Properties of Single Voltage-gated Proton Channels in Human Eosinophils Estimated by Noise Analysis and by Direct Measurement

Vladimir V. Cherny, Ricardo Murphy, Valerij Sokolov, Richard A. Levis, and Thomas E. DeCoursey

1Department of Molecular Biophysics and Physiology, Rush Presbyterian St. Luke’s Medical Center, Chicago, IL 60612
2Frumkin Institute of Electrochemistry, Moscow 117071, Russia

ABSTRACT. Voltage-gated proton channels were studied under voltage clamp in excised, inside-out patches of human eosinophils, at various pHs with pHs 7.5 or 6.5 pipette solutions. H+ current fluctuations were observed consistently when the membrane was depolarized to voltages that activated H+ current. At pH ≤ 5.5 the variance increased nonmonotonically with depolarization to a maximum near the midpoint of the H+ conductance-voltage relationship, g0V, and then decreased, supporting the idea that the noise is generated by H+ channel gating. Power spectral analysis indicated Lorentzian and 1/f components, both related to H+ currents. Unitary H+ current amplitude was estimated from stationary or quasi-stationary variance, σH2. We analyze σH2 data obtained at various voltages on a linearized plot that provides estimates of both unitary conductance and the number of channels in the patch, without requiring knowledge of open probability. The unitary conductance averaged 38 fA at pH 6.5, and increased nearly fourfold to 140 fA at pH 5.5, but was independent of pHo. In contrast, the macroscopic g0 was only 1.8-fold larger at pH 5.5 than at pH 6.5. The maximum H+ channel open probability during large depolarizations was 0.75 at pH 6.5 and 0.95 at pH 5.5. Because the unitary conductance increases at lower pH, more than the macroscopic g0, the number of functional channels must decrease. Single H+ channel currents were too small to record directly at physiological pH, but at pH ≤ 5.5 near Vthreshold (the voltage at which g0 turns on), single channel–like current events were observed with amplitudes 7–16 fA.

KE Y W ORDS: protons • hydrogen ion • ion channels • patch clamp • phagocytes

INTRODUCTION

Voltage-gated proton channels differ in several respects from other voltage-gated ion channels. Indeed, it is still debated whether they are ion channels or carriers. Like ion channels, H+ channels conduct protons passively down their electrochemical gradient, and independently of other ionic species. They probably do not meet a narrow definition of an ion channel as a water-filled pore through which ions diffuse, because the mechanism of permeation is believed to be radically different. Proton channels appear to conduct by a Grothuss-like mechanism in which protons hop across a hydrogen-bonded chain spanning the membrane (Nagle and Morowitz, 1978; DeCoursey, 2003). Several distinctive properties of voltage-gated proton channels are likely a consequence of this unique conduction mechanism. H+ channels are extremely selective for H+ (Pf/Pc > 109) (DeCoursey and Cherny, 1994). H+ conduction has much greater temperature dependence (Byerly and Suen, 1989; Kuno et al., 1997; DeCoursey and Cherny, 1998) and stronger deuterium isotope effects (DeCoursey and Cherny, 1997) than the vast majority of ion channels. Nevertheless, H+ permeates passively down its electrochemical gradient, and H+ channels exhibit time- and voltage-dependent gating, and thus resemble ion channels more than other transporters. If proton channels are genuine voltage-gated ion channels, their gating ought to generate current fluctuations that could be used to estimate the single channel conductance. The H+ current fluctuations described here provide strong evidence of gating, a defining property of ion channels, and thus support the designation of voltage-gated proton channels as genuine ion channels.

Three groups have attempted to detect H+ current fluctuations previously (Byerly and Suen, 1989; Bernheim et al., 1993; DeCoursey and Cherny, 1993). In the best case, the signal-to-noise ratio (S/N = the ratio of H+ current variance to the “background” variance when H+ channels are closed or blocked) was very poor, 0.5 (DeCoursey and Cherny, 1993). Byerly and
Suen (1989) established an upper bound at <50 fS at pH 5.9. No excess fluctuations were seen (S/N = 0), but the data had to be filtered at 1 kHz because of the rapid gating kinetics in snail neurons. Bernheim et al. (1993) filtered at 5 kHz, and from a 6% reduction of variance in the presence of Cd$^{2+}$ (S/N = 0.06), they estimated the conductance to be 90 fS at pH 5.5 (Bernheim et al., 1993). DeCoursey and Cherny (1993) improved S/N to 0.5 and estimated the unitary conductance at pH 6.0 in human neutrophils to be ~10 fS. All of these estimates were compromised by poor S/N and should be considered very rough. Here we report noise measurements in which S/N was routinely >100 and sometimes >1,000. In whole-cell studies, stationary H$^+$ current fluctuations can only be recorded just above $V_{\text{threshold}}$ because prolonged H$^+$ currents deplete intracellular buffer and change pH, (Thomas and Meech, 1982; DeCoursey, 1991). To avoid this problem, we studied noise in excised inside-out patches. An additional benefit is that this approach enables varying pH in the same experiment.

**MATERIALS AND METHODS**

**Eosinophil Isolation**

Venous blood was drawn from healthy adult volunteers under informed consent according to procedures approved by our Institutional Review Board and in accordance with federal regulations. Neutrophils were isolated by density gradient centrifugation as described previously (DeCoursey et al., 2001) with one modification. In the two cycles of hypotonic lysis performed for isolation as described previously (DeCoursey et al., 2001) with one of the solutions described above. Bath temperature was controlled by Peltier devices, and monitored by a resistance thermometer. Experiments were done at 21°C.

**Solutions**

External and internal solutions contained 100 or 200 mM buffer supplemented with tetramethylammonium methanesulfonate (TMAMeSO$_4$) to bring the osmolality to ~300 mosmol kg$^{-1}$. Solutions contained 2 mM MgCl$_2$ and 1 mM EGTA. Solutions were titrated to the desired pH with tetramethylammonium hydroxide (TMAOH) or methanesulfonic acid (for solutions using BisTris as a buffer). A stock solution of TMAMeSO$_4$ was made by neutralizing TMAOH with methanesulfonic acid. The following buffers were used near their $pK_a$ at 20°C for measurements at the following pH: pH 4.1, phosphate (phosphoric acid neutralized with TMAOH); pH 5.0, Homopipes (homopiperazine-N,N-bis-2-(ethanesulfonic acid), $pK_a$ 4.61); pH 5.5–6.0 Mes ($pK_a$ 6.15); pH 6.5 Bis-Tris (bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane: $pK_a$ 6.50); pH 7.5 HEPES ($pK_a$ 7.55). Buffers were purchased from Sigma-Aldrich, except for Homopipes (Research Organics). In about half the experiments, solutions with 200 mM HEPES for pH 7.5 or 200 mM Mes for pH 5.5 and 6.5 were used. These solutions contained 2 mM MgCl$_2$ and 2 mM EGTA and were titrated to the desired pH with n-methyl-D-glucamine or with TMAOH.

**T$\Omega$ seals.** The seal resistance in this study was typically in the TΩ (10$^3$ Ω). Our solutions were designed to minimize extraneous conductances by use of impermeant ions and to maximize control of pH by use of high buffer concentrations. The ionic strength was lower than more conventional solutions, especially for the 200 mM buffer solutions. Consequently, the conductivity of these solutions measured at 25°C with a Fisher Digital Conductivity Meter (Fisher Scientific) was low: 7.2–9.8 mS/cm for the 100 mM buffer solutions and 2.7–7.5 mS/cm for the 200 mM buffer solutions, compared, for example, with 15.9 mS/cm for Ringer’s and 22.4 mS/cm for isotonic K$^+$ Ringer’s solutions.

Both the low conductivity and the paucity of permeant ions may have contributed to the high seal resistances obtained. The seal resistance at voltages negative to the threshold for activating H$^+$ currents averaged 1.33 ± 0.18 TΩ (mean ± SEM, n = 20) in patches studied with 100/100 mM buffer (out/in). With the 200/200 mM buffer solutions, the patch resistance was no higher, 1.32 ± 0.15 TΩ (n = 21). There was no significant difference between resistances measured in any two combinations of solutions. High resistance patches are not cell specific; a 0.9 TΩ resistance was obtained in an alveolar epithelial cell patch studied with similar solutions (Fig. 15 of DeCoursey, 2003). Resistances up to 4 TΩ were reported by Benndorf (1994), using hypertonic solutions (conductivity 36.6 mS/cm) and tiny pipette openings of only 0.2 µm (~70 MΩ pipette resistance even with the highly conductive solution). Our pipettes had “normal” geometry and typically a 5–15 MΩ tip resistance.

**Electrophysiology**

All measurements were made using the inside-out patch configuration. Inside-out patches were formed by obtaining a tight seal and then lifting the pipette into the air briefly. Micropipettes were pulled using a Flaming Brown automatic pipette puller (Sutter Instruments Co.) from 7052 glass (Garner Glass Co.), coated with Sylgard 184 (Dow Corning Corp.), and heat polished. Electrical contact with the pipette solution was achieved by a thin sintered Ag-AgCl pellet (In Vivo Metric Systems) attached to a Teflon-encased silver wire. 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) was passed through a secondary 8-pole lowpass Bessel filter (Frequency Devices model 902LPF) that was used to filter and amplify the signal by 20 dB, generally with a ~3 dB cutoff frequency of 10–20 Hz. The minimum increment of digitization with a gain of 2,090 or 5,000 was ~2.2 fA or ~0.9 fA, respectively. The current was recorded simultaneously on both an Indec Laboratory Data Acquisition and Display System (Indec Corporation) and on a PC-based system with our own software written for the L780 ADC board (Measurement Computing Corp.). Seals were formed with Ringer’s solution (in mM: 160 NaCl, 4.5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 5 HEPES, pH 7.4) in the bath, and the zero current potential established after the pipette was in contact with the cell. Then the bath was exchanged with one of the solutions described above. Bath temperature was controlled by Pellicier devices, and monitored by a resistance temperature detector element (Omega Scientific) in the bath. The experiments were done at 21°C.

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**Abbreviations used in this paper:** HBSS, Hank’s balanced salt solution; TMAOH, tetramethylammonium hydroxide.
To calculate conductance, it is necessary to estimate the reversal potential, \( V_{rev} \). This was done by conventional tail current analysis in patches in which the currents were large enough that tail currents could be resolved. In other patches, \( V_{rev} \) was estimated from the voltage at which \( H^+ \) current first became activated, \( V_{threshold} \), according to the empirical relationship between \( V_{threshold} \) and \( V_{rev} \) reported in Fig. 11 of DeCoursey and Cherny (1997), which appears to apply to voltage-gated proton channels in all cells (Fig. 19 in DeCoursey, 2003).

Conventions. We refer to pH in the format \( \text{pH}_o/\text{pH}_i \). In the inside-out patch configuration the solution in the pipette sets \( \text{pH}_o \), defined as the pH of the solution bathing the original extracellular surface of the membrane, and the bath solution sets \( \text{pH}_i \). Currents and voltages are presented in the normal sense, that is, upward currents represent current flowing outward through the membrane from the original intracellular surface, and potentials are expressed by defining as 0 mV the original bath solution.

Data are presented without correction for leak current or liquid junction potentials.

Evaluation of Adequacy of Noise Sample Duration

If a current sample is too short in relation to the time constants of the kinetic processes responsible for generating the noise, then \( \sigma^2 \) will be underestimated (Diggle, 1990). To estimate the extent to which \( \sigma^2 \) is underestimated by using finite records we pooled successive single records to form longer composite records and then determined \( \sigma^2 \) as a function of sample length for each composite record. Plots like those in Fig. 1 were fitted with the following empirical function:

\[
\sigma^2 = A_0 + A_1(1 - e^{-t/\tau_1}) + A_2(1 - e^{-t/\tau_2}),
\]
or one of its reduced forms (single exponential, exponential plus straight line). Models were chosen by the probability of Student’s \( t \) values for the parameter estimates, followed by visual inspection. The “true” value of \( \sigma^2 \) was then estimated by extrapolating the saturating (exponential) components as \( t \to \infty \). 61 composite records were obtained from seven different patches, and included various suprathreshold voltages at \( \text{pH}_o \) 7.5 and 6.5, and \( \text{pH}_i \) 5.0, 5.5, and 6.5. Single record lengths ranged from 12 to 50 s, but were mostly 12–20 s. Composite-record lengths ranged from 16 to 166 s (two 16 s single records are included).

Values of \( \sigma^2 \) (single-record) \( / \sigma^2 \) (saturated) ranged from 0.72 to 1.34, with a median of 0.99. [Some values of \( \sigma^2 \) (single-record) \( / \sigma^2 \) (saturated) exceed unity because of scatter and/or the presence of a linear component.] Hence, errors in \( \sigma^2 \) resulting from the use of finite (single) records appear to be negligible. Furthermore, there was no evidence that this error varied with \( \text{pH}_o \). The presence of a linear (saturating) component in 20 composite records (e.g., Fig. 1 B) apparently reflects the presence of 1/f noise, since the slope of the linear component was positively correlated with the band-limited variance of 1/f noise (unpublished data).

Spectral Analysis

For the determination of power spectra, the mean current was first subtracted from the current records. Records in which the current increased were fitted by linear or rising exponential functions, which were subtracted to eliminate spurious trends. The resulting residual time series were then analyzed by one of two methods. For \( \text{pH} \) regimes 7.5/5.0 and 7.5/5.5 (relatively fast kinetics), power spectra were obtained with program “spectral” in Press et al. (1992). This program divides the data into at least two segments, estimates the spectral density for each segment using an FFT (fast Fourier transform), and then takes the average. Where several successive records at a given voltage were available, each record was treated as a segment. This gives the lowest frequency obtainable, because equally spaced data are required. Hence, the length of a segment (the reciprocal of which determines the lowest frequency) cannot exceed the length of a record when there are gaps between records. With this lower frequency limit, Lorentzian plateaus often were not detected for \( \text{pH} \) regimes 7.5/6.5 and 6.5/6.5, presumably because the slower gating kinetics shifted the Lorentzian component to lower frequencies than those resolved. Accordingly, a second method for determining power spectra was used for these data that allowed successive records (including gaps) to be treated as a single record, thus reducing the lower frequency limit of the spectrum. Gaps between records pose no problem for the estimation of the autocovariance function, which was calculated according to Eq. 2.5.5 in Diggle (1990). The one-sided spectral density was then obtained as the Fourier cosine transform of the autocovariance (Blackman-Tukey method) as described by Oppen and Enochson (1978). The reliability of both methods for estimating power spectra was checked with simulated data for a two-state kinetic scheme.

![Figure 1](image-url)

**Figure 1.** Effect of sample length (\( \Delta t \)) on estimates of the current variance (\( \sigma^2 \)). (A) \( \text{pH}_o/\text{pH}_i \) 7.5/5.0, \( V = -30 \text{ mV} \); the curve is a least-squares fit of \( \sigma^2 = A_0 + A_1(1 - e^{-t/\tau_1}) + A_2(1 - e^{-t/\tau_2}) \) with \( A_0 = 4.0 \times 10^{-27} \text{ A}^2, A_1 = 1.5 \times 10^{-28} \text{ A}^2, A_2 = 2.0 \times 10^{-27} \text{ A}^2, \tau_1 = 1.1 \text{ s} \) and \( \tau_2 = 9.3 \text{ s} \). (B) \( \text{pH}_o/\text{pH}_i \) 7.5/6.5, \( V = 10 \text{ mV} \); the curve is a least-squares fit of \( \sigma^2 = A_0 + A_1(1 - e^{-t/\tau_1}) + Bt \), with \( A_0 = 7.2 \times 10^{-26} \text{ A}^2, A_1 = 7.6 \times 10^{-28} \text{ A}^2, \tau_1 = 1.5 \text{ s} \) and \( B = 1.3 \times 10^{-26} \text{ A}^{-1} \text{s}^{-1} \). See text for details.
To correct for filtering, spectra were divided by the square modulus of the transfer function for an eight-pole low-pass Bessel filter using equation 3.11 and coefficients in Tietze and Schenk (1978). This had a negligible effect on most of the spectrum, but it did allow curve-fitting up to about the cutoff frequency of the filter (typically 20 Hz); data above this frequency were discarded. The data were then binned in log10(f) intervals of 0.2 (where f is the frequency in Hz) before fitting with various functions by nonlinear least squares. The most general function employed treats the one-sided spectral density $G(f)$ as the sum of Lorentzian, $1/f$ and white components:

$$G(f) = \frac{4\tau_1^2\sigma^2}{1 + (2\pi\tau_f f)^2} + A + B,$$

where $\sigma^2$ is the current variance attributed to open/closed transitions of the channels, $\tau_1$ is the “Lorentzian” time constant defined as $(2\pi f_c)^{-1}$, where $f_c$ is the corner (half-power) frequency, and $A$, $B$, and $\sigma$ are constants. In practice, estimates of $\sigma$ were in the range 0.7 to 1.4; noise of this type is generally described as $1/f$ (Neumcke, 1978; DeFelice, 1981). Various reduced models were then fitted by removing one or more of these adjustable parameters. Initial selection of a minimum-parameter model was made using $F$-tests as described by Gallant (1975) and Walpole and Myers (1978). The fits were then checked visually and a value probabilities for parameter estimates were inspected; in a few cases, the initial choice of minimum-parameter model was overridden.

**Online Supplemental Material**

The online supplemental material (available at http://www.jgp.org/cgi/content/full/jgp.200308813/DC1) evaluates quantitatively the possibility that pH changes due to H$^+$ current might occur under the conditions of these experiments, and we estimate the errors introduced. Analysis of simulated $\gamma g_0 l$ plots (e.g., Fig. 6) suggests that the errors in $\gamma$ and $N$ due to proton depletion/accumulation are relatively small and essentially independent of pHl and pHw. For typical values, $\gamma l$ was underestimated by 1–4% while $N$ was underestimated by 2–10%. We also estimate that local pH changes due to current flow will be established within 0.1–1.2 s.

**RESULTS**

H$^+$ Channel Gating Generates Current Fluctuations

The currents in excised patches were distinctly noisier when the H$^+$ conductance was activated. Fig. 2 illustrates currents recorded during pulses or prolonged depolarizations in the same inside-out patch of membrane from a human eosinophil, at three different pHl (the bath solution faces the intracellular side of the membrane). The threshold voltage, $V_{threshold}$, at which H$^+$ current is first activated is exquisitely sensitive to pH, becoming more negative at lower pHl, as in all other cells with voltage-gated proton channels (Byerly et al., 1984; DeCoursey, 2003). The patch currents exhibit little noise at subthreshold voltages, but become markedly noisier when small outward H$^+$ current is activated just above $V_{threshold}$.

In Fig. 3, chord conductance-voltage ($g_{tr} V$) relationships from the experiment in Fig. 2 are plotted. The curves show that the quasisteady-state $g_{tr} V$ relationship is well described by a Boltzmann function. The slope factors are 5–8 mV, somewhat steeper than most estimates of 7–14 mV in whole-cell studies using shorter pulses (DeCoursey and Cherny, 1994; Cherny et al., 2001). The average slope factors were 8.16 $\pm$ 0.52 mV (mean $\pm$ SEM, n = 13) at pH 7.5/6.5 and 6.99 $\pm$ 0.48 mV (n = 16) at pH 7.5/6.5 (P > 0.1). The present values are probably more reliable for two reasons. First, they are more nearly steady-state, because they were measured after long times at each voltage. Second, H$^+$ currents in excised patches are less distorted than in
whole-cell configuration by pH changes that occur during current flow, due to depletion of protonated buffer (see online supplemental material, available at http://www.jgp.org/cgi/content/full/jgp.200308813/DC1).

As evident in Fig. 3, lowering pH shifts the $g_{H,V}$ relationship toward more negative voltages and increases $g_{H,max}$. The average shift in $V_{threshold}$ between pH 6.5 and 5.5 was $-36 \pm 5$ mV (mean ± SD, $n = 9$) and the average shift in the midpoint of the $g_{H,V}$ relationship was $-31 \pm 9$ mV ($n = 8$). Thus, in most patches, there was a larger shift in $V_{threshold}$ than in the patch illustrated. Although this patch is not ideally representative in all respects, we use it in many figures in this paper because it was a rare patch in which we were able to record extensive data at three pH and it exhibits, at least qualitatively, all of the behaviors that characterize this system. The limiting $g_{H,V}$, $g_{H,max}$ consistently increased at lower pH. Rundown, which was often observed during long experiments (e.g., $g_{H,max}$ might decrease 50%), compromises our comparison of $g_{H,max}$ and of the number of channels in the patch, $N$, at different pH. However, measurements made before and after pH changes indicate that the macroscopic $g_{H,max}$ has a relatively weak dependence on pH. On average, in nine patches in which sufficient data were recorded at both pH, $g_{H,max}$ was $1.78 \pm 0.10$ (mean ± SE) times larger at pH 5.5 than 6.5 in each patch. This comparison was done with pH changes in both directions and the measurements were fairly close together in time to minimize effects of rundown.

Current fluctuations can be quantified by their variance, $\sigma^2$. Variance measured in the patch illustrated in Figs. 2 and 3 is plotted in Fig. 4. Each data point represents a 12-s sample of current, collected during prolonged sojourns at each voltage. Comparison of Figs. 3 and 4 confirms that $\sigma^2$ increases precisely at the voltage at which the $g_{H}$ becomes activated at each pH. At subthreshold voltages the variance is $<10^{-28}$ A$^2$. It is noteworthy that the signal-to-noise ratio (S/N, defined in introduction) at lower pH is $>100$ at many voltages, which is a two-order-of-magnitude improvement over previous studies (Byerly and Suen, 1989; Bernheim et al., 1993; DeCoursey and Cherny, 1993). In some patches, S/N was $>1,000$. 

![Figure 3](http://www.jgp.org/cgi/content/full/jgp.200308813/DC1)

**Figure 3.** Steady-state $g_{H,V}$ relationships for the patch illustrated in Fig. 2. Chord conductance, $g_{H}$, was calculated assuming $V_{rev}$ of $-40$, $-80$, and $-100$ mV, at pH 6.5 (●), 5.5 (■), and 5.0 (▲), respectively. Curves show best-fitting (by nonlinear least squares) Boltzmann functions: $g_{H} = g_{H,max} [1 + \exp(-(V-V_{1/2})/k)]^{-1}$ with midpoints ($V_{1/2}$) of $-10.4$, $-26.9$, and $-43.0$ mV, slope factors ($k$) of 5.2, 7.8, and 8.1 mV, and $g_{H,max}$ of 32.6, 48.7, and 62.2 pS at pH 6.5, 5.5, and 5.0, respectively. ES-2596.

![Figure 4](http://www.jgp.org/cgi/content/full/jgp.200308813/DC1)

**Figure 4.** Voltage dependence of total variance from the same patch as Figs. 2 and 3. Symbols have the same meaning. The midpoints of the $P_{open}$-voltage relationships determined as described in Fig. 7, are indicated by symbols near the X axis. The open symbols show the band-limited Lorentzian component of the $\sigma^2$ in this experiment, $\sigma_{L}^2$, obtained by fitting power spectra with Lorentzian plus $1/f$ components (as described in Fig. 10). There is divergence only at large depolarizations at pH 5.0, where the $1/f$ component became significant. ES-2596.
Several features indicate that the noise is related to the activation of H\(^+\) channels. First, when pH was varied, which shifted the \(g_{\text{H}}V\) relationship, the current always became noisy (i.e., \(\sigma^2\) increased) at \(V_{\text{threshold}}\). Second, no other detectable ion-specific conductance is evident in eosinophil patches under these ionic conditions. During depolarizing pulses, the current turns on from a small initial level, indicating that practically all of the outward current is the result of voltage- and time-dependent gating. Finally, \(\sigma^2\) increases rapidly with depolarization above \(V_{\text{threshold}}\), but at larger depolarizing voltages, \(\sigma^2\) does not increase in proportion, and at pH 5.5 or 5.0, actually decreases. Strikingly, \(\sigma^2\) is maximal near the midpoint of the \(g_{\text{H}}V\) relationship at pH 5.5 or 5.0 (symbols labeled \(V_{1/2}\) in Fig. 4). If the noise were generated by some process other than channel gating fluctuations, one would expect \(\sigma^2\) to increase with the current amplitude (DeFelice, 1981; Kogan, 1996). The 1/\(f\) component of \(\sigma^2\) that is associated with ion transport through open ion channels often increases with \(f^2\) (Conti et al., 1975; DeCoursey et al., 1984). To the extent that we observe a distinct maximum in the \(\sigma^2-V\) relationship (Fig. 4), any such component must be dwarfed by \(\sigma^2\) generated by H\(^+\) channel gating, that is expected to have a Lorentzian spectrum (see Power spectra). In fact, the \(\sigma^2\) contained within the Lorentzian component alone, plotted as open symbols in Fig. 4, is similar to the total \(\sigma^2\) except at large depolarizations. As shown in Fig. 5, the voltage dependence of the current variance behaves precisely as one would predict from a simple gating model.

Ideally, the variance due to H\(^+\) channel gating, \(\sigma_{\text{H}}^2\), would be measured under stationary conditions. At lower pH, the activation time course (turn-on) of H\(^+\) current was relatively rapid and accordingly these data appeared to be stationary. More rigorous tests of stationarity, in which the variance was estimated at different times during prolonged records also revealed no clear trends in \(\sigma^2\). However, activation was much slower at pH 6.5, and up to several minutes were required at some voltages before stationarity was achieved. H\(^+\) current data that increased slowly during each successive record were “corrected” for the slow drift by subtracting a fitted single exponential curve. We discarded the first several records in which \(I_{\text{H}}\) increased more rapidly and used only the last few records, treating each one separately (i.e., with its own \(I_{\text{H}}, P_{\text{open}},\) and \(\sigma_{\text{H}}^2\)). In Fig. 1 (Materials and Methods) we showed empirically that \(\sigma_{\text{H}}^2\) measured during 12–20-s current records included >90% of the total \(\sigma_{\text{H}}^2\) that is expected to be obtained with much longer records.

**Calculation of \(i_{\text{H}}\) from Stationary Variance of H\(^+\) Currents**

Accepting that the current fluctuations originate in H\(^+\) channel gating, we would like to estimate the single channel H\(^+\) current, \(i_{\text{H}}\), and related properties (the number of channels in the patch, \(N\), and the open probability, \(P_{\text{open}}\)). \(i_{\text{H}}\) can be calculated from (Hille, 2001):

\[
I_{\text{H}} = \frac{\sigma_{\text{H}}^2}{I_{\text{H}}(1 - P_{\text{open}})}
\]

where \(\sigma_{\text{H}}^2\) is the H\(^+\) current variance, and \(I_{\text{H}}\) is the mean H\(^+\) current. Except for \(P_{\text{open}}\) these parameters are directly measurable, although it is necessary to distinguish the contributions to current and variance that originate in H\(^+\) channels from background current or noise. The total \(\sigma^2\) was essentially independent of voltage at subthreshold voltages where H\(^+\) channels are closed (Fig. 4). Therefore, we subtracted the average \(\sigma^2\) at subthreshold voltages from that measured at voltages where H\(^+\) channels were open. This approach was used by Byerly and Suen (1989). Another approach is to inhibit H\(^+\) current with Zn\(^{2+}\) or Cd\(^{2+}\) and to consider the resulting \(\sigma^2\) to be the background at that voltage (Bernheim et al., 1993). We avoided this approach because Zn\(^{2+}\) sometimes reduced both the background leak current and \(\sigma^2\) at voltages where H\(^+\) channels were closed, and at high concentrations Zn\(^{2+}\) apparently caused membrane damage that increased \(\sigma^2\) spuriously. In a few patches, the maximal H\(^+\) current was <1 pA and the fluctuations attributable to H\(^+\) channel gating were comparable with background levels (i.e., S/N = 1). However, in most patches, S/N > 100, and sometimes S/N > 1,000, hence the subtraction of background variance had negligible effect. Determination of \(I_{\text{H}}\) required subtraction of leak current. Leak current at subthreshold voltages was usually Ohmic, and we extrapolated it to estimate the leak at each voltage where noise was measured. The most difficult parameter to estimate was \(P_{\text{open}}\). In preliminary analyses, we approximated \(P_{\text{open}}\) as \(g_{\text{H}}\) measured during the noise record normalized to \(g_{\text{H}}\) from a family of pulses, which presumes that the maximum value of \(P_{\text{open}}\) (\(P_{\text{max}}\)) approaches 1.0 for large depolarizations. For measurements just above \(V_{\text{threshold}}\) where \(P_{\text{open}}\) is small, this assumption will cause only a small overestimation of \(i_{\text{H}}\). However, knowledge of \(P_{\text{open}}\) becomes important at more positive voltages where \(g_{\text{H}}\) approaches saturation. For this reason, previous attempts to estimate \(i_{\text{H}}\) were restricted to voltages near \(V_{\text{threshold}}\) (Bernheim et al., 1993; DeCoursey and Cherny, 1993). Eventually, we adopted a procedure of data analysis (see below) that obviates the need to guess \(P_{\text{open}}\).

**What is the Limiting \(P_{\text{open}}\) at Large Depolarizations?**

The calculation of \(i_{\text{H}}\) requires an estimate of \(P_{\text{open}}\) (Eq. 1). Although \(g_{\text{H}}\) may be proportional to \(P_{\text{open}}\) (after correction for rectification), it provides no information about \(P_{\text{max}}\). However, the voltage dependence of the
Figs. 5. Model showing the voltage dependence of variance in a simple two-state channel, for different values of \( P_{\text{max}} \), the maximum \( P_{\text{open}} \) during large depolarizations. The top panel shows the assumed \( P_{\text{open}}-V \) relationships with various \( P_{\text{max}} \) values, as indicated.

All were calculated from a Boltzmann function with midpoint, \( V_{1/2} = -40 \text{ mV} \) and slope factor, \( k = 8 \text{ mV} \). The bottom panel shows the variance expected for 200 channels with \( \gamma_H = 50 \text{ fS} \) and \( V_{rev} = -120 \text{ mV} \), assuming the various \( P_{\text{open}}-V \) relationships plotted above. The curves were generated using Eq. 1 with \( I_H = 1 \text{ nA} \) and \( P_{\text{open}} = \gamma_H (V - V_{rev}) \).}

\[ y = \gamma_H - N^{-1} g_H, \quad (2) \]

where

\[ y = \frac{\sigma_H^2}{I_H (V - V_{rev})}. \quad (3) \]

Hence, provided \( \gamma_H \) is constant (e.g., no rectification) and \( N \) is constant (e.g., no rundown) a plot of \( y \) versus \( g_H \) should be linear. The Y intercept is the unitary conductance, \( \gamma_H \), and the slope is \(-1/N\). The intercept on the X axis is \( \gamma_H N \), the maximum possible \( g_H \) if all channels were open simultaneously (which may be greater than the observed \( g_{H\text{max}} \)). In practice, within the limits of experimental error, linear \( y-g_H \) plots were obtained, as in Fig. 6. For pH 6.5, the slope was often small and sometimes not significantly different from zero. In such cases \( N \) could not be determined reliably, although \( \gamma_H \) could still be estimated.

We adopted the \( y-g_H \) plot because (a) through \( N \), it provides an estimate of \( P_{\text{open}} \) without requiring assuming an...
arbitrary value, and (b) it makes use of all the data over a range of voltages that include low to high $P_{\text{open}}$. Different errors are expected to occur at both extremes of $P_{\text{open}}$ as well as at intermediate values. Near $V_{\text{threshold}}$, both $I_H$ and $\sigma^2$ are close to background levels, and thus are poorly determined. During large depolarizations, $I_H$ might be attenuated due to pH changes that result from prolonged $H^+$ flux through the patch. This error is considered in the online supplemental material (available at http://www.jgp.org/cgi/content/full/jgp.200308813/DC1), and shown to have had little impact on the results. At intermediate voltages, the estimated $I_H$ using Eq. 1 depends strongly on the limiting $P_{\text{open}}$ value. Attempting to use Eq. 1 directly on individual records would require guessing $P_{\text{open}}$. By including all the data in plots such as in Fig. 6, we can estimate $N$, from which $P_{\text{open}}$ can be calculated.

In Fig. 7, $P_{\text{open}}$ is plotted for the same patch as in other figures. To approach true steady-state, we plot only the estimate from the final record at each voltage. The results are quite similar to the simple $g_{\text{ch}}V$ relationships in Fig. 3. The additional information obtained from Fig. 7 is the limiting value for $P_{\text{open}}$, $P_{\text{max}}$. Average values of $P_{\text{max}}$ are given in Table I. Consistent with the behavior predicted from Figs. 4 and 5, $P_{\text{max}}$ is 0.95 at pH 1, $\leq 5.5$, and at pH 6.5, $P_{\text{max}}$ is 0.75. Almost all $H^+$ channels open during large depolarizations at low pH, but $P_{\text{max}}$ decreases at higher pH.

The single-channel conductance, $\gamma_H$, is already determined from the $y_{\gamma_H}$ plot (Fig. 6). However, in Fig. 8 the unitary currents calculated for each noise sample are plotted. The lines are not fitted to these data but simply illustrate the values for $\gamma_H$ obtained from the $y_{\gamma_H}$ plots. Single channel $H^+$ currents are a few femtoamperes at pH 6.5, and increase markedly at lower pH. Mean values obtained for $\gamma_H$ are given in Table I. Lowering pH from 6.5 to 5.5 increased $\gamma_H$ by 3.7-fold on average. Although only a few measurements were made at pH 6.5/6.5, the similar $\gamma_H$ obtained at pH 7.5/6.5 suggests that $\gamma_H$ depends only on pH, and not on pH$_i$.

**Direct Measurement of Single-channel $H^+$ Currents**

Consistent with previous studies (Byerly and Suen, 1989; Bernheim et al., 1993; DeCoursey and Cherny, 1993), we were unable to detect single $H^+$ channel currents directly when pH$_i$ was 6.5 or higher. However, at pH 5.5 there were tiny step-like fluctuations in the current in some patches (Fig. 9). Putative unitary currents could be resolved only near $V_{\text{threshold}}$, where just a few channels opened at a time. In Fig. 9, channel-like events are present at $-60$ and $-70$ mV, but at $-50$ mV too many channels are open to resolve discrete levels. The amplitude of the events at $-60$ mV is $\sim 10$ fA, estimated as the difference in local mean current when the channel is judged to be open or closed. The $\sigma^2_{y_H}$ analysis ($y_{\gamma_H}$ plot) of this patch indicated that $I_H$ was $\sim 5$ fA at $-60$ mV. Apparent single-channel currents in seven
other patches under similar conditions were 7–16 fA, which is about twice as large as estimates obtained from variance analysis, although it is evident that the amplitudes are not clearly resolved. To our knowledge, these are the smallest single-channel currents identified by direct voltage-clamp measurement of any channel. Decker and Levitt (1988) resolved 25-fA H⁺ currents through gramicidin channels.

**Frequency Dependence of H⁺ Current Fluctuations**

Power spectra were obtained from a subset of the data comprising 11 datasets from 7 different patches. Lorentzian spectral components could be identified under all pH regimes (Fig. 10), although detection of the Lorentzian plateau was difficult for pH 6.5 (Fig. 10 B). The Lorentzian noise appeared to “turn on” when Iᵢ was activated, and the variance contained within the Lorentzian component, σ²_L (Fig. 11, ▲), showed similar voltage dependence to the total current variance (σ²_H = open, σ²_L = solid symbols in Fig. 4). Thus, the σ²_H x V relationship exhibited a maximum for pH 5.0 and pH 5.5 (Fig. 11 A), but increased monotonically with V for pH 6.5 (Fig. 11 B). In addition to the Lorentzian noise, 1/f (Fig. 11, ○) and white noise (< Fig. 11) were also often observed. Both of these components increased monotonically with V. The 1/f component dominates at large depolarizing voltages because of the nonmonotonic voltage dependence of σ²_V at low pH. For reference, the sum of the calculated shot noise of current flow and Johnson noise of the 50 GΩ feedback resistor in parallel with the patch resistance (Hainsworth et al., 1994), both of which are white noise, is shown by the dashed lines without symbols in Figs. 10 A and 11. The patch resistance was mainly determined by the H⁺ conductance. The 1/f spectrum at −80 mV in Fig. 10 A approaches this limiting noise level at −20 Hz.

If σ²_L represents the only contribution of open/closed transitions to the current variance, then σ²_L rather than

---

**TABLE I**

**Single H⁺ Channel Conductance (γ_H) and Maximum Open Probability (P_max) at Different pH**

<table>
<thead>
<tr>
<th>pH_o//pH_i</th>
<th>γ_H</th>
<th>P_max</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SEM (n)</td>
<td>mean ± SEM (n)</td>
</tr>
<tr>
<td>7.5//6.5</td>
<td>37.4 ± 4.0 (19)</td>
<td>0.746 ± 0.027 (17)</td>
</tr>
<tr>
<td>6.5//6.5</td>
<td>38.3 ± 4.3 (3)</td>
<td>0.60 (1)</td>
</tr>
<tr>
<td>7.5//5.5</td>
<td>138.9 ± 7.5 (13)</td>
<td>0.954 ± 0.014 (10)</td>
</tr>
<tr>
<td>7.5//5.0</td>
<td>220 (2)</td>
<td>0.95 (2)</td>
</tr>
<tr>
<td>7.5//4.1</td>
<td>400 (1)</td>
<td>0.97 (1)</td>
</tr>
</tbody>
</table>

Estimates of γ_H and N were obtained from γ_yH plots (Fig. 6) and were based on the total H⁺ current variance (σ²_H). If calculated using only the Lorentzian variance (σ²_L), then the estimates of γ_H are about 20% lower, whereas the estimates of N are unaffected (Fig. 12).
are so similar to those based on $\sigma_p^2$ that $\sigma_T^2$ dominates the total variance at small depolarizations. At large depolarizing voltages where the $1/f$ component becomes large (Fig. 11), and hence $\sigma_T^2$ and $\sigma_p^2$ diverge, the effect on the slope and intercept of the $y_{50}$ plot is quite small.

The Lorentzian time constants $\tau_L = (2\pi f)^{-1}$ were smaller than the time constants for $H^+$ current activation ($\tau_{act}$) by roughly an order of magnitude (Fig. 13). However, they showed a qualitatively similar dependence on $pHi$. In contrast to $\tau_{act}$ (which decreases with $V$), no consistent voltage dependence could be discerned for $\tau_L$, although this may have been masked by the considerable scatter. Mean deactivation time constants, $\tau_{dact}$, measured during tail currents at voltages just negative to $V_{rev}$ are plotted in Fig. 13. To compare

---

**Figure 10.** Power spectra of $H^+$ current fluctuations. Test voltages (mV) are shown next to each curve. (A) $pHi = 7.5$, $pH_L = 5.5$, $V_{threshold} = -55$ mV. The subthreshold data ($\triangle$) are fitted with a $1/f$ spectrum while the suprathreshold data sets are fitted with Lorentzian plus $1/f$ spectra. The latter two data sets can be fitted equally well by a Lorentzian plus white noise. The horizontal lines show the calculated sum of the spectral densities of the calculated shot noise of current flow and Johnson noise of the 50 GΩ feedback resistor in parallel with the patch resistance, which were: $3.8 \times 10^{-31}$ (□), $4.4 \times 10^{-31}$ (○) and $1.7 \times 10^{-30}$ (▼) A² Hz⁻¹. (B) $pHi = 7.5$, $pH_L = 6.5$, $V_{threshold} < -30$ mV. The near-threshold data ($\triangle$) are fitted with a Lorentzian plus white noise while the other datasets are each fitted with a Lorentzian plus $1/f$ plus white noise. The data at −10 and 20 mV can be fitted equally well with a $1/f$ spectrum plus white noise, but the exponents ($m$) on $f$ are significantly greater than unity ($1.29 \pm 0.05$ and $1.37 \pm 0.07$, respectively). The sum of Johnson- and shot-noise spectral densities were $1.3 \times 10^{-30}$ (□), $8.4 \times 10^{-30}$ (○) and $2.0 \times 10^{-29}$ (▼) A² Hz⁻¹ (too small to show on this scale). Data in A and B are from two different patches (ES-2596, ES-2579).

**Figure 11.** The Lorentzian variance ($\triangle$) and the band-limited $1/f$ (○) and white noise (▼) variances were determined from fitted power spectra (Fig. 10) and are plotted here against membrane voltage ($V$). The white noise and $1/f$ variances were calculated over the interval [$f_1$, $f_2$], where $f_1$ is the lowest frequency in the spectrum and $f_2$ is the Nyquist frequency. On average, the band-limited Lorentzian variance (open symbols in Fig. 4) was ~10% less than the total Lorentzian variance (which is plotted here). The dashed lines without symbols show the calculated sum of the Johnson- and shot-noise variances (see Fig. 10) over the interval [$f_1$, $f_2$]. (A) $pHi = 7.5$, $pH_L = 5.5$, $V_{threshold} = -55$ mV; (B) $pHi = 7.5$, $pH_L = 6.5$, $V_{threshold} < -30$ mV. Same two patches as in Fig. 10. In A, $1/f$ noise was not detectable between −10 and 20 mV; presumably it was masked by the Lorentzian. On the other hand, only $1/f$ noise was detected at 40 mV; apparently Lorentzian noise had fallen to undetectable levels, suggesting a $P_{max}$ value close to unity. In B, no peak is observed in the Lorentzian variance, suggesting $P_{max} \leq 0.8$ (see text and Fig. 5).
tail and $L$ act with $L$ in the same voltage range, these measured tail values are extrapolated to the midpoint voltage of the $g_{H^-}V$ relationship, assuming exponential voltage dependence with a slope of 40 mV/e-fold change in $g_{H^-}$. The $L$ values are closer to $L$ tail than to $L$ act. At pH 7.5/5.5, we determined the mean open time in five patches in which apparently discrete events occurred near $V_{threshold}$ (e.g., Fig. 9). The mean open time from these patches is similar to $L$ tail.

**DISCUSSION**

**Proton Channel Gating Generates $H^+$ Current Noise**

Distinct $H^+$ current fluctuations were clearly evident in patches of eosinophil membranes. Compelling evidence indicates that these fluctuations originate in the gating of voltage-gated proton channels. First, at various pH$_I$, $\sigma^2$ increases precisely at $V_{threshold}$. Second, $\sigma^2$ increases with voltage to a maximum that occurs near the midpoint of the $g_{H^-}V$ relationship at each pH$_I$. At larger depolarizing voltages, $\sigma^2$ then decreases at pH$_I \leq 5.5$, but increases again gradually at very large depolarizing voltages. Precisely this behavior is predicted by a simple gating model to occur if $P_{max} \approx 0.9$ (Fig. 5), but is exceedingly unlikely to result from fluctuations originating in leak current. Finally, no other conductance is evident under the ionic conditions of this study.

Single $H^+$ channel currents were estimated from stationary and quasistationary $H^+$ current variance, and were also measured directly at low pH$_I$. $H^+$ current fluctuations could be resolved with excellent S/N because proton channels in mammalian cells, especially in phagocytes, gate at low frequencies (DeCoursey, 2003), which allows heavy filtering. In contrast, $H^+$ current fluctuations could not be detected in snail neurons (Byerly and Suen, 1989), because these $H^+$ channels open on a time-scale of a few milliseconds (Byerly et al.,...
of roughly an order of magnitude, but were in the range kHz with S/N of single channel currents (Fig. 13).

Excess white noise evident at high frequencies might contribute low frequency Lorentzian component, \( \sigma^2 \). Excess white noise evident at high frequencies could have been resolved only at low pH. At pH 6.5, clear resolution of such a Lorentzian component (i.e., resolution down to \( f/10 \)) would require stationary current samples \( \sim 70 \) min long. Similarly, if all or part of the 1/f noise were associated with channel gating (e.g., if the 1/f spectrum were actually a superposition of Lorentzians) then estimates of \( \gamma_H \) based on \( \sigma^2_H \) (Fig. 12) will be downwardly biased. The same can be said of the white noise component, although this was very small. On average, however, the estimates of \( \gamma_H \) based on \( \sigma^2_H \) and \( \sigma^2 \) differ by only \( \sim 20\% \).

Unitary \( H^+ \) Currents Increase at Low pH, More Than Macroscopic Conductance

The single channel conductance estimated from \( \sigma^2_H \) was 38 fS at pH 6.5, and 139 fS at pH 5.5 (Table I). A few measurements at lower pH, (where patches were extremely unstable) indicate similar pH dependence. Extrapolating \( \gamma_H \) at pH 5.5 and 6.5 to pH 7.2 gives an estimate of 15 fS for the unitary conductance at physiological pH. Corrected to 37°C assuming a \( Q_0 \) of 2.8 (DeCoursey and Cherny, 1998), \( \gamma_H \) becomes 78 fS. The most surprising result of this study was that the single channel conductance increased 3.7-fold at pH 5.5 compared with pH 6.5, in contrast with the macroscopic conductance, which increased only 1.8-fold. In nearly all existing studies of voltage-gated proton channels, the macroscopic \( g_H \) increased only \( \sim 2\)-fold per unit decrease in \( \mathrm{pH} \) (DeCoursey, 2003). The relative pH independence of the macroscopic \( g_H \) has been considered somewhat paradoxical, both for voltage-gated proton channels (DeCoursey and Cherny, 1994) and for the proton channel of ATP synthase (Junge, 1989). In contrast, the \( H^+ \) conductance of gramicidin channels is almost directly proportional to \([H^+]\) over a wide pH range (Eisenman et al., 1980; DeCoursey, 2003). The unitary conductance found here (Table I) is not proportional to \([H^+]\), but is closer to proportionality than estimates based on macroscopic \( g_H \) measurements.

Because lowering \( \mathrm{pH} \) increased the estimated single-channel conductance substantially more than the macroscopic \( g_{\mathrm{H}, \text{max}} \), the total number of \( H^+ \) channels, \( N \), must decrease. This anomalous behavior has several possible explanations. Although the result might be artificial, consideration of possible sources of error does not lead to likely candidates. The variance at higher pH might be underestimated if we did not sample a sufficiently low frequency range, but the variance-
versus-record length analysis (materials and methods) gave no indication of this. Progressive rundown (loss of functioning channels) at lower pH could contribute in experiments in which measurements were first made at higher pH. However, correction for rundown or reversing the order of pH measurements did not eliminate the phenomenon. The observed pH dependence of $P_{\text{max}}$ does not explain this result, but instead works in the opposite direction. There seems no alternative but to accept that low pH actually reduces the number of functioning H$^+$ channels. Based on the average values of $\gamma_H$, $P_{\text{max}}$, and $g_{\text{fle}}$, N is 2.6 greater at pH 6.5 than 5.5 (or 62% of the channels at pH 6.5 are unavailable at pH 5.5).

Single-channel–like events (7–16 fA) could be observed directly in many patches at pH $\leq$ 5.5, just above $V_{\text{threshold}}$. The amplitude of these events was not well-resolved, but usually was about double the unitary H$^+$ current calculated at the same voltage from $\sigma_H$. It is possible that these events were simultaneous openings of multiple channels, and that the single channels were simply not resolved. Alternatively, H$^+$ channels may have a cooperative gating mechanism. We assume that most H$^+$ current fluctuations occur within the frequency range examined. The adequacy of the bandwidth employed in the low frequency range was demonstrated empirically in Fig. 1, but it is possible that there could be components of gating at higher frequencies than we explored. Of course, it is possible that the discrepancy simply reflects errors in the measurements.

If each channel flickered on a millisecond scale, we would have resolved only the average current. However, from the vantage point of most cellular processes, such as pH regulation, a channel that conducts 5 fA of current would have resolved only the average current. However, extrapolation of this pH dependence to pH 6 predicts <1 fA of H$^+$ current at 100 mV. The present demonstration of distinct H$^+$ current fluctuations attributable to voltage- and pH-dependent gating confirms that voltage-gated proton channels behave like genuine ion channels, in spite of their conductance at physiological pH being low enough to approach the range of carriers. Voltage-gated proton channels are simply channels with a small conductance.

The authors thank Wolfgang Nonner for helpful discussions, and Tatiana Iastrebova and Julie Murphy for excellent technical assistance. Many eosinophils were generously provided by Larry L. Thomas.

This work was supported in part by the Heart, Lung and Blood Institute of the National Institutes of Health (research grants HL52871 and HL61437 to Dr. DeCoursey).

Olaf S. Andersen served as editor.

Submitted: 4 February 2003
Revised: 2 April 2003
Accepted: 2 May 2003

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Effect of Proton Depletion/Accumulation on Estimates of $\gamma_h$ and N Obtained from $y-g_h$ Plots

It is self-evident that H$^+$ current changes pH. In the whole-cell configuration, these pH changes can be large and slowly reversible, even with 100 mM buffer in the pipette solution. The present experiments were done on excised, inside-out patches in order to minimize pH changes. Here we evaluate the magnitude of any pH changes under the conditions of this study, and their effects on estimates of $\gamma_h$ and N. Two types of measurements are affected. First, any pH change during the noise measurement itself will directly alter the result. Second, pH changes during the tail current measurements used to estimate $V_{rev}$ will also affect the outcome. Here we show that systematic errors in $\gamma_h$ and N are relatively small under the conditions of this study.

Proton Depletion and Accumulation in the Steady-state

During gigaseal formation, part of the cell membrane is drawn into the pipette, so that after excision the patch lies some distance from the tip. Outward proton current under voltage clamp leads to a depletion of protons at the intracellular side of the patch and an accumulation at the extracellular side. First, consider the depletion of protons at the intracellular side of the patch under steady-state conditions. Because the proton concentration, [H$^+$], is miniscule compared to the concentration of protonated buffer, [BH], we assume that $I_h$ is sustained almost entirely by the diffusion of BH from the bath to the membrane patch. And since in the steady state the diffusive flux of BH is balanced by an equal and opposite electrodiffusive flux of deprotonated buffer, B$^-$, we have:

$$I_h = -\pi r^2 F D_{BH} \frac{d[BH]}{dx} = \pi r^2 F D_{B^-} \left( \frac{d[B^-]}{dx} - \frac{F [B^-] dV}{RT} \frac{dx}{dx} \right),$$

(A1)

where $r$ is the internal pipette radius at some distance $x$ from the tip, $D_{BH}$ and $D_{B^-}$ are diffusion coefficients, and $F$, $R$, and $T$ have their usual meanings. We assume that activity coefficients are concentration independent (Schultz, 1980). Solving Eq. A1 for [BH] and [B$^-$] gives:
\[ [BH] = \left[ BH \right]_o - \frac{I_{II}}{\pi FD_{BH}} \int_0^x \frac{dx}{r^2}, \quad (A2) \]

\[ [B^-] = [B^-]_o + \frac{I_{II}}{\pi FD_{B^-}} \int_0^x \frac{dx}{r^2} + \frac{F}{RT} \int_0^x [B^-] \left( \frac{dV}{dx} \right) dx, \quad (A3) \]

where \([BH]_o\) and \([B^-]_o\) are the bath concentrations of protonated and deprotonated buffer, respectively. \([BH]\) and \([B^-]\) are related by the buffer dissociation constant:

\[ K_a = \frac{[H^+][B^-]}{[BH]} \quad (A4) \]

whence the degree of dissociation (\(\alpha\)) is given by

\[ \alpha = \frac{[B^-]}{[B_{total}]} = \frac{K_a}{K_a + [H^+]}, \quad (A5) \]

where \([B_{total}] = [BH] + [B^-]\) is the total buffer concentration.

To simplify matters, suppose that \(D_{BH} = D_{B^-} = D_B\) and assume the pipette can be approximated as the frustum of a cone, with \(r_0\) the internal pipette-tip radius, \(r_p\) the patch radius, and \(b\) the internal shank taper (= \(dr/dx\)). Then on defining a parameter

\[ \beta_i = \frac{1}{\pi F D_B [B_{total}]_0} \int_0^p \frac{dx}{r^2} = \frac{1}{\pi b F D_B [B_{total}]_0} \left( \frac{1}{r_0} - \frac{1}{r_p} \right), \quad (A6) \]

where \(p\) is the distance of the patch from the pipette tip, Eq. A2 can be written

\[ [BH]_i = [B_{total}]_0 \left( 1 - \alpha_0 - \beta I_{II} \right) \quad (A7) \]

at \(x = p\), where “i” signifies the intracellular side of the patch and “0” signifies the bath. Furthermore, the second integral in Eq. A3 can be written

\[ \int_0^x [B^-] \left( \frac{dV}{dx} \right) dx = \int_{r_0}^{r_x} [B^-] \left( V_x - V_0 \right), \quad (A8) \]

where \(V_0\) is the electrical potential in the bath, \(V_x\) is the electrical potential at \(x\), and \([B^-]\) is a mean concentration in the interval \([0, x]\). Hence, Eq. A3 becomes

\[ [B^-] = [B_{total}]_0 \left[ \alpha_0 + \beta I_{II} + \frac{F [B^-]}{RT [B_{total}]_0} \left( V_p - V_0 \right) \right], \quad (A9) \]
at $x = p$. Substituting Eqs. A7 and A9 into Eq. A4 and solving for $[\text{H}^+]$ gives the proton concentration adjacent to the intracellular side of the patch:

$$[\text{H}^+] = K_{\text{a,i}} \left( \frac{1 - \alpha_0 - \beta I_{\text{HI}}}{\alpha_0 + \beta I_{\text{HI}}} \right) \left[ 1 + \frac{F[B]}{RT[B_{\text{total}}](\alpha_0 + \beta I_{\text{HI}})} \right]^{-1}. \quad \text{(A10)}$$

Removal of protons from the intracellular side ensures that $V_p < V_0$ so that from Eq. A9 $[B^-] < [B_{\text{total}}](\alpha_0 + \beta I_{\text{HI}})$ provided $[B]_0 \leq [B^-] \leq [B^-]$. It then follows that,

$$\left| \frac{F[B]}{RT[B_{\text{total}}](\alpha_0 + \beta I_{\text{HI}})} \right| < \left| \frac{F(V_p - V_0)}{RT} \right| < \left| \frac{FI_{\text{HI}}R_{\text{pip}}}{RT} \right|, \quad \text{(A11)}$$

where $R_{\text{pip}}$ is the pipette resistance. The second inequality in Eq. A11 arises from the fact that $I_B = I_{\text{HI}}$ is driven partly by the gradient of $[B^-]$ (Eq. A1) and so $|V_p - V_0| < |I_{\text{HI}}R_{\text{pip}}|$. (Also the distributed resistance of the pipette between the tip and the patch membrane is less than $R_{\text{pip}}$.) Since $I_{\text{HI}}$ was generally <50 pA and $R_{\text{pip}} < 15 \, \text{M}\Omega$, $F I_{\text{HI}} R_{\text{pip}} / RT < 0.03$. Hence, from Eq. A11, the term in square brackets in Eq. A10 will be close to unity, and so the expression for $[\text{H}^+]$, reduces to

$$[\text{H}^+] = K_{\text{a,i}} \left( \frac{1 - \alpha_0 - \beta I_{\text{HI}}}{\alpha_0 + \beta I_{\text{HI}}} \right). \quad \text{(A12)}$$

Exactly analogous arguments yield the following expression for the proton concentration at the “extracellular” (“o”) side of the membrane patch, where protons accumulate:

$$[\text{H}^+]_o = K_{\text{a,o}} \left( \frac{1 - \alpha_0 + \beta_o I_{\text{HI}}}{\alpha_0 - \beta_o I_{\text{HI}}} \right), \quad \text{(A13)}$$

where

$$\beta_o = \frac{1}{\pi F D_B [B_{\text{total}}]} \cdot \int_0^p \frac{dx}{r^2} = \frac{1}{\pi b F D_B [B_{\text{total}}]} \cdot \frac{1}{r_p} \quad \text{(A14)}$$

and “•” signifies the pipette-stem lumen.

In order to evaluate $\beta_i$ and $\beta_o$ we need an expression for $r_p$. This is obtained by considering the membrane in the pipette shank (i.e., the patch membrane plus the membrane adhering to the glass between $x = 0$ and $x = p$), the area of which must be some fraction ($q < 1$) of the original cell membrane area. Thus, since $b = dr/dx = (r_p - r_o)/p$, we have

$$\pi p (r_p + r_o) + \pi r_p^2 = \pi (r_p - r_o)(r_p + r_o) + \pi r_p^2 = 4q \pi r_c^2, \quad \text{(A15)}$$
whence,

\[ r_p = \sqrt{\frac{4 b q r_c^2 + r_0^2}{1 + b}} \]  

(A16)

where \( r_c \) is the radius of the cell from which the patch was excised.

**Effect of Proton Depletion/accumulation on \( y \)-\( g_H \) plots**

To estimate the effects of proton depletion/accumulation on \( y \)-\( g_H \) plots, the following equations (Eqs. A17 and A18) were solved numerically for \( I_H \) using a modified linear interpolation algorithm (Gerald and Wheatley, 1984):

\[
E_H = \frac{RT}{F} \log \frac{[H^+]_s}{[H^+]_i} = \frac{RT}{F} \log \frac{K_{a,0}}{K_{a,i}} \left( \frac{1 - \alpha_0 + \beta_0 I_H}{1 - \alpha_0 - \beta_0 I_H} \right) \left( \frac{\alpha_0 + \beta_0 I_H}{1 - \alpha_0 - \beta_0 I_H} \right),
\]

(A17)

\[
I_H = \gamma_H NP_{\text{open}} (V - E_H),
\]

(A18)

where \( E_H \) is the Nernst potential for protons. \( \alpha_0 \) was calculated from Eq. A5 with \([H^+] = [H^+]_o\), while \( \beta_0 \) and \( \beta_0 \) were calculated from Eqs. A6, A14, and A16, given assumed values for \( D_B \), \([B_{\text{total}}]\), \( b \), \( q \), \( r_c \) and \( r_0 \) (see below). \( P_{\text{open}}(V) \) was approximated by a Boltzmann function

\[
P_{\text{open}} = \frac{P_{\text{max}}}{1 + e^{-(V - V_{\text{rev}})/k}}.
\]

(A19)

Values of \( P_{\text{max}} \) were taken from Table 1, while values of \( V_{\text{rev}} \) and \( k \) were obtained by fitting the function

\[
I = \frac{g_{H,\text{max}} (V - V_{\text{rev}})}{1 + e^{-(V - V_{\text{rev}})/k}}
\]

(A20)

to stationary \( I-V \) plots, where \( V_{\text{rev}} \) is an estimate of \( E_H \) derived from tail currents (see below). The mean value of \( k \) was 6.85 ± 0.68 mV (\( n = 11 \)). Consistent with previous studies (DeCoursey and Cherny, 1997; DeCoursey, 2003), \( V_{1/2} \) was a linear function of \( V_{\text{rev}} \): \( V_{1/2} = (45.5 \pm 6.0) + (0.840 \pm 0.075) V_{\text{rev}} \). The dependence of \( \gamma_H \) on \([H^+]_i\) was estimated by interpolating the observed \( \gamma_H \) values in Table 1. Other relevant data are as follows: The molecular weights of the buffers were in the range 200-300, and so from Hobbie (1978) the Stokes radius should be around 0.45 nm. The Stokes-Einstein relation then gives \( D_B = 5 \times 10^{-10} \text{ m}^2 \text{ s}^{-1} \). From measurements on seven pipettes, the external shank taper was around 0.1 and the external tip radius was 1 \( \mu \text{m} \). Then assuming that the nominal internal/external diameter ratio of 0.7 is maintained within the shank, we get \( b = 0.07 \) and \( r_0 = 0.7 \mu \text{m} \). Possibly some pipettes had a smaller tip opening, but given an expected pipette
resistance of 1–5 MΩ when filled with 150 mM KCl (Sakmann and Neher, 1983), it seems unlikely that \( r_0 \) was much less than 0.5 µm. For most types of glass the internal/external diameter ratio increases near the tip (Purves, 1981), so if anything \( r_0 \) has been underestimated. Symmetrical buffer concentrations were used so that \([B_{\text{total}}]_0 = [B_{\text{total}}]^* = [B_{\text{total}}] = 100-200\) mM. The cell radius \((r_c)\) was about 4 µm and the fraction of the cell membrane \((q)\) drawn into the pipette was varied from 0.25 to 0.75. This range of values is somewhat arbitrary, but the form of predicted \( y-g_H \) curves does not depend strongly on \( q \). The reason for this is that as the patch approaches the pipette tip (small \( q \)) intracellular H\(^+\) depletion decreases but extracellular accumulation increases. Conversely as the patch moves away from the tip (large \( q \)) intracellular H\(^+\) depletion increases but extracellular accumulation decreases. Hence the ratio \([H^+]_o/[H^+]_i\) (and therefore \(E_H\)) does not vary greatly with the distance of the patch membrane from the pipette tip.

Having determined \( I_H \) for a particular \( V \), \( \sigma_H^2 \) was calculated from the relation,

\[
\sigma_H^2 = \gamma_H (V - E_H) I_H - I_H^2 / N \tag{A21}
\]

(Sigworth, 1980). The generation of \( y-g_H \) plots also requires an estimate \((V_{\text{rev}})\) of \(E_H\) (Eqs. 2 and 3), which is obtained from the analysis of tail currents, where possible (Materials and Methods). \( V_{\text{rev}} \) was calculated assuming a prepulse to 80 mV above the zero-current \(E_H\) (i.e., \(E_H\) in the absence of proton depletion/accumulation). \( I_H \) and \(E_H\) during the prepulse were then obtained by solving Eqs. A17 and A18 as before, except that \( I_H \) was assumed to reach 50–80% of its stationary value at the end of the prepulse (varying \( I_H \) within this range had little effect on \( y-g_H \) plots). The value of \(E_H\) at that time was set equal to \(V_{\text{rev}}\) in Eqs. 4 and 5 in order to obtain values of \( y \) and \( g_H \). This approach assumes the establishment of a near-steady state by the end of the prepulse; the validity of this assumption is discussed below.

Simulated \( y-g_H \) plots for the 7.5//5.5 and 7.5//6.5 pH regimes are shown in Fig. S1. Values of \( N \) were chosen to obtain average and extreme values of \( g_H \) (as determined experimentally). For each \( g_H \) class the solid line represents no H\(^+\) depletion/accumulation, the dashed curve represents the minimum expected depletion/accumulation \(([B_{\text{total}}] = 200\) mM, \(r_0 = 0.7\) µm, \(q = 0.25\), prepulse \(I_H\) to 50% of its stationary value), and the dashed-dotted curve represents the maximum expected depletion/accumulation \(([B_{\text{total}}] = 100\) mM, \(r_0 = 0.5\) µm, \(q = 0.75\), prepulse \(I_H\) to 80% of its stationary value). The plots are nearly linear over most of the range of \( g_H \) values; certainly the predicted deviations from linearity could not be detected in the experimental data given its scatter.
To quantify the errors in estimates of \( \gamma_H \) and \( N \), regression lines were fitted to the middle one-third of the \( y-g_H \) curves. \( \gamma_H \) was then estimated as the \( y \)-intercept and \( N \) as \(-1/slope\) of the fitted lines. For average values of \( g_H \), \( \gamma_H \) was overestimated by 1-4\% while \( N \) was underestimated by 2-10\%. For extreme values of \( g_H \), \( \gamma_H \) was overestimated by 2-8\% while \( N \) was underestimated by 6-20\%. Similar results were obtained for regimes 7.5//5.0 and 6.5//6.5 (not depicted). Hence, the analysis of simulated \( y-g_H \) plots suggests that the errors in \( \gamma_H \) and \( N \) due to proton depletion/accumulation are relatively small and essentially independent of \( \text{pH}_i \) and \( \text{pH}_o \).

**Rate of Establishment of a Steady-state during \( I_H \) Activation**

Some idea of the time required to establish a steady \( \text{pH} \) at the patch can be obtained by modeling the pipette-shank lumen for \( 0 < x < p \) as a well-stirred compartment of volume \( V \), separated from the bath by a diffusive resistance of effective area \( \pi r_0^2 \) and permeability \( P_s \). The time constant (\( \tau_s \)) for the establishment of a steady state is then:

\[
\tau_s = \frac{V}{\pi r_0^2 P_s}.
\]  

(A22)

Substituting Eq. A6 into A7 and solving for \( I_H/F \) suggests taking

\[
\pi r_0^2 P_s > \pi b D_B \left( \frac{r_0 r_p}{r_p - r_0} \right).
\]  

(A23)

Furthermore,

\[
V < \frac{\pi p}{3} \left( r_0^2 + r_p^2 + r_0 r_p \right) = \frac{\pi (r_p - r_0)}{3b} \left( r_0^2 + r_p^2 + r_0 r_p \right).
\]  

(A24)

Hence given Eq. A16 for \( r_p \), \( \tau_s \) can be expressed as a function of \( D_B = 5\times10^{-10} \text{ m}^2 \text{ s}^{-1} \), \( b = 0.07 \), \( r_0 = 0.5-0.7 \text{ \mu m} \), \( q = 0.25-0.75 \) and \( r_c = 4 \text{ \mu m} \), giving \( \tau_s = 0.1-1.2 \text{ s} \). A small transient “droop” in \( I_H \) was sometimes observed during the first few seconds of depolarizing pulses that elicited large currents at \( \text{pH}_i = 5.0 \) (Fig. 2, top panel). Although this calculation is somewhat crude, it seems clear that proton flux–induced \( \text{pH} \) changes were close to a steady state in inside-out patches during noise measurements (\( \geq 12 \text{ s} \) records). Assumption of a steady state will lead to overestimation of the errors in \( \gamma_H \) and \( N \) in simulated \( y-g_H \) plots. That this is a small effect can be appreciated from the fact that if proton depletion/accumulation during the prepulse is ignored, the \( y-g_H \) curves intercept the \( y \) axis at the true value of \( \gamma_H \) (reducing an already small error to zero), but with little change in slope (and hence little change in the estimate of \( N \)).
References


