Pharmacology of Voltage-Gated Proton Channels

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Abstract: Voltage-gated proton channels are highly proton selective ion channels that are present in many cells. Although their unitary conductance is 1000 times smaller than that of most ion channels, detection of single-channel currents supports their identification as channels rather than carriers. Proton channels are gated by membrane depolarization, but their absolute voltage dependence is also strongly regulated by the pH gradient, ΔpH (pH_o - pH_i). A model of this behavior postulates regulatory protonation sites that are alternately accessible to external or internal solutions. Consequently, proton channels open only when the electrochemical gradient is outward, and serve to extrude acid from cells. No "classical" blockers of proton channels that bind to and physically occlude the channel have been identified. A number of weak bases that inhibit proton currents probably act indirectly, perhaps by changing local pH. The best known and most potent inhibitors are polyvalent cations, especially Zn^{2+} and Cd^{2+}. These cations are coordinated at two or more external protonation sites, most likely His residues where they compete with protons and interfere with gating. In phagocytes, proton channels are required to compensate for the electrogenic action of NADPH oxidase. During the "respiratory burst," i.e., when NADPH oxidase is active, proton channels in these cells adopt an "activated" gating mode. Recently, two labs identified a gene that codes for either the proton channel itself or a protein that is essential for proton channel activity. Expression of this protein results in currents with many similarities to the native channel.

Key Words: Proton current, zinc, ion channels, pH, respiratory burst, phagocytes, NADPH oxidase, electrophysiology.

INTRODUCTION

In this review, the pharmacology of voltage-gated proton channels is brought up to date with a narrower focus than in recent reviews of proton channels [1-5]. A concise version of this review is simply "Zn^{2+}" -- the best known and most potent inhibitor of these channels. The sad fact is that no selective, high affinity inhibitor of proton channels is known. In fact, no "blockers" of proton channels exist, in the traditional sense of compounds that bind to the channel and sterically occlude the ion conduction pathway. We hope to show that despite its sparseness, the pharmacology of proton channels is rich and informative. After a brief history of voltage-gated proton channels, their main properties are summarized. Compounds that have been reported to inhibit proton channels are listed, and the reasons their mechanism of inhibition is unlikely to be traditional block are discussed. The mechanism and pH dependence of Zn^{2+} inhibition of proton current is then discussed at length. Study of the interactions of Zn^{2+} with proton channels has enabled fairly detailed structural deductions about important features of the channel molecule [6] that have been confirmed by the recently discovered channel gene [7]. Finally, current knowledge of the functions of proton channels in several tissues is summarized, with the main intent to convince the reader that it would be profitable to discover potent and selective inhibitors or modulators of proton channels.

History of Voltage-Gated Proton Channel Research

Voltage-gated proton channels were first hypothesized in 1972 to exist in certain bioluminescent marine organisms, dinoflagellates [8]. The membrane of the large central vacuole in these cells (the tonoplast) conducts an action potential that triggers bioluminescence [9, 10], and the ionic basis for this action potential is a proton conductance [11]. The logical deduction from this evidence is that the action potential reflects the activity of voltage-gated proton channels (Fig. **1**), as proposed by J. Woodland Hastings [12, 13]. Thus, in dinoflagellates, protons play the role that Na^+ does in most excitable cells (muscle and nerve) in more evolutionarily advanced organisms, as the ion that generates action potentials. An intriguing parallel is the use of a proton gradient by fungi, algae, and plants to drive secondary active transport, in contrast with the ubiquitous Na^+ gradient that drives secondary active transport in animal cells [14].

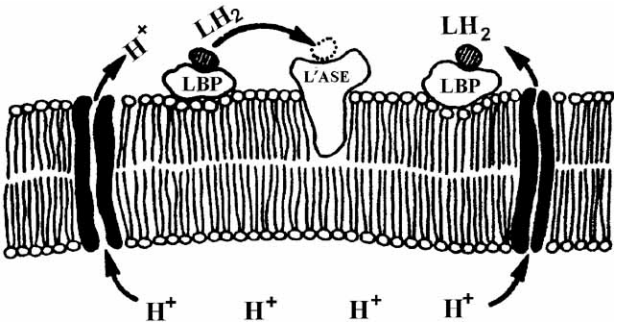

Fig. (1). Proposed mechanism by which voltage-gated proton channels in dinoflagellates mediate action potentials that trigger bioluminescence. Shear at the cell surface initiates an action potential in the tonoplast (membrane surrounding a large central vacuole) [10] that is propagated by proton channels [11]. The electrochemical gradient drives protons from the vacuole, at pH 3.5 [186], into the "scintillon," the light-emitting organelle that is formed by evagination of the tonoplast. In the resting state at a high pH, luciferin is bound to its binding protein (LBP) and luciferase (L'ASE) is inactive [187]. Proton flux into the scintillon both releases luciferin and activates luciferase. Reprinted from [12] with permission from the author and Elsevier.

The first voltage-clamp study of voltage-gated proton currents was in snail neurons, by Roger Thomas and Bob Meech [15]. Lou Byerly, Bob Meech and Bill Moody [16] systematically characterized the voltage-, time-, and pH-dependence of gating. The first mammalian cells shown to have proton channels were rat alveolar epithelial cells [17], in which H^+ current was noticed serendipitously during an investigation of the pH dependence of Cl^- currents. In 1987, Lydia Henderson, Brian Chappell, and Owen Jones postulated that proton channels existed in human neutrophils [18]; this prediction was confirmed six years later in human neutrophils [19] and in other phagocytes [20, 21]. To date, proton channels have been demonstrated in several dozen cell types [1], and it is difficult to find cells that do not express these channels.

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PROPERTIES OF VOLTAGE-GATED PROTON CHANNELS

Voltage-Gated Proton Channels: Molecular Identity

Voltage-gated proton channels recently left the ranks of a shrinking number of ion channels whose genetic and molecular identity is unknown. Two labs have identified a gene that, when expressed, produces currents that exhibit the main properties of voltage-gated proton currents. The official name for the human channel is HVCN1, a gene that codes for a protein of 273 amino acids [7]. Sasaki and colleagues [22] called the analogous protein in mouse and *Ciona intestinalis* a VSOP (voltage sensor only protein), because it has the equivalent of the first four membrane-spanning regions (S1-S4) found in most ordinary voltage-gated K^+, Na^+, or Ca^{2+} channels, including the highly charged S4 region that is believed to be the voltage sensor of both conventional channels [23] and proton channels [7, 22]. In contrast to ordinary ion channels, however, the putative proton channel lacks the S5 and S6 regions that contain the water-filled pore that comprises the permeation pathway of other channels. The absence of an aqueous channel appears to confirm predictions made over the past decade that the voltage-gated proton channel has a distinct conduction mechanism, a hydrogen-bonded chain (HBC) that includes at least one titratable amino acid side group [1, 3, 4, 24-39]. Experimental observations that pointed to such a mechanism include: (1) the weak pH dependence of the H^+ conductance [25-27], (2) the exquisite, if not perfect, selectivity [24, see ref. 1], (3) the large deuterium isotope effect [28], (4) the strong temperature sensitivity [40-43], and even (5) the lack of blockers (no molecules sterically occlude the pore, because there is no pore) [33]. The HBC concept was originally proposed by Nagle and Morowitz [44] as a mechanism for transporting protons across cell membranes through proteins without postulating an explicit aqueous channel.

Cells expressing the putative proton channel gene, HVCN1, have large H^+ selective currents that are opened by depolarization, with gating that is appropriately sensitive to both pH_o and pH_i, and that are inhibited by Zn^{2+} [7, 22]. Furthermore, mutations of charged residues in the S4 region profoundly alter gating kinetics [7, 22]. The evidence strongly implicates HVCN1 as a voltage-gated proton channel. However, alternative explanations exist that cannot be dispelled easily [45]. HVCN1 might be a necessary regulatory component of another protein that forms the actual proton channel (i.e., that contains the permeation pathway). Furthermore, even if the HVCN1 gene codes for a complete proton channel, the wide variation in proton channel gating kinetics, which led to the proposed existence of 4 distinct varieties of proton channels [46], raises the possibility that other isoforms might exist. It would be exceptional to find a gene that codes for a single variety of ion channel. If only one proton channel gene exists, post-translational modification must occur or additional subunits must exist to account for the variations in behavior.

In view of these considerations, we still cannot formally discard the proposal made a decade ago that the $gp91^{phox}$ component (recently renamed Nox2) of the NADPH oxidase of phagocytes (discussed below under **Functions of Voltage-Gated Proton Channels in Phagocytes**) is also a voltage-gated proton channel [29, 31, 36, 38, 47-49]. Subsequently, several other closely related molecules of the Nox/Duox family [50] were also proposed to act as proton channels [37, 51, 52]. However, consideration of a large body of evidence strongly suggests that none of the Nox molecules mediates voltage-gated proton currents [1]. The authors are of the opinion that most studies fail to support the idea that any member of the Nox/Duox family is responsible for voltage-gated proton currents. Nevertheless, its proponents have not abandoned the idea that $gp91^{phox}$ and its Nox relatives can function as proton channels [4, 38, 39, 49, 53]. The controversy has been summarized at great length from various perspectives in several reviews [1, 3, 4, 36, 54-57]. Consequently, only a few key studies and recent developments will be mentioned.

Genetic absence of $gp91^{phox}$ (Nox2) in chronic granulomatous disease does not reduce or detectably alter proton currents in unstimulated human leukocytes [32, 58, 59]. Knockout of $gp91^{phox}$ in PLB-985 cells affects neither the resting proton conductance nor the increase in proton conductance after stimulation [59]. Expression of $gp91^{phox}$ in COS-7 cells that lack native proton currents does not result in the appearance of detectable proton current [60], although contrary results have been published [37, 49]. This discrepancy has been ascribed to intermittent expression of $gp91^{phox}$ at the plasma membrane in many $gp91^{phox}$ transfected COS-7 cells [49]; however, an antibody to an extracellular epitope of $gp91^{phox}$ stained 96-98% of the transfected cells [60]. It was reported that a short transcript of Nox1 is a proton channel [51]; however, this transcript does not exist as a natural product of the Nox1 gene, and the protein could not be detected in cells with high Nox1 expression [61]. Comparison of two human airway epithelial cell lines revealed high expression of Duox-1 and Duox-2 in hTE cells, whereas Calu-3 cells lacked Duox-1 and had weak Duox-2 expression; yet both had proton currents of similar amplitude and properties [62].

The determination of the single channel proton conductance (γ_H 38 fS) and maximum open probability, $P_{open,max}$ 0.75 [63], allows calculation of the number of proton channels in a single eosinophil, given the maximum H^+ conductance $g_{H,max}$ 0.92 nS [64] (all values measured at pH_i 6.5). From $g_{H,max}/(P_{open,max} \times \gamma_H)$, each human eosinophil has ~32,000 proton channels. However, each eosinophil has >10 times more active NADPH oxidase complexes, ~375,000, based on the directly measured electron current, -18 pA [32] divided by the elementary charge to convert to the number of electrons s^{-1}, divided by the turnover rate of one NADPH oxidase complex 300 s^{-1} [65, 66]. (The total number of $gp91^{phox}$ in one human neutrophil is much larger, 800,000-1,000,000 [67], but not all of these are active simultaneously.) A comparable discrepancy (5-11 times more NADPH oxidase complexes than proton channels) results from a calculation based on I_e and I_H values measured in individual patches of eosinophil membranes [53]. This calculation requires extrapolation of γ_H to pH_i 7.5 (~10 fS), ignoring the uncertain mechanism of the increased g_H in activated membranes, and assuming an arbitrary value (300 s^{-1}) for the turnover rate for NADPH oxidase under the particular conditions employed (room temperature, saturating [NADPH], and excised membrane patch). In any case, if each active NADPH oxidase complex were also a functioning proton channel, the proton currents in stimulated eosinophils would be an order of magnitude larger than they are. All things considered, the newly discovered gene is by far the most likely candidate to be the proton channel.

One factor that complicates attempts to distinguish the two molecules is that there appear to be functional interactions between them [reviewed recently by ref. 4]. When the NADPH oxidase is active in phagocytes, proton current deactivation kinetics is slowed [32, 59, 68, 69], although this does not occur with arachidonic acid as a stimulus [70]. There is also evidence for functional interactions in airway epithelial cells [62]. A proposed mechanism that may explain the interactions in both tissues is through the effects of intracellular protons generated by NADPH oxidase activity [62, 68]. In the gating model (Fig. **5**) proposed by Cherny and colleagues [25], increased local intracellular proton concentration inhibits the first step in channel closing, namely, deprotonation at an intracellular site.

Permeation Mechanism

Voltage-gated proton channels are ion channels, although it is possible to quibble about the definition of a "channel." They categorically are not pumps, because they do not use ATP and because they mediate only passive H^+ movement down its electrochemical gradient. They are not symporters or antiporters, because they require no co- or counter-ions [24]. They are passive low-resistance pathways that allow protons to permeate membranes at high rates. They open and close like other ion channels, giving rise to conduc-

tance fluctuations [19, 63, 71] and discrete single channel currents [63]. The opening and closing, or "gating," of these channels is tightly regulated by membrane potential and pH; behavior that is typical of ion channels and unequivocally defines them as proteins. They are perfectly selective for protons and deuterons [28]. This extreme selectivity has been attributed to a "hydrogen-bonded chain" mechanism of conduction [1, 24]. A hydrogen-bonded chain is a general mechanism that allows efficient proton conduction across membranes through proteins [44]. This chain may comprise any combination of water molecules (a chain formed exclusively of water molecules is called a water wire) and side groups of amino acids [44, 72-74]. As knowledge of protein structures has advanced, the proton pathways in a number of proton-conducting molecules have been reduced to a water-filled channel that is interrupted by one or a few titratable amino acid residues [1]. These titratable residues in the permeation pathway comprise the selectivity filter of proton channels, conferring upon them high selectivity [1]. In contrast, the permeation pathway of most ion channels is thought to be an aqueous pore that contains many water molecules and occasional ions, with at least part of the pathway narrow enough to enforce single-file movement of water and ions. For example, the highly-studied gramicidin channel is a cation selective channel that can conduct protons [75]. The gramicidin channel contains ~12 water molecules in single file [76]. Proton conductance in gramicidin greatly exceeds that of other cations [75, 77-79], reflecting the ability of protons to hop across the water wire in the channel, whereas ordinary cations must wait for the waters in the pore to permeate.

Significant differences between proton permeation through gramicidin channels and through voltage-gated proton channels have been interpreted to mean that their conduction pathways are qualitatively different. In addition to the high selectivity of voltage-gated proton channels, several other properties also suggest that the permeation pathway is not a simple water wire. Deuterons permeate with a conductance just ~50% that of protons [28]. This isotope effect is larger than that for conduction in bulk water [80] and than that for H^+ or D^+ conduction through water-filled gramicidin channels [81, 82]. Proton channels have unusually strong temperature dependence. Both the conductance [40-42] and the kinetics of gating [42] are more temperature dependent than almost any other known ion channel. The open-channel conductance has a Q_{10} of 2-5 or activation energy, E_a, of 12-27 kcal/mol [40, 42, 43], which greatly exceeds that of proton diffusion in bulk solution [83] or that of proton flux through gramicidin channels [81, 84]. The large activation energy supports the idea of a conduction mechanism more complex than a simple water wire [42]. Gating is even more sensitive to temperature with Q_{10} 6-9 (E_a 30-38 kcal/mol) for all three kinetic parameters extracted (the activation time constant τ_{act}, the deactivation or tail current time constant τ_{tail}, and the delay preceding activation), suggestive of a rate-limiting, first-order, energetically-demanding conformational change in each of several channel subunits [42].

Histidine modifying reagents inhibit proton conduction [32, 35], which might indicate interruption of a HBC involving one or more His residues, but might also reflect less specific damage to the channel. Henderson [29] proposed that three His located in the membrane-spanning region of $gp91^{phox}$ (a His-X-X-X-His-X-X-X-His motif) formed part of the proton conduction pathway. Mutation of three His to Leu in $gp91^{phox}$ (or mutation of His^{115} alone) reportedly inhibited proton conduction in cells expressing the mutant protein [29, 31, 37]. This result would be a more convincing argument that His residues form part of the conduction pathway, if $gp91^{phox}$ and other Nox molecules were really proton channels.

If the permeation mechanism is a hydrogen-bonded chain, then it is possible that the entrance to the channel might simply comprise a titratable group on the channel. Several proton-conducting proteins have titratable amino acids near the mouth of the proton pathway, which appears to enhance their conductance [1], as discussed in the next section. In contrast, "normal" ion channels often have large, funnel-shaped vestibules at both entrances that are ideally suited to accept a complementary toxin molecule that binds with high affinity and sterically blocks conduction. These considerations may explain why no proton channel blockers have been found.

Small Single Channel Conductance

The single-channel proton conductance is small, 140 fS at pH_i 5.5 and 38 fS at pH_i 6.5, which extrapolated to physiological pH_i 7.2 is 15 fS at 21°C [63]. The single-channel currents in Fig. (**2**) are only ~10 fA at pH_i 5.5, were barely detectable under optimal conditions [63], and are the smallest directly-recorded unitary currents reported to date. Although ~1000 times smaller than most ion channels, single-channel proton currents are probably as large as can be expected, given the miniscule concentration of protons in physiological solutions [1, 24]. Certain other proton pathways in proteins, such as carbonic anhydrase [85-88] and the bacterial reaction center [89, 90], evidently increase their proton conductance by means of a titratable amino acid residue at the mouth of the channel. Similarly, the proton conductance of gramicidin is increased by an order of magnitude by formic acid, which is believed to bind near the pore mouth and enhance the supply of protons [91]. In the case of carbonic anhydrase, there is also direct proton transfer from buffer to the channel [92-96], resulting in a turnover rate of 10^6 s^{-1} [96, 97], the fastest known enzyme, and also several orders of magnitude faster than predicted by simple diffusion of free protons [1]. In contrast, the current through voltage-gated proton channels was only weakly dependent on buffer concentration from 1 mM to 100 mM [98], indicating that direct proton transfer between buffer and the channel is not rate determining.

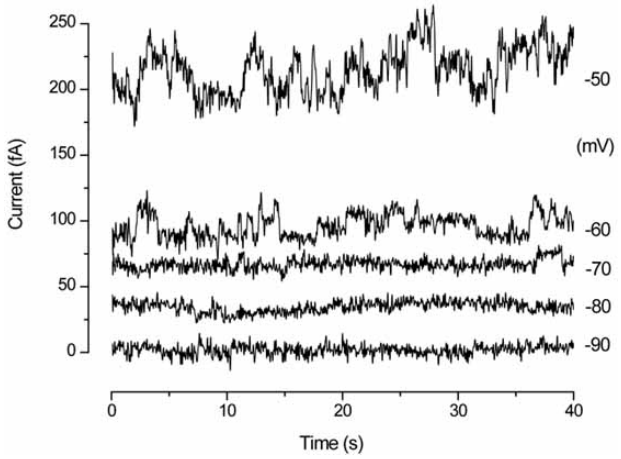

Fig. (2). Single proton channel currents at pH_o 7.5 and pH_i 5.5 in an inside-out patch from a human eosinophil. Step-like events (outward H^+ current when the channel is open is shown as an upward deflection) that may reflect the opening and closing of individual proton channels cold be detected only near threshold voltages at which only a few channels opened. Each record is displaced vertically by 20 fA above its true position relative to the record underneath; the current scale is positioned arbitrarily. Low frequency noise is minimized by a high seal resistance [188], which was 5 TΩ in this patch, filter 10 Hz. Reproduced from [63] by copyright permission of The Rockefeller University Press.

Distinctive Gating Properties

Voltage-gated proton channels behave in most respects like traditional voltage-gated ion channels. They open upon depolarization with a sigmoid time course, and even display a Cole-Moore effect [24] like that of squid delayed rectifier K^+ channels [99]. During a large depolarization, 95% of the proton channels open at pH_i 5.5 or lower, with the maximum open probability decreasing to 75% at pH_i 6.5 [63]. Two distinctive features of the gating of proton channels, pH dependence and dual gating modes, are important for their function.

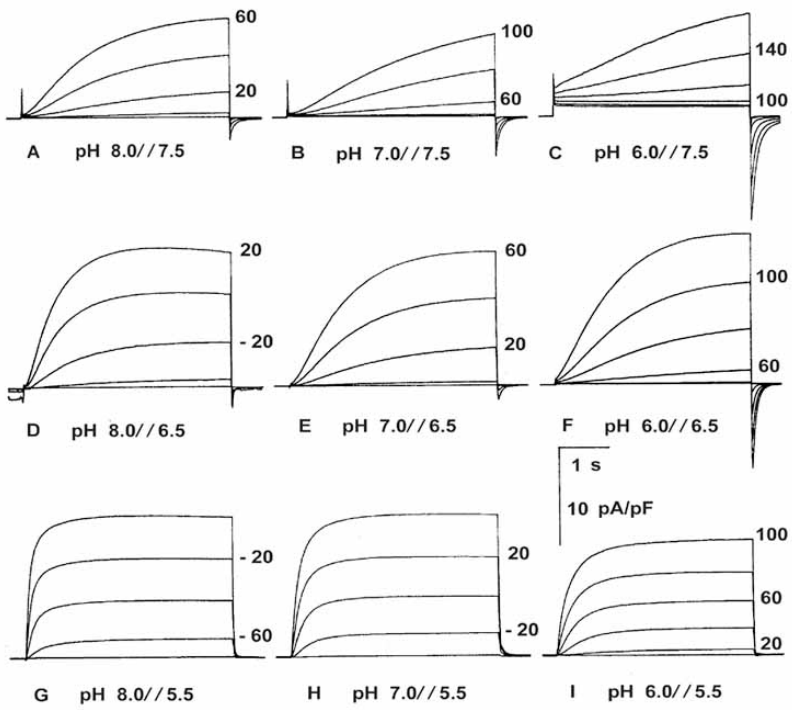

Fig. (3). Families of proton currents in rat alveolar epithelial cells studied over a wide range of pH_o and pH_i, labeled as pH_o//pH_i. Pulses were applied in 20 mV increments as indicated. The currents in three cells (A-C, D-F, and G-I) were scaled to membrane area, assessed by capacitance. Reproduced from [25] by copyright permission of The Rockefeller University Press.

The voltage dependence of proton channels is not absolute, but is exquisitely sensitive to the pH gradient, ΔpH, defined as pH_o - pH_i. Lowering pH_i or increasing pH_o shifts the voltage-activation curve toward more negative voltages by 40 mV/Unit [1, 25, 28]. These shifts are in the same direction as those observed for pH effects on other voltage-gated channels, which are often interpreted in terms of surface potential neutralization [23, 100]. However, the pH effects on proton channels are larger than expected for this kind of mechanism, and suggest direct interaction of protons with the channel gating mechanism [16]. In contrast, most voltage-gated ion channels have the same absolute voltage dependence regardless of permeant ion concentration. For example, the *Shaker* K^+ channel voltage sensor can be converted to a proton channel by mutation of a single amino acid to His [101]; however, the voltage dependence remains largely independent of pH.

The effects of pH on voltage-gated proton channels are illustrated in Figs. (**3** and **4**). Fig. (**3**) shows families of proton currents in alveolar epithelial cells over a 4 Unit range of ΔpH. Although the threshold voltage, $V_{threshold}$ ranges from -80 mV to +80 mV, only outward H^+ current is activated. In other words, these channels open only when doing so will result in proton extrusion from cells (i.e., at voltages more positive than E_H). Once the channel is open, it can readily conduct inward current; inward tail currents after repolarization are clearly evident in Fig. (**3**). The open channel rectifies only weakly [25]; hence, the ΔpH dependence is entirely due to pH effects on gating. The origin of this property can be appreciated in Fig. (**4**), in which proton currents from similar measurements are plotted against voltage. With a large outward pH gradient (ΔpH = 2.5, pH_o 8.0//pH_i 5.5), $V_{threshold}$ is -80 mV (Fig. **4**). Decreasing ΔpH progressively shifts $V_{threshold}$ by 40 mV/Unit up to +80 mV at ΔpH = -1.5 (pH_o 6.0//pH_i 7.5). Although E_H changes by more than 40 mV/Unit, $V_{threshold}$ is positive to E_H over the entire range [25, 28]. The result of this extraordinary ΔpH dependence is that voltage-gated proton channels in resting cells (cf. **Two Modes of Gating** below) open only when there is an outward electrochemical gradient (i.e., at voltages positive to E_H). It is universally accepted that channels possessing this elaborate pH dependence must function to extrude acid from cells.

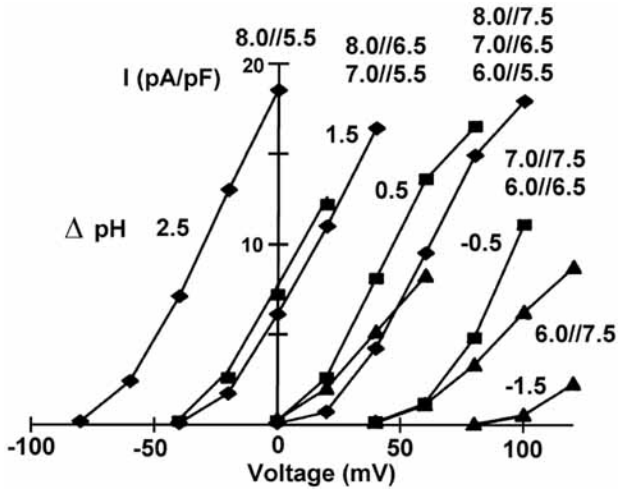

Fig. (4). Average proton current-voltage relationships measured from experiments like those illustrated in Fig. (**3**). The position of the voltage-activation curve is determined by the pH gradient, ΔpH. Currents were normalized to capacitance of the cells to adjust for variation in cell size. Symbols indicate pH_i 5.5 (♦), 6.5 (■), or 7.5 (▲). Reproduced from [25] by copyright permission of The Rockefeller University Press.

Fig. (**5**) illustrates a model that shows how the voltage- and ΔpH-dependence of gating could arise [25]. Each cartoon depicts the same model. Several assumptions are required. Each channel is comprised of multiple subunits or protomers. Each protomer can be protonated from either the external or internal solution, but the protonation site is accessible only to the solution on one side of the membrane at a time. Protonation from the internal solution favors the open state, whereas protonation from the external solution stabilizes the closed channel conformation. The accessibility of the site is switched by a conformational change that can occur only when the site is deprotonated. This model reproduces the main features of voltage- and pH dependent gating of proton channels. It provides a

first guess at the essential working parts of the channel, any of which would make logical drug targets. The recent discovery of proton channel genes [7, 22] opens the way for structure-function studies that may identify the sites involved in pH sensing.

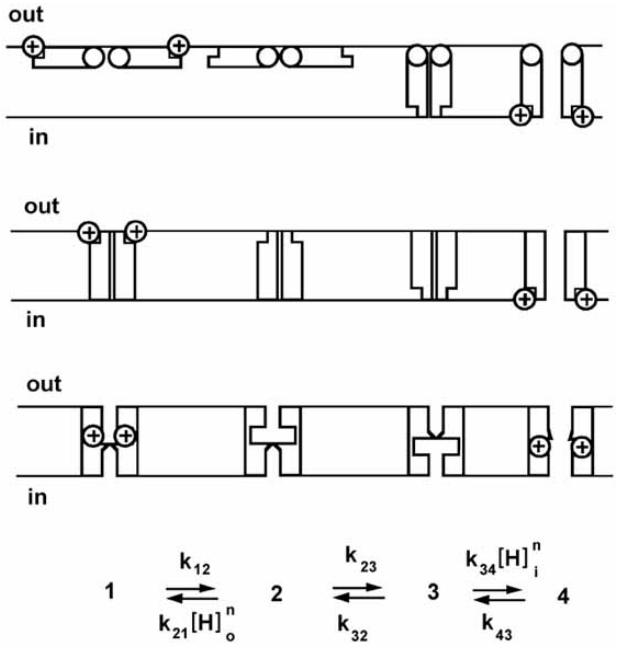

Fig. (5). Model of the pH and voltage dependence of proton channel gating. The three cartoons illustrate the same model that is shown at the bottom. Gating is regulated by extent of protonation of sites on the channel molecule. Protonation from the inside opens the channel; protonation from the outside closes it. The required assumptions are listed in the text. Reproduced from [25] by copyright permission of The Rockefeller University Press.

The second unusual feature is that voltage-gated proton channels, at least in phagocytes, have two distinct modes of gating, whose functional importance will be discussed below in Figs. (**11** and **12**). The concept of modal gating was introduced to describe a behavior of voltage-gated calcium channels, which at infrequent intervals shift from one internally-consistent pattern of gating kinetics to another [102]. The two modes of proton channel gating are so distinctive they were at first thought to reflect the existence of two different channel molecules [32]. Subsequent evidence indicates that most likely the same channel molecule has radically different gating kinetics when it is "activated," apparently by phosphorylation [53, 68, 69, 103-105]. In the "activated" gating mode, the channels are much more likely to open at a given voltage; with consequences that are discussed below (**Proton Channels in Phagocytes**). This conclusion is strengthened substantially by the recent demonstration that proton channel modulation is reversible [53, 105].

PHARMACOLOGY OF VOLTAGE-GATED PROTON CHANNELS

Organic Inhibitors

In general, voltage-gated proton channels are resistant to block. One could even say that no blocker exists, at least in the traditional sense of a substance that binds to the channel and prevents current flow. Numerous inhibitors of other transporters have been tested and found to have no effect [1]. The classical inhibitors of proton channels, polyvalent cations, are discussed in the next section. Several organic compounds that have been reported to inhibit or "block" proton channels fall mainly into two classes, weak bases and tetraethylammonium (TEA^+). Inhibition by TEA^+ exhibits exceedingly peculiar properties. Concentrations of 1, 10, and 52 mM TEA^+ all reduced the H^+ current by 29-35% [16, 71, 106]. Furthermore, robust proton current is observed in symmetrical [19, 107, 108] or external only [109, 110] isotonic TEA^+ solutions. One might speculate that TEA^+ partitions partway into the membrane or binds to the channel where it exerts some kind of effect, but the mechanism is clearly not traditional block. Inhibition by weak bases exhibits other, but similarly peculiar features. Compounds such as 4-aminopyridine [15, 106, 107, 111, 112], amantadine [24], amiloride [113], D600 [111], nicardipine [114], and rimantadine [24] all inhibit H^+ currents in what has been called a "voltage dependent" manner. The inhibitors shift the voltage dependence in a positive direction, which is consistent with their increasing pH_i [111]. Because increasing pH_i itself reduces proton current during a given test pulse, it is not clear that any other mechanism is required to explain their effects. Preliminary evidence suggests that when pH_o is varied, weak bases inhibit H^+ currents roughly in proportion to the concentration of neutral form of the drug, consistent with the idea that the effect is mediated by local pH changes as the neutral drug crosses the membrane [24]. This interpretation has never been tested rigorously. No known inhibitor of H^+ current has been shown to act by binding to the pore and abolishing conduction by steric occlusion (in the manner of a traditional channel blocker).

Mechanism of Inhibition by Divalent Cations

The classical inhibitors of voltage-gated proton channels are Zn^{2+} and Cd^{2+}, but many other polyvalent cations have similar effects when applied to the extracellular solution [1]. Published data in a variety of cells suggests the following 'consensus' potency sequence among divalent metal inhibitors [46]:

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

The trivalent cations La^{3+}, Gd^{3+}, and Al^{3+} also inhibit H^+ current [15, 24, 64, 106, 111], with La^{3+} being nearly as potent as Zn^{2+} [106], as do Be^{2+}, Pb^{2+}, and Hg^{2+} [1, 24, 40]. Ba^{2+} [106], Ca^{2+}, and Mg^{2+} are weak inhibitors [71]; or completely ineffective [16, 40, 46]. Although the relative potency of polyvalent cations appears similar among different cells and species, the absolute potency of Zn^{2+} is about an order of magnitude greater in human eosinophils [69] than in rat alveolar epithelial cells [6] or Jurkat cells [115], based on the magnitude of voltage shift produced (see below). It was reported that proton channels in PMA-stimulated eosinophils were more sensitive to Zn^{2+} than those in unstimulated cells, suggesting that two distinct channel types existed [32]. However, this result was shown to reflect the methods used to estimate block; proton channels in human eosinophils have identical Zn^{2+} sensitivity before and after stimulation [69].

In all cases, the primary effect of $Zn^{2+}$is to shift the voltage-activation curve in a positive direction. In the presence of increasing $ZnCl_2$ concentrations (Fig. **6**), identical families of depolarizing voltage pulses elicit proton current at progressively more positive voltages. Essentially all voltage-gated ion channels are affected by divalent metal cations in this manner [23]. A classical interpretation of this phenomenon is that the presence of a divalent cation at the external side of the channel alters the membrane potential perceived by the channel's voltage sensor such that additional depolarization is required to produce the same level of activation [100]. Polyvalent cations must bind directly to the proton channel, rather than acting by a simple charge screening mechanism, because different cations have very different potency [16].

An additional effect of Zn^{2+} is to slow proton channel opening. Slowing would be expected to occur as a result of a local potential mechanism – indeed, the slowing produced by Cd^{2+} and Ni^{2+} in epithelial cells was entirely accounted for by the shift in the g_H-V relationship [6]. However, the slowing effects of Cd^{2+} or Zn^{2+} in snail neurons [16, 116], Cd^{2+} in axolotl [109], and Zn^{2+} in rat epithelium [6] are too large to be explained by this mechanism. Therefore, certain metal cations must interact directly with the channel gating mechanism [16]. As observed for other voltage-gated chan-

nels [23], not all voltage dependent parameters are shifted equally; the opening rate is affected more than the closing rate both for squid K^+ channels [117] and H^+ channels [6]. Gilly and Armstrong [117] explained this phenomenon by proposing that the externally accessible Zn^{2+} binding site was exposed only when the channel was closed. This proposal coincides remarkably with the proton channel model shown in Fig. (**5**), if one proposes that Zn^{2+} binds to the same groups that mediate pH_o dependence of gating. In the model, the external sites must be deprotonated before the channel can open, and upon opening, the external sites disappear as the internal sites become available for protonation from the inside.

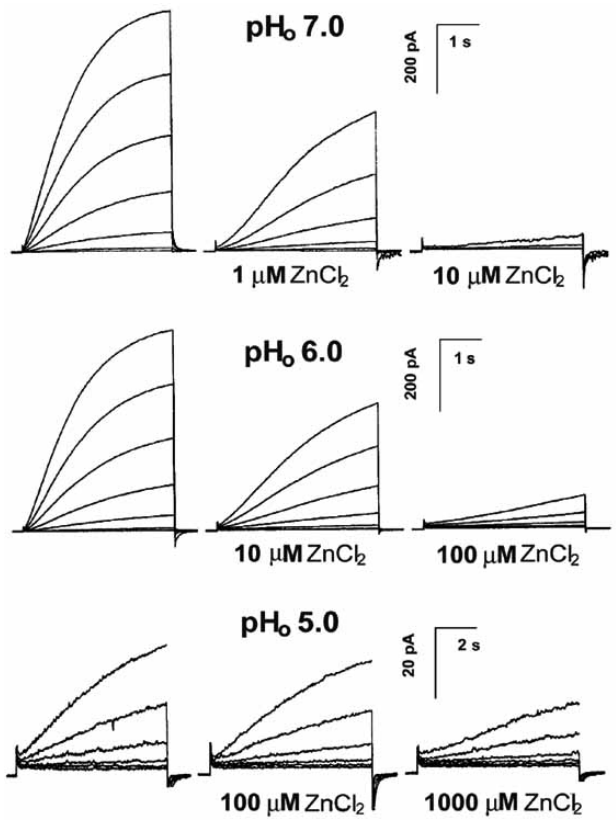

Fig. (6). The inhibition of voltage-gated proton currents by Zn^{2+} in rat alveolar epithelial cells depends strongly on pH_o. In each row, identical families of pulses were applied in the presence of the indicated concentrations of $ZnCl_2$. Note that the turn-on of the current is slowed dramatically and that the voltage at which H^+ current first turns on, $V_{threshold}$, is shifted positively. Reproduced from [6] by copyright permission of The Rockefeller University Press.

Some authors have analyzed the effects of Zn^{2+} on proton currents in terms of voltage dependent block [53, 71, 108]. We prefer to describe the effects as modulation of gating. These interpretations are not equivalent, and understanding the mechanism of interaction is an important consideration in drug design. Voltage dependent block implies that Zn^{2+} enters partway through the channel to reach its block site, as has been explicitly proposed [53], although voltage dependent accessibility of the binding site could not be formally ruled out. Five arguments against the idea of genuine voltage dependent block of proton channels by Zn^{2+} are discussed at length elsewhere [pp. 829-830, ref. 6] and will be summarized here only briefly. (1) There is no evidence for rapidly reversible open-channel block, because the instantaneous current-voltage relationship was simply scaled down by Zn^{2+}. (2) If the slower activation of proton current in the presence of Zn^{2+} is viewed as reflecting slow unblock, then the unblock rate is concentration dependent, contrary to normal drug-receptor interactions. (3) If the steady-state g_H-V relationship in the presence of Zn^{2+} is considered to reflect the voltage dependence of block, then the steepness factor indicates that at the electrical distance of its binding site, Zn^{2+} must sense more than twice the entire membrane electrical field. (4) It is certainly true that if one adopts the usual procedure of plotting the ratio of current in the presence and absence of Zn^{2+}, apparent voltage dependence results. However, this is precisely the expectation if the mechanism of Zn^{2+} effects were a shift of the g_H-V relationship along the voltage axis. In this case, the apparent voltage-dependence of block will have identical steepness to the g_H-V relationship itself, as has been observed [6]. (5) If any part of the conduction pathway through the proton channel comprises a hydrogen-bonded chain including a titratable amino acid residue, then Zn^{2+} could not permeate the pore. The recently identified proton channels appear to lack anything resembling an aqueous pore [7, 22]. These considerations indicate that Zn^{2+} binds to an external site that does not sense the membrane electrical field, where it interferes with the opening of proton channels.

Internally applied Zn^{2+} has much weaker activity and produces qualitatively different effects on gating. Kinetics of activation was unaffected, but channel closing (tail current decay) was 2-3-fold slower and the current amplitude was reduced [6]. The attenuation of H^+ current appears to be somewhat greater at pH_i 7.5 [53] than at pH_i 6.5 [6], suggesting that competition between protons and Zn^{2+} may occur also at internal sites. However, Zn^{2+} may simply be more effective on proton channels in eosinophils than in alveolar epithelial cells, as was observed for external application.

Elevated levels of intracellular free Ca^{2+}, $[Ca^{2+}]_i$, reportedly enhanced proton currents in human eosinophils [64, 108]. However, the enhancement was substantially smaller when $[Ca^{2+}]_i$ was controlled rigorously using buffered solutions. In other studies, increased $[Ca^{2+}]_i$ did not detectably enhance proton currents in snail neurons [16], HEK-293 cells [52], or human neutrophils [118], or in inside-out patches from human lymphocytes [115], rat alveolar epithelial cells [Cherny & DeCoursey, unpublished data], or human eosinophils [53]. These observations may be reconciled by assuming that Ca^{2+} has no direct effect on the channel itself, and that changes in $[Ca^{2+}]_i$ in intact cells affect proton currents indirectly [1].

Competition between Zn^{2+} and H^+ Reveals Structural Features of Proton Channels

A remarkable property of the interaction of Zn^{2+} with proton channels is that its apparent potency is greatly reduced at low pH (Fig. **6**). The effects of Zn^{2+} are quantified as the size of the shift in the g_H-V relationship in Fig. (**7**). For example, $V_{threshold}$ is shifted roughly 20 mV by 100 nM Zn^{2+} at pH_o 8, or by 10 mM Zn^{2+} at pH_o 5, a difference in sensitivity of 5 orders of magnitude. Evidently there is intense competition between protons and Zn^{2+} for a binding site on the channel. Consideration of various possibilities led to the conclusion that the externally-accessible Zn^{2+} binding site comprises multiple (most likely three) titratable groups with apparent pK_a 6-7, suggestive of His residues [6]. A single site with competition between Zn^{2+} and a single proton would exhibit at most a 10-fold reduction in apparent Zn^{2+} potency per Unit decrease in pH_o, thus multiple titratable groups must coordinate the binding of one Zn^{2+} atom to account for the ≥100-fold reduction in potency between pH 6 and 5 (Fig. **7**). Similar results were obtained when the Zn^{2+} effect quantified was the slowing of τ_{act}, the time constant of the turn-on of proton current during a depolarizing pulse. Again, it was necessary to assume that 2-3 titratable residues form the Zn^{2+} binding site, and the pK_a for all such models ranged 6.2-6.6, again suggestive of His residues [6]. Similar interactions occur in the bacterial reaction center, in which Cd^{2+} competes with two protons for two His residues near the proton channel entrance [119].

The recent identification of the proton channel gene has produced astonishing confirmation of these predictions. Fig. (**8A**) illustrates the sequence of the human proton channel, with four pre-

dicted membrane-spanning regions. Ramsey *et al.* [7] mutated two externally-facing His residues to Ala either singly, H140A or H193A, or both at the same time. As evident in Fig. (**8B**), the affinity of Zn^{2+} for the proton channel was reduced roughly an order of magnitude for single His replacements (by 9- and 39-fold, respectively). The double mutant was nearly Zn^{2+} insensitive.

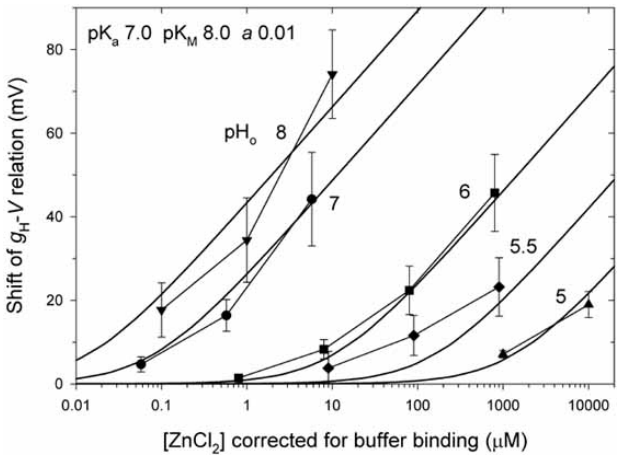

Fig. (7). Voltage shift produced by Zn^{2+} at various pH_o. Data points are connected by lines at each pH. The curves all were drawn from a simple model in which the external Zn^{2+} binding site comprises three titratable residues with apparent pK_a 7.0. Zn^{2+} binds with a K_M of 10^{-8} M. It is assumed that the Zn^{2+} affinity is reduced by a factor a = 0.01 (i.e., 100-fold) when one group is protonated. The model incorporates typical gating kinetics for voltage-gated proton channels and assumes that the proton channel cannot open when Zn^{2+} is bound. Reproduced from [6] by copyright permission of The Rockefeller University Press.

Sasaki *et al.* [22] identified analogous proton channel genes in mouse and sea squirt, *Ciona intestinalis*, and found that the mouse channel is 30 times more sensitive to Zn^{2+} than is the *Ciona* channel (Fig. **9A**). Intriguingly, examination of the positions corresponding to the two His residues that confer Zn^{2+} sensitivity upon the human channel reveals that the mouse channel has three His at roughly comparable positions, whereas the *Ciona* channel has only one His (Fig. **9B**). However, the Zn^{2+} binding site in these species has yet to be determined.

Zinc exists in several chemical forms in solution, whose concentrations depend strongly on pH [120]. The reduction of the apparent potency of zinc at low pH suggested that $ZnOH^+$ might be the active form. However, the concentration of $ZnOH^+$ decreases only 10-fold per Unit decrease in pH, whereas the apparent potency of zinc is reduced by ≥100-fold/Unit at low pH. Other zinc forms whose concentration decreases with pH are anionic and are present at $<10^{-12}$ M [120]. Thus, the active species is most likely Zn^{2+} [6].

The external His residues in the human proton channel are obvious targets for pharmacological intervention, because of their profound ability to regulate proton channel gating. In addition, because protons and Zn^{2+} produce similar changes in proton channel gating, it was speculated that the titratable sites that coordinate Zn^{2+} might be the same sites that regulate the pH_o dependence of gating in the model (the sites in the left-most column in Fig. (**5**)) [6]. If this were the case, it would provide an additional reason to target these His residues.

FUNCTIONS OF VOLTAGE-GATED PROTON CHANNELS

General and Specific Functions in Various Cells

The general function of voltage-gated proton channels is to extrude acid from cells. Several situations and tissues in which acid extrusion has been documented will be described. The specialized function of charge compensation in phagocytes is well established and will be discussed at greater length. Acid extrusion is classically observed in cells that have been acid loaded, generally by an NH_4^+ prepulse [121]. Removal of NH_4^+ after a period of equilibration results in the membrane permeable NH_3 exiting the cell, effectively leaving a proton behind. When other mechanisms of acid extrusion (such as Na^+/H^+-antiport) are precluded, recovery is slowed or prevented by proton channel inhibitors, usually Zn^{2+}. By this method, a contribution of voltage-gated proton channels to the recovery from an acid load has been demonstrated in human neutrophils [122, 123], rabbit osteoclasts [112, 124], murine mast cells [41], rat hippocampal neurons [125], and rat alveolar epithelial cells [126]. Proton currents were first detected in snail neurons by their participation in recovery of pH_i from HCl injection [15]. Any cell with proton channels has a "safety valve" that (under appropriate conditions) can turn on automatically when confronted with a sudden acid load.

In excitable cells, proton current may serve to remove metabolic acid during action potentials [71, 110, 111, 127].

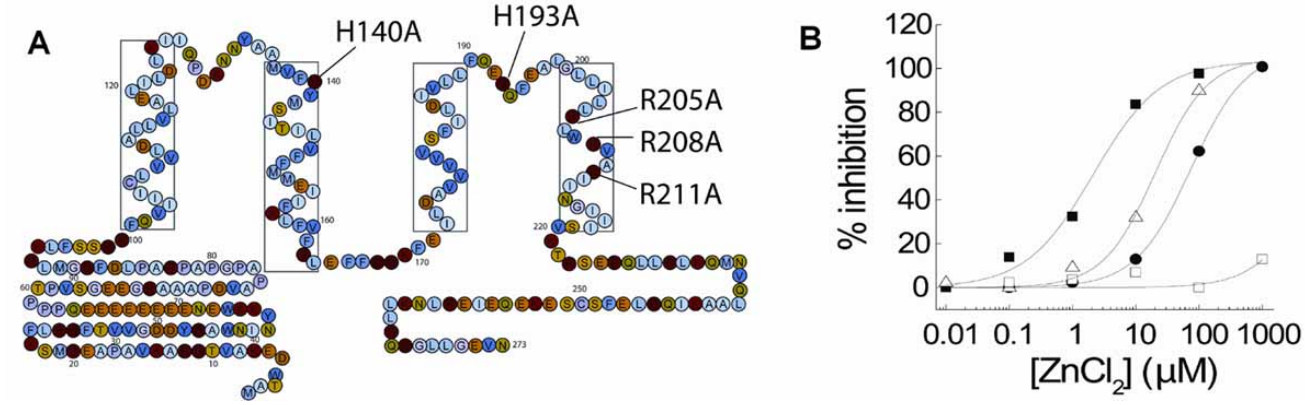

Fig. (8). Origin of the zinc sensitivity of the human proton channel Hv1, expressed in HEK-293 cells. (A) The amino acid sequence is shown with the putative membrane-spanning regions in boxes. The two His residues that face the extracellular solution and appear to form the Zn^{2+} binding site are indicated. The Arg residues in the S4 region influence gating kinetics [7]. (B) Concentration-response curves for inhibition of H^+ current (measured at the end of 2-s pulses in symmetrical pH 6.5 solutions) by Zn^{2+} (■ = wild type, Δ = H193A, ● = H140A, □ = double mutant H140A/H193A). Reproduced with permission from *Nature* and the authors [7].

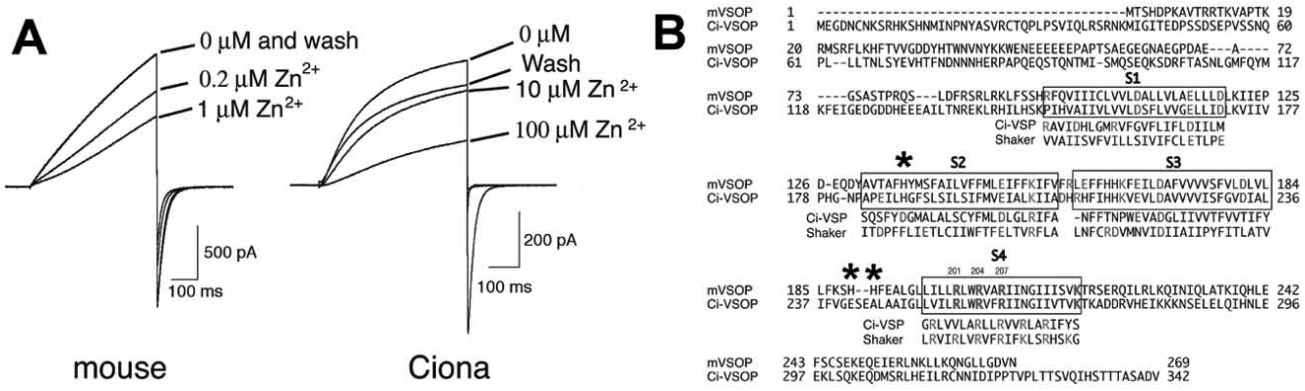

Fig. (9). Different zinc sensitivity of mouse and *Ciona* proton channels. (A) Currents during pulses to +100 mV in the absence or presence of the indicated concentrations of Zn^{2+} are superimposed. (B) Sequences of the mouse (mVSOP) and *Ciona* (Ci-VSOP) proton channel proteins, aligned for comparison with homologous regions of the voltage-sensor-containing phosphatase of *Ciona intestinalis*, Ci-VSP [189] and the Shaker K^+ channel. Putative membrane spanning regions S1-S4 are in boxes, and the asterisks indicate three His residues that roughly correspond to the His that bind Zn^{2+} in the human channel. Reproduced from [22] with permission from AAAS and the authors.

Several types of amphibian oocytes have large proton currents [107, 109]. H^+ channel expression changes during maturation in *Ambystoma* [128]. In *Rana esculenta* oocytes, H^+ currents have been proposed to regulate $[Ca^{2+}]_i$ oscillations [129].

In rat hippocampal neurons, proton channels appear to contribute to cytoplasmic alkalinization during responses to anoxia [125, 130]. Anoxia results in both cytoplasmic acidification and membrane depolarization, both of which tend to open proton channels. The resulting alkalinization is inhibited by Zn^{2+} or Cd^{2+} [125, 130].

The high level of expression of proton channels in alveolar epithelium led to a hypothesis that some fraction of the CO_2 eliminated by the lung might dissociate into HCO_3^- and H^+ inside alveolar epithelial cells. The H^+ might then pass through proton channels and recombine with HCO_3^- in the alveolar space [131]. Indirect evidence contrary to this proposal has been presented [132-134], but a direct test has not been attempted.

Proton channels have been shown to mediate acid secretion in airway epithelium. The pH of airway secretions is maintained at a low value. Acid secretion is stimulated by ATP or histamine, and this secretion is inhibited by Zn^{2+}, thus implicating proton channels [135]. This story has become even more intriguing with a recent exploration of the relationship between proton channels and NADPH oxidase in airway epithelium [62], two molecules that have a relationship of crucial importance in phagocytes (*below*). Ciliated airway epithelial cells acidify the airway fluid, whereas submucosal gland cells secrete alkaline fluid. These cell types were modeled by Schwarzer *et al*. [62] by the hTE and Calu-3 cell lines, respectively. Although both cell lines had similar proton currents, only the acid secreting hTE cells expressed a full array of NADPH oxidase subunits. Inhibitors of either proton channels or NADPH oxidase reduced acid secretion by hTE cells, but had no effect on the much smaller acid extrusion by Calu-3 cells. The authors proposed that acid secretion was facilitated by intracellular acid production that results from NADPH oxidase activity (as depicted in Fig. (**10**)).

FUNCTIONS AND PHYSIOLOGICAL REGULATION OF VOLTAGE-GATED PROTON CHANNELS IN PHAGOCYTES

Voltage-gated proton channels play a crucial and well-established role in phagocytes. Proton currents have been observed in every leukocyte or related cell line that has been studied appropriately, including neutrophils [19], eosinophils [64, 108], basophils [43], macrophages [20], microglia [106], osteoclasts [136], osteoblasts [112], T lymphocytes [115], B lymphocytes [115], HL60 cells [21], THP-1 cells [27], Jurkat cells [115], mast cells [41], PLB-985 cells [59], K-562 cells [24], BV-2 cells [34], GM1-R1 cells [137], and MLS-9 cells [34]. The proton current amplitude is roughly correlated with NADPH oxidase activity in these cells, and is large enough in each cell type to establish its kinetic competence to perform the presumed function of charge compensation [1, 34], which is described next.

Fig. (**10**) illustrates the role of proton channels in phagocytes. A key component of innate immunity is NADPH oxidase, an enzyme complex that produces superoxide anion, $O_2^{\cdot-}$, a precursor to a variety of other reactive oxygen species generally thought to contribute to killing of bacteria and other invaders [5, 138-142]. The active enzyme complex is electrogenic [18]; it removes electrons from intracellular NADPH and translocates them across the membrane *via* an electron transport pathway [1, 2, 143-145] to reduce extracellular or intraphagosomal O_2 to $O_2^{\cdot-}$. This electron movement depolarizes the membrane [18] by 100 mV or more in intact human neutrophils [146-148]. Electrogenic proton efflux occurs in phagocytes under conditions in which NADPH oxidase is active [18, 149]. As originally proposed [18], this proton flux is mediated by voltage-gated proton channels, which certainly are present in phagocytes [19-21]. The function originally proposed for proton channels was charge compensation [18]. It was confirmed recently that NADPH oxidase activity is sensitive to membrane potential, thus supporting a need for charge compensation, and that extreme depolarization in itself can abolish NADPH oxidase activity [150, 151]. Furthermore, inhibitors of proton currents, such as Zn^{2+} and Cd^{2+}, exacerbate the depolarization [4, 18, 32, 148] and (consequently) inhibit the activity of NADPH oxidase [5, 104, 142, 150, 152-159]. It thus seems clear that charge compensation by proton channels is required to sustain NADPH oxidase activity in human neutrophils and eosinophils.

Two Modes of Proton Channel Gating

Agonists that activate NADPH oxidase also radically alter the properties of voltage-gated proton channels [32, 68, 70]. The changes produced all increase the likelihood of proton channel opening. As illustrated in Fig. (**11**), after stimulation with the phorbol ester, PMA, the proton currents are larger, activate more rapidly, deactivate more slowly, and turn on at 40 mV more negative voltages. These changes are so profound that it was first concluded that a second, novel type of proton channel had appeared [32], but the simpler interpretation is that the enhanced gating mode reflects the behavior of channels modified by phosphorylation or some other biochemical enhancement [53, 68-70, 103-105]. Proton channels after stimulation by PMA or other agonists are often described

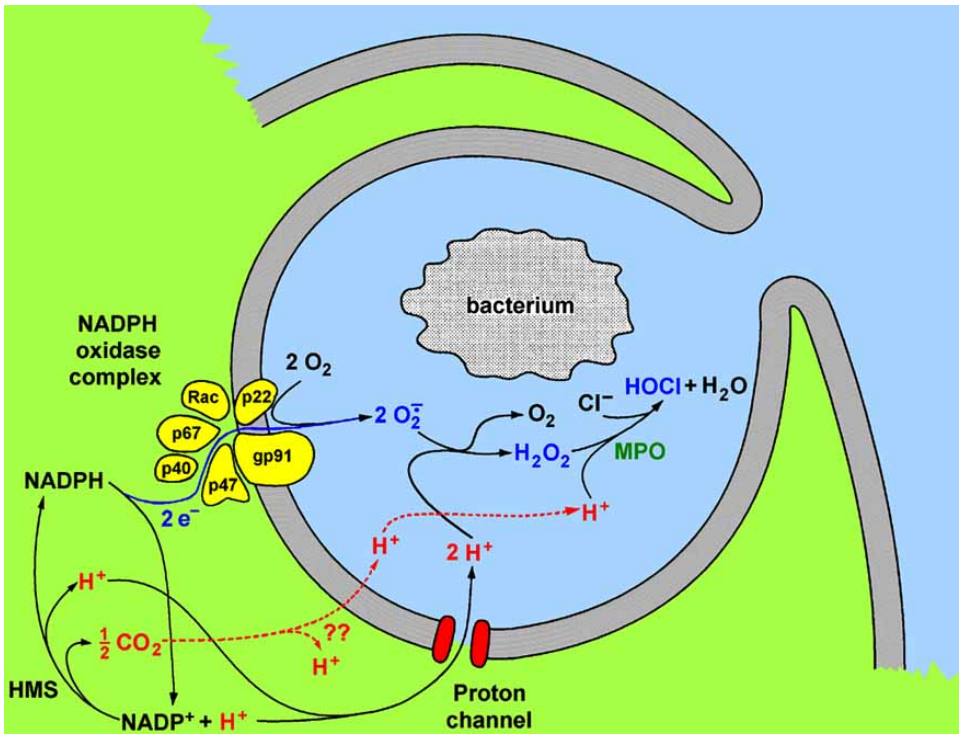

Fig. (10). Diagram of the key components of the respiratory burst in phagocytes, from the viewpoint of proton husbandry. Gray is plasma membrane; green indicates cytoplasm; blue is extracellular fluid, part of which will become intraphagosomal. Stimulation by bacteria or artificial agonists induces the assembly of the multi-component NADPH oxidase complex. This enzyme transfers electrons from intracellular NADPH across the membrane to reduce extracellular or intraphagosomal (depending on the stimulus) O_2 to superoxide anion, $O_2^{\cdot-}$. Protons are conducted from the cytoplasm into the phagosome (or extracellular fluid) independently of the electron movement, but still nearly stoichiometrically, approaching one proton per electron [190-192]. Reproduced from [5] with permission from Elsevier.

as being "activated," meaning that they are in an enhanced gating mode. Unfortunately, this terminology is ambiguous, because it is customary to describe the opening of ion channels as "activation" [160]. Proton channels in the activated gating mode are far more likely to open than those in the resting mode, but they still require an appropriate combination of depolarization and ΔpH to open.

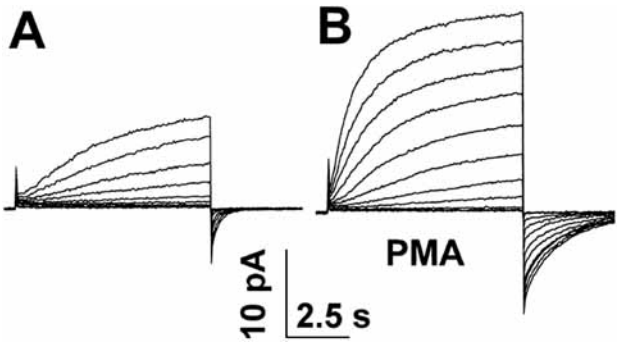

Fig. (11). Families of proton currents in a human neutrophil in perforated-patch configuration during identical families of depolarizing pulses, illustrating (A) "resting" mode and (B) "activated" mode gating behavior. **A** is an unstimulated cell; **B** is the same cell several minutes after stimulation with the phorbol ester, PMA. The membrane was held at -40 mV and then stepped to -20 to +80 mV in 10-mV increments. After stimulation, activation (channel opening) is faster, tail current deactivation (channel closing) on repolarization is slower, the currents are larger, and the threshold voltage is shifted negatively. Reproduced with permission from [68].

Phenomenologically, a divalent metal sensitive proton conductance is activated during the respiratory burst [18, 149, 158], based on measurements of pH and membrane potential. To some extent, the enhanced gating mode of voltage-gated proton channels must contribute to the activation of this conductance. However, the intense depolarization due to electron transport by NADPH oxidase is undeniably a powerful stimulus to open proton channels. For this reason, experimental evidence that a proton conductance is activated does not necessarily mean that the enhanced gating mode has been activated, unless direct voltage-clamp evidence confirms this conclusion. Quantitative modeling (see below: **Proton Channels Compensate Charge**) indicates that if proton channels retained their resting gating mode behavior during the respiratory burst, the membrane potential of neutrophils would depolarize 25-30-mV more, which would attenuate I_e and consequently $O_2^{\cdot-}$ production by 15-20% [5].

Mechanism of "Activation" of Proton Channels

The biochemical pathways that shift proton channel gating modes have not been clearly established. Elucidation of these pathways would enable a rational approach to developing pharmacological intervention in the regulation of proton channels, as opposed to direct alteration of the channel itself. The enhanced gating mode of proton channels occurs not only with PMA, but also when phagocytes are stimulated with more physiological agonists such as arachidonic acid (AA) [70], interleukin-5 (IL-5), leukotriene B_4 (LTB_4) [104], fMetLeuPhe [105], or adherence, which is the most likely cause of spontaneous activation [69]. Activation of proton channels by PMA suggest that a likely mechanism is phosphorylation of the channel protein, perhaps by protein kinase C (PKC) [149, 158]. However, PKC could be just one element in a signaling cascade. Evidence exists to supports each possibility, as well as various combinations, such as phosphorylation leading to AA release, or AA stimulated phosphorylation [105, 161, 162]. Support for activation by PKC-mediated phosphorylation is that PMA-activated H^+ flux is prevented by PKC inhibitors such as staurosporine [149, 158, 163] and GF109203X [103-105]. However, staurosporine concentrations that fully inhibit PKC did not abolish the proton flux stimulated by chemotactic peptides, whereas tyrosine kinase

inhibitors did, implicating tyrosine phosphorylation without a requirement for PKC [118]. In support of the latter pathway, a phosphotyrosine phosphatase antagonist, vanadate, activated the proton conductance [164]. It appears that distinct phosphorylation sites activate the proton channel and NADPH oxidase, because rottlerin, which is considered specific for the PKCδ isoform, inhibits activation of NADPH oxidase but not of proton currents [104]. In a recent study, proton currents in excised membrane patches were stabilized by ATP applied to the intracellular solution, but this effect appeared to reflect ATP binding rather than phosphorylation [165].

It has also been proposed that AA is the final activator of proton channels [166]. Substantial evidence supports the idea that AA released by the action of phospholipase A_2 (PLA_2) is a necessary step in proton channel activation. PLA_2 inhibitors prevent proton channel activation assessed indirectly [64, 167, 168]. Similarly, the proton conductance was not activated in a $cPLA_2\alpha$ deficient cell line, and this was overcome by exogenous AA [159, 169]. AA directly enhances proton currents in the whole-cell configuration [19, 64, 108, 168, 170], despite the fact that pathways involving diffusible second messengers are typically disrupted. This observation suggests a direct pharmacological action of AA on proton channels. However, more profound enhancement by AA was observed in perforated-patch studies in which many signaling pathways are preserved [70], suggesting an additional role for diffusible second messengers. In most respects, the enhanced gating state produced by AA resembled that seen with other stimuli.

Despite this evidence, three details raise doubts that AA is a required final activator of proton channels. First, the effects of AA were more pronounced in perforated-patch than in whole-cell experiments [70], suggesting that some additional factor beyond AA itself contributes to the response. Second, when stimulated by PMA or by adherence, the tail current decay was slowed drastically in human neutrophils and eosinophils [59, 68, 69], whereas when AA was the stimulus, there was little slowing [70], suggesting that the AA response is not identical to that produced by other agonists. Finally, although it is indisputable that exogenous AA promotes some degree of proton channel activation, that AA is produced by phagocytes during the respiratory burst, and that AA can stimulate a respiratory burst, it cannot be concluded on this basis alone that AA is produced in the correct concentrations and locations *in vivo*. Recent studies indicate that proton channel activation, that is, induction of the enhanced gating mode, was not inhibited by selective $cPLA_2\alpha$ inhibitors [105]. Furthermore, PMA- or fMetLeuPhe-activation of proton channels was identical in granulocytes from control and $cPLA_2\alpha$ knockout mice [105]. In summary, the signaling pathways leading to proton channel activation in living phagocytes during the respiratory burst are not yet clearly established.

Proton Channels Compensate Charge

Fig. (**12**) illustrates the membrane potential response of a neutrophil during the respiratory burst, calculated using a model based entirely on relevant empirically-determined electrophysiological properties [5]. The activity of NADPH oxidase results in a rapid and large depolarization in human phagocytes [4, 18, 146-149, 171-175] and also in tobacco plant cells [176]. In the model (Fig. **12**), after activation of NADPH oxidase, the membrane rapidly depolarizes toward the Nernst potential for protons (E_H), because the proton conductance is the predominant conductance in this voltage range [5, 32, 142], and then more slowly exceeds this value, reaching levels similar to those observed experimentally.

The model can be "asked" what benefit might accrue to the cell to shift proton channels into their enhanced gating mode. One result evident in Fig. (**12**) is that the extent of depolarization during the PMA response ("PMA") of a human neutrophil is attenuated by 26 mV compared with the response if proton channels retained their resting properties ("rest"). Thus, the enhanced gating mode requires less depolarization to activate sufficient proton conductance to balance the electron flux. Minimizing depolarization is beneficial to the cell, because the oxidase is inhibited by depolarization [150, 151]. In the model, the enhanced gating mode improves the efficiency of NADPH oxidase (the integral of the calculated electron current) by ~18% [5].

If we model the effects of 1 mM Zn^{2+} based on its known effects of slowing activation and shifting the voltage-dependence of H^+ channel opening, the resulting prediction is extreme depolarization (Fig. (**12**), "Zn^{2+}"), which reduces enzyme activity by 93% over 10 min [5], similar to the inhibition observed experimentally [150]. Eosinophil membrane patches in which NADPH oxidase is active in the presence of Zn^{2+} depolarize to +180 mV [4]. The model thus confirms quantitatively that the most likely mechanism by which Zn^{2+} inhibits NADPH oxidase activity is through its effects on proton channels. Zn^{2+} has no direct effect on NADPH oxidase activity [150, 156, 177]. By preventing proton channels from opening, Zn^{2+} prevents charge compensation, resulting in extreme depolarization that inhibits the enzyme [150].

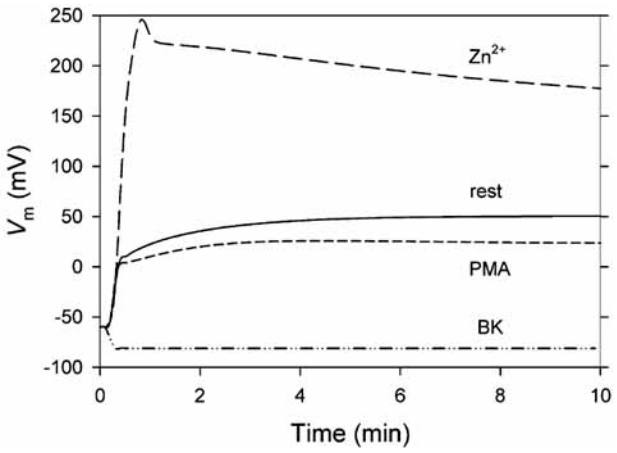

Fig. (12). Calculated membrane potential responses in a model of a human neutrophil. The resting potential was set at -60 mV, which is a typical value based on numerous studies [1]. NADPH oxidase activity was simulated by assuming that electron current is activated with a sigmoid time course, as observed experimentally. The membrane potential response (V_m) is plotted, assuming that proton channels retain their unstimulated behavior ("rest"), that proton channels shift into their activated mode of gating ("PMA"), that 1 mM Zn^{2+} is present ("Zn") during PMA stimulation, or that there is also a large K^+ conductance ("BK") as reported in one recent study [193]. A large K^+ conductance is not compatible with the depolarization that occurs during the respiratory burst. The model reproduces the main features of experimental data if the proton conductance is the only significant conductance present. Reproduced from [5] with permission from Elsevier.

Proton Channels Perform Other Important Tasks

It is often overlooked that proton efflux serves several crucial functions during the respiratory burst in phagocytes in addition to charge compensation [5]. The tendency of electron flux to acidify the cytoplasm and alkalinize the phagosome is counterbalanced by proton flux. If one proton is moved with each electron, the result does not change pH on either side of the membrane; in contrast, if electron flux is compensated electrically with any other ion, pH_i decreases and $pH_{phagosome}$ increases [5, 178]. It is important to keep pH_i near neutral to optimize NADPH oxidase activity [179] and phagosomal pH needs to be optimized for the bactericidal activity of proteolytic enzymes released by granules [139, 180, 181]. In addition, in view of the enormity of electron transport during the respiratory burst, charge compensation by protons is favorable because it prevents the massive osmotic swelling of the phagosome (e.g., 10-20-fold) that would result if electronic charge were compensated by other ions [5]. When protons compensate electron flux, the end products include H_2O, O_2, and HOCl, all of which are

membrane permeable [182], which means that less obligate swelling will result. Finally, protons are required substrates both for the dismutation of $O_2^{\cdot-}$ to H_2O_2 and for the conversion of H_2O_2 into HOCl (and H_2O) by myeloperoxidase (Fig. **10**). Even if charge compensation were not necessary, proton flux into the phagosome would still be essential to achieve the additional functions of pH regulation, volume regulation, and to provide substrate for reactive oxygen species formation.

CONCLUSION

The functional roles that have been identified for proton channels in a variety of tissues make this channel an important target for pharmaceutical development. Proton channels contribute to acid secretion in airways and in excitable cells and facilitate the continuous activity of NADPH oxidase in phagocytes. In the latter role, proton channels are potential targets for pharmacological modulation of the production of reactive oxygen species, which are important not only as bactericidal agents, but also in signaling and as possible causes of microglia-mediated self injury in diseases such as in Alzheimer's [183] and HIV-1 associated dementia [184, 185]. Pharmacological studies have revealed a number of important features of voltage-gated proton channels. However, the discovery of a specific high-affinity inhibitor of proton channels would represent a breakthrough. Elucidation of the biochemical signaling pathways that govern the switch between resting and activated gating modes would provide an additional target for pharmacological intervention.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

AA	=	Arachidonic acid
DPI	=	Diphenylene iodonium
ΔpH	=	pH Gradient (pH_o - pH_i)
g_H	=	Proton conductance
γ_H	=	Single proton channel conductance
I_e	=	Electron current (due to NADPH oxidase)
$O_2^{\cdot-}$	=	Superoxide anion
pH_i	=	Intracellular pH
pH_o	=	Extracellular pH
PKC	=	Protein kinase C
PLA_2	=	Phospholipase A_2
PMA	=	Phorbol myristate acetate
P_{open}	=	Open probability of the proton channel
$P_{open,max}$	=	Maximum P_{open} of the proton channel
TEA^+	=	Tetraethylammonium ion
TΩ	=	Teraohm = 10^{12} Ohm
V	=	Voltage

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