The Voltage-activated Hydrogen Ion Conductance in Rat Alveolar Epithelial Cells Is Determined by the pH Gradient

VLADIMIR V. CHERNY,* VLADISLAV S. MARKIN,† and THOMAS E. DECOURSEY*

From the *Department of Molecular Biophysics and Physiology, Rush Presbyterian St. Luke’s Medical Center, Chicago, Illinois 60612; †Frunkin Institute of Electrochemistry, Russian Academy of Sciences, Moscow, Russia; and ‡Department of Cell Biology and Neuroscience, University of Texas, Dallas, Texas 75235-9039

ABSTRACT Voltage-activated H⁺ currents were studied in rat alveolar epithelial cells using tight-seal whole-cell voltage clamp recording and highly buffered, EGTA-containing solutions. Under these conditions, the tail current reversal potential, \( V_r \), was close to the Nernst potential, \( E_H \), varying 52 mV/U pH over four \( \Delta pH \) units (\( \Delta pH = pHo - pHi \)). This result indicates that H⁺ channels are extremely selective, \( P_H/P_T > 10^7 \), and that both internal and external pH, \( pHi \) and \( pHo \), were well controlled. The H⁺ current amplitude was practically constant at any fixed \( \Delta pH \), in spite of up to 100-fold symmetrical changes in H⁺ concentration. Thus, the rate-limiting step in H⁺ permeation is pH independent, must be localized to the channel (entry, permeation, or exit), and is not bulk diffusion limitation. The instantaneous current-voltage relationship exhibited distinct outward rectification at symmetrical pH, suggesting asymmetry in the permeation pathway. Sigmoid activation kinetics and biexponential decay of tail currents near threshold potentials indicate that H⁺ channels pass through at least two closed states before opening. The steady state H⁺ conductance, \( g_H \), as well as activation and deactivation kinetic parameters were all shifted along the voltage axis by ~40 mV/U pH by changes in \( pHi \) or \( pHo \), with the exception of the fast component of tail currents which was shifted less if at all. The threshold potential at which H⁺ currents were detectably activated can be described empirically as ~20–40(pHo-pHi) mV. If internal and external protons regulate the voltage dependence of \( g_H \) gating at separate sites, then they must be equally effective. A simpler interpretation is that gating is controlled by the pH gradient, \( \Delta pH \). We propose a simple general model to account for the observed \( \Delta pH \) dependence. Protonation at an externally accessible site stabilizes the closed channel conformation. Deprotonation of this site permits a conformational change resulting in the appearance of a protonation site, possibly the same one, which is accessible via the internal solution. Protonation of the internal site stabilizes the open conformation of the channel. In summary, within
the physiological range of pH, the voltage dependence of \( H^+ \) channel gating depends on \( \Delta p \text{H} \) and not on the absolute pH.

**I**ntroduction

A highly \( H^+ \)-selective conductance, \( g_H \), is activated by membrane depolarization in a number of cells. Described first in snail neurons (Thomas and Meech, 1982), its presence in mammalian cells and evidence suggesting a role in metabolic acid dissipation during phagocytosis in human neutrophils (reviewed by DeCoursey and Cherny, 1994b) have stimulated renewed interest in this conductance. The single-channel conductance based on noise measurements (Byerly and Suen, 1989; DeCoursey and Cherny, 1993; Bernheim, Krause, Baroffio, Hamann, Kaelin, and Bader, 1993) corresponds with a turnover rate in the range of carriers and pumps. We use the term channel because \( H^+ \) is conducted passively down its electrochemical gradient, \( H^+ \) permeation is not coupled with the movement of any other ion, ATP is not required, and the \( g_H \) displays time- and voltage-dependent gating. The mechanism of permeation probably differs significantly from that of other ion channels.

A characteristic feature of voltage-activated \( H^+ \) currents is their dependence on \( p\text{H}_o \) and \( p\text{H}_i \). Increasing \( p\text{H}_o \) or lowering \( p\text{H}_i \) shifts the voltage dependence of activation of the \( g_H \) to more negative potentials. The magnitude of these effects varies substantially among existing studies (DeCoursey and Cherny, 1994b), and the relationship between \( p\text{H}_o \), \( p\text{H}_i \), and the voltage dependence of \( g_H \) activation has not been formally described. A significant source of variability is imperfect control of \( p\text{H}_i \), in particular, and perhaps also \( p\text{H}_o \). It is reasonable to assume that \( V_{\text{rev}} \) reliably indicates the actual pH gradient across the membrane in the vicinity of the \( H^+ \) channel. The reversal potential, \( V_{\text{rev}} \), of \( H^+ \) currents has been found to vary less steeply than the Nernst potential for protons, \( E_H \), in most studies when \( p\text{H}_o \) or \( p\text{H}_i \) were changed (Mahaut-Smith, 1989a; DeCoursey, 1991; Demaurex, Grinstein, Jacconi, Schlegel, Lew, and Krause, 1993; Kapus, Romanek, Rotstein, and Grinstein, 1993; Bernheim et al., 1993; DeCoursey and Cherny, 1994a). In the present study, \( V_{\text{rev}} \) was near \( E_H \) over the range \( p\text{H}_o \), 5.5–8.0 and \( p\text{H}_i \), 5.5–7.5, demonstrating that both \( p\text{H}_o \) and \( p\text{H}_i \) were well controlled. Several factors contributed to this more consistent data. High buffer concentrations (100 mM) were used in all solutions, improving pH control and accelerating recovery of \( p\text{H} \) after pulses (Byerly and Moody, 1986; DeCoursey, 1991; Demaurex et al., 1993; Kapus et al., 1993). We avoided substrates for the \( Na^+-H^+ \) antiporter which indirectly inhibit \( H^+ \) currents (DeCoursey and Cherny, 1994a), as well as TEA+ which alters the behavior of \( H^+ \) currents (Byerly, Meech, and Moody, 1984; Meech and Thomas, 1987; DeCoursey and Cherny, 1994b). Finally, we show here that addition of EGTA to the extracellular solution, even with excess \( Ca^{2+} \) to keep \([Ca^{2+}]_o \) constant, significantly enhances the \( g_H \), with the main effect being a shift of the voltage dependence to more negative potentials, perhaps due to chelation of metal contaminants. We conclude that these conditions allow us to observe the fundamental effects of \( p\text{H}_o \) and \( p\text{H}_i \) on the \( g_H \). We extend the description of \( H^+ \) current behavior to include both activation and deactivation kinetics over a wide range of \( p\text{H}_o \) and \( p\text{H}_i \). The results constrain the possible types of mechanisms involved in \( H^+ \) permeation.

A remarkable pattern which emerged was that for a constant pH gradient, \( \Delta p\text{H} = \)
pH_o-pH_i, most properties of the \( g_H \) were practically independent of the absolute pH, over the range pH_o 6.0-8.0 and pH_i 5.5-7.5. The H^+ current amplitude increased only twofold when the H^+ concentration was increased by 100-fold symmetrically at fixed \( \Delta pH \). Both pH_o and pH_i had similar potency in shifting the H^+ current-voltage relation and the voltage dependence of the kinetics of activation and deactivation of the \( g_H \). The kinetic equivalence of changes in H^+ concentration and membrane potential is reminiscent of "ion well" or "access channel" models for the H^+-ATPase (Mitchell and Moyle, 1974; Läuger, 1991) or the Na^+ /K^+-ATPase (Gadsby, Rakowski, and De Weer, 1993; De Weer, Rakowski, and Gadsby, 1994; Hilgemann, 1994). In contrast with these ATPases in which the ion well is accessible from only one side of the membrane, voltage-activated H^+ channels would need bilateral proton wells to account for the effects of changes in both pH_o and pH_i. The dependence of several gating properties on \( \Delta pH \) led us to suggest a simple physical model in which H^+ channel gating is regulated by a site which may be protonated either from the external or internal solution, but is not accessible to both simultaneously. The accessibility of this site to protons from one or the other side of the membrane is switched by a conformational change in the channel molecule which can occur only when the site is deprotonated. Protonation of this site from the external side of the membrane stabilizes the closed channel conformation, while protonation from the inner side of the membrane stabilizes the open conformation. Calculations using this model reproduce the main features of the results.

**METHODS**

**Cells**

Type II alveolar epithelial cells were isolated from adult rats using enzyme digestion, lectin agglutination, and differential adherence, as described elsewhere (DeCoursey, Jacobs, and Silver, 1988; DeCoursey, 1990). These experiments were undertaken with the purpose of advancing knowledge. The rats were treated humanely, experienced no pain, and were killed under deep anesthesia, in compliance with law and with the NIH Guide for the Care and Use of Laboratory Animals. H^+ currents were studied in cells up to several weeks after isolation. As described previously, the properties of H^+ currents appeared to be independent of time in culture. Approximately spherical cells were selected for study.

**Whole-Cell Recording**

Conventional whole-cell patch clamp technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) was used. Experiments were done at 20°C, with the bath temperature controlled by Peltier devices and monitored continuously by a thin film platinum RTD (resistance temperature detector) element (Omega Engineering, Stamford, CT) immersed in the bath solution. Micropipettes were pulled in several stages using a Flaming Brown automatic pipette puller (Sutter Instruments, San Rafael, CA) from EG-6 glass (Garner Glass Co., Claremont, CA), coated with Sylgard 184 (Dow Corning Corp., Midland, MI), and heat polished to a tip resistance ranging typically between 2–6 MΩ. Electrical contact with the pipette solution was achieved by a thin sintered Ag-AgCl pellet (In Vivo Metric Systems, Healdsburg, CA) attached to a silver wire covered by a Teflon tube. A reference electrode made from a Ag-AgCl pellet was connected to the bath through an agar bridge made with Ringer's solution. The current signal from the patch clamp (List Electronic, Darmstadt, Germany) was recorded and analyzed using.
an Indec Laboratory Data Acquisition and Display System (Indec Corporation, Sunnyvale, CA). Data acquisition and analysis programs were written in BASIC-23 or FORTRAN.

Solutions

Most internal and external solutions were made with 100 mM buffer and 80 mM tetramethylammonium methanesulfonate (TMAMeSO₄) titrated with tetramethylammonium hydroxide (TMAOH). A stock solution of TMAMeSO₄ was made by neutralizing TMAOH with methanesulfonic acid. External solutions also included 3 mM CaCl₂ and 1 mM EGTA, except for experiments described in Fig. 1, in which EGTA was omitted. The pH 6.0 solution included 1 mM EGTA and 2 mM CaCl₂ because EGTA does not buffer Ca²⁺ well at low pH (Martell and Smith, 1974; McGuigan, Lüthi, and Buri, 1991). The intent was to keep [Ca²⁺]₀ constant at ~2 mM in all external solutions. Solutions at pH 5.5, 6.5, and 7.5 included 1 mM EGTA and 2 mM MgCl₂ and were used both in the pipette and externally. No difference was observed between H⁺ currents bathed in 2 mM Ca²⁺ and 2 mM Mg²⁺. Another solution used in many experiments at pH 5.5 included NMG⁺ instead of TMA⁺, and had 3.7 mM EGTA, 0.74 mM CaCl₂, and 119 mM buffer. Buffers (Sigma Chemical Co., St. Louis, MO), which were used near their pK, were: pH 5.5-6.0 MES (2-[N-Morpholino]ethanesulfonic acid), pH 6.5 BIS-TRIS (bis[2-Hydroxyethyl]iminotrismethanomethylene), pH 7.0 BES (N,N-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid), pH 7.5 HEPES (N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]), pH 8.0 TRICINE (N-tris[Hydroxymethyl)methylglycine), pH 9.0 CHES (2-[N-Cyclohexylamino]ethanesulfonic acid).

Seals were formed with Ringer’s solution (160 NaCl, 4.5 KCl, 2 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.4) in the bath, and the zero current potential established after the pipette was in contact with the cell. The combined correction for the liquid junction potentials at the initial pipette/bath interface and subsequent bath/reference electrode interface derived from measured values amounts to 2-3 mV and is nearly identical for each combination of pipette and bath solutions used. Therefore, no junction potential correction has been applied to the data. Raw data are presented without correction for leak current. To quantitate H⁺ current and g_H amplitudes, a usually small linear leak conductance was subtracted based on currents during subthreshold pulses. Under certain extreme conditions, when a cell became leaky or an additional time-independent and possibly nonlinear conductance was present (e.g., at extreme positive potentials in pH 6.0/7.5 in Fig. 3 C), the H⁺ current was defined as the time-dependent component. No other time-dependent conductances were observed consistently under the ionic conditions employed.

Special Experimental Procedures

Cells were placed initially in a bath containing Ringer’s solution. H⁺ currents were detectable in Ringer’s solution, but increased substantially at any given potential upon changing the bath solution to a Na⁺-free solution, due to the removal of indirect inhibition of H⁺ currents by Na⁺-H⁺ antiport (DeCoursey and Cherny, 1994a). Over the next several minutes, the bath solution was changed repeatedly until no further increase in H⁺ current during a test pulse took place. Although the behavior was fairly stable after this point, there was still a tendency as experiments progressed for the H⁺ currents to become larger and to activate faster and at more negative potentials. Byerly et al. (1994) noted that H⁺ current activation in snail neurons also tended to become more rapid during long experiments.

Enhancement of H⁺ Currents by External EGTA

Fig. 1 illustrates that addition of EGTA to external solutions significantly enhanced H⁺ currents in alveolar epithelial cells. Families of H⁺ currents at pH 7.0/6.5 are plotted in a cell bathed in
solutions identical except that in Fig. 1A there was 2 mM CaCl₂ and no EGTA, and in Fig. 1B there was 3 mM CaCl₂ and 1 mM EGTA. Although the free [Ca²⁺]ₐ was about the same, the H⁺ currents are obviously different. The most obvious effect of EGTA was to shift the voltage-activation curve of the gH to more negative potentials by ~15–20 mV. The gH was clearly activated at +20 mV in the EGTA-free solution, but at 0 mV in the EGTA-containing solution. In addition, the time course of H⁺ current activation was faster at any given potential, and appeared somewhat less sigmoid in EGTA-containing solution. Similar effects were observed in ~12 cells studied systematically; and these effects were largely reversible. Because [Ca²⁺]ₐ and pHₑ were the same, the effects of EGTA are attributable either to pharmacological effects directly on H⁺ channels, or to chelation by EGTA of contaminants in our solutions originating in either the water or the chemicals. The effects of several polyvalent cations on H⁺ currents are essentially identical to those of EGTA removal: smaller Iₜ₀ at a given potential, slower activation, and activation at more positive potentials (Byerly et al., 1984; Barish and Baud, 1984; Meech and Thomas, 1987; Byerly and Suen, 1989; Mahaut-Smith, 1989b; DeCoursey, 1991; Demarex et al., 1993; DeCoursey and Cherny, 1993; Kapus et al., 1993; Bernheim et al., 1993). Detectable effects of external Ca²⁺ on H⁺ currents have been reported in myotubes (Bernheim et al., 1993), but not in snail neurons (Byerly and Suen, 1989). We saw little further effect, beyond that described in Fig. 1 for addition of EGTA, of complete removal of [Ca²⁺]ₐ, using a solution with 5 mM EGTA and no added calcium, but which included 2 mM MgCl₂ (data not shown). While this experiment does not rule out small effects of [Ca²⁺]ₐ on H⁺ currents, it shows that small differences in [Ca²⁺]ₐ are not likely responsible for the large effects of EGTA. In subsequent experiments EGTA was included in both internal and external solutions.

Figure 1. Enhancement of H⁺ currents by external EGTA, in a cell at pH 7.0/6.5. Families of currents in a solution containing 2 mM CaCl₂ (A) and 3 mM CaCl₂ and 1 mM EGTA (B), with other components of solutions identical (in millimolar: 100 BES, 80 TMAMESO₃, and TMAOH added to bring the pH to 7.0). Pipette contained pH 6.5 solution (Methods), 20°C, filter 200 Hz, pulses applied from Vhold = -40 mV in +20-mV increments at 36-s intervals.

RESULTS

The gH Is Highly Selective

The selectivity of the gH can be demonstrated by measurement of the reversal potential, Vrev. Examples of tail current records used to determine Vrev will be illustrated in Fig. 9, and are not repeated here. Fig. 2 illustrates that Vrev was very close to the Nernst potential for H⁺, Eₜ₀, over a wide range of pHₑ and pHᵦ, varying >200 mV over a range of pH gradients spanning 4 U. Linear regression indicates a slope of 52.4 mV/U pH (dashed line), close to the Nernst prediction of 58.2 mV/U pH at 20°C (solid line). In most previous studies, including our own studies of alveolar epithelial cells (DeCoursey, 1991), the observed Vrev varied in the same direction as
but typically by only ~40 mV/U pH or less (Mahaut-Smith, 1989a; Demaurex et al., 1993; Kapus et al., 1993; Bernheim et al., 1993). The small deviation of \( V_{\text{rev}} \) from \( E_H \) in the present data could be attributable to: (a) finite permeability to TMA\(^+\), corresponding with a relative permeability \( P_{\text{H}^+}/P_{\text{TMA}^+} > 10^7 \); (b) experimental error; or (c) a small linear \( \text{H}^+ \) leak, resulting in small differences in the actual \( \Delta \text{pH} \) which in the most extreme case at pH 8.0/5.5 was <0.3 U. There is no clear indication in Fig. 2 that better agreement occurred near neutral pHi as had been observed in some earlier studies (Byerly et al., 1984; Demaurex et al., 1993). The present data indicate that under the conditions employed, (a) the \( g_H \) is extremely selective for \( \text{H}^+ \); (b) \( \text{H}^+ \) efflux during the prepulse of tail current measurements did not in itself significantly alter pHi; and (c) over a wide range there were no significant deviations from the nominal pH of either external or internal solutions.

\[ V_{\text{rev}} \text{ of H}^+ \text{ currents measured at a range of pH}_0 \text{ in cells perfused with pH}_i 7.5 (\triangle), 6.5 (\square), \text{ and } 5.5 (\bigcirc). \]

(a) \( E_H \) calculated by the Nernst equation (slope 58.2 mV/U \( \Delta \text{pH} \)); (dashed line) linear regression on the points, with a slope of 52.4 mV/U \( \Delta \text{pH} \). Mean \( \pm 1 \) SD is plotted for 2-18 determinations (total = 83) at each of the 12 different pH\(_0/\text{pH}_i \) (two and three points are plotted at \( \Delta \text{pH} = -0.5 \text{ and } 0.5 \), respectively, but cannot be distinguished); in most cases the SD is within the point size. When multiple measurements were made at the same pH in the same cell they were averaged and included as a single datum in the mean value plotted here.

The Main Effects of pH\(_i\) and pH\(_0\) Are Shifts in the Voltage Dependence of H\(^+\) Currents

H\(^+\) currents appeared qualitatively similar over a wide range of pH\(_0\) and pH\(_i\) as evident in Fig. 3. Only outward currents can be seen because the voltage-dependent activation process is shifted by pH\(_0\) or pH\(_i\) such that activation occurs only positive to \( E_H \). The effects of pH\(_i\) and pH\(_0\) over the range explored in this figure are of approximately equivalent intensity. The threshold voltage was shifted by 40-50 mV/U pH\(_0\) (comparisons within rows), and was shifted similarly but in the opposite direction when pH\(_i\) was varied (comparisons within columns). Another way of viewing this phenomenon is to say that at a fixed pH gradient, \( \Delta \text{pH} \), the voltage-activation relationship is essentially constant. This can be seen by comparing diagonal families in Fig. 3, B with F; A, E, and I; D with H.
At all pHo and pHi studied, H⁺ currents increased slowly during depolarizing pulses, and H⁺ current activation was faster at more positive potentials. Two general trends apparent in Fig. 3 indicate that H⁺ channel gating kinetics do not shift precisely with ΔpH. Activation appears to become progressively faster as pHi is decreased. Within a given cell activation also appears faster as pHo is increased. These trends were consistently observed. All of the described effects of pHo were reversible.

![Graphs showing pH dependence of H⁺ currents](image)

**Figure 3.** Families of H⁺ currents at pHo 8, 7, and 6 in three cells with pHi 7.5 (A–C), 6.5 (D–F), and 5.5 (G–I). To facilitate comparison, the currents have been scaled according to input capacity, 30, 51, and 86 pF, respectively. All current families were obtained by applying 4-s voltage pulses in +20-mV increments at 36-s intervals. The holding potentials were set negative to the threshold for activating the gH under the specific conditions of each measurement, and were −20 mV (B, C, F), −40 mV (A, E, I), −60 mV (H), or −100 mV (D, G). The nearly time-independent component of outward current at pH 6.0/7.5 (C) was also seen in some but not all other cells studied under this condition and is thus unrelated to the voltage-gated and time-dependent gH. In each case the lowpass filter was at 200 Hz, data points were collected at 10-ms intervals.

Over the duration of individual experiments (typically up to 1–2 h), H⁺ currents at a given potential often gradually became larger and activated faster. For example, the currents at pHo 8 and 7 in the cell in Fig. 3, G and H, were recorded late in the experiment, whereas H⁺ currents from an earlier run at pHo 7 (data not shown) were smaller and more closely resembled the currents at pHo 6 (Fig. 3 I), also recorded
early in the experiment. We therefore placed more emphasis on measurements taken temporally close to each other in a given experiment, and periodically returned to the same solution to monitor any slow changes in the $g_H$.

**The Voltage Dependence of $H^+$ Current Activation Depends on pH$_i$ and pH$_o$ but the Effect of pH$_o$ Saturates**

Average values of the $H^+$ current, $I_{H}$, at the end of pulses like those in Fig. 3 are plotted for a number of cells in Fig. 4. At any given pH$_o$ the average $I_{H}$-$V$ relationship was shifted by $\sim 40$ mV/U pH in cells studied at pH$_i$ 5.5, 6.5, or 7.5 (Fig. 4, A-C). The $I_{H}$-$V$ relationship appears to become less steep at pH$_i$ 7.5 in each case. However, the significance of this result is complicated by the fact that the driving force ($V$-$V_{rev}$) is smallest at high pH$_i$ because $V_{rev}$ is very near the threshold for activation of H$^+$ currents, whereas at lower pH$_i$ there is a larger separation between $V_{rev}$ and threshold.

Fig. 4 D illustrates average $I_{H}$-$V$ relationships from cells studied at pH$_i$ 7.5 and four different pH$_o$. Between pH$_o$ 6 and 8, the $I_{H}$-$V$ relationships shifted essentially identically as when pH$_i$ was varied, namely by $\sim 40$ mV/U pH. The shift between pH$_o$ 8 and 9 was distinctly smaller. This result may indicate the approach of pH$_o$ to the pKa of a protonation site which regulates the voltage dependence of $g_H$ gating (see Theoretical section).

The data at different pH$_o$ and pH$_i$ are superimposed in Fig. 4 E to illustrate that at any given $\Delta$pH the $I_{H}$-$V$ relationship was nearly identical. Within the scatter of the data, the voltage dependence of $H^+$ current activation appears to depend only on $\Delta$pH. In other words, pH$_o$ and pH$_i$ are equally effective in regulating the voltage dependence of $g_H$ activation. Comparing the effects of pH$_i$ at a fixed $\Delta$pH, rather than at a constant pH$_o$ as has been done in previous studies, avoids complications due to the varying driving force. Although the H$^+$ currents at pH$_i$ 7.5 ($\Delta$) for large depolarizations tended to be smaller than at lower pH$_i$, the largest difference at any potential and any $\Delta$pH was < twofold, and in no case was the difference significant ($P > 0.05$). Considering that the concentration of H$^+$ is 100 times smaller at pH$_i$ 7.5 than 5.5, the H$^+$ current amplitudes were remarkably similar.

**Instantaneous Current-Voltage Relationship**

Although single H$^+$ channel currents are too small to have been observed directly, open channel rectification can be explored by measuring the instantaneous current-voltage ($I_o$-$V$) relationship. As illustrated in Fig. 5 A, a prepulse which activated the $g_H$ was followed by a test pulses to various potentials. At negative potentials the "tail" current decays essentially completely whereas at large positive test potentials further activation occurs during the test pulse. Because the same number of H$^+$ channels is open at the end of each identical prepulse, the instantaneous current, $I_0$, measured at the beginning of the test pulse reflects the ability of these channels to conduct current at that potential. The $I_0$-$V$ relationship in a cell studied in symmetrical pH 6.5/6.5 is plotted in Fig. 5 B. A simple resistor would produce an ohmic, or linear, $I_0$-$V$ relationship, as would be predicted by the GHK current equation. Instead, there was distinct outward rectification, such that the slope conductance for large outward currents was about twice that for inward currents. Moderate outward rectification was
FIGURE 4. The effects of $pHi$ and $pHo$ on the $H^+$ current, $I_{H}$, (mean ± SEM) measured at the end of 4-s pulses in families of pulses like those in Fig. 3. In each part, $pHi$ is indicated by symbol shape, $pHi$, 5.5 = ●, $pHi$, 6.5 = ■, and $pHi$, 7.5 = ▲. Note that although the voltage spans the same 160-mV range in each part A–D, the absolute potentials are different. Effect of $pHi$ on $H^+$ currents is compared in cells with $pHo$, 8.0 (A), $pHo$, 7.0 (B), and $pHo$, 6.0 (C). Numbers of cells (n) for A: pH 8/5.5, n = 4–6, pH 8/6.5, n = 5, pH 8/7.5, n = 9, for B: pH 7/5.5, n = 5–10, pH 7/6.5, n = 6–9, pH 7/7.5, n = 5–16, for C: pH 6/5.5, n = 3–5, pH 6/6.5, n = 3–5, pH 6/7.5, n = 5–6. At pH 6.0/7.5 only two cells (one of which is illustrated in Fig. 3 C) survived pulses up to +140 mV, thus this extreme voltage range is not represented in the mean data. (D) Effect of $pHo$ on average $I_{H}$ for cells studied with $pHi$, 7.5, labeled according to $pHo$, with n = 5–6 at $pHo$, 9, and other $pHo$ data as plotted in A–C. Note that the shift between $pHo$, 8 and 9 is smaller than at lower $pHo$. (E) The data from parts A–C are superimposed on the same graph to show the similarity of $I_{H}$ at each $\Delta pH$. No significant difference exists between $I_{H}$ data at any $\Delta pH$ and voltage ($P > 0.05$).

observed in this and other cells studied at several $\Delta pH$ from −0.5 to 1.5 (at $\Delta pH > 1.5$ the amplitude of inward tail currents could not be reliably determined). Rectification appeared to be more pronounced at larger $\Delta pH$. 

The $G_{H}$–V Relationship

The chord conductance, $g_{H}$, at the end of depolarizing pulses was calculated using $V_{rev}$ values measured in each solution in each cell. This procedure corrects the $I_{H}$ data for variations in the driving force for $H^+$ currents. In Fig. 6, the average specific conductance, $G_{H}$, normalized to the input capacity in each cell is plotted. Over the
range of pH studied, $G_H$ approached similar values at large depolarizations above $V_{rev}$. Remarkably, $G_H$ was practically the same at any $\Delta pH$ regardless of absolute pH. As for $I_H$, the $G_H$ values for large depolarizations were slightly smaller at pH 7.5 than at lower pHo, however this difference was never more than a factor of two, and was not statistically significant ($P > 0.05$). The position of the $G_H-V$ relationship on the voltage axis evidently is determined by $\Delta pH$ just as was the $I_H-V$ relationship, shifting $\sim 40$ mV/U $\Delta pH$ whether $pH_o$ or $pH_i$ was changed. If $pH_o$ and $pH_i$ exert independent regulatory control via separate protonation sites, then these sites must be considered equally effective in setting the position of the $G_H-V$ relationship.

The voltage dependence of ion channel gating in general can be evaluated from the steady state conductance-voltage relationship. The conductance will be propo-
lack of convincing saturation) were consistently observed. Furthermore, different parameters were obtained from the fits if more or fewer data points were included. The fit of $g_H-V$ data to a Hodgkin-Huxley parameter raised to an exponent (2 or 4) was statistically better (lower Hamilton's R) but exhibited the same consistent deviation.

Correcting the data for instantaneous rectification only slightly improved the fit to Eq. 1 (Fig. 7 B). Each $I_H$ value was divided by $I_0$ measured at the same potential. Thus evaluated in the same solutions and cells, the $g_H-V$ relationship was steepened slightly, with $V_{\text{slope}} \sim 1$ mV smaller, and the midpoints, $V_{1/2}$, a few mV more negative.

![Graph](image)

**Figure 6.** Average chord conductance $G_H$ (normalized by dividing $g_H$ by the input capacity) for a range of $pH_o$ and $pH_i$. Mean ± SEM is plotted with numbers of experiments given in Fig. 4, with the same meaning for symbol shape, $pH_i$ 5.5 = ◆, $pH_i$ 6.5 = □, and $pH_i$ 7.5 = △. Similar shading of symbols indicates the same $\Delta pH$, which is indicated on the figure near each set of symbols. Only two pairs of data points are significantly different at $P < 0.05$; ◆ < □ at $-40$ mV and ◆ < ■ at $+20$ mV.

Several other complications ($H^+$ depletion/accumulation phenomena, slow gating kinetics) limit the quantitative usefulness of $g_H-V$ data (discussed in DeCoursey and Cherny, 1994a, b). Nevertheless, the slope of the $P_{\text{open}}-V$ relationship is likely closer to values obtained by this procedure. We did not observe any consistent pattern of effects of pH on $V_{\text{slope}}$, but the arbitrariness of the fit weakens this result.

**Activation Kinetics: Maximum Rate-of-Rise of $H^+$ Currents ($I_{H}$)**

The time course of activation, or turn on, of $H^+$ currents in most mammalian cells is distinctly sigmoid (DeCoursey and Cherny, 1994b). In human neutrophils a Hodgkin-
Huxley parameter raised to the 1.5–2.0 power gave a reasonable fit to the rising phase of H+ currents (DeCoursey and Cherny, 1993). In alveolar epithelial cells, the time course of H+ currents could not be fitted with a gating parameter raised to any fixed exponent. The optimal exponent in a given cell varied from ~1.1 to 2.0 at various potentials, or when pHo was varied. For this and other reasons, we used the maximum rate-of-rise of H+ currents, Ih, as an indicator of activation kinetics. Fig. 8A illustrates the average Ih-V relationship at four pHo in cells perfused with pH 7.5 solution. Over the range pHo 6–8 Ih was shifted by ~45 mV/U pHo, slightly larger than the shifts of IH-V and gH-V. There was a smaller shift between pHo 8 and 9 of ~20 mV. This result is analogous to the saturation of the effect of pHo on IH-V relationships in Fig. 4D. The Ih parameter is a mixture of activation kinetics, driving force (V-Vr~), and open probability. When pHo was changed from 8 to 9, Eh was shifted much more than was the voltage dependence of activation. (Vr~ could not be accurately estimated in experiments at pH 9.0/7.5 because inward tail currents were not convincingly resolved, but must have been more negative than ~60 mV, at which potential tail currents in three cells were still detectably outward.) The substantially increased driving force at pHo 9 for a given level of activation results in a vertical as well as a horizontal shift of the Ih-V relationship.
Voltage Dependence of $H^+$ Current Activation Is Set by $\Delta pH$

Figure 8. (A) Dependence of the maximum rate-of-rise of $H^+$ currents, $I_{H1}$, on $pH_o$ (labels on the graph) in cells studied with $pHi$, 7.5. The values were normalized according to input capacity of each cell and then averaged. Numbers of experiments are given in Fig. 4 D.

(B) Dependence of maximum rate-of-rise of $H^+$ currents, $I_{H1}$, on $\Delta pH$. Symbol shape indicates $pHi$ as in other figures, $pH_i$, 5.5 = ◊, $pH_i$, 6.5 = □, and $pH_i$, 7.5 = △, shading indicates $\Delta pH$ as labeled near each set of data points. Mean ± SEM are plotted for the numbers of experiments in Fig. 4. The symbols plotted near the abscissae show the mean $V_{rev}$ ± SD measured in the same cells for which $I_{H1}$ data are plotted, using matching symbols. Note that $V_{rev}$ is near the threshold for activation of $H^+$ currents at $\Delta pH = -1.5$ ($pH_6.0//7.5$) and becomes progressively more negative than threshold as $\Delta pH$ increases. For $\Delta pH = 2.5$ ($pH_8.0//5.5$) $V_{rev}$ was $-128 ± 3.2$ mV ($n = 3$) and is not plotted.
Average $I_{Hi}-V$ relationships at pH$_o$ 6.0–8.0 and pH$_i$ 5.5–7.5 are plotted in Fig. 8 B. The most striking result is that at any given $\Delta$pH (indicated on the figure), $I_{Hi}$ was essentially identical, that is, independent of the absolute pH. The magnitude of the voltage shift with $\Delta$pH appears to be 45–50 mV/U $\Delta$pH, slightly larger than the 40 mV observed for $I_H$ and $g_H$, but part of this difference can be ascribed to progressive changes in driving force, as discussed for Fig. 8 A. The mean $V_{rev}$ measured in those cells for which data are plotted in Fig. 8 B are indicated near the abscissae. Because $V_{rev}$ changed by $\sim$52 mV/U $\Delta$pH (Fig. 2), whereas the voltage dependence of $g_H$ activation shifted by only $\sim$40 mV, the driving force for a given level of activation increased progressively as $\Delta$pH was increased. Thus, $V_{rev}$ was nearly at the threshold for activating H$^+$ currents at pH 6.0/7.5, but was $\sim$50 mV negative to threshold at pH 8.0/5.5. Due in part to this difference, at high $\Delta$pH the values of $I_{Hi}$ are larger for a given level of activation of the $g_H$, and thus there is a tendency for the $I_{Hi}-V$ curves to shift upward as well as to more negative potentials as $\Delta$pH is increased. This situation makes it difficult to make quantitative comparisons of the effects of either pH$_o$ or pH$_i$ individually, but do not affect comparisons when both are changed symmetrically, i.e. when $\Delta$pH is held constant. As with the $G_H-V$ relationships, at fixed $\Delta$pH there was a only a small reduction of $I_{Hi}$ when the H$^+$ concentration was reduced symmetrically by 100-fold. The similarity of $I_{Hi}$ at any fixed $\Delta$pH at various absolute pH is more striking than are any subtle differences.

**Deactivation Kinetics**

After H$^+$ current has been elicited by a depolarizing pulse, repolarization results in a tail current, which decays with a time course reflecting the rate that H$^+$ channels close at that potential. Examples of tail currents at pH$_o$ 7.5, 6.5, and 5.5 in a cell perfused with pH$_i$ 6.5 are illustrated in Fig. 9 A–C. Tail currents after depolarizing prepulses usually decayed exponentially at voltages around and more negative than $V_{rev}$. Like nearly all voltage-gated ion channels, H$^+$ channels close more rapidly at more negative potentials. At more positive voltages approaching the threshold for activating H$^+$ currents, a slower component of current decay became obvious. The presence of two distinct kinetic components in the tail currents was most obvious at a high $\Delta$pH, for example at pH 7.5/6.5 in Fig. 9 A, and was less pronounced at low $\Delta$pH, as at pH 5.5/6.5 in Fig. 9 C; however, the decay was still biexponential. This pattern suggests that the two kinetic components may not shift equally with changes in $\Delta$pH.

Tail currents were fitted with a single exponential function and the time constant of decay, $\tau_{tail}$, plotted at several pH$_o$ and pH$_i$ in Fig. 9 D. The average $\tau_{tail}-V$ relationship was approximately linear on semilog axes, consistent with an exponential voltage dependence, especially at large negative potentials. At $\Delta$pH = 1.5 the averaged $\tau_{tail}$ changed e-fold in 50–55 mV. This slope is flatter than observed in many individual cells, $\sim$35–40 mV/e-fold change, perhaps due in part to smearing of the data by averaging. At more positive potentials, near threshold potentials for activating the $g_H$, $\tau_{tail}$ appeared to become more steeply voltage dependent. However, this voltage range coincides with the appearance of the slower component of current decay, thus the apparent increase in steepness may reflect contamination by the slower component. Fig. 9 D shows that $\tau_{tail}$ was essentially the same at a given $\Delta$pH.
Figure 9. Tail currents in a cell at pH 6.5, at three different pHo. (A) Tail currents at pH 7.5/6.5. Vhold was -60 mV, a 1.5-s prepulse to +10 mV was followed by 2.5-s test pulses to -100 mV through 0 mV in 10-mV increments. Note that at more positive potentials distinct rapid and slow decay phases are apparent, and that at 0 mV the rapid decay of H+ current is followed by a slower rising phase. (B) Tails at symmetrical pH 6.5/6.5 from Vhold-40 mV, with 2-s prepulses to +70 mV followed by 2-s test pulses to -50 through +30 mV. (C) Tails at pH 5.5/6.5 from Vhold -20 mV with 1.5-s prepulses to +120 mV followed by 2.5-s test pulses to 0 mV through +70 mV.

(D) Voltage dependence of tail current time constants, $\tau_{\text{tail}}$, at three different $\Delta$PH. Tail currents like those in A–C were fitted by a single exponential, and the mean ± SEM is plotted. At the more positive potentials of each data set, when a slower component of decay became evident, the fit was biased toward the faster component, and when the slower component became large data were excluded. Shape of symbols indicates pHi (pHi 5.5 = ○, pH 6.5 = □, and pH 7.5 = Δ), and shading of symbols indicates the $\Delta$PH as indicated on the graph. Data points just above the abscissa indicate mean ± SD $V_{\text{rev}}$ observed in the same cells in which $\tau_{\text{tail}}$ was measured. Numbers of experiments for $\Delta$PH = 1.5 (pH 7.0/5.5, n = 4–10; pH 8.0/6.5, n = 3), for $\Delta$PH = 0.5 (pH 6.0/5.5, n = 3–5; pH 7.0/6.5, n = 3–9; pH 8.0/7.5, n = 4–6), and for $\Delta$PH = -0.5 (pH 6.0/6.5, n = 4; pH 7.0/7.5, n = 4–8).

(E) Voltage dependence of fast ($\tau_{\text{fast}}$) and slow ($\tau_{\text{slow}}$) components of tail currents in the same cell as in parts A–C, at several pHo as indicated on the figure. Tail currents were fitted by a single exponential at relatively negative potentials, or to the sum of two exponentials at more positive potentials. Separate lines connect $\tau_{\text{fast}}$ (or the single exponential $\tau_{\text{tail}}$ in the more negative range) and $\tau_{\text{slow}}$ for each pHo studied. The slower component was more steeply voltage- and pHo-dependent than $\tau_{\text{fast}}$. Note that the $\tau$ axis spans a wider range than in D.
regardless of the absolute pH. H⁺ current deactivation kinetics thus exhibited ΔpH dependence, as did \( g_{H^+} \), \( I_{H^+} \), and \( I_{H} \). However, the shift of the average \( \tau_{\text{tail}}-V \) relationships with ΔpH was at most only ~20 mV/ΔpH, much less than that of the other parameters.

Tail currents in several experiments were analyzed further by double exponential fits at relatively positive potentials (Fig. 9 E). The time constant of the faster component, \( \tau_{\text{fast}} \), corresponded with \( \tau_{\text{tail}} \) values for the single exponential decay at potentials negative to \( V_{\text{rev}} \) and was only weakly voltage dependent. In contrast, the time constant of the slower component, \( \tau_{\text{slow}} \), was steeply voltage dependent, changing e-fold/8–15 mV, and shifted by ~40 mV/ΔpH. In the experiment illustrated in Fig. 9 E, pH₀ over a wide range did not convincingly alter \( \tau_{\text{fast}} \) at all. In view of this result, we cannot rule out the possibility that the faster component may be completely insensitive to pH₀, and that the small shifts in the averaged \( \tau_{\text{tail}} \) data in Fig. 9 D are due to contamination of the fit by the strongly pH dependent slower component. \( \tau_{\text{slow}} \) ranged up to several seconds, about an order-of-magnitude slower than \( \tau_{\text{fast}} \). The slower component thus appears to be related kinetically to the activation process, in that both are slow and shift ~40 mV/ΔpH₀ when pH₀ is varied.

**THEORETICAL**

**Hypotheses**

It seems reasonable to suggest that the conductance of the open H⁺ channel is independent of the proton concentration at either side of membrane (Fig. 6) and of membrane voltage (ignoring for now the rectification in Fig. 5 B). Then all pH and voltage dependencies should be ascribed to the channel open probability alone. There are closed and open conformations of the channel, and protons outside the cell stabilize the closed state while protons in the cell stabilize the open state. If the ratio of the open-state probability to the closed one were proportional to \( [H^+]_{\text{in}}/[H^+]_{\text{out}} \), the open probability could depend on ΔpH only. One tentative modeling idea that could explain this observation incorporates the following hypotheses: (a) the conductance of the open channel is independent of proton concentration; (b) the transition between open and closed states of the channel is regulated by proton adsorption at either side of membrane to a site associated with the channel; (c) this regulatory site is accessible from only one side of membrane at a time; (d) the regulatory site can change orientation (accessibility) in deprotonated form only; and (e) the orientation of the regulatory site and/or proton adsorption is voltage dependent.

**The "Butterfly" Model**

Let us visualize the voltage-activated proton-regulated H⁺ channel as a multimeric structure consisting of \( j \) protomers, or wings (Fig. 10 A). The wings can move independently of each other and only if they are not protonated. When they are protonated, they are immobilized in the corresponding position. The channel conducts only when all wings are down and protonated (state 4). As follows from the analysis below, we have to consider at least two \( (j = 2) \) protomers or wings that can move up to the horizontal position and down to the vertical position where they can
form a conducting pathway or a pore. Fig. 10, B and C, illustrate alternative depictions of the proposed gating mechanism, all of which are mathematically equivalent. Some properties may be more readily visualized using one picture or another. For convenience, we will describe the model using the image in Fig. 10 A.

The minimal state diagram for each wing includes the four states shown in Fig. 10 with corresponding rate constants. The rates of transitions of a single wing between different states $r_{ik}$ depend on proton concentrations inside and outside the cell and on membrane potential. The transition of a single wing from state 2 to state 1 involves adsorption of $n$ protons, and hence, it is pH dependent. If H$^+$ adsorption occurs at the surface of membrane, it might not be sensitive to membrane potential. But if the adsorption site is immersed in a proton well, then the protons experience the effect of some fraction of the membrane potential. For external protons we...
denote this fraction \( \delta_{\text{out}} \) and for internal protons \( \delta_{\text{in}} \). In this case channel opening becomes voltage dependent.

Another kind of voltage dependence can arise if the wings are charged, i.e., if the transition from state 2 to state 3 involves the movement of effective charge \( m e_0 \). In a general case, both proton wells and charged wings can contribute to the voltage dependence of the proton channel.

Predicting steady state behavior demands fewer assumptions from the model than does kinetics, therefore, we first present the steady state dependencies and only afterwards address the kinetic data. The equations describing this model are derived in the Appendix and their solution provides the steady state conductance. In the general case, even the steady state conductance is a rather involved function but by making reasonable assumptions described in the Appendix, this conductance reduces to the form of a Boltzmann function of potential:

\[
\frac{g_H}{g_{H,\text{max}}} = \frac{1}{1 + \exp \left(\frac{(V_{1/2} - V)}{V_{\text{slope}}}\right)}
\]

with half-activation potential:

\[
V_{1/2} = \frac{k_B T \left(0.881 + \ln K_w + 2.3 n (\Delta p K - \Delta p H)\right)}{e_0 [m + n (\delta_{\text{in}} + \delta_{\text{out}})]}
\]

and slope factor:

\[
V_{\text{slope}} = \frac{k_B T}{1.17 e_0 [m + n (\delta_{\text{in}} + \delta_{\text{out}})]}
\]

where \( k_B \) is Boltzmann's constant, \( T \) is the absolute temperature, and \( e_0 \) is the elementary charge. As one can see from Eq. 2, in this approximation the conductance depends only on \( \Delta p H \) and does not depend on the magnitude of \( \text{pH} \) at each side. When \( \Delta p H \) is changed the half activation potential shifts as:

\[
\Delta V_{1/2} = \frac{-2.3 n k_B T}{e_0 [m + n (\delta_{\text{in}} + \delta_{\text{out}})]} \Delta p H = -2.7 n V_{\text{slope}} \Delta p H
\]

Kinetic processes in this model are described by more complicated equations and in practice should be found numerically, if the parameters of the model are known.

**Parameters of the Model**

Comparing the steady state predictions of the theory with experimental observations, we can find the most important parameters of the model: \( m, n, \delta_{\text{in}}, \) and \( \delta_{\text{out}} \). To a first approximation, the experimental dependence of the \( g_H \) on potential (Fig. 7 B) can be described by a Boltzmannian with a slope factor \( V_{\text{slope}} = 10 \text{ mV} \). When \( \Delta p H \) is changed the \( g_H - V \) curve shifts \(~-40 \text{ mV}/\text{U pH}\). We can conclude from Eq. 4 that \( n = 1.5 \), so that the whole complex is regulated by the adsorption of three protons.

From Eq. 3 we find that:

\[
m + n (\delta_{\text{in}} + \delta_{\text{out}}) = 2.14.
\]

There are two possible sources of voltage dependence of channel opening: displace-
ment of the wing charge \( m e_0 \) accompanying wing movement (the transition between states 2 and 3) and/or proton adsorption in wells where the protons "feel" some fraction of the membrane potential. As one can see from the preceding equations, both charges enter the equations in a similar manner and in that sense they are almost indistinguishable in steady state measurements. Nevertheless, we can find characteristic parameters for both cases.

**Figure 11.** (A) Steady state \( g_H \) calculated using the model parameters as in Fig. 10, for conditions like those in Fig. 6. (Solid curves) pH 5.5; (dotted curves) pH 6.5; and (dashed curves) pH 7.5 curves are labeled with \( \Delta pH \). Note that these are true steady state \( g_H \), whereas the data were sampled at the end of 4-s pulses, which in many cases clearly was before steady state level had been achieved (see text for further discussion). Assumed \( g_{H,\text{max}} \) was 140 pS/pF.

(B) Simulated \( I_H' \) relationships calculated for conditions like those in Fig. 8 B, with curves coded as in A. Note that pH 9.0/7.5 is plotted here but not in Fig. 8 B, to show the saturation of the effect of the external protonation site at high pH, as was illustrated in Fig. 8 A. The calculated \( I_H' \) is in arbitrary units.

*Charged wings.* Suppose that protons adsorb at the membrane surfaces and \( \delta_{\text{in}} = \delta_{\text{out}} = 0 \). Then the whole voltage dependence will originate from the movement of the wings and from Eq. 5, \( m = 2.14 \), i.e., the effective charge of a wing is 2.14 \( e_0 \). In this case the model successfully reproduces experimental observations on \( V_{\text{slope}} \) and on the shift of the \( g_H-V \) curve with changes in \( \Delta pH \). Over a broad range of pH the conductance depends on \( \Delta pH \) only rather than an absolute value of pH. When pH approaches the pK of the regulatory site, the conductance is described by the general
formulae from the Appendix and the ΔpH-dependent voltage shift displays saturation at high pHo.

Proton wells. Suppose that the wings do not carry charge, m = 0, and the whole voltage dependence originates from proton adsorption in wells inside the membrane potential field. In that case δin + δout = 1.42, or, if the inner and outer wells are similar, δin = δout = 0.71. The calculated dependence of gH-V on pH is shown in Fig. 11A, which resembles the data in Fig. 6, as well as the results for the charged-wings model. Both versions of the model predict similar ΔpH dependence of the steady state conductance. The only difference is that at saturation (pHo > 9) the slope of the gH-V curve becomes two times shallower in the proton well version.

Kinetics. Having found these basic parameters we addressed the kinetic processes of channel opening and closing. We found it impossible to reach a reasonable agreement between theory and experiment if the wings were supposed to be identical. We tried to make the difference between wings as small as possible. Therefore, we kept all the equilibrium constants the same, and the only differences we introduced were different rates of protonation and deprotonation of the two wings at the inner side of membrane. All the other parameters of the wings are the same. Because the agreement between theory and experiment happened to be better in the proton well version of the butterfly model, this was used to generate the simulations shown here. The calculated time-courses of H+ currents at different potentials, pHo, and pHi in Fig. 12 reproduce all of the major features of the real data in Fig. 3. The calculated Ih-V relationships in Fig. 11B are similar at any given ΔpH, as were the
experimental relationships in Fig. 8B. The calculated $I'_H$ at pH 7.5 diverges increasingly from calculated values for large depolarizations at the same ΔpH but lower absolute pH, with the divergence becoming marked at pHo 9 (left-most dashed curve). The smaller shift between pHo 8 and 9 is also evident in this figure (two left-most dashed curves), which corresponds with the data in Fig. 8A, and reflects the approach of pHo to pKout. Finally, tail currents generated with the model (Fig. 13) reproduce the main features of the tail current data in Fig. 9. All of these curves were calculated with the same set of parameters (Fig. 10). We believe that the model is able to account for the fundamental properties of both steady state and kinetic observations.

**DISCUSSION**

We have characterized the pHo and pHi dependence of H+ current gating over a wide ΔpH range. By using high buffer concentrations and extracellular EGTA, we observed behavior more consistent than in most previous studies. The close correlation of measured $V_{rev}$ and $E_H$ indicates a very high H+ selectivity ($P_{TMA}/P_H < 10^{-7}$). In previous studies, $V_{rev}$ generally deviated from $E_H$ in being more positive (DeCoursey, 1991; Demaurex et al., 1993; DeCoursey and Cherny, 1993; Bernheim et al., 1993; Kapus, Romanek, and Grinstein, 1994), consistent with the interpretation that H+ efflux during the prepulse increased pHi. This explanation cannot be applied to the present data, because the small deviation of $V_{rev}$ from $E_H$ appeared to be directly proportional to ΔpH (Fig. 2). Conceivably TMA+ might be weakly permeant, but more likely, the local pH near H+ channels deviates slightly from the bulk pH. Even so, the largest combined deviation of pHo and pHi was 0.3 U at pH 8.0/5.5, indicating that over a wide range the local pH was well controlled and similar to nominal bulk values in both bath and pipette solutions. Thus, measurement of $V_{rev}$ is a good indicator of the actual pH gradient across the membrane.

**The $G_H$ Amplitude is Practically Independent of H+ Concentration**

Remarkably, the amplitude of the H+ conductance was nearly independent of pHi over the range 5.5–7.5. The data are compatible with a small effect of pHi on the amplitude of H+ currents. For large depolarizations both $I_H$ and $G_H$ were up to ~50% smaller at pH 7.5 than at pH 6.5 or 5.5 (Figs. 4 and 6). When one considers that the permeant ion concentration was varied 100-fold bilaterally, this seems a small effect indeed. In previous studies $g_H$ or $g_{H,max}$ was increased by lowering pHi, either by a small amount (Byerly et al., 1984; Mahaut-Smith, 1989a; Demaurex et al., 1993) as in the present results, or substantially (Kapus et al., 1993). However, when direct comparison was made at fixed ΔpH, by means of simultaneous bilateral variation of pH, $I_H$ and $g_H$ were practically constant over a wide range of pH encompassing physiological values. In conclusion, lowering pHi in an intact cell will tend to activate the $g_H$ at any given membrane potential, however, the mechanism primarily responsible is a shift in the voltage dependence of the $g_H-V$ relation.
Is the Single-Channel Conductance pH Independent?

The near constancy of the $g_H$ between pH 5.5–7.5 at constant $\Delta$pH has intriguing implications. Either the single-channel H$^+$ conductance is constant over this pH range, or the number of conducting channels must increase by the same factor that the unitary conductance decreases, i.e., by an order-of-magnitude/U pH if the GHK equation is used to predict the unitary current. The latter possibility might apply if H$^+$ blocked or otherwise reduced the effective number of conducting H$^+$ channels: 99% of the H$^+$ channels that are open at pH 7.5 would have to be blocked or closed at pH 5.5. Such a mechanism appears to require that H$^+$ both activates and inhibits H$^+$ channel opening. Given the approximate $\Delta$pH dependence of most of the behavioral features of H$^+$ currents, and the similarity of the limiting $g_H$ over a wide range of pHo and pHi, a simpler hypothesis is that the number of H$^+$ channels available to open and the single channel H$^+$ conductance are essentially constant over the entire pH range studied. This conclusion suggests that the rate-limiting step in H$^+$ conduction is pH-independent.

The pH Independence of the $g_H$ Is Not Due to Diffusion Limitation

Possible rate-limiting steps in H$^+$ permeation include those considered for ordinary ion channels by Andersen (1983): diffusion through the aqueous phases to and from the channel, channel entry and exit, and channel permeation. Special properties of protons present additional possibilities: buffer deprotonation/protonation reactions, hydrolysis (cf Kasianowicz, Benz, and McLaughlin, 1987), or breaking hydrogen bonds between neutral water molecules (Nagle, 1987). Diffusion of H$^+$ (or H$_3$O$^+$) in bulk solution would occur far too slowly (due to [H$_3$O$^+$] being four to six orders of magnitude lower than [BH]) to support the magnitude of $I_H$ observed (DeCoursey, 1991; DeCoursey and Cherny, 1994b). Therefore, buffer shuttles protons to the vicinity of the membrane (cf Gutknecht and Tosteson, 1973). If diffusion of buffer through the tip of the pipette were rate-limiting (cf Mathias, Cohen, and Oliva, 1990), manifestations would have a relatively slow time course (seconds), $I_H$ would not simply scale down at all potentials, and furthermore the limiting current would depend on tip geometry and not on cell size.

If hydrolysis, the diffusion of buffer to the vicinity of the membrane, or diffusion-limited buffer deprotonation/reprotonation reactions (Eigen, 1964) were rate limiting, the effect would be manifest as saturation of the H$^+$ current at a voltage-independent limit during large pulses (cf Andersen, 1983). This is so because all of these mechanisms place the limitation to the unitary H$^+$ conductance mainly outside the membrane potential field. We observed precisely this phenomenon in alveolar epithelial cells perfused with 5 mM pipette buffer (DeCoursey, 1991). However, in the present experiments with 20-fold higher buffer concentrations, we did not observe current limiting up to as large voltages as the membrane tolerated; the $I_o-V$ relation was superlinear at symmetrical pH up to +140 mV (Fig. 5). Therefore, the $I_H$ amplitude must be determined at the level of the channel and must involve a voltage-dependent process, either permeation itself or a voltage-dependent step in channel entry or exit.
How Does $H^+$ Permeation Work?

It appears likely that $H^+$ permeates all channels, from gramicidin to the $F_0$ component of $H^+$-ATPases, by a two-step (hop-turn) mechanism in which protons hop across a hydrogen-bonded chain, after which the molecules or groups forming the chain must reorient before another proton can enter the chain (Nagle and Morowitz, 1978; Nagle and Tristram-Nagle, 1983). In ice, the hopping of a proton across a hydrogen-bonded chain is very rapid and reorientation is rate determining (Nagle and Tristram-Nagle, 1983). By analogy with $H^+$ conductance in ice, where 36% of the full protonic charge translocation occurs during the turning step and the rest during the hopping step (Scheiner and Nagle, 1983), both steps are likely voltage dependent. Because entry of the turning defect into the hydrogen-bonded chain is initiated by the breaking of hydrogen bonds between neutral water molecules, it is essentially pH independent (Nagle, 1987). A logical conclusion is that the rate-limiting step in $H^+$ permeation may be the entry of the turning defect into the putative hydrogen-bonded chain.

The kinetic competence of hydrogen-bonded chains to conduct protons much faster than the turnover rate of $H^+$-ATPases, $\sim 1,200$ $H^+/s$ (Lauger, 1991), or a 10 fS $H^+$ channel conductance ($10^4$ $H^+/s$ at 150 mV), has been demonstrated by theory, using assumptions favorable to rapid permeation (Nagle and Morowitz, 1978; Schulten and Schulten, 1985), and by experiment. The $H^+$ conductance of the water-filled pore of gramicidin is prodigious, with single-channel $H^+$ currents saturating at 140 pA at $>3$ M HCl (Akeson and Deamer, 1991). The apparently much lower conductance of voltage-activated $H^+$ channels seems incompatible with a water-filled pore, and suggests instead a hydrogen-bonded chain mechanism involving protein side groups, allowing tight control by a gating process and a conductance which saturates at five to six orders of magnitude smaller [$H^+$].

How Large Can $H^+$ Channel Currents Be?

The unitary $H^+$ current through gramicidin channels appears to be diffusion-limited (Decker and Levitt, 1988) and is directly proportional to $[H^+]$ over a wide range from pH 0 to pH 4 (references in DeCoursey and Cherny, 1994b), to pH 7.5 (Krishnamoorthy, 1986) or even pH 8.5 (Gutknecht, 1987). Extrapolation of unitary $H^+$ currents measured at much lower pH in a variety of other $H^+$ permeable channels, on the assumption that the conductance is proportional to $[H^+]$, gives <1 fA at pH 6 and <0.1 fA at pH 7 for a 100 mV driving force (DeCoursey and Cherny, 1994b). The convergence permeability of an $H^+$ channel calculated for conditions used here according to a model which includes a small enhancement by buffer (by a factor of 1.26–1.42) predicts the maximum single-channel $H^+$ current to be limited by diffusion to 21 fA at pH 5.5, 2.3 fA at pH 6.5, and 0.23 fA at pH 7.5 (Nunogaki and Kasai, 1988).

Based on $H^+$ current fluctuation measurements, the unitary $H^+$ channel conductance is <50 fS at pH 5.9 (Byerly and Suen, 1989), $\sim 10$ fS at pH 6.0 (DeCoursey and Cherny, 1993), and <90 fS at pH 5.5 (Bernheim et al., 1993). The signal-to-noise ratio was poor in all of these studies, so the estimates must be considered very approximate. These values are comparable with the equivalent $H^+$ efflux of Na$^+$-$H^+$
antiporters at their maximum turnover rate, 0.5 fA and 1.7 fA, at pH 6.0 in normal and transformed human fibroblasts, respectively (Siczkowski, Davies, and Ng, 1994). Given the apparent pH-independence of the \( g_H \) and the estimate of 10 fS for the unitary conductance of H\(^+\) channels at pH 6, the unitary H\(^+\) current at pH 7.5 is evidently significantly larger than the diffusion-limited predictions above (0.1–0.23 fA). The \( F_0 \) component of H\(^+\)-ATPase also is a proton channel with a unitary conductance independent of pH over the range pH 5.6–8.0 (Althoff, Lill, and Junge, 1989; Wagner, Apley, and Hanke, 1989) and of similar magnitude, 10 fS (Schoenknecht, Junge, Lill, and Engelbrecht, 1986) up to 1 pS (Junge, 1989) depending on assumptions. Several mechanisms which were not included in the above calculations have been discussed in the context of attempts to explain how such large proton fluxes can be sustained by diffusion at physiological pH: local concentration of H\(^+\) by charges on the membrane or channel, a large vestibule, buffering by membrane lipids, hydrolysis, or rapid surface conduction of protons (Haines, 1983; Nachliel and Gutman, 1984; Schulten and Schulten, 1985; Nagle and Dilley, 1986; Prats, Tocanne, and Teissie, 1987; Kasianowicz et al., 1987; Althoff et al., 1989; Junge, 1989; Wagner et al., 1989). Electrodiffusion may further enhance the supply of protons (but not of neutral BH) to the channel, increasing the effective pore radius by one Debye length (Peskoff and Bers, 1988).

**THE VOLTAGE DEPENDENCE OF H\(^+\) CHANNEL GATING IS SET BY ΔpH**

\( p_H \) and \( p_H \), Shift \( H^+ \) Current Activation According to ΔpH

A defining characteristic of voltage-activated H\(^+\) channels is that their voltage-activation curve is shifted to more negative potentials by increased \( p_H \) or decreased \( p_H \). The magnitudes of these effects varied in previous studies, and no quantitative relationship had been described. We show here that over the range of \( p_H \) 5.5–7.5 and \( p_H \) 6.0–8.0, \( I_{H-V} \) relationships shifted by ~40 mV/U pH, and were essentially identical at a given ΔpH. In other words, the shift was directly proportional to ΔpH, and \( p_H \) and \( p_H \) were equally effective in shifting the voltage dependence of H\(^+\) channel gating. The effect of \( p_H \) exhibited saturation, such that the shifts of \( I_{H-V} \), \( g_H-V \), or \( I_{H-V} \) between \( p_H \) 8 and 9 were less than at lower pH. This result is consistent with an externally accessible protonation site with a \( pK \) ~8.5 (cf Fig. 11B). The \( g_H-V \) relation in snail neurons was shifted ~46 mV between \( p_H \) 6.4 and 7.4, but no further shift was seen at \( p_H \) 8.4 (Byerly et al., 1984) consistent with a similar mechanism with a lower \( pK \) than in alveolar epithelial H\(^+\) channels. Qualitatively similar saturation of the \( g_H-V \) shift at high \( p_H \) occurs in *Ambystoma* (Barish and Baud, 1984) and murine macrophages (Kapus et al., 1993).

Another ion channel whose voltage-dependent gating depends on permeant ion concentration is the inward rectifier K\(^+\) channel (Hagiwara and Takahashi, 1974). Both activation and deactivation kinetics of some inward rectifier channels shift precisely with \( V-V_K \) when [K\(^+\)]\(_{o}\) is varied (Saigusa and Matsuda, 1988; Silver and DeCoursey, 1990); in other cells the shifts are unequal (Pennefather, Oliva, and Mulrine, 1992). For H\(^+\) channels, \( V_{rev} \) measured under identical conditions in the same cells varied by 52 mV/U ΔpH, a significantly larger shift than that of steady
state gating or kinetic parameters. Thus, H⁺ channel gating cannot be defined strictly as (V-E_H) dependent, but as ΔpH dependent. The magnitude of the shift of various parameters with ΔpH varied. The voltage dependence of I_H, g_H, the rate of activation (I_{th}), and the slow component of deactivation (τ_{slow}) all shifted ~40 mV/ΔpH. In contrast, τ_{fast} was much less sensitive, shifting only ~20 mV/ΔpH or less. I_H appeared to increase with ΔpH in addition to shifting along the voltage axis. To a first approximation, however, the behavior of the g_H was the same at any given ΔpH. Given these general similarities, it is not surprising that our model shares some features of models proposed to account for gating of inward rectifier K⁺ channels (Ciani, Krasne, Miyazaki, and Hagiwara, 1978; Pennefather et al., 1992; Pennefather and DeCoursey, 1994). The equivalence of voltage and pH suggests a parallel with the concept of ion wells or access channels as proposed for the H⁺-ATPase (Mitchell and Moyle, 1974; Läuger, 1991) and the Na⁺/K⁺-ATPase (Gadsby et al., 1993; Hilgemann, 1994; De Weer et al., 1994). In the latter case, the access channel for Na⁺ senses ~70% of the membrane potential (Gadsby et al., 1993). The proton well version of our model incorporates voltage-dependent proton binding in external and internal proton wells, each sensing 71% of the membrane potential.

Practical Consequences: Predicting g_H Activation in Intact Cells

By extrapolating the present results to behavior in intact cells, the following relationship was distilled from the data in order to predict the "threshold" potential at which activation is first detectable:

\[ V_{threshold}(mV) = V_0 - 40ΔpH \]  \hspace{1cm} (6)

where ΔpH is defined as used in this paper: ΔpH = p_{Ho} - p_{Hi}, and V_0 is V_{threshold} at symmetrical pH (i.e., when ΔpH = 0). In many cells V_0 was +20 mV, but sometimes it was +40 mV (we normally used 20-mV voltage increments). There was cell-to-cell variability in V_0, but the shift in any given cell of ~40 mV/U p_{Ho} was quite consistent. Recognizing that no sharply distinguishable threshold potential actually exists, V_{threshold} provides a useful if arbitrary guide. Considering the absence of depolarization-activated inward currents (DeCoursey et al., 1988), the resting potential of intact alveolar epithelial cells probably does not exceed V_{threshold} by much, and the voltage dependence of the g_H is steep enough that within a few mV enough H⁺ channels will be activated to increase pH_i or to repolarize the membrane potential. The relationship in Eq. 6 applies only to the conditions of our study; the pH sensitivity of the g_H may vary in different cell types or may be modulated by cytokines. For example, arachidonic acid enhances the g_H in phagocytes (Henderson and Chappell, 1992) by shifting the voltage-activation curve to more negative potentials (DeCoursey and Cherny, 1993; Kapus et al., 1994).

An intriguing prediction of Eq. 6 is that at a large negative ΔpH steady state inward H⁺ currents should occur. In fact, V_{threshold} was close to V_{rev} at pH 6.0/7.5 (ΔpH = -1.5), where activation of the g_H could best be detected indirectly, by observing the tail current after depolarizing pulses. In an intact cell inward I_H would depolarize the membrane and further activate the g_H like the regenerative activation of Na⁺ channels during action potentials in excitable tissues. It is obviously unlikely that an intact cell could achieve these conditions, so it remains true that over the
physiologically relevant range of pH and membrane potentials H⁺ channels only conduct outward steady state currents.

**PROPERTIES OF H⁺ CHANNELS**

**Instantaneous Rectification**

The $I_0$-$V$ relationship rectified outwardly at symmetrical pH, with a slope conductance about twice as large at positive than at negative potentials (rectification ratio = 2). Rectification was more pronounced at higher $\Delta$pH, in the direction expected from the GHK current equation, e.g., at $\Delta$pH = 1.5 the rectification ratio increased to $\geq 3$. However, the GHK equation predicts that changing $\Delta$pH from 0 to 1.5 ought to increase the rectification ratio from 1.0 to 31.6, and therefore cannot be used to predict rectification of H⁺ currents. Previously, the $I_0$-$V$ relation was described as approximately linear or slightly outwardly rectifying. Because the rectification is fairly gradual, over a narrow voltage range the $I_0$-$V$ relation appears to be approximately linear. For this reason, correcting the $G_{H^+}$-$V$ relation for rectification had only a subtle effect. That H⁺ moves outwardly more readily than inwardly suggests that the H⁺ permeation pathway is asymmetrical.

**H⁺ Channel Gating**

The data provide a number of empirical constraints on the mechanism of H⁺ channel gating. The sigmoid time course of mammalian H⁺ current activation suggests that the channels traverse at least two closed states before opening (DeCoursey and Cherny, 1994b). We could not fit the activation time course with a Hodgkin-Huxley parameter raised to a fixed exponent, which argues against the simplest case of independent conformational changes in two or more identical channel protomers to form a conducting pore. The next most parsimonious scheme is two sequential kinetically distinct transitions:

$$C_1 \leftrightarrow C_2 \overset{\alpha}{\Rightarrow} O.$$ \[\text{slow} \quad \text{fast}\]

H⁺ current deactivation was noted to be faster at more negative potentials in previous studies (Barish and Baud, 1984; DeCoursey, 1991; Kapus et al., 1994), but its voltage and pH dependence was not investigated systematically. Tail currents after depolarizing prepulses decayed exponentially at potentials well negative to $V_{rev}$, but a second, slower component of decay became evident at potentials near threshold, which coincidentally is the most physiologically relevant range. Two components of $I_H$ decay indicate the existence of at least three states. Because $\tau_{fast}$ and $\tau_{slow}$ differed at some potentials by an order of magnitude or more, the two steps evidently have widely different kinetics, again inconsistent with an $n^2$ model in which the $\tau$'s would differ only by a factor of two (Bernasconi, 1976; Zagotta, Hoshi, Dittman, and Aldrich, 1994). At large negative potentials $\tau_{tail}$ reflects mainly $\beta$, because $\alpha$ is likely small when $P_{open} \approx 0$. Single exponential decay with $\tau \approx 1/\beta$ would be expected if $C_1 \leftrightarrow C_2$ transitions are relatively slow. The voltage dependence of the $\tau_{tail}$ in this
region was weak, 40–60 mV/e-fold change in potential, and was approximately exponentially voltage dependent. In contrast, $\tau_{\text{slow}}$ was steeply voltage dependent, changing e-fold in 8–15 mV. These results suggest that the process reflected in $\tau_{\text{slow}}$ includes most of the charge movement of gating.

An intriguing result is that $\tau_{\text{fast}}$ was the least sensitive of any parameter studied to changes in $\Delta p\text{H}$, shifting at most $\sim 20$ mV/U $\Delta p\text{H}$. In light of its shallow voltage dependence and interference by the slower component, it is possible that this step may be completely independent of pH. $\tau_{\text{slow}}$ was as sensitive to $p\text{H}_0$ as were kinetic and equilibrium indicators of activation. Evidently most of the pH dependence of gating resides in the slower transition as well.

The slower component of tail current decay which becomes pronounced near threshold potentials presumably is attributable to the $C_1 \leftrightarrow C_2$ transition. The faster $\tau$ related to the $O \rightarrow C_2$ transition is still present, but because $\alpha$ is now closer to $\beta$ some channels will reopen and thus a slower component will appear as channels gradually disappear via the $C_2 \rightarrow C_1$ path. Given the above simple three-state scheme, the first step, $C_1 \leftrightarrow C_2$, could not be rapid because then its relaxation would be “filtered” through the slower $O \rightarrow C_2$ transition. At equilibrium the $C_1 \rightarrow C_2$ transition must occur to some extent at more negative potentials than the $C_2 \rightarrow O$ transition. Depolarizing prepulses below threshold reduce the sigmoidicity of activation (DeCoursey and Cherny, 1994b), suggesting that a significant fraction of channels can undergo the $C_2 \rightarrow C_1$ transition without opening. As shown in Fig. 9A, at some potentials above threshold, the tail current relaxation has a rapidly decaying component and a slower rising component. In other words, the two relaxations proceed in opposite directions, given the same preconditions. This nonmonotonic relaxation argues that the faster component reflects transitions nearer the open state.

In summary, any realistic gating model for $H^+$ channel activation must have at least two distinct steps (≥ three states). A transition remote from the open state appears to be slower and more steeply voltage dependent than a transition nearer the open state. It is not likely that the simplest three-state model can account for all details of gating kinetics.

**Gating Model**

The proposed model represents a first attempt to demonstrate a mechanism capable of reproducing the main features of $H^+$ channel gating, specifically the dependence of most parameters on $\Delta p\text{H}$, independent of the absolute pH. We kept the assumptions as simple as possible. The voltage dependence of $H^+$ channel gating is modulated by internal and external protons acting at a site which can be alternately accessible to either solution. The $pK$ of the proposed modulatory site was kept the same at the internally and externally accessible configurations ($pK_{\text{in}} = pK_{\text{out}}$), although the $pK$ of the same site could change when the molecule of which it is a part is in a different conformation, like the Schiff base of bacteriorhodopsin (Stoeckenius, Lozier, and Bogomolni, 1979) and as shown by ab initio modeling (Scheiner and Hillenbrand, 1985). The data at this stage do not adequately constrain the model to define the optimum number of wings, or the number of protonation sites per wing. The types of data required for these refinements are being collected. However, the general type of model proposed is feasible. The assumption of alternate access of the
protonation site seemed simpler than proposing distinct protonation sites at either face of the membrane. To account for the large and uniform voltage shifts observed over 2 U pHo and pHi, it would be necessary to postulate several independent protonation sites with a range of pK's at both the outer and inner ends of the channel molecule with internal and external sites having equivalent effectiveness in altering the transmembrane potential sensed by the channel's voltage sensor.

Fig. 10 illustrates three physical representations of the mathematical formulation of the gating mechanism in the model. The postulated gross movement of the wings across the entire membrane (Fig. 10 A) resembles proposed mechanisms of voltage-dependent gating of a variety of small channel-forming molecules: alamethicin (Woolley and Wallace, 1992), monazomycin (Heyer, Muller, and Finkelstein, 1976), melittin (Kempf, Klausner, Weinstein, Renswoude, Pincus, and Blumenthal, 1982), colicin (Raymond, Slatin, Finkelstein, Liu, and Levinthal, 1986), and synthetic channels (Chung, Lear, and DeGrado, 1992), as well as the larger inward rectifier K+ channel (Ciani et al., 1978). Another embodiment of the model (Fig. 10 B) invokes distinct external and internal regulatory sites, the protonation of either allosterically preventing protonation of the other. A subtle conformational change could also be envisioned (Fig. 10 C), e.g., a regulatory protonation site located within bilateral proton wells could by various mechanisms become alternately accessible to the solution on one or the other side of the membrane. Alternating-access mechanisms are considered more plausible for ion pumps than conformational changes requiring large molecular movements (Läuger, 1991). To adequately simulate the data, it was necessary to make the conformational change (between states 2 and 3) more rapid than the protonation/deprotonation steps, consistent with its entailing a small movement. Together with the better fit to the data when the proton well version of the model was assumed (Theoretical section), and the empirical conclusions that most of the voltage dependence and pH dependence of gating reside in a slow step, we conclude that most of the voltage dependence of gating occurs in protonation/deprotonation steps rather than in the conformational change.

That the rapid component of deactivation (τfast) shifted much less with pHo than the other parameters is intriguing in light of our interpretation that τfast represents the first (or an early) closing transition. If the regulatory protonation site were in the conduction pathway (analogous to the pore of a normal ion channel) it might be unable to sense pHo and pHi when the channel is open, because the open channel H+ conductance is essentially pH independent. All other kinetic transitions occur between closed states, i.e., in the absence of H+ efflux, and these parameters shifted uniformly ∼40 mV/U ΔpH. Therefore, it seems possible that the regulatory protonation site(s) may be in the conduction pathway itself.

APPENDIX

The Model and Constitutive Equations

In the section Theoretical we introduced the butterfly model of a proton channel with four states for each wing defined in Fig. 10. We consider here an arbitrary number of wings, j. Because of electrogenic properties of transitions between different states, the rates of these transitions can be presented (Markin and Chizmadzhev, 1974; Markin,
Pastushenko, and Chizmadzhev, 1987) in the following way:

\[ r_{12} = k_{12} \exp \left( \frac{n_e \delta_{\text{out}} V}{2k_BT} \right), \]  
(A.1)

\[ r_{21} = k_{21}[H^+]_{\text{out}} \exp \left( -\frac{n_e \delta_{\text{out}} V}{2k_BT} \right), \]  
(A.2)

\[ r_{23} = k_{23} \exp \left( \frac{meV}{2k_BT} \right), \]  
(A.3)

\[ r_{32} = k_{32} \exp \left( -\frac{meV}{2k_BT} \right), \]  
(A.4)

\[ r_{34} = k_{34}[H^+]_{\text{in}} \exp \left( \frac{n_e \delta_{\text{in}} V}{2k_BT} \right), \]  
(A.5)

\[ r_{43} = k_{43} \exp \left( -\frac{n_e \delta_{\text{in}} V}{2k_BT} \right). \]  
(A.6)

The variation of the probabilities of different states \( P_1, P_2, P_3, \) and \( P_4 \) (for each wing considered alone) are determined by the following equations:

\[ \frac{dP_1}{dt} = -r_{12}P_1 + r_{21}P_2, \]  
(A.7)

\[ \frac{dP_2}{dt} = r_{12}P_1 - (r_{21} + r_{23})P_2 + r_{32}P_3, \]  
(A.8)

\[ \frac{dP_3}{dt} = r_{23}P_2 - (r_{32} + r_{34})P_3 + r_{43}P_4, \]  
(A.9)

\[ P_1 + P_2 + P_3 + P_4 = 1. \]  
(A.10)

Because each of \( j \) protomers can be in four states, the total number of states is \( 4^j \). But the channel is open only if all \( j \) wings are in the fourth state, i.e., they are down and protonated. To permit assigning different \( H^+ \) affinity or kinetic properties for the different wings, we distinguish the corresponding probabilities: \( P_4^{(1)}, P_4^{(2)}, \ldots, P_4^{(j)} \).

The probability of the open state of the channel is the product of corresponding probabilities: \( P_{\text{open}} = P_4^{(1)} P_4^{(2)} \ldots P_4^{(j)} \). If the limiting conductance is \( g_{H\text{,max}} \), then the conductance at a given voltage is

\[ g_{H}(V) = g_{H\text{,max}} P_4^{(1)} P_4^{(2)} \ldots P_4^{(j)}. \]  
(A.11)

Let us introduce also equilibrium proton dissociation constants

\[ K_{\text{out}} = \frac{k_{12}}{k_{21}} \quad \text{and} \quad K_{\text{in}} = \frac{k_{43}}{k_{34}}. \]  
(A.12)
and an equilibrium wing conformation constant

$$K_w = \frac{k_{32}}{k_{23}}.$$  \hfill (A.13)

The latter constant gives the ratio between "up" and "down" (or out and in) conformations of deprotonated wings in the absence of electrical field (i.e., at 0 mV). It may be convenient to use the pK of acid groups inside and outside membrane:

$$pK_{in} = -\frac{1}{n} \log K_{in} \quad \text{and} \quad pK_{out} = -\frac{1}{n} \log K_{out}. \hfill (A.14)$$

We shall use a pH difference in the form

$$\Delta pH = pH_o - pH_i, \hfill (A.15)$$

and the Nernst potential for protons

$$E_H = -\frac{2.3k_B T}{e_0} \Delta pH. \hfill (A.16)$$

**Steady State Regime**

For the beginning let us consider our system in a steady state. Solving the set of Eqs. A.7-A.10 one obtains:

$$P_1 = P_4 K_w \exp \left\{ \frac{2.3n(pH_i - pH_o - pK_{in} + pK_{out})}{k_B T} - \frac{me_0 V}{k_B T} \frac{n \delta_{in}}{k_B T} \right\}, \hfill (A.17)$$

$$P_2 = P_4 K_w \exp \left\{ \frac{2.3n(pH_i - pK_{in})}{k_B T} - \frac{me_0 V}{k_B T} \frac{n \delta_{in}}{k_B T} \right\}, \hfill (A.18)$$

$$P_3 = P_4 \exp \left\{ \frac{2.3n(pH_i - pK_{in})}{k_B T} - \frac{me_0 V}{k_B T} \frac{n \delta_{in}}{k_B T} \right\}, \hfill (A.19)$$

$$P_4 = \left\{ 1 + \exp \left\{ \frac{2.3n(pH_i - pK_{in})}{k_B T} - \frac{me_0 V}{k_B T} \frac{n \delta_{in}}{k_B T} \right\} \right\}^{-1} \left\{ 1 + K_w \exp \left\{ -\frac{me_0 V}{k_B T} \left[ 1 + \exp \left\{ 2.3n(pK_{out} - pH_o) - \frac{ne_0 \delta_{out} V}{k_B T} \right\} \right] \right\} \right\}^{-1}. \hfill (A.20)$$

Let us suppose for the sake of simplicity that equilibrium constants of all wings are the same. Then substituting these formulas into Eq. A.11 one can find conductance of the system:

$$\frac{g_H}{g_{H, max}} = \left\{ 1 + \exp \left\{ \frac{2.3n(pH_i - pK_{in})}{k_B T} - \frac{me_0 V}{k_B T} \frac{n \delta_{in}}{k_B T} \right\} \right\}^{-1} \left\{ 1 + K_w \exp \left\{ -\frac{me_0 V}{k_B T} \left[ 1 + \exp \left\{ 2.3n(pK_{out} - pH_o) - \frac{n e_0 \delta_{out} V}{k_B T} \right\} \right] \right\} \right\}. \hfill (A.21)$$
This equation gives a steady state conductance as the function of membrane voltage and proton concentrations at both sides of membrane. These rather cumbersome expressions drastically simplify in a practically important particular case. Suppose that the $pK$'s of acidic groups are rather high so that proton concentrations at both sides exceed the dissociation constant:

$$pK_{in} > pH_i \quad \text{and} \quad pK_{out} > pH_o.$$  \hspace{1cm} (A.22)

In that case, acidic groups are almost saturated and the expression for probability $P_4$ simplifies to

$$P_4 = \left\{ 1 + \exp \left[ \ln K_u + 2.3n(\Delta pK - \Delta pH) - \frac{me_0 + ne_0(\delta_{in} + \delta_{out})}{k_BT} \right] \right\}^{-1}.$$  \hspace{1cm} (A.23)

This is a Boltzmann function of the type $1/(1 + e^{-bV})$ with parameters

$$a = \ln K_u + 2.3n(\Delta pK - \Delta pH),$$  \hspace{1cm} (A.24)

and

$$b = \frac{me_0 + ne_0(\delta_{in} + \delta_{out})}{k_BT}.$$  \hspace{1cm} (A.25)

Open probability simplifies to

$$\frac{g_H}{g_{H,max}} = P_{open} = \left\{ 1 + \exp \left[ \ln K_u + 2.3n(\Delta pK - \Delta pH) - \frac{me_0 + ne_0(\delta_{in} + \delta_{out})}{k_BT} \right] \right\}^{-j}.$$  \hspace{1cm} (A.26)

This expression is not an exact Boltzmannian, but it is very close to this function, though with different parameters. If the butterfly has only one wing, then $P_{open} = P_4$. With increasing numbers of wings, the open probability shifts to the right and slope steepens. If open probability is presented in the form

$$\frac{g_H}{g_{H,max}} = \frac{1}{1 + \exp \left( \frac{V_{1/2} - V}{V_{slope}} \right)},$$  \hspace{1cm} (A.27)

with the half-activation potential $V_{1/2}$ and slope factor $V_{slope}$, then one can establish the relationship between these factors and $a$ and $b$:

$$V_{1/2} = \frac{a - \ln (2^{1/j} - 1)}{b} \quad \text{and} \quad V_{slope} = \frac{1}{2j(1 - 2^{-1/j})b}.$$  \hspace{1cm} (A.28)

For $j = 1$ these two factors, as expected, are correspondingly $a/b$ and $1/b$.

For $j = 2$ they are $(a + 0.881)/b$ and $1/(1.17b)$.

For $j = 3$ they are $(a + 1.347)/b$ and $1/(1.23b)$.

For $j = 4$ they are $(a + 1.665)/b$ and $1/(1.27b)$.

For $j = 5$ they are $(a + 1.906)/b$ and $1/(1.29b)$ and so on.

Therefore, if the $pK$'s are high enough, the conductance is a Boltzmann function of membrane potential and of $\Delta pH$ and it does not depend on the magnitude of $pH$ at
each side. With variation of $\Delta p$ the half activation potential shifts as

$$\Delta V_{1/2} = \frac{2.3n k_B T}{\epsilon_0[m + n(\delta_{in} + \delta_{out})]} \Delta p.$$

(A.29)

Interestingly enough, this shift does not depend on the number of wings. But the slope of voltage dependence of conductance does depend on the number of wings:

$$V_{slope} = \frac{k_B T}{2j(1 - 2^{-1/j})\epsilon_0[m + n(\delta_{in} + \delta_{out})]}.$$

(A.30)

This gives the opportunity to establish relationship between $\Delta V_{1/2}$ and $V_{slope}$:

$$\Delta V_{1/2} = -4.6j(1 - 2^{-1/j})nV_{slope}\Delta p.$$

(A.31)

Because $\Delta V_{1/2}/\Delta p$ and $V_{slope}$ can be found in experiment, Eq. A.31 permits finding the number of protons per one wing:

$$n = \frac{1}{4.6j(1 - 2^{-1/j})V_{slope}} \frac{\Delta V_{1/2}}{\Delta p}.$$

(A.32)

The numerical coefficient $4.6j(1 - 2^{-1/j})$ in the denominator of this equation has an interesting structure: it does not change very much with the number of wings. At $j = 1$ it is equal to 2.3, at $j = 2$ to 2.70, at $j = 3$ to 2.83, at $j = 4$ to 2.93 and at $j = 5$ to 2.98. Even if $j$ goes to infinity the limiting value of this coefficient is not very large: it is 3.188. Therefore the number of protons adsorbed at one wing can be estimated from experimental data reliably enough even if the number of wings is unknown.

Relatively simple equations derived above were obtained under supposition that pH inside and outside the cell does not exceed the pK of binding sites for protons. In this case both charge of the wing and charge of the protons adsorbed in the wells enter the equations in the same single combination $\epsilon_0[m + n(\delta_{in} + \delta_{out})]$. Therefore in this domain both versions of the butterfly model predict the same potential dependence of the steady state conductance. But in a more general case the steady state conductance can be obtained from general Eq. A21. The difference between proton wells and charged wing versions on the butterfly model manifests itself at saturation: the proton wells version saturates at higher pH and the slope of conductance changes at saturation. Although the behavior of the $g_{H^+} - V$ data at high pH, more closely resembles that of the charged wings model, the kinetics of $H^+$ currents was much better predicted by the proton well version.

Therefore, steady state measurements permit finding the number of protons per wing, the depth of proton wells, and the charge of the wings. But these measurements are rather insensitive to the number of wings in the channel complex and of course they do not give the opportunity to find the rate constants. These parameters can be found in the kinetic measurements.

**Kinetics**

Some general conclusions can be derived from the analysis of the general kinetic equations, but the most detailed information was obtained as a result of numerical
solution of these equations. The results are presented in Figs. 11 B, 12, and 13. It was found impossible to simulate with one wing only.

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