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DIVERSITY OF VOLTAGE GATED PROTON CHANNELS

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1. ABSTRACT

Voltage gated proton channels were first discovered in snail neurons and recently have been found in many mammalian cells. As their name suggests, H^+ channels are sensitive to voltage, with an open probability that increases with membrane depolarization. Many properties that are shared by voltage-gated proton channels make them unique among ion channels. They show high selectivity for protons, strongly pH dependent gating, and a tiny single channel conductance. Although they are inhibited by divalent cations, including zinc and cadmium, no effective blockers exist. There is sufficient evidence to suggest that they are not water filled pores, unlike many other membrane bound ion channels. Instead, protons probably are conducted by a "hydrogen bonded chain" mechanism that resembles the Grotthuss mechanism in water. Differences in activation and deactivation kinetics of H^+ currents in different cells suggest that there may be at least 4 isoforms of voltage gated proton channels. Gating kinetics may reflect specific functions. Voltage gated proton channels are well suited to extrude acid from cells and also may function in the extrusion of metabolic acid in the form of CO_2 from the lungs. The best established function of H^+ channels is in mammalian phagocytes, where they extrude protons to compensate for the charge separation created by the movement of electrons across the membrane by the bactericidal enzyme NADPH oxidase.

2. INTRODUCTION

Voltage gated proton channels (VGPC) were discovered in *Helix aspersa* (snail) neurons (1) and subsequently have been identified in a wide variety of cell types. They are found in many mammalian cells, including alveolar and renal epithelial cells, cultured skeletal muscle, and a variety of immune cells (2-9). To date these channels have not been isolated and sequenced, and their structure is not known. However, VGPC have been characterized extensively by studies using patch clamp techniques and internal pH (pH_i) measurements. Many of their properties are unique among ion channels.

The function of VGPC is to extrude protons from cells. Because activation of H^+ channels can increase pH_i very rapidly, they can play a role in cellular pH homeostasis (10). Through the extrusion of protons, these channels also function in charge compensation in activated immune cells (11,12) and possibly CO_2 elimination by the lungs (13). Channel opening depends on membrane

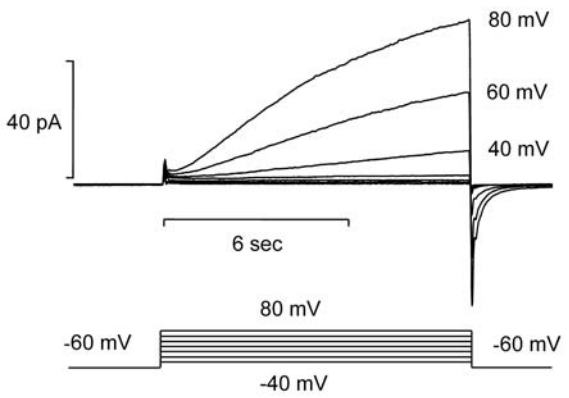

Figure 1. A family of proton currents at symmetrical pH 7. Outward currents in a voltage-clamped eosinophil with bath and intracellular solutions of pH 7. The cell is held at a potential of –60 mV, roughly the resting potential of an intact cell, before being stepped to more positive voltages shown in the diagram below the family of currents. Under these conditions, H^+ channels open at 20 mV. Larger depolarization increases the rate of channel opening and increases the overall outward current. When the voltage is stepped back to –60 mV, H^+ channels close rapidly and allow the brief inward passage of protons, which produces the characteristic "tail" current.

potential, but the voltage dependence of activation also depends profoundly on pH_i and extracellular pH (pH_o) (14,15,15-17).

A typical whole cell recording of a family of proton currents in an eosinophil is shown in Figure 1. The intracellular and extracellular solutions are identical containing 100 mM tetramethylammonium methanesulphonate (TMA^+ $MeSO_3^-$) as a replacement for Na^+, K^+ and Cl^- ions and buffered to pH 7. In order to isolate H^+ currents, ions that permeate other ion channels are often omitted; however, H^+ currents can be seen with physiological solutions. No current is seen below 20 mV, which is the voltage required to activate proton channels ($V_{threshold}$) in a cell at symmetrical pH. At voltages below $V_{threshold}$, the channels are closed. When the voltage is stepped above $V_{threshold}$ the open probability of H^+ channels increases, resulting in an outward current. The current reflects the passage of protons outward across the cell membrane. VGPC channels do not open immediately after depolarization, but instead, they open in a probabilistic manner at a rate that is dependent on voltage and pH. Upon depolarization, the H^+ current amplitude increases after an initial delay, with an (approximately) exponential time course that reflects a progressive increase in the number of open channels. The kinetics of channel opening, or activation, is often quantified by Tau_{act}, the time constant of an exponential curve fitted to the current. The opening rate is $\sim 1/Tau_{act}$. With higher voltages the open probability increases, channels open faster, and the maximum H^+ current is larger. Returning the voltage to below threshold causes the channels to close. As with the opening process, H^+ channels do not close instantaneously, but with a time course that reflects the rate of channel closing. "Tail currents" (visible at -60 mV in Figure 1) are transient currents seen upon hyperpolarisation as protons pass across the cell membrane before all of the channels are closed. The presence of inward tail currents shows that proton channels can pass protons in either direction when they are open, depending on the electrochemical driving force (V-V_{rev}). The time constant of the tail currents indicates the rate of deactivation (channel closing) of VGPC, $\sim 1/Tau_{tail}$.

3. THERE ARE SEVERAL TYPES OF VGPC

VGPC characteristics in different cells vary substantially, suggesting that there may be several types of VGPC. Whereas all VGPCs share many other properties, their gating kinetics varies greatly.

Snail neurons have the fastest opening rate, achieving steady state current in <25 ms (14,18-20). Proton channels in oocytes are generally slower, reaching steady state current within a few hundred milliseconds (15,21,22). Epithelial cells are slower still with Tau_{act} of a few seconds (2,16). The proton currents in immune cells (phagocytes, lymphocytes, monocytes and microglial cells) and muscle cells show the slowest rate of activation (4,7,8,23-25). Although it is possible that some of the differences in Tau_{act} might be attributed to variable regulation of VGPC by cytosolic factors, several observations make this hypothesis unlikely. Excised membrane patches (patches of membrane removed from the cell and cytosol) of alveolar epithelial cells show somewhat different kinetics than whole-cell currents (26). However, this subtle difference could not account for the differences between gating kinetics of VGPC in epithelial cells compared to neurons, for example. In other cells, H^+ currents in excised membrane patches resemble whole-cell currents in the same cell type (27,28). Differences in VGPC in whole-cell measurements are not likely attributable to cytosolic factors, because diffusible cytosolic constituents diffuse into the pipette upon membrane patch rupture. Furthermore, H^+ currents in eosinophils in the permeabilized patch configuration (29,30), in which the cytoplasm remains intact, are similar to those in whole cell configuration (7,31). Therefore, the differences in gating are likely due to different channel structures.

Channel activation occurs after a delay in some cells, which results in a sigmoidal activation time course in epithelial cells (2,16,26,32,33) and in immune cells (7,8,24). In oocytes, the activation of VGPC follows a single exponential time course (15,21,22). The sigmoidicity of activation was increased by hyperpolarization of the membrane preceding the test pulse (33), like the Cole-Moore effect in K^+ channels (34). Presumably, the channel must pass through several sequential closed states before it reaches the open state, and occupancy of 'deeper' closed states is favored by membrane hyperpolarization (33). Variability in the activation time course in VGPC from different cells could reflect different transition rates between states, or a different number of steps.

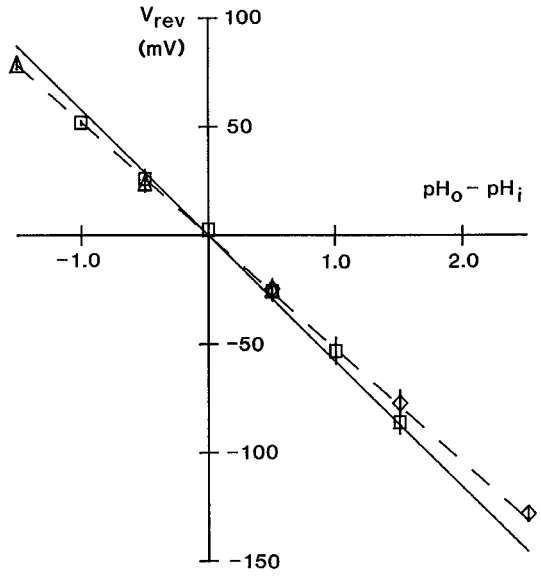

Figure 2. Comparison of V_{rev} with E_H values predicted by Nernst equation. Mean reversal potentials of H^+ currents from rat alveolar epithelial cells at a range of pH_o. The pH_i was 7.5 (8), 6.5 (□) and 5.5 (◇). The dashed line shows a linear regression of the data points and has a slope of 52.4 mV/U pH. Plots are mean ± SD for 2-18 determinations. The solid line indicates E_H as calculated by the Nernst equation and has a slope of 58.2 mV/U pH. V_{rev} is very close to E_H indicating a high selectivity of VGPC. Taken from (16).

The rate of channel closing, or deactivation ($1/Tau_{tail}$), also appears to differ among cell types. Snail neurons have Tau_{tail} of the order of a few milliseconds (14). Deactivation kinetics seems to follow the same pattern as activation. Oocyte VGPC have slower Tau_{tail} than snail neurons but faster than epithelial and immune cells.

It is possible to separate VGPC into four groups based on their gating kinetics. Named according to the cells in which they occur: type **n** for snail neurons, type **o** for oocytes, type **e** for epithelial cells, and type **p** for phagocytes, myotubes, and immune cells (35). This classification is arbitrary; a closer examination of the proton channels in immune cells indicates subtle as well as major variability. Deactivation kinetics in murine macrophages appear to be somewhat dependent on pH_i (36) but in basophils Tau_{tail} was insensitive to pH_i (25). More importantly, when phagocytes are stimulated, the properties of VGPC change dramatically (29,30,37,38). The VGPC in activated phagocytes are so different from "normal" they were at first proposed to comprise a distinct variety of H^+ channel (37), but recent studies show that stimulation of phagocytes simply modifies the properties of the VGPC (29,30,38-40). The modulation of VGPC properties in activated phagocytes is reversible. However, the properties of "activated" H^+ channels (detectable in permeabilized-patch experiments upon stimulation of the cells) revert toward "resting" properties if the patch membrane is ruptured and whole-cell configuration is achieved (30). In a sense, this property precludes strict classifications based on gating kinetics.

4. PROPERTIES OF VOLTAGE GATED PROTON CHANNELS

4.1. VGPC are highly selective for H^+

VGPC are almost perfectly selective for H^+. The only other ion to have detectable permeability is deuterium (41). By comparing the reversal potential (V_{rev}) at different pH_o and pH_i with the predictions of the Nernst equation, one can evaluate selectivity. If the channel is selective for protons, V_{rev} should follow the equilibrium or Nernst potential for H^+ ($E_H = RT/zF \log([H^+]_o/[H^+]_i)$), where $[H^+]$ is the H^+ concentration. E_H is 0 mV at symmetrical pH, and changes 58 mV per unit change in the pH gradient at 20°C. In experiments where pH_i was controlled by high buffer concentrations (e.g., 100 mM), V_{rev} was usually close to E_H, indicating a high selectivity VGPC for H^+ (7,15,16,24,28,31,41-43), as shown in Figure 2. Several studies have shown that V_{rev} deviates substantially from E_H when lower buffer concentrations (5-20 mM) are used (2,5,8,23). The implication of this result is that deviations of V_{rev} from E_H are most likely the result of inadequate control of pH, rather than genuine permeability of VGPC to other cations. The relative permeability of VGPC to H^+ compared with other ions present can be calculated from the Goldman-Hodgkin-Katz voltage equation (44,45). In most studies such calculations indicate a permeability ratio $P_H/P_X > 10^6$ where X is the predominant cation present (7,16,24,28,31-33,41-43), indicating extremely high selectivity. Measurements in heavy water (D_2O) indicated $P_D/P_X > 10^8$ (41).

Ion substitution experiments suggest that the true H^+ selectivity may in fact be even higher than that estimated from absolute V_{rev} measurements. If VGPC were permeable to other ions, substituting one ion for another would alter V_{rev}. In studies where the species of cation (Na^+, K^+, Li^+, Cs^+, tetraethylammonium (TEA^+), tetramethylammonium (TMA^+), N-methyl-D-glucamine (NMG^+)) or anion (Cl^-, aspartate$^-$, glutamate$^-$, methanesulfonate$^-$, and isethionate$^-$) was substituted at constant pH, there was no change in V_{rev} (3,4,8,15,19,23,32,41,46,47). In some experiments, Na^+ or Li^+ did change V_{rev} but this was due to Na^+/H^+ antiport altering pH_i (21,32). Therefore, VGPC do not conduct any ions except protons and deuterons (41).

H^+ as opposed to OH^- is most likely the major charge carrier through the channel. The proton current amplitude (I_H) increases at lower pH_i. If OH^- were the charge-carrying species then decreasing pH_i would not necessarily affect the current, because the source of the current would be external OH^-, which is unchanged. Furthermore, in rat alveolar epithelial cells, the conductance of VGPC was found to be ~50% lower in D_2O compared to H_2O, implicating H^+ as the charge carrier (41). If the current carrier were OD^- as opposed to OH^-, the isotope effect would be expected to lower the conductance by only 6%.

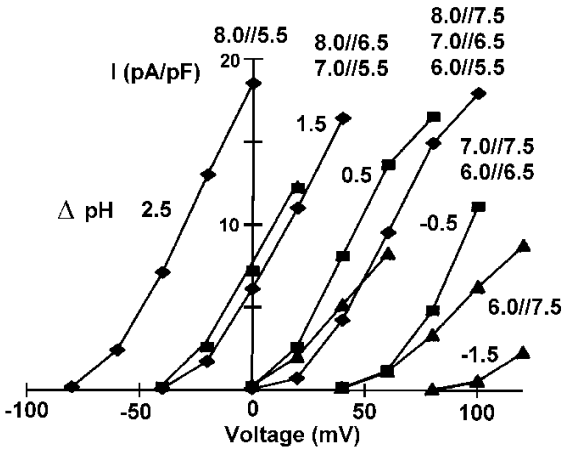

Figure 3. Current voltage relationship of proton channels. The diagram shows the current/voltage relationships of H^+ channels in alveolar epithelial cells at a range of pH_i and pH_o. The graph shows that the position of the current/voltage curve is dependent on the pH gradient. Lower pH inside shifts the curve to more negative (left) values whereas a higher internal pH shifts the curve to more positive (right) values. The curve shifts roughly 40 mV per unit pH in both directions. The data were obtained using 4-s depolarizing pulses and are normalized for cell size (estimated by capacitance). Symbols indicate pH_i 5.5 (Λ), pH_i 6.5 (!) and pH 7.5_i (7). Labels indicate pH gradient following the convention $pH_o//pH_i$. Modified from (16).

The deuterium replacement studies also solidified the notion that H^+ current flows through a specialized conductance mechanism, rather than through the membrane itself. The permeability of deuterons through a phospholipid bilayer is similar to that of protons (48,49). The lower conductance of D^+ than H^+ through VGPC provides evidence that VGPC are specific transport molecules (41). The conductance mechanism is probably a membrane spanning protein, although this has not yet been confirmed.

4.2. H^+ channels are gated by membrane potential

Essentially as a result of Ohm's law, any pathway that translocates ions across a membrane is voltage dependent, in the sense that the ion flux is different at different voltages. However, this voltage dependence is distinct from voltage-dependent gating. VGPC, like all ion channels, are believed to have at least two conformational states, "closed" in which little or no current is conducted, and "open" in which ions flow through the channel continuously until the channel closes again. Changes in membrane potential are probably transduced by a voltage sensor contained within the channel that alters its open probability. The open probability of H^+ channels is essentially zero at potentials negative to E_H. Depolarization to more positive potentials increases the open probability, so that H^+ channels open, resulting in outward H^+ current. Voltage dependent gating is a key feature that distinguishes VGPC from other H^+ channels, such as the M_2 viral proton channel (50,51), bacteriorhodopsin (52,53), and cytochrome *c* oxidase (54), all of which are voltage dependent but do not have voltage dependent gating. Another difference is that VGPC probably form a continuous pathway across the entire membrane, whereas the proton channels of complex molecules like H^+-ATPases, cytochrome *c* oxidase, and bacteriorhodopsin conduct the proton only partway across the membrane to an active site where the proton engages in chemistry.

4.3. H^+ conductance exhibits weak pH dependence

The current through most ion channels increases when the permeant ion concentration is increased. The H^+ conductance (g_H) of gramicidin channels increases in parallel with the H^+ concentration, $[H^+]$, from pH ~5 to very low pH (55-59). A surprising property of VGPC is the weak effect of changes in pH_i on g_H. The limiting g_H for large depolarizations, $g_{H,max}$, increases ~2-fold/Unit decrease in pH_i between pH 5.5 and 7.5 (14,20,23,24,26,35,60). That g_H does not change in direct proportion with $[H^+]$ suggests that the rate-limiting step in H^+ permeation probably occurs within the channel itself, rather than in bulk solution.

4.4. Activation and gating of VGPC are highly sensitive to pH gradients

The voltage dependence of VGPC is not absolute, but depends strongly on pH_i and pH_o, as can be seen in the H^+ current-voltage plots in Figure 3. With an increase in pH_o or a decrease in pH_i, the conductance-voltage relationship (g_H-V) is shifted negatively. This pH dependence occurs in every cell that contains VGPC (2-4,8,9,14-16,23-25,31,46). The voltage at which H^+ channels first open, $V_{threshold}$, is a convenient indicator of the position of the g_H-V relationship. At symmetrical pH, $V_{threshold}$ is ~20 mV. Lowering pH_i by one-unit causes a 40 mV negative shift in $V_{threshold}$. An identical shift is seen when the pH_o is increased one unit (16). Therefore, the voltage-dependence of gating of VGPC is equally sensitive to pH_i and pH_o. This pH gradient dependence is seen over the range pH_i 5.5-7.5 and pH_o 6-8 (16). The effect of the pH gradient on the g_H-V relationship appeared to saturate at high pH_o in several studies (14-16,61). However, a subsequent study showed that no saturation was seen when $V_{threshold}$ was plotted against V_{rev} (41). The saturation of the shift of the g_H-V relationship occurred at high pH_o where V_{rev} also deviated substantially from E_H. Evidently at very high pH_o there is loss of control over pH_i (41).

In addition to shifting the g_H-V relationship of VGPC, pH also modulates rate of channel opening. At a given potential, a decrease in pH_i or an increase in pH_o speeds H^+ channel activation, decreasing Tau_{act}. Qualitatively, this would occur if all kinetic parameters were shifted equally by pH changes. The extent to which the Tau_{act}-V relationship parallels the g_H-V relationship seems to vary among cells. This shift is parallel in oocytes (15), lymphocytes (28), and alveolar epithelial cells (16,32), but not in snail neurons (14) or murine macrophages (5). A decrease in pH_i not only shifts the voltage dependence of Tau_{act} negatively; it also increases the rate of channel opening 3-7 fold/Unit pH in rat alveolar

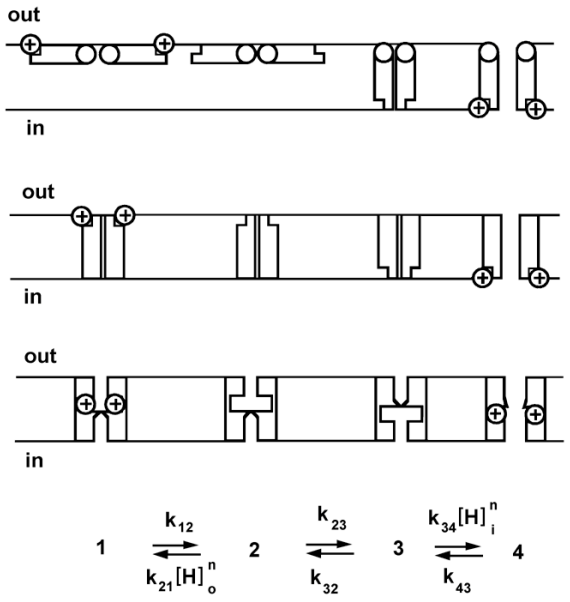

Figure 4. Proposed model of regulation of proton channel activation by voltage and pH. The diagram shows three different cartoons that represent the same state diagram and rate constants. A) A "butterfly" model in which the protons bind to "wings" on the channel subunits. B) Distinct binding sites on the internal and external surfaces of the subunits. Protonation of the internal site causes a conformational change in the protein "hiding" the external proton-binding site and vice versa. C) Protonation sites in proton "wells." The accessibility of the protonation sites depends on the conformation of the protein, which depends on proton binding. In each case, the proton channel requires a conformational change in each channel subunit that only occurs when the regulatory site is deprotonated. Binding of protons at the external site stabilizes the closed channel and binding at the internal site stabilizes the open channel. The conformational change between the closed and open state is rapid. The theoretical I_H traces were produced with the following parameter values: $k_{12} = 1000\ s^{-1}$ $k_{32} = 10^6\ s^{-1}$, $k_{43,\ slow} = 3\ s^{-1}$, $k_{43,fast} = 0.05\ s^{-1}$. Taken from (16).

epithelial cells (26) and human basophils (25). This effect is not seen in snail neurons (14). These data seem to suggest that the effects on gating kinetics of the regulatory protonation sites on the internal and external surface of VGPC are not equivalent. Furthermore, the different effects of pH_i and pH_o on gating in different cells suggest that there are multiple channel types.

In studies that have examined the effect of pH on deactivation kinetics, quantified as the tail current time constant, Tau_{tail}, channel closing was relatively insensitive to changes in pH_o. Alveolar epithelial cells (16,41) and murine macrophages (5) exhibited a small dependence on pH_o. In THP-1 cells, a change in pH_o from 5.5 to 7.5 caused no change in Tau_{tail} (24).

A model has been proposed to account for the voltage and pH dependence of VGPC gating (16). Several assumptions were required to reproduce experimental data. Because changes in pH_o and pH_i produce similar shifts in the g_H-V relationship, the model proposes that the pH gradient (pH_o - pH_i), rather than absolute pH, sets the position of g_H-V relationship. In the model, protons could bind to external or internal sites on the channel, but each binding site is not accessible from the opposing side, and the external and internal sites are not accessible simultaneously. The changes in channel function are due to changes in open probability (p_{open}). Binding of protons to the external site decreases p_{open} whereas internal proton binding increases p_{open}. Furthermore, proton binding is voltage dependent. Protonation of a regulatory site "locks" the channel in a particular conformation. Deprotonation of the site is then necessary for any conformational changes to occur.

Using these assumptions, the model in Figure 4 was derived. Each cartoon shows a channel with at least 2 subunits that have protonation sites that are alternately accessible to each side of the membrane. Accessibility of the protonation sites to only one side of the membrane at a time, as opposed to distinct protonation sites for each side, was necessary for the model to work. Protonation of the internal site stabilizes the open conformation, allowing H^+ current. With a change of voltage or pH_i the internal binding site loses a proton and undergoes a rapid conformational change, exposing the binding site to the outside surface of the membrane. Binding of a proton to the external binding site stabilizes the closed channel, preventing current (16).

4.5. VGPC have evolved to extrude H^+

VGPC have the ability to pass inward as well as outward current, as demonstrated by the transient inward "tail" currents seen upon repolarisation to a membrane potential negative to V_{rev}. However, based on the voltage- and pH-dependent regulation of channel gating, VGPC evidently evolved to extrude H^+ from cells. At all physiological pH gradients, $V_{threshold}$ is positive to V_{rev} (41), so that the H^+ channel opens only when there is an outward electrochemical gradient for H^+. In other words, H^+ channels only open when doing so will result in acid extrusion from cells. This has always been accepted as the primary function of VGPC.

Figure 5 shows $V_{threshold}$ plotted against V_{rev} in rat alveolar epithelial cells at different pH gradients. The relationship is linear and does not saturate at either extreme (41). VGPC in all cells appear to obey the same relationship (62). Because changes in pH_o and pH_i shift the g_H-V relationship less than E_H, the relationship in Figure 5 predicts that at extreme negative pH gradients (>~-1.7 pH/U) proton channels would pass H^+ inward. Gu and Sackin (60) demonstrated small inward H^+ currents at +50 mV at pH_i 8 and pH_o 6.5. Inward currents are seen at more physiological pH gradients in phagocytes in which NADPH oxidase is active (30,37,38). However, under these circumstances there appears to be a drastic modulation of the properties of H^+ channels (discussed below).

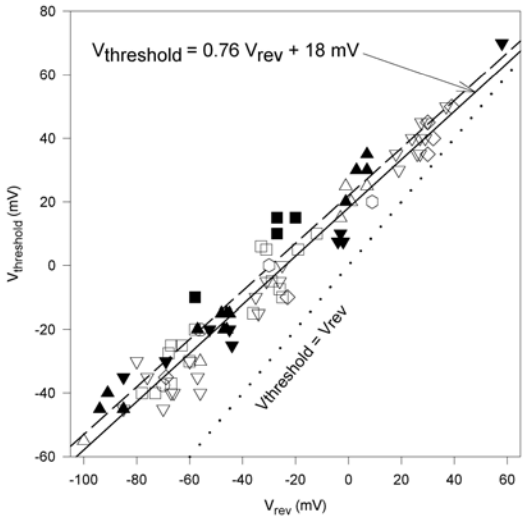

Figure 5. Relationship between $V_{threshold}$ and V_{rev}. The potential at which H^+ currents were first detectably activated ($V_{threshold}$) was plotted against V_{rev} measured in the same cell and same solution. Open symbols indicate H_2O solutions and closed symbols show D_2O solutions. Across the range of pH studied, V_{rev} was always negative to $V_{threshold}$. The dotted line indicates $V_{threshold} = V_{rev}$. Pipette solutions are indicated by the shape of the symbol used pD 7.0 (8), pD 8.0 (X), pD 9 (v), pH 7.5 (◇), pH 5.5 (○) 50 mM NH_4^+ (□). Taken from (41).

4.6. Gating is modulated by divalent cations

No known compounds completely block VGPC. A few reduce H^+ current when added to external solutions, such as TEA^+ (4,10,14), 4-aminopyridine (10), amiloride (32), rimantadine, and amantadine (33). Concentrations of TEA^+ between 10 – 52 mM caused a ~30% reduction in the H^+ current in snail neurons and human muscle myotubes (4,10,14). However, this "block" appears to be independent of concentration. Furthermore, large H^+ currents can be recorded in isotonic TEA^+ solutions (3,15). Thus, TEA^+ cannot be considered an effective blocker. It may act by binding to negative charges near H^+ channels (33). The "block" of H^+ current by the weak bases listed above has been attributed to permeation of the neutral form through cell membranes and altering the buffering power of the intracellular solution (10) or changing the local pH (32,33).

A ubiquitous feature of VGPC is that divalent metals inhibit I_H and slow Tau_{act} when added to the external bath solution. The most potent inhibitory cations are Cu^{2+} and Zn^{2+}, with the potency decreasing through $Ni^{2+} > Cd^{2+} > Co^{2+} > Mn^{2+} > Ba^{2+} > Ca^{2+}$ and Mg^{2+} (1,2,4,10,19,27,31,47). Because different divalent metal ions are effective at very different concentrations, the inhibition is not likely due to simple screening of negative surface charges, but rather must result from specific binding. The effect of divalent metals on VGPC has been described either as voltage dependent block (4,7) or modulation of the voltage dependence of gating (2,3,14,15,24,32,61). The two mechanisms are not equivalent. Voltage dependent block usually involves binding of the blocker within the channel, occluding the pore. However, divalent cations did not produce classical voltage-dependant block, and thus Zn^{2+} did not bind within the membrane voltage field. Instead, Zn^{2+} scaled down the instantaneous I-V curve with no detectable voltage dependence (17). Furthermore, the slope of the g_H-V relationship before and after Zn^{2+} block was the same, consistent with a simple voltage shift. In the case of Ni^{2+} and low Cd^{2+} concentrations, the slowing of Tau_{act} by divalent metals was accounted for by a shift in the Tau_{act}-V relationship in accordance with the shift of the g_H-V relationship. However, Zn^{2+} and high Cd^{2+} concentrations altered the Tau_{act}-V relationship more than could be accounted for by the shift in the g_H-V relationship. An additional slowing of activation by some metals must occur. H^+ channel closing appeared faster in the presence of these metals, but the effects of external divalent metals on Tau_{tail} were minor compared with their profound effects on Tau_{act}.

The inhibition of VGPC by divalent metals is extraordinarily sensitive to pH_o. At lower pH_o, the concentration of Zn^{2+} that was required to achieve the same inhibition increased dramatically. A 1.3-fold increase in Zn^{2+} at pH_o 7 produced effects comparable to those seen at pH_o 8, a 14-fold higher $[Zn^{2+}]$ was required at pH_o 6 compared with pH_o 7, and 129-fold higher $[Zn^{2+}]$ was required at pH_o 5 compared with pH_o 6. These data suggest that protons and Zn^{2+} compete for a common binding site. A model in which the Zn^{2+} receptor comprises three protonatable groups with pKa 6-7 reproduced the strong competition between H^+ and Zn^{2+}. The assumption was that the channel could not open when Zn^{2+} was bound to the receptor. The external Zn^{2+} receptor of VGPC was therefore proposed to comprise three His residues (17). There was no detectable effect of pH_i on external divalent metal ion inhibition of VGPC. In addition, Zn^{2+} added to pipette solutions had a less dramatic effect on H^+ currents, producing minor changes only at large concentration (17).

These results suggest that divalent metals bind to a site on the VGPC that does not interfere directly with the proton conduction pathway but merely interferes with the pH sensor on the molecule (preventing channel opening). Because divalent metals have similar effects on proton channel gating as an increase in external $[H^+]$ (increased Tau_{act}, a positive shift in g_H-V relationship, and modest effects on Tau_{tail}), it was proposed (17) that divalent metals compete for the same protonation sites previously hypothesized to regulate channel opening (16).

4.7. Proton channels are not water filled pores

Most ion channels comprise a pore that is filled with a single-file row of water molecules. Each ion that permeates must 'wait' for the waters in front to permeate. In the case of gramicidin, the pore contains a string of 6-12 water molecules (63). It is generally accepted that H^+ permeates gramicidin by a mechanism distinctly different from other ions, which resembles the Grotthuss mechanism of H^+ conduction in bulk water (64). As proposed by Onsager (65) and developed explicitly by Nagle and Morowitz (66), H^+ permeates gramicidin and other proton

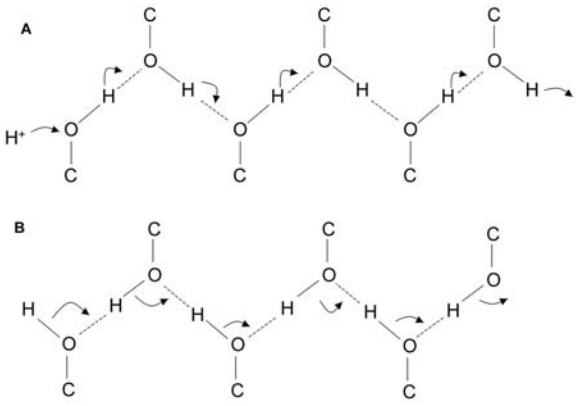

Figure 6. Transport of protons along a hydrogen-bonded chain. The diagram illustrates the mechanism of proton conduction along a hydrogen-bonded chain. In this example, the hydrogen bonds are formed between hydroxyl moieties that are part of amino acid side chains (e.g. serine residues). As an excess proton in sequence A (left proton) associates with the first oxygen, the hydrogen ion covalently bonded to the first oxygen bonds covalently to the next oxygen atom in the sequence. The arrows show the switching of hydrogen bonds to covalent bonds. The switching of bonds is a result of the second proton moving to the right side of a double minimal potential well that exists between two oxygen atoms in the chain. Protons sequentially change their bond state to the right until a proton is ejected into solution at the right end (into the extracellular fluid). The consequence of the hopping process in A is a steric rearrangement of the side chains. In order for the channel to pass another proton in the same direction, the hydroxyl groups must all reorient. As shown in sequence B, the hydroxyls rotate (indicated by the arrows) so that the protons face the original direction and proton conduction can continue. Diagram is redrawn from (66).

pathways by being shuttled along a hydrogen-bonded chain. As shown in Figure 6, a hydrogen-bonded chain transports protons by a hop-turn mechanism (66). Such a pathway could be formed by a combination of amino acid side-groups and intercalated water molecules (66,67). In passing down this water wire, protons hop from a hydronium ion (H_3O^+) to a neighboring water molecule, which becomes a hydronium ion. The proton then hops to the next water molecule and so on. After losing a proton, the water molecules must reorient before they can accept another proton from the same direction. A hydrogen-bonded chain would allow the passage of protons with extreme selectivity, because other molecules would not be able to jump along the chain (67). This mechanism could also explain the low pH dependence of the VGPC and the strong temperature and isotope effects. The calculated mobility of protons inside gramicidin channels is close to that in bulk water (59). The single-channel proton current in gramicidin is proportional to $[H^+]$ from pH ~5 to ~0 (68), increasing ~10-fold/Unit decrease in pH (55-59). In contrast, the conductance of VGPC is relatively pH independent. This comparison with gramicidin, a water-filled pore, seems inconsistent with idea that VGPC might also be water filled pores.

Other evidence that argues against the channel being a water-filled pore includes the extreme H^+ selectivity, studies using D_2O substitution for water, and the temperature dependence. The ratio of H^+ to D^+ current was ~1.9 (41), much larger than expected if buffer diffusion or H_3O^+ diffusion (69) were rate limiting, suggesting that the rate limiting step occurs within the channel (6,41). Furthermore, because this isotope effect was higher than that in the water-filled gramicidin channel (58), it is unlikely that the permeation mechanism consists of a water filled pore. Both the conductance and gating kinetics of VGPC are highly temperature dependent (8,27,70). The Q_{10} (the relative increase in rate for a 10°C increase in temperature) for H^+ current through VGPC was 2-3 in a range of cell types (70). Expressed as activation energy (E_a) this is 12-19 kcal/mol. This temperature dependence greatly exceeds that for H^+ conduction in aqueous solution, and is larger than for the conductance of other ion channels. This strong temperature dependence further indicates that the rate-limiting step is not diffusion to the channel but an event within the channel itself (70). In contrast, the Q_{10} for H^+ current through gramicidin is <1.34 (58,71). Thus, it unlikely that VGPC are water filled pores.

In summary, it is unlikely that VGPC are water filled pores. The high selectivity, weak dependence of g_H on pH, high temperature dependence, and large isotope effects all argue against the notion that the channels are water filled pores. It is probable that a "proton wire" comprised in part of amino acid side groups forms the conduction pathway.

4.8. VGPC have very small single-channel currents

Single channel currents of VGPC detected directly under voltage-clamp were reported recently (72). Poorly-resolved tiny channel-like events 7-16 fA in amplitude (the smallest directly-measured single-channel currents reported to date) were observed just above $V_{threshold}$ at low pH_i of 5.0-5.5 (72). More extensive data were based on stationary noise analysis. The steady state H^+ current variance (Var_H) attributable to H^+ channel gating events can be used to calculate i_H, the unitary current: $i_H = Var_H [I_H (V-V_{rev})(1-p_{open})]^{-1}$ (112). This calculation thus requires knowledge of the open probability (p_{open}) of VGPC. Three early studies estimated the single channel conductance of VGPC to be <100 fS at pH 5.5-6.0, based on stationary variance measured near $V_{threshold}$, where p_{open} can be assumed to be ~0 (3,4,27). In all three studies, the signal-to-noise ratio was poor and therefore the values obtained are rough approximations. A recent study has improved the signal-to-noise ratio dramatically and used an analysis method that obviates the need to guess p_{open}. The unitary conductance was 38 fS at pH_i 6.5 and 138 fS at pH_i 5.5 (72). This conductance is very small compared to other channels, probably reflecting the exceedingly low concentration of protons compared with other physiological ions.

5. FUNCTIONS OF H^+ CHANNELS

5.1. General functions of VGPC

VGPC can increase pH_i dramatically, alkalinizing the cytoplasm up to 100 times faster than other pH regulatory mechanisms such as Na^+/H^+-antiport (33). The regulation of their voltage-dependence by pH ensures that VGPC open only when there is an outward electrochemical gradient for H^+. Because of this property, it seems likely that VGPC evolved to dissipate cytosolic acid. Cells need to regulate pH_i because many enzymes and processes within the cell are sensitive to H^+ concentration, and most metabolic processes generate acid as a byproduct. In most cells, pH_i ranges from 6.8-7.4 (73). At pH_o 7.4, $V_{threshold}$ for H^+ channels is between –4 mV and +20 mV, according to the relationship in Figure 5. Typically, the resting membrane potential is much more negative than this, thus VGPC are unlikely to be open in resting cells. This is of course beneficial, in that the normal electrochemical gradient for H^+ is inward, and an open H^+ channel would acidify the cytoplasm. Cells have many mechanisms to control pH aside from proton channels, such as Na^+/H^+ exchange and Na^+/HCO_3^- symport (73). Most likely, VGPC serve as a safety valve to allow rapid acid extrusion during periods of high metabolic activity. This role has been demonstrated most clearly in phagocytes. In several cells, H^+ channels have been shown to contribute to pH_i recovery after an acid load (1,8,43,74,75).

A speculative function of VGPC may be the regulation of pH near the membrane. Cytoplasmic pH gradients have been generated in myocytes (76), enterocytes (77), and HT29 cells (78) and therefore it is reasonable to suggest that proton concentration near the membrane may differ from that in the bulk cytoplasm. Because H^+ channels are sensitive to local pH, they must respond to any accumulation of protons near the membrane, which could result in H^+ channel activation by the local acidification (4). During large depolarizations, H^+ efflux though channels could increase local pH above the bulk intracellular solution, allowing membrane proteins to function at high pH.

Although H^+ current changes pH, it also changes the membrane potential by moving charge across the membrane. In fact, a given H^+ current changes the membrane potential ~1000 times faster than it changes pH_i (62). As will be discussed, the most crucial function of H^+ channels in phagocytes is charge translocation, not regulation of pH.

5.2. Function of H^+ channels in neuronal cells

A specific role for VGPC was proposed in snail neurons. After the action potential, which results in Ca^{2+} influx in these cells, Ca^{2+} is extruded by a Ca^{2+}/H^+ exchange mechanism, resulting in local concentration of protons near the membrane. VGPC would open as a result of this local proton accumulation, serving to restore pH_i changed by an action potential (10).

Byerly *et al.* (14) suggested that the VGPC might compensate for the intracellular acid load in neurons that occurs during trains of action potentials. Under these conditions, the membrane potential can reach 40 mV (79).

5.3. H^+ channels and CO_2 elimination in the lungs

Alveolar epithelial cells have a high density of VGPC (2) that is exceeded only by eosinophils, basophils, and neutrophils (3,7,25,29,31). As yet, no specific function has been demonstrated. However, it has been hypothesized that proton channels might be involved in the elimination of metabolically produced acid in the form of CO_2 (13). The hypothesis is shown diagrammatically in Figure 7. CO_2 diffuses from the blood into the epithelial cells via the endothelium and the interstitial space. As the CO_2 passes into the epithelial cells across the basolateral membrane, it is converted by carbonic anhydrase into H^+ and HCO_3^-, thereby facilitating diffusion across the cytoplasm. The protons pass through VGPC in the apical membrane and HCO_3^- exits the cell through apical anionic channels or Cl^-/HCO_3^- exchange. This process is electroneutral. The H^+ and HCO_3^- recombine spontaneously to form CO_2, which is expelled into the alveolar gas phase, and H_2O, which is reabsorbed. The traditional assumption is that CO_2 permeates cell membranes freely. However, certain epithelial cells have low CO_2 permeability (80). In addition, the CO_2 permeability in *Xenopus* oocyte membranes is increased after expression of aquaporin-1 channel (81), indicating that the membrane was a significant barrier. One possible advantage of the proposed mechanism of CO_2 extrusion would be strong rectification of CO_2 extrusion.

Although there have been no direct studies to examine the validity of this hypothesis, the necessary elements appear to be present. Indirect evidence suggests that H^+ channels reside on the apical membrane, because H^+ current is seen in membrane patches of alveolar epithelial cells grown on cover slips (26) and such cells appear to orient with the apical side up (26,82-84). The transmembrane potential that exists across the epithelial barrier (85,86) would contribute to selective depolarization of the apical membrane. Furthermore, alveolar epithelium contains carbonic anhydrase (87), the inhibition of which reduces the diffusion of CO_2 (88). In one study (89), perfusion of $ZnCl_2$ through the pulmonary vasculature did not impair CO_2 flux, but it is not clear that sufficient Zn^{2+} reached the apical surface of the alveolar epithelium to inhibit VGPC. Although there are many uncertainties in this hypothesis, the high expression of VGPC in alveolar epithelial cells suggests that they have a specific function. Further experiments are needed to elucidate the functions of these channels.

5.4. Proton channels and NADPH oxidase

Although the proposed functions of VGPC in snail neurons, myotubes, alveolar epithelium, and oocytes are mainly speculative, their main function in phagocytes is well established (12,90,91). Phagocytes (particularly neutrophils and eosinophils) express high levels of the enzyme NADPH oxidase. This enzyme translocates electrons from intracellular nicotinamide adenine dinucleotide phosphate (NADPH) across the cell membrane to reduce extracellular O_2 to superoxide anion (O_2^-).

Superoxide is released into the phagosome and is transformed into hypochlorite (HOCl), which kills bacteria. As illustrated in Figure 8, NADPH oxidase is a multicomponent enzyme comprised of two membrane bound subunits (gp91^{phox} and p22^{phox}) and at least four cytosolic components (p67^{phox}, p47^{phox}, p40^{phox} and Rac1). The membrane-bound part contains two haem groups and binding sites for a flavin group and NADPH, which together form an electron transport chain. Activation of the enzyme entails the migration of the cytosolic component to the membrane (90).

NADPH oxidase is electrogenic (11,12), and electron currents due to NADPH oxidase activity can be measured directly in voltage-clamped cells, either by including NADPH and ATP in intracellular solutions in whole cell configuration (37,92) or by using the permeabilized patch technique (29,30,38). Without a compensating charge mechanism, the membrane potential in eosinophils would depolarize by ~1 kV min^{-1} during peak NADPH oxidase activity. From a resting potential of –60 mV (7) granulocytes would depolarize to +200 mV in ~20 ms. Depolarization to this extent completely inhibits the enzyme (91). It was proposed that VGPC were responsible for the compensation of this charge movement (11,12). Experiments showing that Zn^{2+} or Cd^{2+} could inhibit both proton currents (1,3,19) and superoxide production by phagocytes (11,93-95) supported this proposed role for proton channels. However, there was a discrepancy between concentration of Zn^{2+} needed to inhibit VGPC (1 microM) and the concentration required to inhibit superoxide production (>100 microM).

Recently, we determined the complete voltage dependence of NAPDH oxidase-generated electron current by utilizing permeabilized patch configuration in human eosinophils (91). This technique allows recording currents under voltage-clamp while maintaining intact cytosol and enables the study of NADPH oxidase-generated electron current, I_e (38). We showed that the NADPH oxidase was completely inhibited by depolarization of the cell to >190 mV. The voltage dependence of the enzyme was highly nonlinear and displayed a voltage insensitive region from –100 mV to ~50 mV. This voltage insensitive region explains the discrepancy between the Zn^{2+} efficacy on VGPC compared to NADPH oxidase function. The activity of NADPH measured directly as I_e is insensitive to Zn^{2+} under voltage clamp conditions. Therefore, Zn^{2+} inhibits I_e indirectly by preventing compensating charge movement through H^+ channels. Because Zn^{2+} does not block VGPC but merely shifts the voltage dependence to more positive voltages, Zn^{2+} will inhibit the NADPH oxidase only if it shifts $V_{threshold}$ of H^+ channels into the region where the voltage is inhibitory to the NADPH oxidase. Thus, Zn^{2+} would have to shift $V_{threshold}$ from –20 mV (in activated phagocytes at symmetrical pH (38)) to > +50 mV. Such a shift requires ~300 microM Zn^{2+} (17), thus accounting for the discrepancy between the apparent Zn^{2+} sensitivity of VGPC and NADPH oxidase. The contribution of VGPC to charge compensation of NADPH oxidase was further underscored because the inhibition of superoxide release from granulocytes by Zn^{2+} was partially restored by the H^+ ionophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (91), but not by the K^+ ionophore, valinomycin (12). This showed that proton efflux rather than K^+ efflux was responsible for charge compensation, contradicting a recent suggestion that K^+ efflux compensates for the charge movement (96). This provides strong support for the proposed role of VGPCs in granulocytes.

Further evidence of a close relationship between NADPH oxidase and VGPC came from permeabilized patch studies in which neutrophils and eosinophils were stimulated with arachidonic acid, AA, or phorbol myristate acetate, PMA, to elicit electron current (29,30,38). Activation of the electron current coincides with activation of VGPC (29,38). Agents that activate NADPH oxidase, such as AA and PMA (97,98), also activate VGPC (7,29,36,38). AA is particularly intriguing, because it activates VGPC directly, without intracellular signaling molecules. Thus, in whole cell studies, AA increases $g_{H,max}$, doubles H^+ channel opening and closing rates, and shifts the g_H-V relationship negatively by ~15-20 mV (3,7,31,36). PMA had no effect on VGPC in whole cell conditions, but in permeabilized patch configuration, PMA activated VGPC in a similar manner to AA. PMA, when added to neutrophils or eosinophils, increased I_H, accelerated Tau_{act} and shifted the g_H-V relationship negatively. PMA also slowed Tau_{tail}, an effect that did not occur with AA (3,29,30,38). The slowing of Tau_{tail} by PMA was correlated with the presence of electron current. Diphenyleneiodonium (DPI) inhibits NADPH oxidase activity by binding to the FAD groups and one of the haem groups (99,100). Addition of this inhibitor to an activated cell inhibits electron current. This inhibition did not reverse the shift of the g_H-V relationship, faster Tau_{act}, and larger I_H of activated neutrophils and eosinophils (30,38). However, DPI restored Tau_{tail} to its unstimulated value, showing that the H^+ channel could "sense" the activity of the oxidase. Thus, there is a close functional relationship between NADPH oxidase and VGPC. The similar effects of AA and PMA are consistent with the idea that they share some common signaling pathways. It is possible that AA is the final activator of both NADPH oxidase and VGPC (29,97,101-103), and that PMA increases cytosolic concentration of AA through the indirect action of phospholipase A_2 (104). This may allow them to function in a coordinated fashion (103).

The close relationship of NADPH oxidase and VGPC could be explained by a number of different mechanisms. One proposal is that the H^+ channel is part of the gp91^{phox} subunit of the NADPH oxidase (37,103,105-107). The gp91^{phox} is a member of the NOX family of cytochrome oxidase molecules and it has been postulated that each family member, NOX 1 to NOX 5, also contains a VGPC (108). However, experiments showing the presence and activation of VGPC in cells without gp91^{phox} refute this hypothesis (39,109). Furthermore, in recent experiments, we transfected a fully functional oxidase into COS-7 cells that lack endogenous proton current. Although these cells produced superoxide, we did not detect proton current (40). These direct studies demonstrate that gp91^{phox} is not a

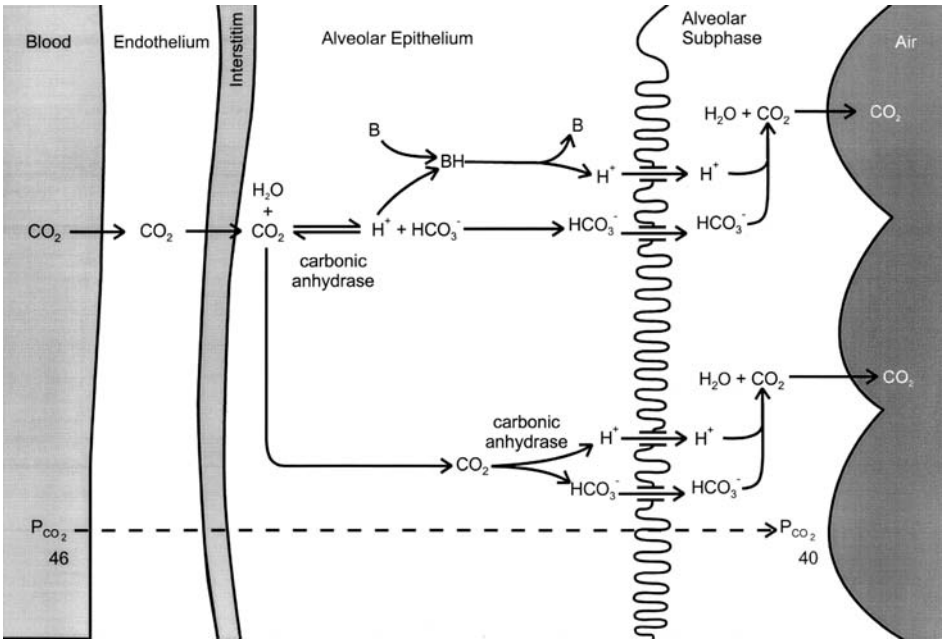

Figure 7. Proposed involvement of VGPC in CO_2 elimination by the lung. CO_2 diffuses passively from the alveolar capillary across the endothelium, the interstitium, and into alveolar epithelial cells. Inside the epithelial cell, CO_2 is converted into H^+ and HCO_3^- by carbonic anhydrase (upper pathway). The H^+ is picked up by buffer, diffuses to the apical membrane, and then enters into the alveolar subphase through VGPC. HCO_3^- diffuses through anion channels into the alveolar subphase, spontaneously recombines with H^+ to form CO_2. CO_2 then diffuses into the gas phase of the lung. In an alternative mechanism (lower pathway), CO_2 diffuses through the cell to the apical membrane before being converted to H^+ and HCO_3^-. Taken from (13).

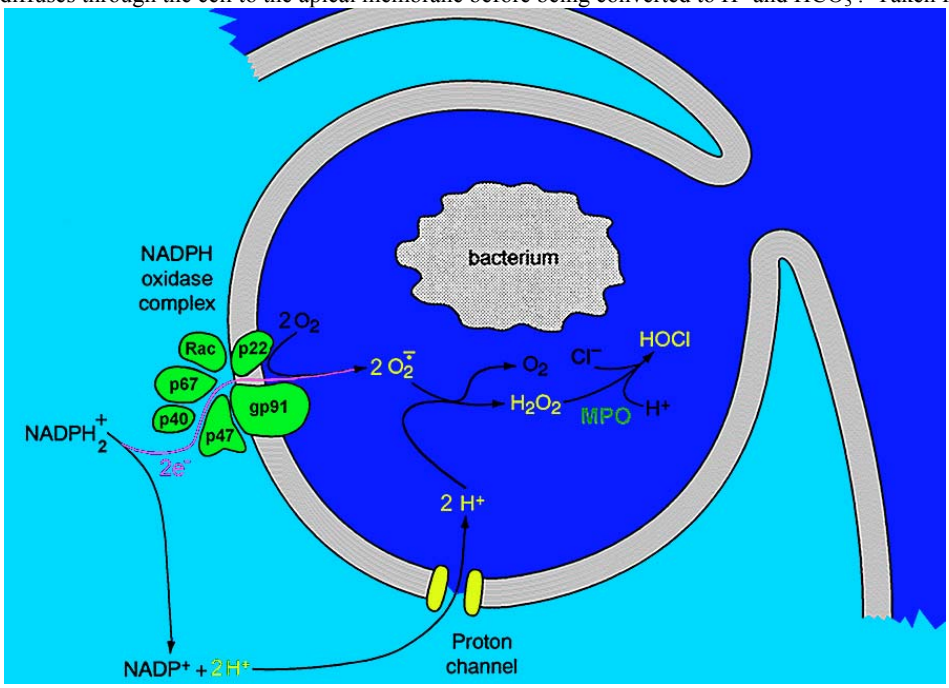

Figure 8. NADPH oxidase and VGPC in phagocytes. The diagram shows the NADPH oxidase complex in the membrane of a phagosome in a phagocyte. The bacterium is in the center of a phagosome, which is closing around it. Two subunits of the enzyme, gp91^{phox} and p22^{phox}, reside in the membrane, whereas the other four subunits (p67^{phox}, p47^{phox}, p40^{phox}, and Rac) reside in the cytosol. When the enzyme is activated, the cytosolic components assemble with the membrane-bound components to form a functional enzyme. NADPH is then oxidized resulting in two electrons that are passed through the enzyme complex to extracellular O_2. This creates superoxide (O_2^-), which dismutates spontaneously to H_2O_2, which in turn is converted to hypochlorite by myeloperoxidase (MPO). These reactive oxygen species, especially HOCl, participate in the killing of bacteria. The enzyme is electrogenic and to compensate for the charge carried by electron movement, protons are passed through VGPC. Taken from (111).

VGPC. In studies that claimed VGPC function, gp91^{phox} was transfected by itself, without any other NADPH oxidase subunits into HEK-293, CHO, and COS-7 cells (105-107,110). Because the activation of VGPC may be related to activation of NADPH oxidase (30,37-39), any function of gp91^{phox} observed when the full oxidase is not present might reflect nonphysiological behavior. The two direct studies that reported patch clamp data from gp91^{phox} transfected CHO cells (107) and HEK-293 cells (110) describe putative H^+ currents that differ greatly from the characteristics of VGPC in phagocytes (38). The currents in CHO cells were much less sensitive to Zn^{2+}, Zn^{2+} did not slow Tau_{act}, no current was seen at pH_i 7.5 even though currents at pH_i 6.9 were larger than in any native cell, and activation was at least an order of magnitude more rapid (107). The putative H^+ currents in transfected COS-7 cells (110) reversed >100 mV positive to the Nernst potential for H^+, showing that they were not H^+ selective.

The simplest explanation for the close relationship of the relationship of NADPH oxidase and VGPC is that they are two separate entities that are activated either in coordination or by the same stimulus. Bánfi *et al.* (37) proposed that there were two types of channel, one in resting cells and a second "novel" variety that is activated when NADPH oxidase is active. The novel VGPC differs from normal VGPC in activating at more negative voltages, having faster Tau_{act}, slower Tau_{tail}, and being more sensitive to Zn^{2+}. We have confirmed most of these changes in the behavior of VGPC when phagocytes are stimulated (38). However, these changes appear to reflect modulation of the properties of the preexistent channels, rather than the appearance of a new type of channel. We have seen no evidence of multiple kinetic components and we observe identical Zn^{2+} sensitivity between VGPC in resting and activated cells (30,38). We conclude that there is one type of H^+ channel that becomes modulated upon stimulation of phagocytes and this channel is responsible for charge compensation of electrons that are passed across the membrane by NADPH oxidase. However, the identity of this proton channel remains unknown.

6. SUMMARY AND FUTURE PROSPECTS

VGPC appear to be designed to extrude acid from cells. The dependence of the g_H-V relationship upon the pH gradient, the activation of H^+ currents positive to V_{rev}, and their very high selectivity make VGPC ideally suited to this function. The properties of the voltage gated proton channels differ in different cells, which may reflect differences in their respective functions. Voltage gated proton channels in snail neurons may help control pH after an action potential due to their rapid activation, whereas in granulocytes, the high expression of proton channels allow a large passage of H^+ suitable for compensating for charge movement due to electron flux. The lack of high affinity ligands for H^+ channels makes their isolation a challenging prospect. Many uncertainties will remain until the molecules are identified. Important questions regarding the specific functions of VGPC in different cell types are yet to be addressed.

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