (41, 50), and tail currents decay exponentially with faster kinetics at more negative voltages. Effects of polyvalent cations and pH are qualitatively similar; extracellular metals and protons slow channel opening and shift the *g-V* relationship positively. However, proton channels distinguish themselves by the exquisite pH dependence of their gating. Increasing pH_o or decreasing pH_i shifts the g_H-V relationship by 40 mV/unit pH toward more negative voltages (33, 51). This relationship can be expressed as:

$$V_{threshold} = k\, V_{rev} + V_{offset} \qquad (1)$$

where $V_{threshold}$ is the voltage at which proton current is first detectable, V_{rev} is the reversal potential, and V_{offset} is a constant. The slope factor, k, was 0.79 for a large series of native proton channels (meaning that $V_{threshold}$ changes somewhat less steeply than V_{rev}) and

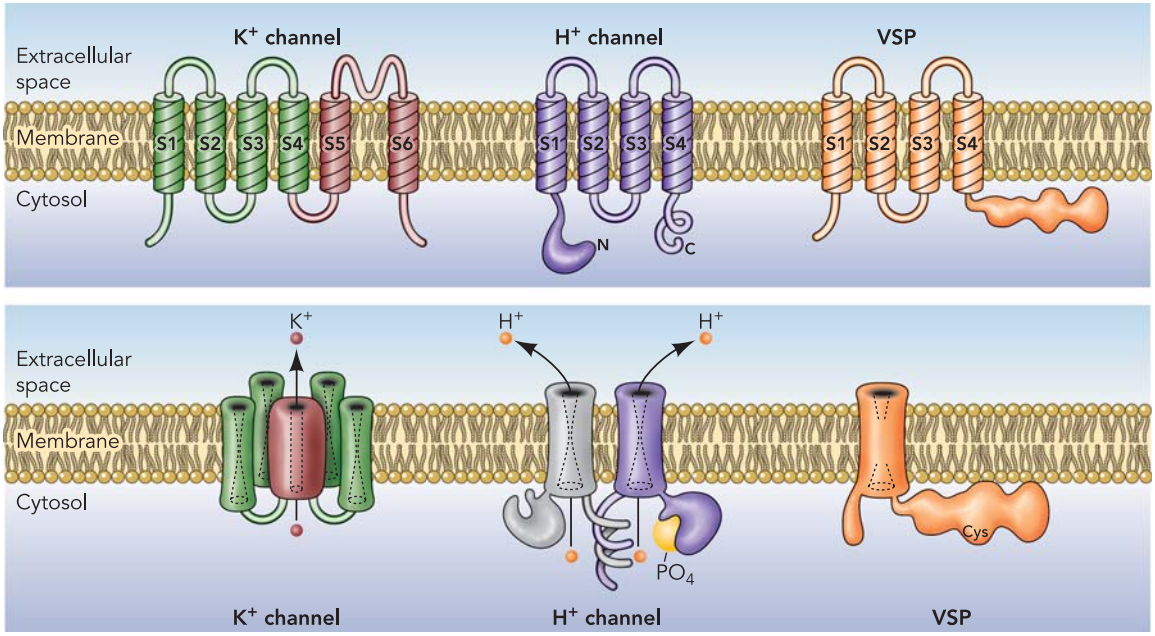

FIGURE 2. **Comparison of architectural features of K^+ channels, H^+ channels, and voltage-sensing phosphatases**

K^+ channels are tetramers of subunits that each contain six membrane-spanning regions, of which S1–S4 comprise the voltage-sensing domain (VSD) and S5–S6 form the pore. The S5–S6 regions from each subunit together form a single central pore, which is surrounded by the four VSDs. The voltage-gated proton channel contains S1–S4 regions that are quite similar to the K^+ channel VSD but lacks the pore domain S5–S6 (173, 184). The proton channel is a dimer in which each monomer has its own conduction pathway (107, 116, 212). The main site of attachment is the COOH-terminal coiled-coil domain (107, 116, 212). The NH_2-terminal intracellular region contains a phosphorylation site thought to convert the channel from "resting mode" to "enhanced gating mode" (56, 136, 152). The voltage-sensing phosphatase shares similar S1–S4 regions with the others but lacks conduction. Instead, it senses membrane potential and modulates phosphatase enzyme activity accordingly (147, 148).

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the offset was +23 mV (so that at symmetrical pH, $V_{threshold}$ is positive to V_{rev}). *Equation 1* holds over all physiological pH and appears to apply to all native proton currents (45). Thus, under physiological conditions, $V_{threshold}$ is positive to V_{rev}, and the proton conductance activates only when outward proton flux will result. This elaborate regulation of gating seems designed to facilitate acid extrusion from cells.

The mechanism by which ΔpH regulates the voltage dependence of gating is not known but presumably involves one or more titratable groups that can sample pH on both sides of the membrane, perhaps with alternating access, reminiscent of a carrier. The main features of pH- and voltage-dependent gating can be explained by a model that postulates a modulatory site that is accessible to only one side of the membrane at a time, its accessibility switches by a conformational change that can occur only when the site is deprotonated, and protonation from the inside promotes opening while protonation from the outside stabilizes the closed state (33).

For reasons that remain mysterious, both human (H_V1) and mouse (mVSOP) proton channel gene products in heterologous expression systems (COS-7 or HEK-293 cells) exhibit altered voltage dependence compared with native proton channels. Expressed proton channels obey the same 40 mV/unit pH rule summarized in *Eq. 1*, with similar *k*, but V_{offset} is roughly –10 mV, so the entire g_H-V relationship is shifted by roughly –30 mV compared with native proton currents. Consequently, at symmetrical pH ($pH_o = pH_i$), inward currents can be detected negative to E_H (155), something never reported in (resting) native proton channels.

Inhibition by Zn^{2+}

The most potent inhibitor of proton current is Zn^{2+} (123, 211); numerous other polyvalent cations are also effective. No high-affinity peptide inhibitors exist, although hanatoxin (from tarantula spider venom) at micromolar concentrations shifts the g_H-V relationship positively (5). A number of weak bases produce vague inhibition in the direction expected if the neutral form of the drug enters the cell and increases pH_i near the membrane (45, 50, 53, 130). Antibodies to the channel protein have been developed, but thus far none that binds to the channel and inhibits conduction has been reported. The effects of Zn^{2+} resemble the effects of divalent cations on other voltage-gated ion channels (74): activation slows and the *g*-*V* relationship shifts toward more positive voltages. The effects of Zn^{2+} are greatly attenuated at lower pH_o, suggesting competition between H^+ and Zn^{2+} for binding site(s) on the channel (30). The data were described by assuming a Zn^{2+} binding site comprising two to three titratable groups with pK_a ~6.5, suggesting His residues. When the human proton channel gene was identified, Ramsey et al (173) found that there were two externally accessible His residues, His^{140} and His^{193} (FIGURE 1), both of which contribute to inhibition by Zn^{2+}. Recently, it was proposed that Zn^{2+} may bind simultaneously to one His in each monomer rather than to both His in each monomer (157).

"Proton currents look remarkably like delayed rectifier K^+ currents, although their gating is generally slower."

Physiological Modulation of Proton Channel Function: The Enhanced Gating Mode

Proton channels are not sensitive to intracellular Ca^{2+} concentration (22, 53, 170, 186). In fact, very few agents affect proton currents studied in whole cell configuration. However, in perforated-patch studies, a variety of stimuli produce a constellation of dramatic effects on proton channel gating, called the "enhanced gating mode." Despite intriguing quantitative differences among cell types (154), the pattern is so stereotyped that it must reflect a common mechanism or pathway. FIGURE 3, A AND B, illustrate families of proton currents in a human eosinophil before and after stimulation by the phorbol ester PMA. The enhanced currents are larger, activate faster, and deactivate more slowly, and the g_H-V relationship is shifted negatively by 40 mV. This enhanced gating mode, first described in human eosinophils (9), occurs nearly identically in human neutrophils (56), monocytes (154), and PLB-985 cells (55), and in murine osteoclasts (141) and granulocytes (136). Enhanced gating with reduced features (a smaller shift of the g_H-V relationship and little or no slowing of τ_{tail}) is seen in human basophils (156), neutrophils from human subjects with chronic granulomatous disease, and PLB-985 cells lacking $gp91^{phox}$ (55). Intriguingly then, a full response is seen in cells with robust NADPH oxidase activity, and a weaker response occurs in cells lacking this enzyme (154). Proton currents in some cells have little or no PMA response, including rat alveolar epithelial cells (56) and HEK-293 or COS-7 cells transfected with *Hvcn1* (155).

Strengthening the apparent connection between proton channels and NADPH oxidase, agonists that induce enhanced gating also activate NADPH oxidase. These include PMA, arachidonic acid, oleic acid, LTB4 (leukotriene B4), IL-5 (interleukin-5), and fMLF (a chemotactic peptide) (11, 32, 54–56, 136, 141). Some, but not all, of the interactions between proton channels and NADPH oxidase (48, 154) may be explained by their common activation by phosphorylation. FIGURE 3, BOTTOM, illustrates the induction of enhanced proton channel gating after stimulation by the PKC activator, PMA, and the parallel activation of

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inward electron current that reflects the activity of NADPH oxidase. The PKC inhibitor GFX (GF109203X) reverses the effects on both molecules at least partially. Mutation of Thr^{29} in the intracellular NH_2 terminus of H_V1 abolished PKC and GFX responses, suggesting that phosphorylation of the channel itself, and not an accessory molecule, produces enhanced gating (152).

Functions of Voltage-Gated Proton Channels

Acid extrusion

In view of the exquisite regulation of their gating by voltage and the pH gradient, ΔpH, the primary function of voltage-gated proton channels has universally been considered to be acid extrusion. Intense metabolic activity produces intracellular acid, which promotes proton channel opening. Substantial evidence supports this function in a variety of cells. Recovery of pH by cells that are loaded with acid by HCl injection (211) or by the NH_4^+ prepulse approach (176) is slowed by inhibiting proton channels with Zn^{2+} in numerous cells (29, 59, 62, 110, 142, 150, 160, 164, 165, 194, 211). Depolarization by elevated $[K^+]_o$ to activate proton channels produces rapid alkalinization of TsA201 cells transfected with the murine proton channel but not in nontransfected cells (184). In cells that extrude metabolically produced acid, proton channels may be activated by the lower pH_i rather than by depolarization. This may be more evident in an alternative form of *Eq. 1*:

$$V_{threshold} = k\,(pH_o - pH_i) + V_{offset} \qquad (2)$$

in which, for alveolar epithelial cells,

$$k = -40 \text{ mV and } V_{offset} = 20 \text{ mV} \qquad (3).$$

Acid extrusion by proton channels is required during histamine secretion by human basophils (156). Zn^{2+} inhibits histamine-stimulated acid secretion by airway epithelial cells (69, 93, 188). Acid secretion in excised human sinonasal mucosa is inhibited 71% by Zn^{2+}, implicating proton channels in this process (38). The Zn^{2+}-sensitive fraction decreased to ~57% in chronic rhinosinusitis (38).

Specialized functions

Although acid extrusion is inevitable when proton channels are activated, other consequences of proton channel activity may be equally important in some situations. A contribution to cell volume regulation has been proposed in microglia (142) and chondrocytes (182). Facilitation of reactive oxygen species (ROS) production by DUOX1 in airways, analogous to that described below in phagocytes, has been suggested (68). Proton channels are highly expressed in human B lymphocytes (186) where they participate in B-cell receptor-mediated signaling cascades and antibody responses, most likely by enabling ROS production (18, 25). Possible facilitation of CO_2 elimination by the lung has been hypothesized (44). A contribution to pH and membrane potential regulation in cardiac fibroblasts, especially during ischemia, has been proposed (64). Elimination of protons during action potentials has been suggested in snail neurons (130) and human skeletal muscle myotubes (13). Proton channel activity was demonstrated in localized pH microdomains in snail neurons during electrical activity (190).

Proton Channels Facilitate the Respiratory Burst in Phagocytes by Four Mechanisms

Charge compensation in phagocytes

The best established specialized function of proton channels is charge compensation in phagocytes (FIGURE 4). Reactive oxygen species (ROS) produced by phagocytes contribute to microbial killing (7, 80, 99, 105, 106, 162, 177, 217). In other cells, ROS play a plethora of roles related to signaling but may also contribute to chronic disease (114, 163). NADPH oxidase produces superoxide anion ($O_2^-\bullet$), a precursor to numerous other ROS, and thus plays a central role in innate immunity. Of the several components that comprise the oxidase complex, $gp91^{phox}$ or Nox2 is the site of most of the action, containing an electron transport chain comprising NADPH in its binding site, FAD, and two heme groups that pass electrons sequentially to O_2 at an external binding site where $O_2^-\bullet$ is produced. The enzyme is electrogenic, because the electrons extracted from cytoplasmic NADPH are translocated to extracellular or intraphagosomal O_2 that is reduced to $O_2^-\bullet$ (84). In essence, the enzyme conducts electrical current in the form of electrons, just like a copper wire. Schrenzel et al. (187) demonstrated electron current in eosinophils, directly confirming that NADPH oxidase is electrogenic.

The necessity for charge compensation can be appreciated by considering the magnitude of the task. The electron current is roughly 35 pA in human eosinophils at 37°C (138). Most NADPH oxidase assembles at the plasma membrane in eosinophils (111), whose job description may include killing helminthes and other parasites too large to engulf (178). The 35-pA electron current charges the 2.5-pF plasma membrane capacitance (187) at a rate of 14 V/s. In fact, it has been proposed to use NADPH oxidase to power biofuel cells in implantable devices (180). Without charge compensation, the membrane would depolarize to +200 mV in <20 ms, at which voltage NADPH oxidase ceases to function (57). This depolarization reflects efflux of ~4×10^6 electrons, which constitute just 0.01% of the ~4.6×10^{10} electrons extruded in a brief (5 min) respiratory burst stimulated by the chemotactic peptide fMetLeuPhe (221). Considering the extreme sensitivity of membrane

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potential to net charge separation, it is understandable that early studies generally concluded that depolarization preceded NADPH oxidase activity and was, in fact, hypothesized to trigger the respiratory burst (24, 40, 100, 108, 192, 199, 216). The subsequent discovery that NADPH oxidase is electrogenic (84) made it clear that depolarization is a consequence of NADPH oxidase activity rather than its cause.

Protons are extruded from human neutrophils in 1:1 stoichiometry, with O_2 consumed during the respiratory burst (17, 75, 208, 213), indicating that proton efflux is responsible for the bulk of charge compensation (FIGURE 4). Evidence that voltage-gated proton channels mediate the bulk of the proton flux is extensive. Na^+/H^+ antiport is electroneutral (104, 196) and cannot compensate charge. Similarly, proton removal by CO_2 diffusion is not electrogenic. Inhibiting the (electrogenic) H^+-ATPase does not directly inhibit ROS production in neutrophils or macrophages (15, 59, 95, 206). Activation of NADPH oxidase produces a large depolarization, ~100 mV above the resting potential in human neutrophils (77, 94, 140, 172). The depolarization is exacerbated by Zn^{2+} (9, 10, 61, 84, 112, 172), indicating that a Zn^{2+}-sensitive mechanism compensates charge.

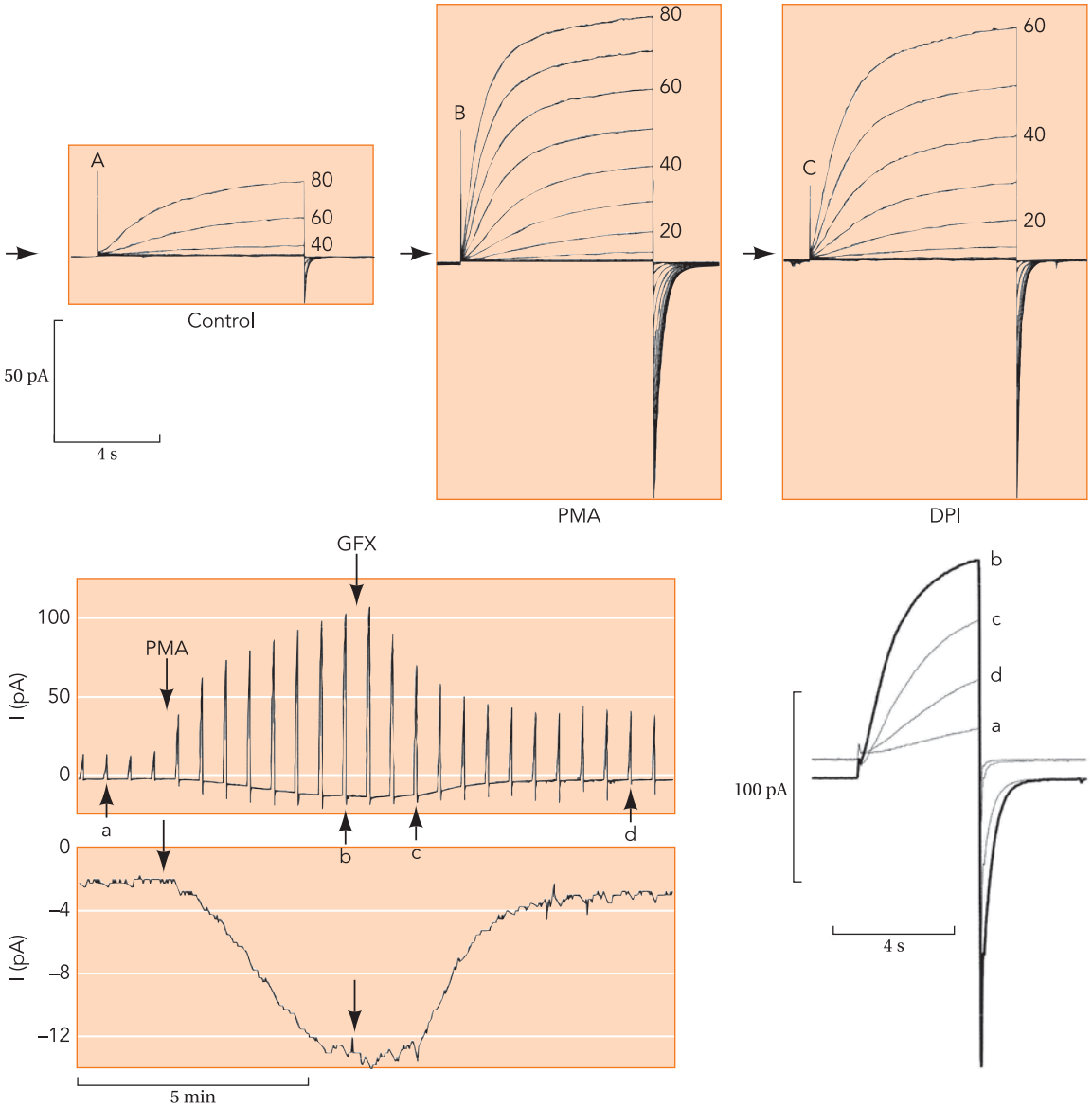

FIGURE 3. **Enhanced gating mode of proton channels in phagocytes**
Conversion of proton channels in human eosinophils from resting mode (*A*) to enhanced gating mode (*B*) by PMA stimulation in perforated-patch configuration. Families of pulses were applied from a holding potential of –60 mV in 20-mV (*A*) or 10-mV steps (*B*–*C*) as indicated (mV). Arrows indicate zero current; note the small inward electron current elicited by PMA (*B*). Inhibition of NADPH oxidase with diphenylene iodonium (DPI; *C*) eliminates the inward electron current, without immediate effect on the proton current. *Bottom*: the time course of the responses to PMA and GFX in a different eosinophil. Outward proton currents during pulses to +60 mV every 30 s are shown (above), with individual records identified by lower-case letters superimposed on the right at an expanded time scale. Net inward current at –60 mV at higher gain (below) reflects electron current through NADPH oxidase. PMA activates PKC, and GFX inhibits PKC; thus enhanced proton channel gating and NADPH oxidase are both regulated by phosphorylation. Figure reprinted with permission from the *Journal of Physiology* (54, 136).

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If charge compensation is necessary and is performed by proton channels, then inhibiting proton current should inhibit NADPH oxidase activity. Metals that inhibit proton current potently, including Zn^{2+}, Cd^{2+}, and La^{3+}, inhibit NADPH oxidase activity in neutrophils, eosinophils, monocytes, PLB-985 cells, macrophages, and microglia, from humans, mice, dogs, rats, and pigs, measured by cytochrome *c* reduction (superoxide anion production), by Amplex Red assays (H_2O_2 generation), or by O_2 consumption (11, 14, 39, 57, 67, 85, 86, 101, 121, 153, 166, 172, 174, 198, 201, 210, 222). The effects of Zn^{2+} cannot be ascribed to direct effects on NADPH oxidase, because there is no inhibition of enzyme activity in a cell-free system (222) or when electron currents are recorded under voltage clamp (57, 187). In both of these experimental situations, there is no need to compensate charge: membranes are permeabilized in the former, and the voltage-clamp amplifier provides compensation in the latter. These data show not only that proton channel activity occurs during the respiratory burst but that proton flux performs a function that is necessary for efficient ROS production, presumably charge compensation.

The creation of *Hvcn1*-deficient [proton channel knockout (KO)] mice has enabled non-pharmacological approaches to this question. In leukocytes from KO mice, NADPH oxidase activity is reduced by up to 75% (25, 65, 168, 174). This result indicates that the effects of Zn^{2+} in previous studies were mainly attributable to effects of proton channels rather than to some other Zn^{2+}-sensitive molecule. That some activity remains in KO cells demonstrates that proton channels are not indispensible, although they are clearly the mechanism preferred by phagocytes. This result is not surprising, because COS^{phox} cells (COS-7 cells transfected stably with the main NADPH oxidase components) have no proton currents, but produce detectable levels of superoxide anion (139). The impaired NADPH oxidase activity in KO cells suggests that proton channels contribute significantly and that the function they perform cannot readily be assumed by another molecule.

Despite the extensive evidence that proton flux through voltage-gated proton channels provides the bulk of charge compensation in neutrophils, other transporters might facilitate charge compensation in different cells. Also proposed to contribute to charge compensation in leukocytes are the ClC-3 Cl^-/H^+ antiporter (113, 134, 191), CLIC-1 Cl channels (132), TRPV1 nonselective cation channels (185), SK2 and SK4 Ca^{2+}-activated K^+ channels (103), and Kv1.3 delayed rectifier K^+ channels (72). SK channels appear to facilitate mitochondrial (NADPH oxidase independent) ROS production in granulocytes (66), illustrating that partial reduction of ROS production by an inhibitor does not always reflect reduction of NADPH oxidase activity.

The magnitude of the NADPH oxidase (Nox2) activity in phagocytes, hence the enormous need for charge compensation, strongly implicates protons as the main charge carrier (see below). The numerous other cells that use ROS for signaling generally produce orders of magnitude lower levels than phagocytes, often by other Nox or Duox isoforms. In most of these cells, the mechanism of charge compensation is unknown, although ROS production by B lymphocytes is defective in *Hvcn1* KO mice (25). In fact, there exists no direct demonstration that other Nox isoforms are electrogenic, although this seems exceedingly likely. Because the evidence supporting a role of proton channels in compensating for the electrogenic activity of NADPH oxidase in neutrophils is compelling (45, 61, 63), there is a tendency to assume a similar role for proton channels in other cells with Nox activity. That other conductances can compensate charge to some extent is clear from the studies of COS^{phox} cells (139) and *Hvcn1* knockout mice (25, 65, 168, 174) discussed above. Ultimately, it will be necessary to determine how charge is compensated in each cell.

Phagocytes produce enormous quantities of $O_2^-\bullet$, and ~95% of charge compensation is via proton channels (151). The cumulative concentration of electron equivalents (i.e., negative charges) in the phagosome (counting each $O_2^-\bullet$ produced as one electron) during a respiratory burst lasting a few minutes is 4 M (175). Despite $O_2^-\bullet$ being produced in the phagosome at a rate of 5–10 mM/s (80), neither electrons nor their initial product, $O_2^-\bullet$, accumulates in phagosomes. The $O_2^-\bullet$ rapidly dismutates into H_2O_2 (FIGURE 4) either spontaneously or catalyzed by myeloperoxidase (MPO) acting as a dismutase (102, 218). In turn, H_2O_2 is converted by MPO into HOCl, the active ingredient in household bleach. Most of the efforts of NADPH oxidase result in extensive HOCl production: one-third to two-thirds of the O_2 consumed is converted into HOCl (27, 71, 75, 97, 209, 215). HOCl is highly reactive (105) and can oxidize bacterial components as well as other phagosome contents or it can be converted into other ROS. The pK_a of HOCl is 7.5, and the neutral undissociated acid form can permeate the phagosome membrane and enter the cytoplasm, effectively shuttling protons out of the phagosome (FIGURE 4). MPO inhibitors attenuate the acidification that occurs after phagocytosis, indicating that HOCl diffusion from phagosome into cytoplasm constitutes a significant acid load (135).

The converse of charge compensation is control of the membrane potential by proton channels. If other conductances are minimized, the resting membrane potential approaches the Nernst potential for protons (E_H), for example, when NADPH oxidase is active in human eosinophils (9) or when pH is changed in cardiac fibroblasts (64). Even in physiological solutions, the strong depolarization produced by NADPH oxidase activity in human neutrophils or eosinophils (10, 61, 77, 84, 94, 135, 172), combined with a dearth of other voltage-gated ion channels (46, 67), results in the membrane potential depolarizing to a level at which proton and electron currents are balanced (61, 84, 151, 172).

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Cytoplasmic pH regulation during the respiratory burst

Although pH regulation is equivalent to acid extrusion, discussed above, special circumstances apply in the context of the phagocyte respiratory burst. NADPH oxidase activity in the phagosome membrane tends to lower cytoplasmic pH but increase phagosomal pH. Matching proton flux stoichiometrically with electron flux would prevent pH changes in either compartment. Maintaining pH is important because NADPH oxidase has strong pH_i dependence, with an optimum at pH_i 7.0–7.5 (137). In the presence of Zn^{2+}, pH_i in phagocytosing human neutrophils decreases rapidly: to 6.5 after 2 min and 6.0 after 10 min (135), at which pH_i NADPH oxidase activity is reduced by 50 or 80%, respectively (137). In *Hvcn1* KO mouse phagocytes, pH_i drops during phagocytosis to the same level seen in WT cells in the presence of Zn^{2+} (135). These results indicate that, without H^+ efflux through proton channels, pH_i would drop to levels that would impair NADPH oxidase activity drastically. This raises a semantic question. Is the main role of proton channels during the respiratory burst charge compensation or pH regulation? This question cannot be answered unambiguously, because H^+ efflux performs both functions simultaneously.

Substrates for ROS production: The problem of Cl^-

Phagocytes compensate charge with protons partly to supply the phagosome with the protons required for stoichiometric combination with $O_2^-\bullet$ to produce H_2O_2 (FIGURE 4). Another proton is consumed in the conversion of H_2O_2 to HOCl, which, as discussed above, is the predominant product of the respiratory burst.

In addition to protons, a key substrate for production of HOCl is Cl^-, which would initially be present in the phagosome at its concentration in extracellular fluid (~0.1 M) and has been measured to be 73 mM (169). The problem is that ~1 M of HOCl is produced in the phagosome. Some Cl^- may be introduced when granules and vesicles fuse with the phagosomal membrane. However, when the phagosome membrane depolarizes, as will tend to occur when NADPH oxidase begins to work, this will drive ClC-3, a Cl^-/H^+ antiporter that is expressed primarily in secretory vesicles and phagosome membranes in neutrophils (134), to compensate charge in the direction shown in FIGURE 4, namely introducing one H^+ into the phagosome and removing 2 Cl^- per cycle. The result will be rapid loss of Cl^-, which will terminate MPO activity by loss of substrate. Although some Cl^- is regenerated when target molecules are oxidized by HOCl, chlorinated targets would deplete Cl^- (218). Furthermore, diffusion of HOCl from the phagosome (FIGURE 4) also removes Cl^-. Confounding the problem of supply of Cl^-, a number of studies indicate a massive efflux of cytoplasmic Cl^-, which in resting neutrophils is present at the unusually high concentration of 80 mM (197), into the extracellular medium (21, 131, 195). When extracellular Cl^- is removed, the phagosomal Cl^- concentration drops from 76 to 6 mM within 15 min (169), strongly suggesting that loss of Cl^- from the cytoplasm would exacerbate the depletion of Cl^- from the phagosome.

Preventing osmotically induced swelling of the phagosome

Because of the magnitude of the charge compensation problem in neutrophil phagosomes, the choice of ion to compensate charge is critical. For example, if charge were compensated by K^+ flux into the phagosome, the resulting 4 M K^+ would swell the phagosome to ~25

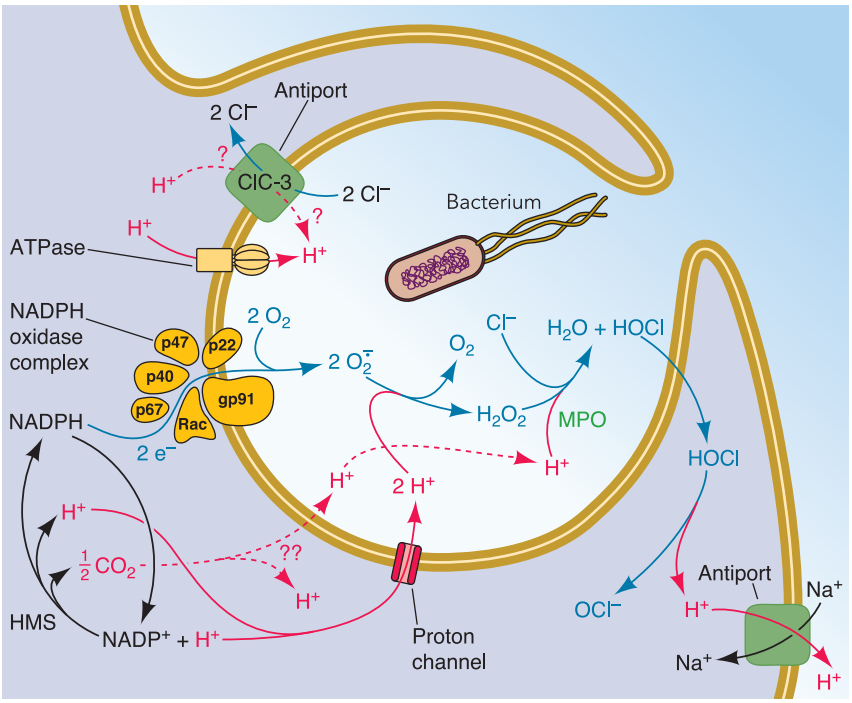

FIGURE 4. **The main molecules and transporters thought to participate in charge compensation and pH regulation during the respiratory burst**
A phagocyte is depicted engulfing a bacterium into a nascent phagosome, which will proceed to close and become intracellular. NADPH oxidase assembles preferentially in the phagosomal membrane in neutrophils (19, 167) and begins to function before the phagocytic cup has sealed (119). In eosinophils (111), macrophages (98), and neutrophils stimulated with soluble agonists (16), most NADPH oxidase assembles at the plasma membrane. Charge compensation is required wherever the enzyme is located. The entire system is driven by NADPH oxidase activity. Electrons from cytoplasmic NADPH are translocated across a redox chain to reduce O_2 to superoxide anion ($O_2^-\bullet$) inside the phagosome or extracellularly. The electrogenic activity can be measured directly as electron current (Fig. 3; Refs. 56, 187). For each electron removed from the cell, approximately one proton is left behind. Thus NADPH oxidase activity tends to depolarize the membrane, decrease pH_i, and increase pH_o or $pH_{phagosome}$. NADPH is regenerated continuously by the hexose monophosphate shunt (HMS) during the respiratory burst. Many of the transporters are functionally unidirectional. An exception is ClC-3 (Table 1), which is shown moving H^+ into the phagosome and Cl^- out, as is expected to occur at depolarized potentials that exist during the respiratory burst (113). In endosomes lacking Nox activity, ClC-3 is thought to operate in the reverse direction, removing H^+ and injecting Cl^- into the interior to compensate for electrogenic H^+-ATPase activity (96). HOCl reacts rapidly with phagosomal constituents but is membrane permeant (214) and also reacts with cytoplasmic contents such as taurine (129) or glutathione (217). For present purposes, HOCl shuttles protons out of the phagosome (135). Note that the H^+ in any compartment are equivalent; e.g., protons derived from HOCl dissociation are not preferentially removed by Na^+/H^+ antiport. (Figure is expanded from Ref. 151.)

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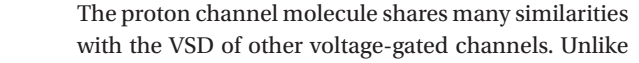
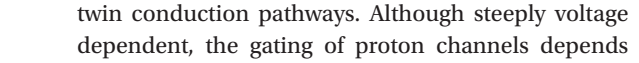
times its original volume, which does not occur (175). In contrast, when H^+ compensates charge, the products are mostly membrane permeant (H_2O, H_2O_2, and HOCl), consequently reducing their deleterious osmotic effects. When phagosomal Cl^- is depleted, which likely occurs rapidly due to the confusticatingly positioned ClC-3, the multitalented MPO can act as an oxidoreductase (218):



$$H_2O_2 + 2O_2^- + 2H^+ \rightarrow 2O_2 + 2H_2O$$

or a catalase:



$$2H_2O_2 \rightarrow O_2 + 2H_2O$$

In both cases, $O_2^-\bullet$ and H^+ eventually combine to form O_2 and H_2O, which are relatively inert both osmotically and physiologically. Depletion of Cl^- from the phagosome by ClC-3 activity may serve to terminate the more toxic aspects of the respiratory burst, perhaps limiting the damage done to the neutrophil or to surrounding tissues.

Summary

The proton channel molecule shares many similarities with the VSD of other voltage-gated channels. Unlike most channels, the proton channel is a dimer with twin conduction pathways. Although steeply voltage dependent, the gating of proton channels depends strongly on the pH gradient, with the result that it almost invariably extrudes acid when it opens. Proton channels are uniquely suited to their job in phagocytes, where they optimize NADPH oxidase activity by simultaneously compensating charge, minimizing pH changes both in cytoplasm and phagosome, minimizing osmotic imbalance, and providing substrate protons for ROS production in the phagosome. ■

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