Deuterium Isotope Effects on Permeation and Gating of Proton Channels in Rat Alveolar Epithelium

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Abstract: The voltage-activated H\(^+\) selective conductance of rat alveolar epithelial cells was studied using whole-cell and excised-patch voltage-clamp techniques. The effects of substituting deuterium oxide, D\(_2\)O, for water, H\(_2\)O, on both the conductance and the pH dependence of gating were explored. D\(^+\) was able to permeate proton channels, but with a conductance only about 50% that of H\(^+\). The conductance in D\(_2\)O was reduced more than could be accounted for by bulk solvent isotope effects (i.e., the lower mobility of D\(^+\) than H\(^+\)), suggesting that D\(^+\) interacts specifically with the channel during permeation. Evidently the H\(^+\) or D\(^+\) current is not diffusion limited, and the H\(^+\) channel does not behave like a water-filled pore. This result indirectly strengthens the hypothesis that H\(^+\) (or D\(^+\)) and not OH\(^-\) is the ionic species carrying current. The voltage dependence of H\(^+\) channel gating characteristically is sensitive to pH\(_i\) and pH\(_o\) and was regulated by pD\(_o\) and pD\(_i\) in an analogous manner, shifting 40 mV/U change in the pD gradient. The time constant of H\(^+\) current activation was about three times slower (\(\tau_{act}\) was larger) in D\(_2\)O than in H\(_2\)O. The size of the isotope effect is consistent with deuterium isotope effects for proton abstraction reactions, suggesting that H\(^+\) channel activation requires deprotonation of the channel. In contrast, deactivation (\(\tau_{deact}\)) was slowed only by a factor \(<1.5\) in D\(_2\)O. The results are interpreted within the context of a model for the regulation of H\(^+\) channel gating by mutually exclusive protonation at internal and external sites (Cherny, V.V., V.S. Markin, and T.E. DeCoursey. 1995. J. Gen. Physiol. 105:861–896). Most of the kinetic effects of D\(_2\)O can be explained if the pK\(_i\) of the external regulatory site is ~0.5 pH U higher in D\(_2\)O.

Key words: ion channels • proton transport • pH • pneumocytes • membrane transport

Introduction

Voltage-gated H\(^+\) channels conduct H\(^+\) current with extremely high selectivity and exhibit voltage-dependent gating that is strongly modulated by both extracellular and intracellular pH (pH\(_i\) and pH\(_o\), respectively). Here we explore the effects of substituting heavy water (deuterium oxide, D\(_2\)O), for water (protium oxide, H\(_2\)O), on both the conductance and the pH dependence of channel gating. The isotope effect on conductance should provide insight into the mechanism by which permeation occurs. Isotope effects on the regulation by pH (or pD) of the voltage dependence and kinetics of gating provide clues to the possible protonation/deprotonation reactions that have been proposed to play a role in channel gating (Byerly et al., 1984; Cherny et al., 1995).

Several chemical properties of D\(_2\)O and H\(_2\)O are compared in Table I. From the perspective of this study, the main differences between D\(_2\)O and H\(_2\)O are: (a) the viscosity of D\(_2\)O is 25% greater than H\(_2\)O, (b) the conductivity of H\(^+\) in H\(_2\)O is 1.4–1.5 times that of D\(^+\) in D\(_2\)O, (c) H\(^+\) has a much greater tendency than D\(^+\) to tunnel, (d) D\(^+\) weights twice as much as H\(^+\), and (e) D\(^+\) is bound more tightly in D\(_2\)O\(^+\) and in many other compounds than is H\(^+\). Three main types of deuterium isotope effects are recognized: general solvent effects and primary and secondary kinetic effects. General solvent isotope effects reflect the different properties of D\(_2\)O and H\(_2\)O as solvents, such as viscosity or dielectric constant. As seen in Table I, these differences are rather moderate, and their effects are accordingly usually moderate as well. Kinetic isotope effects reflect involvement of protons or deuterons in chemical reactions. Primary kinetic isotope effects occur when H\(^+\) directly participates in a rate-determining step in the re-
action, for example a protonation/deprotonation or $H^+$ transfer reaction. For example, ionization of a number of bases is typically three to seven times slower in $D_2O$ (Bell, 1973). Secondary isotope effects reflect $D^+$ for $H^+$ substitution at some site distinct from the primary reaction center. Secondary kinetic isotope effects tend to be small, 1.02–1.40 (Kirsch, 1977).

The conductivity of $H^+$ is about five times higher than that of other cations with ionic radii like that of $NH_4^+$ (Robinson and Stokes, 1965). This anomalously high conductivity for $H^+$ has been ascribed to conduction by a mechanism in which $H^+$ jumps from $H_2O^+$ to a neighboring water molecule (Danneel, 1905; Hückel, 1928; Bernal and Fowler, 1933; Conway et al., 1956). $H^+$ hopping can occur faster than ordinary hydrodynamic diffusion (i.e., bodily movement of an individual $H_2O^+$ molecule analogous to the diffusion of ordinary ions). After one $H^+$ conduction event, a structural reorientation of the hydrogen-bonded water lattice is necessary before another proton can be conducted (Danneel, 1905; Bernal and Fowler, 1933; Conway et al., 1956). Proton conduction through channels is believed to occur by an analogous two-step “hop-turn” process through a hydrogen-bonded chain or “proton wire” spanning the membrane (Nagle and Morowitz, 1978; Nagle and Tristram-Nagle, 1983).

The mobility (measured as conductivity) of $H^+$ in $H_2O$ is 1.41 times that of $D^+$ in $D_2O$ (Table I); nevertheless, the mobility of $D^+$ is still 4 times that of $K^+$ in $D_2O$ (Lewis and Doody, 1933). Thus, $D^+$ also exhibits abnormally large conductivity, even though tunnel transfer of $D^+$ is 20 times less likely than for $H^+$ and one might have expected simple hydrodynamic diffusion of $D_2O^+$ to play a larger role for $D^+$, which would accordingly have a conductivity similar to that of other cations (Bernal and Fowler, 1933). Evidently the reorientation of hydrogen-bonded water molecules (the turning step of a hop-turn mechanism) is rate limiting for both $H^+$ and $D^+$ conduction. The nature of this rate-determining step has been proposed to be the reorientation of hydrogen-bonded water molecules in the field of the $H_2O^+$ ion (Conway et al., 1956), “structural diffusion” or formation and decomposition of hydrogen bonds at the edge of the $H_2O_4^+$ complex (i.e., the hydronium ion with its first hydration shell) (Eigen and DeMaeyer, 1958), or more recently, the breaking of an ordinary second-shell hydrogen bond converting $H_2O_4^+$ to $H_2O_2^+$ (Agnon, 1995, 1996). Some such reorganization of hydrogen bonds may also be the rate limiting step in proton translocation across water-filled ion channels such as gramicidin (Pomès and Roux, 1996).

A characteristic feature of voltage-gated $H^+$ currents is their sensitivity to both $pH_o$ and $pH_i$. Increasing $pH_o$ and decreasing $pH_i$ shift the voltage-activation curve to more negative potentials in every cell in which these parameters have been studied (reviewed by DeCoursey and Cherny, 1994). This effect of $pH$ is reminiscent of its effects on many other ion channels, which may reflect the neutralization of negative surface charges (see Hille, 1992). However, the magnitude of the $pH$-induced voltage shifts for $H^+$ currents has led to the suggestion that protonation of specific sites on or near the channel allosterically modulate gating (Byerly et al., 1984). In alveolar epithelial cells (Cherny et al., 1995), as well as in other cells (DeCoursey and Cherny, 1996a; Cherny et al., 1997), the shift produced by internal and external protons ($H^+_i$ and $H^+_e$) is quite similar, 40 mV/U change in $\Delta pH$, within a large $pH$ range encompassing physiological values. Thus the position of the voltage activation curve can be predicted from the $pH$ gradient, $\Delta pH$, rather than by $pH_o$ and $pH_i$ independently. This behavior was explained by a model (Cherny et al., 1995) in which there exist similar protonation sites accessible from either the internal or external solution, but not both simultaneously. Protonation from the outside stabilizes the closed channel, whereas protonation from the inside stabilizes the open channel. Here we show that $H^+$ channels are regulated in a similar manner by $D^+$, but that $D^+$ binds more tightly to the modulatory sites on the channel molecule.

We follow Bell’s (1973) rationale for expressing isotope effects as a ratio that increases as the magnitude of the isotope effect increases. The ratio of the parameter value in $D_2O$ to that in $H_2O$ is given, but the inverse ratio is given when this results in a ratio $>1.0$. Note that the mobility values reflect measurements of the mobility of $H^+$ (or $D^+$) in the solution, and that the conductivity of a salt solution in $H_2O$ compared with $D_2O$ will differ by a smaller ratio (e.g., 1.17 for KCl, Lewis and Doody, 1933) because the salt will short-circuit the conductivity due to $H^+$ or $D^+$ which are present at much lower concentrations. Technical details of the two mobility estimates are discussed on p. 369 of Lengyel and Conway (1983). *In- terpolated value. ‡At 25°C. §The ratio given for the dielectric relaxation time, 1.28, is based on the actual measurements of Collie et al. (1948) at 29°C, rather than the “smoothed value” (1.27) given in their Table 6.

<table>
<thead>
<tr>
<th>Property</th>
<th>$D_2O$/$H_2O$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity (shear)</td>
<td>1.245</td>
<td>Hardy and Cottingham, 1949</td>
</tr>
<tr>
<td>Viscosity (volume)</td>
<td>1.09</td>
<td>Jarzynski and Davis, 1972</td>
</tr>
<tr>
<td>Mobility (conductance)</td>
<td>1.41–1.44</td>
<td>Lewis and Doody, 1933</td>
</tr>
<tr>
<td>Dielectric constant</td>
<td>1.005</td>
<td>Schowen, 1977</td>
</tr>
<tr>
<td>Dielectric relaxation</td>
<td>1.28, 1.29</td>
<td>Grant and Shack, 1969</td>
</tr>
<tr>
<td>Density</td>
<td>1.108</td>
<td>Schowen, 1977</td>
</tr>
<tr>
<td>Vapor pressure</td>
<td>1.15–1</td>
<td>Brooks, 1937</td>
</tr>
</tbody>
</table>

We follow Bell’s (1973) rationale for expressing isotope effects as a ratio that increases as the magnitude of the isotope effect increases. The ratio of the parameter value in $D_2O$ to that in $H_2O$ is given, but the inverse ratio is given when this results in a ratio $>1.0$. Note that the mobility values reflect measurements of the mobility of $H^+$ (or $D^+$) in the solution, and that the conductivity of a salt solution in $H_2O$ compared with $D_2O$ will differ by a smaller ratio (e.g., 1.17 for KCl, Lewis and Doody, 1933) because the salt will short-circuit the conductivity due to $H^+$ or $D^+$ which are present at much lower concentrations. Technical details of the two mobility estimates are discussed on p. 369 of Lengyel and Conway (1983). *Interpolated value. ‡At 25°C. §The ratio given for the dielectric relaxation time, 1.28, is based on the actual measurements of Collie et al. (1948) at 29°C, rather than the “smoothed value” (1.27) given in their Table 6.
Materials and Methods

Alveolar Epithelial Cells

Type II alveolar epithelial cells were isolated from adult male Sprague-Dawley rats under sodium pentobarbital anesthesia using enzyme digestion, lectin agglutination, and differential adherence, as described in detail elsewhere (DeCoursey et al., 1988; DeCoursey, 1990). Briefly, the lungs were lavaged to remove macrophages, elastase and trypsin were instilled, and then the tissue was minced and forced through fine mesh. Lectin agglutination and differential adherence further removed contaminating cell types. The preparation at first includes mainly type II alveolar epithelial cells, but after several days in culture, the properties of the cells become more like type I cells. No obvious changes in the properties of H+ currents have been observed. H+ currents were studied in approximately spherical cells up to several weeks after isolation.

Solutions

Most solutions (both external and internal) contained 1 mM EGTA, 2 mM MgCl₂, 100 mM buffer, and TMA(MeSO₃) added to bring the osmolarity to ~300 mosM, and titrated to the desired pH with tetramethylammonium hydroxide or methanesulfonic acid (solutions using BisTris as a buffer). The pH 8, 9, and 10 solutions contained 3 mM CaCl₂ instead of MgCl₂. A stock solution of TMAMeSO₃ was made by neutralizing tetramethylammonium hydroxide with methanesulfonic acid. Buffers (Sigma Chemical Co., St. Louis, MO), which were used near their pK in the following solutions, were: pH 5.5, pD 6.0 Mes; pH 6.5, pD 7.0 Bis-Tris (bis[2-hydroxyethyl][iminio-tris[hydroxymethyl]methane); pH 7.0, pD 7.0 BES (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid); pH 7.5, pD 8.0 HEPES; pH 8.0 Tricine (N-tris[hydroxymethyl]methylglycine); pH 9.0, pD 9.0 CHES (2-[N-cyclohexylamino]ethanesulfonic acid); pH 10, pD 10 CAPS (3-[cyclohexylamino]-1-propanesulfonic acid). The pH (or pD) of all solutions was checked frequently.

A series of solutions containing NH₄⁺ was made to impose a defined pH gradient across the cell membrane, as described by Grinstein et al. (1994). The principle is that if neutral NH₃ molecules permeate the membrane rapidly enough to approach identical concentrations on both sides of the membrane, then:

\[ \text{pH}_i = \text{pH}_o - \log \left( \frac{[\text{NH}_4^+]_i}{[\text{NH}_4^+]_o} \right) \]  

because the bath solution is heavily buffered (100 mM buffer) and diffuses freely but the pipette solution (for these measurements) is weakly buffered and diffusion is slowed by the pipette tip. The shift of pH occurs because [H⁺] = \( pK_a - \log \left( \frac{[\text{NH}_4^+]_i}{[\text{NH}_3]_i} \right) \). The extracellular solutions were made with 100 mM HEPES, 2 mM MgCl₂, 1 mM EGTA, and various concentrations of (NH₄)₂SO₄ at pH 7.5. TMAMeSO₃ was added to bring the osmolarity to ~300 mosM. The pipette solution, which was also used externally, included 25 mM (NH₄)₂SO₄, 5 mM BES, 2 mM MgCl₂, 1 mM EGTA, and TMAMeSO₃, brought to pH 7.0 with tetramethylammonium hydroxide.

We assume that when NH₄⁺ diffuses from the pipette into the cell, if D₂O is present in the bath (and hence inside the cell) there will be rapid exchange of D⁺ for H⁺ in NH₄⁺, and that therefore efflux of ND₃ will occur, leaving D⁺ rather than H⁺ behind inside the cell. Deuterons in deuterio-ammonia, ND₃, exchange rapidly with protons (Cross and Leighton, 1958).

The osmolarity of the solutions was measured with a Wescor 5500 Vapor Pressure Osmometer (Wescor, Logan, UT). Deuterium oxide (99.8% or 99.9%) was purchased from Sigma Chemical Co. A liquid junction potential of ~2 mV was measured between solutions identical except that D₂O replaced H₂O. If water did not permeate the cell membrane, correction for this junction potential would make the transmembrane potential 2 mV more negative. However, as described in Fig. 1, we feel that water permeates the cell membrane, and thus there would be offsetting junction potentials at the pipette tip and bath electrode even in whole cell configuration. Therefore no junction potential correction has been applied.

pD Measurement

The reading taken from a glass pH electrode, pHnom, deviates from the true pD of D₂O solutions by 0.40 U, such that pD = pHnom + 0.40 (Glasoe and Long, 1960). Another estimate of this difference is 0.43 ± 0.03 (Dean, 1985), and even more disparate values can be found in early studies. Given the uncertainty about the precise value, we tested our pH meter (Radiometer Ion83 Ion meter; Radiometer, Copenhagen, Denmark) following the approach taken by Glasoe and Long (1960). Our pH meter read 0.402 ± 0.006 (mean ± SD, n = 3) higher when 0.01 M HCl was added to H₂O than when added to D₂O. We therefore corrected the pD in D₂O solutions by adding 0.40 to the nominal reading of our pH meter.

Estimation of the pKₐ of the Buffers in H₂O and in D₂O

Most simple carboxylic and ammonium acids with pKₐ between 4 and 10 have a pKₐ 0.5–0.6 U higher in D₂O than in H₂O (Schonen, 1977). We titrated the buffers used in this study at room temperature (20–25°C). 10 mmol of buffer was added to 20 ml of H₂O or D₂O and titrated with 10 N NaOH, or 10 N HCl in the case of Bis-Tris. The resulting contamination of D₂O by the H⁺ from the base or acid titrating solutions is <3%. We corrected for this error in two ways. First, we increased the apparent change in pKₐ assuming a linear mole-fraction dependence (cf. Glasoe and Long, 1960), which increased the pKₐ in D₂O by ≤0.02 U. We also carried out some titrations using deuterated acids and bases (DCl and NaOD, both from Aldrich Chemical Co, Milwaukee, WI). The results by these two methods were similar. The averages of two to three separate determinations for each buffer are given in Table II.

Electrophysiology

Conventional whole-cell, cell-attached patch, or excised inside-out patch configurations were 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. 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 3–10 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 us-
<table>
<thead>
<tr>
<th>Buffer</th>
<th>( pK_a ) (H\textsubscript{2}O)</th>
<th>( pK_a ) (D\textsubscript{2}O)</th>
<th>( pK_{aD})/( pK_{aH} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mes</td>
<td>6.15*</td>
<td>6.15</td>
<td>--</td>
</tr>
<tr>
<td>BisTris</td>
<td>6.50*</td>
<td>6.57</td>
<td>--</td>
</tr>
<tr>
<td>BES</td>
<td>7.15*</td>
<td>7.10</td>
<td>--</td>
</tr>
<tr>
<td>HEPES</td>
<td>7.55*</td>
<td>7.57</td>
<td>8.14\dagger</td>
</tr>
<tr>
<td>Tricine</td>
<td>8.15*</td>
<td>8.15</td>
<td>--</td>
</tr>
<tr>
<td>CHES</td>
<td>9.30*</td>
<td>9.34</td>
<td>--</td>
</tr>
<tr>
<td>CAPS</td>
<td>10.40\dagger</td>
<td>10.47</td>
<td>--</td>
</tr>
</tbody>
</table>

Average \( pK_a \) values are for 2-3 paired measurements (H\textsubscript{2}O and D\textsubscript{2}O done the same day) for each buffer. See Materials and Methods for details of titrations and corrections applied (e.g., \( pK_a \) values in D\textsubscript{2}O were corrected by adding 0.4 to the value read on the pH meter). Literature values and measurements are at room temperature (\( \sim \)25°C) except Sigma values are at 25°C. The titration was less accurate at high pH, so the values obtained for CAPS in particular should be considered tentative. The average of all individual \( pK_{aD}/pK_{aH} \) values in 0.66 ± 0.05 (mean ± SD, n = 17) or excluding the doubtful CAPS data, 0.67 ± 0.04 (n = 14). *Good et al. (1966). ‡Sigma Chemical Company. §Roth and MacKinnon (1994).

where \( I_0 \) is the amplitude of the decaying part of the tail current, or to the sum of two exponentials:

\[
I(t) = A_1 \exp \left( -\frac{t}{\tau_1} \right) + A_2 \exp \left( -\frac{t}{\tau_2} \right),
\]

where \( A_n \) are amplitudes and \( \tau_n \) are time constants.

**Conventions**

We refer to the pL in the format pL\textsubscript{w}//pL\textsubscript{b}. In the inside-out patch configuration the solution in the pipette sets pL\textsubscript{w}, which is defined as the pL of the solution bathing the original extracellular surface of the membrane, and the bath solution is considered pL\textsubscript{b}. 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. Current records are presented without correction for leak current or liquid junction potentials.

As discussed in detail in Strategic Considerations and in Fig. 1, when the bath solvent differs from that in the pipette, the effective pH\textsubscript{i} (or pD\textsubscript{i}) will differ from the nominal value of the pipette solution by \( \sim \)0.5 U. Therefore, when bath and pipette solvents differ, we provide values for the presumed effective internal H\textsuperscript{+} or D\textsuperscript{+} concentration, e.g., pH\textsubscript{eff} 6.5 indicates a pD 7.0 pipette solution in any H\textsubscript{2}O solution in the bath. The majority of experiments were done with D\textsubscript{2}O rather than with H\textsubscript{2}O pipette solutions because we wanted the measurements in D\textsubscript{2}O to be contaminated as little as possible by H\textsubscript{2}O.

**RESULTS**

**Strategic Considerations**

The nature of the problem under investigation introduces several complications, which require explanation, as well as a perhaps less-than-obvious approach. Ideally we would like to compare the behavior of the proton conductance in the same cell under identical conditions while varying only the solvent (D\textsubscript{2}O or H\textsubscript{2}O) on one side of the membrane and keeping pL\textsubscript{w} and pL\textsubscript{b} constant (pL\textsubscript{w} refers to either pH\textsubscript{i} or pD\textsubscript{i}). However, the high membrane permeability of water means that only symmetrical solvent studies can be contemplated. Less obviously, due to the increased \( pK_a \) of buffer in D\textsubscript{2}O (Table II), it is impossible to compare directly in the same cell identical pH\textsubscript{i} and pD\textsubscript{i} by simply changing the external solvent, without at the same time changing pL\textsubscript{w}. However, it is desirable to make comparisons in the same cell, because H\textsuperscript{+} currents vary substantially from cell to cell. We therefore adopted two strategies. First, we compare currents measured with the same pH or pD gradient (e.g., pH 6.5//pH 6.5 and pD\textsubscript{i} 7.0//pD\textsubscript{i} 7.0), because the gradient, ΔpH, appears to be a fundamental determinant of H\textsuperscript{+} channel gating (Cherny et al., 1995). This approach has the drawback of comparing the effects of different absolute concentrations of protons and deuterons, and there is some indication that H\textsuperscript{+} channel gating kinetics depend on the absolute pH\textsubscript{i}, rather than ΔpH alone (DeCoursey and Cherny, 1995). The second approach (see MATERIALS...
AND METHODS) overcomes this shortcoming by controlling pH, by applying a known NH4+ gradient (Roos and Boron, 1981), as illustrated by Grinstein et al. (1994). Varying the NH4+ gradient allows resetting pH (or pD) in cell under whole-cell voltage-clamp, and ideally, comparison of currents at the same pH and pD.

Only symmetrical solvent is possible. In these experiments we varied the solvent in the pipette and bath solutions. Because water has a high membrane permeability, it seemed likely that the solvent in the bath solution would enter the cell much faster than solvent would diffuse from the pipette, and thus the solvent in the bath would also be present in the cell, regardless of the pipette solution. This expectation was tested theoretically and experimentally.

• How fast does water enter the cell? A critical question in the interpretation of the data is whether solvent in the bath diffuses across the cell membrane fast enough to dominate the intracellular solution in spite of the presence of the pipette tip which is a continuous source of solvent from the pipette solution. The water permeability, $P_{\text{osm}}$, of planar lipid bilayers or liposomes ranges from $10^{-4}$ cm/s to $10^{-2}$ cm/s; $P_{\text{osm}}$ in various epithelial cell membranes similarly ranges from $10^{-4}$ cm/s to $10^{-2}$ cm/s (Tripathi and Boulpaep, 1989). Because both HgCl2-sensitive and HgCl2-insensitive water channels occur in lung tissue (Folkesson et al., 1994; Hasegawa et al., 1994), it is likely that $P_{\text{osm}}$ is relatively high in alveolar epithelial cells, at least in situ. Osmotic water permeability ($P_w$) is $1.7 \pm 10^{-2}$ cm/s and diffusional water permeability, $P_d$, is $1.5 \pm 10^{-5}$ cm/s across the alveoli of intact mouse lung (Carter et al., 1996). However, $P_w$ was probably grossly underestimated because of unstirred layer effects (Finkelstein, 1984; Carter et al., 1996). We calculated the steady-state distribution of normal or heavy water when one species was in the pipette solution and the other in the bath solution. The compartmental diffusion model used has been described in detail previously (DeCoursey, 1995), and simplifies the calculation by placing the pipette tip at the center of a spherical cell. The diffusion coefficient of H2O was taken as $2.1 \times 10^{-5}$ cm$^2$/s (Robinson and Strokes, 1965), the pipette tip was assumed to have a diameter of 1.0 μm, the cell diameter was 20 μm, and we assume that D2O and H2O have similar membrane permeabilities (Perkins and Cafiso, 1986; Deamer, 1987; Gutknecht, 1987). A range of $P_{\text{osm}}$ was assumed. For $P_{\text{osm}} > 10^{-3}$ cm/s the membrane presented essentially no barrier to diffusion, and the solvent in the bath was the main solvent inside the cell. Nevertheless, because the pipette is a constant source, there is always a finite concentration of the pipette solvent. For the pipette tip at the center of a 20 μm diameter cell, the limiting submembrane concentration at infinite $P_{\text{osm}}$ is $\sim$2% due to that in the pipette. Lowering $P_{\text{osm}}$ to $10^{-4}$ cm/s caused the membrane to become a significant diffusion barrier, with the steady-state concentration of solvent near the inside of the membrane 24% due to the pipette and 76% due to the bath. The fraction of solvent near the membrane originating in the pipette would be larger in a smaller cell but would be smaller if the pipette tip diameter were smaller. In conclusion, the pipette solvent is present in the cell at significant levels only for a quite conservative estimate of $P_{\text{osm}}$, and in all likelihood the solvent in the bath permeates the membrane rapidly enough that most of the solvent near the membrane originated in the bath. We therefore assume that the membrane is exposed to nearly symmetrical solvent, with a finite but small contribution from the pipette.

• What is the pL (pH or pD) inside the cell? The actual pL, can be deduced from knowledge of pL, and the reversal potential, $V_{\text{rev}}$. In the experiment illustrated in Fig. 1, the pipette contained pD 7.0 solution, and the tail current reversal potential, $V_{\text{rev}}$, was measured in several different bath solutions. $V_{\text{rev}}$ was near 0 mV when the bath contained pD 7.0 (Fig. 1A) or pH 6.5 (Fig. 1C), and was $-27$ mV at pH 7.0 (Fig. 1B). In eight cells, $V_{\text{rev}}$ was $29.9 \pm 4.5$ mV (mean $\pm$ SD) more negative at pH 7.0 than at pH 7.0, both with pD 7.0. Reversal near 0 mV is expected for symmetrical pH 7.0/7.0. Why was $V_{\text{rev}}$ near 0 mV at pH 7.0, 6.5 but not at pH 7.0, under nominally symmetrical bi-ionic conditions? The explanation arises from the fact that many molecules bind D+ more tightly than H+. Most simple carboxylic and ammonium acids with pKa, between 4 and 10, including buffers, have a pK between 0.5–0.6 U higher in D2O than in H2O (Schowen, 1977). We confirmed this generalization by titrating the buffers used in this study in both H2O and D2O and found pKa shifting 0.60–0.69 U (Table II). Fig. 1D illustrates diagrammatically the effect of this pKa difference on a cell studied in the whole-cell configuration. The cell nominally contains the pipette solution with its buffer titrated to some pH or pD, in this example pD 7.0. If the solvent in the bath differs from that in the pipette, the bath solvent will replace the pipette solvent inside the cell, as discussed above. Because H+ has a lower affinity for buffer than does D+, fewer H+ will be bound to buffer than were D+, and hence the actual pH will be lower by $\sim$0.5 U than was the pD of the pipette solution. This is true regardless of the actual value of $p\text{H}_{\text{p}}$ because it results from the solvent dependence of the pKa of the buffer. The chart in Fig. 1 summarizes the experiment illustrated. Given the bath and pipette solutions, the observed $V_{\text{rev}}$ agrees well with $E_{\text{rev}}$ calculated with the assumptions that (a) the solvent in the bath completely replaces that in the cell, and (b) the effective pH will be $\sim$0.5 U lower than pH in the pipette when H2O replaces D2O in the bath. By similar logic, when H2O is in
the pipette solution and D$_2$O is in the bath, the actual pD$_i$ will be $\sim$0.5 U higher than pH$_i$ with H$_2$O in the bath.

- $V_{rev}$ measurements are consistent with high water permeability and the 0.5 U pK$_a$ correction for intracellular buffer in D$_2$O. We proposed above that the bath solvent will “fill” the cell regardless of the pipette solvent and that when the bath solvent differs from that in the pipette, pL$_i$ will change by $\sim$0.5 U from its nominal value. To a first approximation these assumptions seem reasonable, but two possible sources of error should be considered. First, some finite fraction of solvent in the cell is derived from the pipette. We could not determine from

<table>
<thead>
<tr>
<th>BATH</th>
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<th>PIPETTE</th>
</tr>
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<tbody>
<tr>
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<td>pL</td>
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</tr>
<tr>
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<tr>
<td>B</td>
<td>H$_2$O</td>
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</tr>
<tr>
<td>C</td>
<td>H$_2$O</td>
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Figure 1. The solvent in the bath replaces the solvent in the cell. Measurement of the tail current reversal potential, $V_{rev}$, is illustrated in a cell studied with a pipette solution containing D$_2$O at pH 7.0. The bath solution was D$_2$O at pH 7.0 (A), or H$_2$O at pH 7.0 (B) or pH 6.5 (C). (D) Schematic diagram of a didactic experiment in which the pipette solution is D$_2$O at pH 7.0 and the bath contains H$_2$O at pH 7.0 (or pH 6.5). The solvent in the bath permeates the membrane and fills the cell faster than the pipette solvent diffuses into the cell. Buffered pipette solution enters the cell, but H$_2$O replaces D$_2$O, and the effective pH$_i$ is 0.5 U lower than was the original pD$_i$ of the pipette solution. In the table the composition of the bath and pipette solutions, and the presumed effective composition of the solution in the cell is given, and a comparison of observed $V_{rev}$ values with the Nernst potential, $E_L$, was calculated assuming that the bath solvent fills the cell and that the pK$_a$ of all buffers is 0.5 U higher in D$_2$O than in H$_2$O (see text for details). (E) Instantaneous current-voltage relationships for the measurements in parts A (■), B (○), and C (▼). The amplitude of a single exponential fitted by eye to the tail current at each voltage is plotted. $V_{rev}$ was determined by interpolation.
our data the extent of this “contamination.” Second, we assume that the buffer $pK_a$ increases exactly 0.5 U when D$_2$O replaces H$_2$O, although the true change may be slightly higher and may differ for different buffers. Our titration of several buffers used (Table II) revealed an average $pK_a$ shift of 0.67 U in D$_2$O. To test the adequacy of our approximation of a 0.5 U shift, we compared the value for $V_{rev}$ measured in the same cell in D$_2$O and in H$_2$O at 0.5 U lower $pL_e$. The difference in $V_{rev}$ averaged 2.9 ± 0.7 mV (mean ± SEM, $n = 21$) for $pD_o$ 6.0–$pH_i$ 5.5, $pD_o$ 7.0–$pH_i$ 6.5, and $pD_o$ 8.0–$pH_i$ 7.5. We could not detect any significant difference between buffers in this respect. By this measure the actual $pH_i$ may be ~0.05 U more acidic than our assumed value, i.e., $pH_i$ may be 0.55 U lower than $pD_o$. However, considering that the slope of the $V_{rev}$ vs. $pD$ relationship in water was 52.4 mV (Cherny et al., 1995) compared with 58.2 mV for $E_{hi}$, possibly indicating a ~10% attenuation of the $pD$ applied across the membrane, one might suggest that the change in buffer $pK_a$ should also be attenuated by 10% for internal consistency.

A complementary comparison can be made between $V_{rev}$ measured in the same bath solution, but with H$_2$O or D$_2$O in the pipette solution. At $pD_o$ 7, $V_{rev}$ averaged +4.5 ± 1.2 mV (mean ± SEM, $n = 4$) with $pH_i$ 6.5 and +4.3 ± 0.8 mV ($n = 12$) with $pD_i$ 7. At $pH_i$ 6.5, $V_{rev}$ averaged +2.0 ± 1.6 mV ($n = 4$) with $pH_i$ 6.5, and +0.5 ± 1.1 mV ($n = 10$) with $pD_i$. Thus, no systematic difference was observed in $V_{rev}$ with D$_2$O or H$_2$O in the pipette. Together these data support the validity of the assumptions used to interpret these experiments.

Reversal Potential of D$^+$ Currents

Values of $V_{rev}$ obtained from tail current measurements, such as those illustrated in Fig. 1, A–C, in bilateral D$_2$O are plotted as a function of the $pD$ gradient in Fig. 2. In most experiments, $V_{rev}$ was slightly positive to the calculated Nernst potential for D$^+$, $E_{hi}$ (dark line), reminiscent of the small positive deviations of $V_{rev}$ from $E_{hi}$ reported in most studies of H$^+$ currents. Most of the data points for each $pD_i$ parallel $E_{hi}$, clearly establishing the selectivity of this conductance for D$^+$. The largest deviation occurred at $pD_o$ 10//pD_i 8. Parallel experiments in H$_2$O solutions (not shown) produced a similar but more exaggerated result—$V_{rev}$ followed $E_{hi}$ closely up to $pH_i$ 8, with a smaller shift at $pH_i$ 9, and no further shift at $pH_i$ 10. The simplest interpretation of this result is that at high $pH_i$ there is a loss of control over $pH_i$.

A more traditional but less accurate interpretation of the deviations of $V_{rev}$ from $E_{hi}$ is that the selectivity of the conductance for D$^+$ is not absolute, and that at high $pL$ the permeability to some other ion (e.g., TMA$^+$) is increased. However, the observed deviations are not consistent with a constant permeability of TMA$^+$ relative to D$^+$, because they were roughly the same at a given $pD$ gradient, $pD_i$, at various absolute $pD$. Thus, the ratio $P_{TMA^+}/P_D$ calculated using the GHK voltage equation was $2 \times 10^{-7}$, $2 \times 10^{-8}$, and $5 \times 10^{-9}$ at $pD_i$ 6, $pD_i$ 7, or $pD_i$ 8, respectively, all at $pD$ = 2.0. Barring a bizarrely concentration-dependent permeability ratio, it appears that the conductance is extremely selective for D$^+$ (or H$^+$), with a relative permeability >$10^8$ greater for D$^+$ than for TMA$^+$.

Behavior of the Proton Conductance in D$_2$O

Effects of changes in $pD_o$. After complete replacement of water with heavy water, D$^+$ currents behaved qualitatively like H$^+$ currents in normal water. Typical families of currents are illustrated in Fig. 3, with $pD_i$ 6 and $pD_o$ 8, 7, or 6. At relatively negative potentials only a small time-independent leak current was observed. During depolarizing pulses a slowly activating outward current appeared. The current has a sigmoid time course, and activation was faster at more positive potentials. Decreasing $pD_o$ produced two distinct effects on the currents. The voltage at which the conductance was first activated, $V_{threshold}$ became more positive by about 40 mV/U decrease in $pD_o$, and the rate of current activation became slower. The shift in the position of the voltage-activation curve is more apparent in Fig. 4. The currents measured at the end of 8s pulses are plotted (solid symbols), as well as the amplitude extrapolated.
from a single-exponential fit to the rising phase (open symbols). This latter value corrects for the fact that the currents did not always reach steady state by the end of the pulses, as well as correcting for any time-independent leak current. In this example, and in other experiments, the shift in the current-voltage relationship was very nearly 40 mV/U decrease in pH. These effects are quite similar to those of changes in pH in water (Cherny et al., 1995).

Another effect of changes in pH evident in Fig. 3 is that the conductance was activated more slowly at lower pH. The time course of activation of H+ or D+ currents was fitted by a single exponential after a delay (Eq. 2). In some cases the fit was good, as in the example shown in the inset to Fig. 5, but sometimes the time course was more complex, with fast and slow components. Deviations from an exponential time course seemed most pronounced at large positive voltages and when there was a large pH gradient. Activation time constants, $\tau_{act}$, in the same cell at pH 8, 7, and 6 are plotted in Fig. 5. At each pH, $\tau_{act}$ is clearly voltage dependent, decreasing with depolarization. Lowering pH appears to shift the $\tau_{act}$-V relationship to more positive potentials and upwards, slowing activation in addition to shifting the voltage dependence. Similar results were obtained in other cells. Although the magnitude of $\tau_{act}$ varied from cell to cell, the effects of changes in pH in each cell were quite similar to those illustrated.

Effects of changes in pH. The effects of pH on D+ currents were studied both in whole-cell experiments and in excised patches. Studying patches allows a direct comparison in the same membrane. Fig. 6 illustrates D+ currents in an inside-out patch at pH 8.0 and pH 6.0 (A) or pH 7.0 (B). In this and in several other patches, voltage threshold was shifted by about −40 mV/U decrease in pH. Time-dependent outward current first appeared at −40 mV at pH 6.0 and at 0 mV at pH 7.0. The small amplitude of most patch currents in D2O limits the quantitative accuracy of any conclusions. However, the conductance approximately doubled when pH was reduced 1 U, comparable with the 1.7-fold increase/U decrease in pH reported previously in inside-out patches (DeCoursey and Cherny, 1995). It is also obvious that activation was much faster at lower pH.
The effects of changes in pD i in whole-cell experiments were explored in individual cells by varying the NH4 + gradient across the cell membrane (Materials and Methods). Fig. 7 illustrates families of D + currents in a cell at two NH4 + gradients. In each case pD o was 7.5, but pD i decreased as the NH4 + in the bath was lowered. With a 1//50 NH4 + gradient (A) V rev was −66 mV, and with a 15//50 NH4 + gradient (B) V rev was −27 mV. On the basis of this change in V rev, pD i was <0.7 U lower in A than in B. At lower pD i, the currents activated more rapidly and the conductance appeared to be increased. Qualitatively similar effects of changes in pH i were seen in H2O solutions at various NH4 + gradients in alveolar epithelium (not shown) and in macrophages (Grinstein et al., 1994).

Deuteron Isotope Effects on H + (D +) Currents

Families of currents in the same cell in H2O and D2O are illustrated in Fig. 8. To keep ΔpL approximately constant, we compared pH i, 6.5//pH ext 6.5 and pD o,7//pD i, 7 (Fig. 8, A and B, respectively). In D2O the currents are smaller and activate more slowly.

Voltage-gated current amplitude. The average ratios of the current measured in individual cells both in effectively symmetrical H2O and symmetrical D2O are plotted in Fig. 9. The “steady-state” current amplitudes were obtained by extrapolation of single exponential fits (Eq. 2). At all potentials the currents were substantially larger in H2O. The ratio decreased at more positive potentials, but two sources of error would tend to cause a voltage-independent effect to deviate in this direction. First, during large depolarizations there is depletion of protonated (or deuterated) buffer from the cell, which tends to reduce the currents in a current-dependent manner. Because the currents were larger in H2O, there would be more attenuation than in D2O. Second, to the extent that the position of the voltage-activation curve may be shifted slightly positive in D2O relative to H2O (e.g., see Figs. 10 and 11), a smaller fraction of the total conductance would be activated in D2O, and this would mainly affect smaller depolarizations to the steep part of the g f V relationship. Thus, it is not clear whether this effect was voltage dependent. The average ratio at +80 and +100 mV was 1.92 at pD 8 compared with pH 7.5, 1.91 at pD 7 compared with pH 6.5, and 1.65 at pD 6 compared with pH 5.5. In summary, the current carried by H + through proton channels is about twice as large as that carried by D +.
Comparison of the $g_H$-voltage and $g_D$-voltage relationships.

In symmetrical D$_2$O the conductance-voltage relationship shifted about 40 mV/U change in $\Delta pD$ just as in H$_2$O. However, the absolute voltage dependence might be different in the two solvents. To address this possibility we compared similar $\Delta pH$ and $\Delta pD$ in the same cell, varying the NH$_4^+$ gradient to regulate pLi. Fig. 10 illustrates a typical experiment. Measurements were made in D$_2$O (filled symbols) and in water (open symbols) at 1/50 mM NH$_4^+$, 3/50 mM NH$_4^+$, and 15/50 mM NH$_4^+$. At each NH$_4^+$ gradient, the $g_D$ against $V$ relation was shifted 10–15 mV positive to the corresponding $g_H$ relation. Moreover, $V_{rev}$ was consistently more positive in D$_2$O as at any given NH$_4^+$ gradient. Apparently NH$_4^+$ gradients were less effective at clamping pLi in D$_2$O, perhaps reflecting the higher viscosity of D$_2$O (Fig. 10). We could not detect any difference in this limiting slope in D$_2$O and H$_2$O. Measured at $10^{-2}$ to $10^{-3}$ of its maximal value, the conductance changed e-fold in 4.65 ± 0.16 mV (mean ± SEM, $n = 22$) in D$_2$O and H$_2$O combined; the lines drawn through the data in Fig. 10 illustrate this average slope. The conductance near threshold potentials changed e-fold in 4–5 mV at each NH$_4^+$ gradient. We could not detect any difference in this limiting slope in D$_2$O and H$_2$O. Measured at $10^{-2}$ to $10^{-3}$ of its maximal value, the conductance changed e-fold in 4.65 ± 0.16 mV (mean ± SEM, $n = 22$) in D$_2$O and H$_2$O combined; the lines drawn through the data in Fig. 10 illustrate this average slope. This slope corresponds with the translocation of 5.4 charges across the membrane during gating, which should be considered a lower bound for the actual gating charge movement.

Finally, examination of the limiting maximum conductance at large depolarizations (Fig. 10) reveals that over the range of pLi studied, the conductance was about twice as large in H$_2$O as in D$_2$O. This result is an important corroboration of the conclusion drawn from Figs. 8 and 9, because those comparisons were at ~0.5 U different absolute pLi. The higher conductance in H$_2$O than in D$_2$O in Fig. 10 cannot be ascribed to different pLi and must reflect a fundamental difference in the rate at which D$^+$ and H$^+$ permeate the channel.
the average limiting slope of 4.65 mV/e. For H2O and D2O, Thomas (1988) ob-
doubtedly by linear regression yielded identical slopes varying the NH4z0.2%.

obtained at pHo 6.5–10.0 and pDo 7–10 are included, as effectively symmetrical H2O and D2O fitted indepen-
suggestion of saturation at either extreme. The data for data describe a remarkably linear relationship, with no

Relationship between Vthreshold and Vrev. The potential at which the H+ conductance is first activated by depolar-
ization, Vthreshold, is plotted in Fig. 11 as a function of Vrev in H2O (open symbols) and in D2O (filled symbols). Data
obtained at pH6, 6.5–10.0 and pD7–10 are included, as well as from experiments in which pLi was changed by
varying the NH4+ gradient across the membrane. The data describe a remarkably linear relationship, with no suggestion of saturation at either extreme. The data for effectively symmetrical H2O and D2O fitted independently by linear regression yielded identical slopes (0.76 for H2O and 0.75 for D2O). Thomas (1988) ob-
served a similarly linear relationship between Erev and Vrev in snail neurons, over a range of pH6 ∼7–8. This result
shows clearly that the fundamental determinant of the position of the voltage-activation curve of the proton conductance is
fixed in a very similar manner by ∆pD as by ∆pH.

Deuterium slows channel opening. The time-course of H+ or D+ current activation during depolarizing pulses was
fitted by a single exponential after a delay to obtain τact, as was the inset in Fig. 5. Mean values for τact at various pD (solid symbols) and pH (open symbols) are plotted in Fig. 12, all for ∆pL = 0. It is unclear from these data whether there might be some effect of the absolute value of pL on τact. However, all the mean τact values in D2O are slower at each potential than any of the values in H2O. The average of the ratios at all potentials ±60 mV of the mean τact values in D2O to H2O at 0.5 U lower pL was 2.17 at pD 8, 3.19 at pD 7, and 2.96 at pD 6. In summary, D2O slows τact by about threefold.

FACT RESULTING from the greater difficulty in detecting small currents in D2O because the conductance is smaller and activation is slower. In any case, there was little or no solvent dependence of the relationship be-
tween Vrev and Vthreshold suggesting the position of the voltage-activation curve of the proton conductance is
fixed in a very similar manner by ∆pD as by ∆pH.
Because there was substantial variability of $\tau_{\text{act}}$ from one cell to another, comparisons were also made in individual cells at effectively symmetrical pH or pD. The average ratio of $\tau_{\text{act}}$ in D$_2$O to that in H$_2$O plotted in Fig. 13 reveals that $\tau_{\text{act}}$ was 2.0–3.6 times slower in D$_2$O. The slowing was not noticeably voltage dependent. There is a suggestion that the slowing effect was greater at higher pD (or pH). If the ratios at all voltages in each solution are averaged, the slowing effect was 2.17 at pD 6 compared with pH 5.5, 3.06 at pD 7 compared with pH 6.5, and 3.21 at pD 8 compared with pH 7.5. The solid symbols include only cells studied with D$_2$O pipette solutions, the open squares show data from cells with H$_2$O in the pipette. The slowing of $\tau_{\text{act}}$ by D$_2$O appears to be attenuated in these cells, possibly reflecting the small amount of H$_2$O inside the cell, although the difference is not significant. In summary, D$_2$O slows $\tau_{\text{act}}$ about threefold, and this effect appears to be voltage independent.

Deuterium does not strongly affect deactivation kinetics. The channel closing rate was examined by fitting the time course of the decay of tail currents (materials and methods), such as those illustrated in Fig. 1, A–C. The average values of $\tau_{\text{tail}}$ obtained in effectively symmetrical solutions are plotted in Fig. 14. There is a suggestion in the data that $\tau_{\text{tail}}$ was slightly slower at higher pL, and in D$_2$O compared with H$_2$O. The average ratios at all potentials of the mean $\tau_{\text{tail}}$ data for essentially symmetrical pL are 1.31 (pD 8/pH 7.5), 1.04 (pH 7.5/pD 7), 1.29 (pD 7/pH 6.5), 1.05 (pH 6.5/pD 6), and 1.51 (pD 6/pH 5.5). The apparent slowing by D$_2$O was thus 23–51%, and some part of this effect may be ascribable to increasing pLi.

In some cells $\tau_{\text{tail}}$ is independent of pH$_i$ (DeCoursey and Cherny, 1996a; Cherny et al., 1997), but the effects of pH$_i$ have not been clearly determined. Therefore, we attempted to compare $\tau_{\text{tail}}$ in H$_2$O and D$_2$O at similar pL$_i$ in the same cell by varying the NH$_4^+$ gradient. Increasing pH$_i$ in individual cells at constant pH$_o$ consistently slowed $\tau_{\text{tail}}$ by a small amount (not shown). When D$_2$O was compared with H$_2$O at a constant NH$_4^+$ gradient, i.e., at nearly constant pL$_i$ (see above), there was also a consistent slowing of $\tau_{\text{tail}}$ in nearly every cell, by roughly 50%, consistent with the average values given above.

Deuterium effects in cell-attached patches. Fig. 15 illustrates putative H$^+$ currents in a cell-attached patch. The cell was bathed with isotonic KMeSO$_3$ solution to depolar-
DeCoursey and Cherny

izate the membrane to near 0 mV. During depolarizations positive to 0 mV, there are slowly activating outward currents that resemble H$^+$ currents (cf. DeCoursey and Cherny, 1995), as well as brief discrete openings of some other channel(s). When H$_2$O in the bath was replaced with D$_2$O, the outward currents became much smaller and appeared to activate even more slowly. This isotope effect is comparable to the effects seen in whole-cell configuration, but larger than reported for other ion channels (Table III). Therefore, we conclude that the slowly activating outward currents were in fact H$^+$ currents.

Absolute H$^+$ or D$^+$ permeability of the cell membrane (not through proton channels). The “leak” current at subthreshold voltages usually decreased when D$_2$O replaced H$_2$O. However, it appears extremely unlikely that the leak is carried primarily by H$^+$ or D$^+$. Attempts to calculate the H$^+$ permeability, $P_{H^+}$, of the leak current using the Goldman-Hodgkin-Katz (GHK) current equation (Goldman, 1943; Hodgkin and Katz, 1949):

$$I_{H^+} = P_{H^+} \frac{EF}{RT} \left[ \left[ H^+ \right] - \left[ H^- \right] \right], \exp \left( \frac{-zFE}{RT} \right)$$

where $I_{H^+}$ and $P_{H^+}$ are expressed normalized to membrane area estimated assuming that the specific capacitance is 1 $\mu$F/cm$^2$, revealed numerous inconsistencies with this idea. The slope conductance of leak currents (defined as time-independent currents at subthreshold potentials) rarely changed by more than twofold/U change in pH or pD, and not always in the same direction. For a large pL gradient (e.g., pD 8//6), leak currents at negative potentials but positive to $E_L$ were inward, giving a negative calculated $P_L$. Calculated values for $P_L$ decreased substantially at low pL, even when the observed leak slope conductance was increased. Finally, the apparent reversal potential of the leak current, which was not well defined because the leak currents were often small, was usually closer to 0 mV than to $E_L$, and did not always change in the “right” direction when pL was varied. In summary, there is no evidence that H$^+$ carries a significant fraction of the leak current. An upper limit on the passive membrane permeability to H$^+$ or D$^+$ can be given as $<<10^{-4}$ cm/s at pH 5.5 or pD 6. By comparison, when the $g_H$ is fully activated, $P_{H^+}$ exceeds 1 cm/s at pH 8.0//7.5 (calculated from data in Cherny et al., 1995).

Figure 14. Tail current time constant, $\tau_{tail}$ at effectively symmetrical pH (open symbols) or pD (solid symbols). Symbols indicate pH, 8.0//pD, 8.0 (▲), pH, 7.5//pH$_{eff}$, 7.5 (△), pH, 7.0//pD, 7.0 (■), pH, 6.5//pH$_{eff}$, 6.5 (□), pH, 6.0//pD, 6.0 (○), or pH, 5.5//pH$_{eff}$, 5.5 (◇). Plotted is the mean ± SEM of $\tau_{tail}$ obtained by fitting the decay of the tail current with a single exponential (Eq. 3). Means are for 4–10 cells for each condition, with fewer measurements at some potentials.

Figure 15. H$^+$ and D$^+$ currents in a cell-attached patch. During 16s depolarizing pulses, there are slowly increasing outward currents, which we interpret as H$^+$ currents. In both parts $V_{hold}$ was −60 mV relative to the membrane potential, and pulses were applied to −40 mV through +100 mV in 20mV increments. The bath contained KMMeSO$_4$ solution, intended to clamp the membrane potential to near 0 mV, and the pipette contained pD 8.0 solution. (B). When the bath was changed to D$_2$O instead of H$_2$O, the outward currents were much smaller and, if anything, even slower to activate. The H$^+$ currents are small, consistent with a small patch area and the membrane being near the pipette tip (cf. DeCoursey and Cherny, 1995). The completeness of exchange of solvent near the membrane cannot be determined, but the altered behavior when D$_2$O in the bath replaced H$_2$O suggests that the solvent near the membrane was changed substantially.
**Table III**

Deuterium Isotope Effects on Other Channels (temperature, °C)

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<th>τtail (D2O/H2O)</th>
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<td>1.39 (0–2)</td>
<td>1.86 (0–2)</td>
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</tr>
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<td>Na+</td>
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<td>~1.0 (9.5)</td>
<td>&gt;1 (95)</td>
<td>Alicata et al., 1990</td>
</tr>
<tr>
<td>Na+</td>
<td>1.35 (2–4)</td>
<td>1.43 (2–4)</td>
<td>~1.0 (5–14)</td>
<td>1.5–2.6 (3–6)</td>
<td>Schaft and Bullock, 1980, 1982</td>
</tr>
<tr>
<td>K+</td>
<td>1.47 (2–4)</td>
<td>1.40 (2–4)</td>
<td>1.20 (12–14)</td>
<td>~1.4 (11–14)</td>
<td>Schaft and Bullock, 1980</td>
</tr>
<tr>
<td>K+, gramicidin (Li+, Cs+)</td>
<td>1.18 (5–25)</td>
<td></td>
<td></td>
<td></td>
<td>Pottosin et al., 1993</td>
</tr>
<tr>
<td>gramicidin (K+, Cs+, Cs+, NH4+)</td>
<td>1.16 (25)</td>
<td></td>
<td></td>
<td></td>
<td>Andersen, 1983</td>
</tr>
<tr>
<td>gramicidin (H+)</td>
<td>1.2–1.35 (22)</td>
<td>0.85 (12)</td>
<td>1.17 (12–14)</td>
<td></td>
<td>Akeson and Deamer, 1991</td>
</tr>
<tr>
<td>AChR</td>
<td>1.49 (12)</td>
<td>0.85 (12)</td>
<td></td>
<td></td>
<td>Lewis, 1985</td>
</tr>
<tr>
<td>cyclic nucleotide-gated</td>
<td>1.36 (20)</td>
<td>0.85 (12)</td>
<td></td>
<td></td>
<td>Root and MacKinnon, 1994</td>
</tr>
<tr>
<td>α-Toxin</td>
<td>1.13–1.2 (24)</td>
<td>1.17 (12–14)</td>
<td></td>
<td></td>
<td>Kasianowicz and Bezrukov, 1995</td>
</tr>
<tr>
<td>H+ currents</td>
<td>1.9 (20)</td>
<td>~3 (20)</td>
<td>≤1.5 (20)</td>
<td>none</td>
<td>this study</td>
</tr>
</tbody>
</table>

Values listed for τtail in some cases include time-to-half-peak or other measures of activation, τact reflects deactivation as tail current decay or single-channel lifetime, τrel is the inactivation time constant. Na+ channel gating current kinetics were not affected by solvent substitution (Meves, 1974). The effects on channel gating kinetics are clear at low temperatures (<10°C), but decrease as temperature increases, vanishing by 15–20°C (Schaft and Bullock, 1982; Alicata et al., 1990).

**Discussion**

The deuterium isotope effects observed provide information about H+ permeation as well as the regulation of gating by protons (or deuterons). The main results are: (a) D+ permeates proton channels. (b) The relative permeability of proton channels is >10^8 greater for D+ than for TMA+. (c) The H+ conductance through proton channels is ~1.9 times that of D+. (d) D+ regulates the voltage dependence of H+ channel gating much like H+. (e) The threshold for activating the proton conductance is a linear function of Vrev, and changes 40 mV/U change in ΔpH or ΔpD. (f) D+ currents activate with depolarization ~3 times slower than H+ currents, but deactivation is at most 1.5-fold slower in D2O. (g) At least 5.4 equivalent gating charges move across the membrane field during proton channel opening in D2O and in H2O. (h) The upper limit of any proton leak conductance of the membrane of rat alveolar epithelial cells must be <<10^-4 cm/s. When the gH is fully activated, P_H exceeds 1 cm/s.

**Properties of Proton Channels**

Proton channels are extremely selective. At high pD, the D+ permeability was >10^8 greater than the TMA+ permeability. The calculated permeability ratio P_{TMA}/P_D decreased as pD increased, by about 10-fold/U change in pD. Although a concentration dependent permeability ratio cannot be ruled out, it seems more reasonable to suppose that deviations of Vrev from E_D are due to imperfect control of pD, rather than to finite permeability of the channel to other ions. Several other H+ channels have been reported to have comparably high selectivity for H+, including the F_0 component of H+-ATPase (Althoff et al., 1989; Junge, 1989) and the M2 viral envelope protein (Chizhmakov et al., 1996). Protons rather than hydroxide ions carry the current. The substantially lower conductance of proton channels in D2O than in H2O suggests that the charge-carrying species is H+ (or D+) rather than OH− (or OD−). The isotope effect for D+ is large because its mass is twice that of H+, but OD− is only 6% heavier than OH−, and thus a much smaller isotope effect is to be expected: 41% for D+ vs. 3% for OD− for a classical square-root dependence on the mass of reactants (Glassstone et al., 1941). A similar argument can be made against H2O+ which would have a predicted isotope effect of just 8% over D2O+. However, the extremely high selectivity of the gH has been ascribed to a Grothuss-type or proton-wire permeation mechanism, which could exist for L+ or OL−, but not H2O+. Additional evidence supporting H+ rather than OH− as the charge carrying species is that the gH increases ~1.7-fold/U decrease in pH over the range pH 7.5–4.0 (DeCoursey and Cherny, 1995, 1996a), i.e., as [H+] increases and [OH−], decreases and [OH−], remains constant. Finally, the reduction of outward current in cell-attached patches when the bath solvent is changed from H2O to D2O (Fig. 15), is consistent with L+ efflux across the membrane from the cell to the pipette, but not OL− influx from the pipette into the cell.
Voltage-gated H⁺ and D⁺ currents pass through channels, not the phospholipid bilayer membrane: the g_H is not a membrane leak. The finding that the voltage-activated and time-dependent H⁺ conductance is clearly larger than the D⁺ conductance provides further support for the idea that this conductance occurs through specialized membrane transporters, presumably proteins, and not simply through leaks in the bilayer. The conductance of phospholipid bilayers to D⁺ is similar to that of H⁺ (Perkins and Cafiso, 1986; Deamer, 1987; Gutknecht, 1987). The proton (or OH⁻) permeability, P_H, of lipid bilayer membranes is several orders of magnitude higher than its permeability to other cations. Reported values for P_H vary widely, from 10⁻⁹ to <10⁻³ cm/s in lipid bilayers and from 10⁻⁵ to 10⁻³ cm/s in biological membranes (reviewed by Deamer and Nichols, 1985). At least part of this variability is due to a dependence on the nature of the membrane and the pH gradient, ΔpH (Perkins and Cafiso, 1986)—at ΔpH = 1.0 in membranes of varying lipid composition, P_H ranged from 2.0 × 10⁻⁷ to 1.8 × 10⁻⁵ cm/s (Perkins and Cafiso, 1986). We suspect that no more than a very small fraction of our leak current at subthreshold potentials is carried by H⁺ or D⁺. This leak current provides an upper limit of P_H ≪<10⁻⁴ cm/s in rat alveolar epithelial cells, providing no indication of any unusual H⁺ permeability of these particular biological membranes. Even if the leak were carried entirely by H⁺ or D⁺, P_H increases by 3–4 orders of magnitude during depolarization from subthreshold to large positive potentials. It is difficult to imagine that a transient water-wire spanning the membrane would exhibit consistent, well-defined voltage- and time-dependent gating.

If we convert the observed voltage-gated H⁺ current to permeability, P_H, using the GHK current equation (Goldman, 1943; Hodgkin and Katz, 1949), P_H increases with depolarization approaching a limiting value at any given ΔpH. However, the value calculated for P_H is much larger at high pH, because of the relative insensitivity of the H⁺ conductance, g_H, to absolute pH (Cherny et al., 1995; DeCoursey and Cherny, 1995). The limiting value for P_H is about 1.1 × 10⁴ cm/s at pH 8.0/7.5, 1.7 × 10⁻¹ cm/s at pH 7.0/6.5, and 1.4 × 10⁻² cm/s at pH 6.0/5.5 (recalculated from data in Cherny et al., 1995). Clearly, the GHK formalism is not a useful means of expressing P_H through the voltage-activated g_H, because its value is nowhere near being concentration-independent. That the P_H values obtained for the voltage-gated g_H are 3–9 orders of magnitude greater than those for H⁺/OH⁻ conductivity through lipid bilayers makes it clear that the voltage-activated g_H requires a special transport molecule and cannot reasonably be ascribed to H⁺ permeation through the phospholipid component of the cell membrane.

Deuterium Permeation

What is the rate-limiting step in H⁺ permeation? The ratio of H⁺ current to D⁺ current was 1.65, 1.91, and 1.92 at pH 6, 7, and 8, respectively. Nearly all the H⁺ that carry current during a depolarizing pulse are derived from buffer molecules that were protonated before the pulse (DeCoursey, 1991). If diffusion of protonated buffer to the channel were rate limiting, one would predict a smaller isotope effect on the conductance. Protonated or deuterated buffer should have almost identical diffusion coefficients. However, the 25% greater viscosity of D₂O than H₂O (Table I) would impede the diffusion of buffer molecules. That the g_H is reduced by almost 50% in D₂O is inconsistent with buffer diffusion being rate determining. We have shown recently that above 10 mM buffer there is negligible limitation of H⁺ current by the diffusion of buffer at either side of the membrane (DeCoursey and Cherny, 1996b). In contrast, the smaller deuterium isotope effect on the conductance of most ion channels is consistent with diffusion of permeant ions being the rate-determining factor (Table III).

If H⁺ permeation were set by the hydrodynamic mobility of H₂O⁺, then the H⁺/D⁺ conductance ratio should similarly correspond with the relative viscosities and dielectric constants of H₂O and D₂O (Lengyel and Conway, 1983). In fact, the relative mobility of H⁺ in H₂O to D⁺ in D₂O is significantly larger, namely 1.41 compared with 1.17 for KCl in H₂O vs. D₂O at 20°C (interpolated from the data of Lewis and Doody, 1933), indicating that a more rapid transfer mechanism for H⁺ exists, namely the “Grotthuss” mechanism in which protons hop from one water molecule to another. An isotope effect of 1.4–1.5 might therefore be expected if H⁺ or D⁺ conduction to the mouth of the pore were rate determining, or if permeation through the channel involved a mechanism like H⁺ or D⁺ diffusion in bulk water. Indeed, the relative conductance of H⁺ to D⁺ through gramicidin is of this magnitude, 1.34 at 10 mM L3O⁺, consistent with the approach of L3O⁺ to the channel being rate limiting, and 1.35 at 5 M L3O⁺ where the gramicidin channel current is saturated and the ratio presumably reflects that of permeation mechanism (Akeson and Deamer, 1991). The g_H/g_D ratio in voltage-gated H⁺ channels was larger than can be accounted for by diffusion of either buffer or L3O⁺ molecules, strongly suggesting that the rate-determining step in permeation occurs in the channel itself. Furthermore, the larger isotope effect in voltage-gated channels than in gramicidin suggests that H⁺ permeates by a different mechanism than gramicidin, in which H⁺ is believed to hop across a continuous hydrogen-bonded chain of water molecules filling the pore (Myers and Haydon, 1972; Levitt et al., 1978; Finkelstein and Andersen, 1981; Akeson and Deamer, 1991). Perhaps voltage-gated H⁺ channels are not simple wa-
ter-filled pores, but include amino acid side groups in the hydrogen-bonded chain, as proposed previously to account for their high selectivity and nearly pH-independent conductance (DeCoursey and Cherny, 1994, 1995; Cherny et al., 1995), by analogy with the proton wire mechanism proposed by Nagle and Morowitz (1978) to explain H⁺ transport through the “proton channel” component of mitochondrial and chloroplast H⁺-ATPases and bacteriorhodopsin. In summary, although the permeation of H⁺ through gramicidin behaves in a manner consistent with the behavior of H⁺ in bulk water solution, the permeation of H⁺ through voltage-gated channels appear to behave differently.

To explain the apparent pH independence of the H⁺ conductance of bilayer membranes, Nagle (1987) suggested that the rate-determining step might be the breaking of hydrogen bonds between water molecules. Applied to H⁺ channel currents, the H⁺ conductance might have an activation energy like that of hydrogen bond cleavage. The isotope effect for cleavage of an ordinary hydrogen bond in liquid water is ~1.4 (Warraf et al., 1996). The observed ratio of H⁺ to D⁺ current, ~1.65–1.92, is significantly larger, suggesting that the rate determining step resides elsewhere. If a quantum-mechanical tunnel transfer within the pore were rate determining, then a much larger isotope effect would be expected, for example, 6.1 calculated for the relative mobilities calculated for tunnel transfers in water (Conway et al., 1956). Although H⁺ tunneling may occur in the channel, it evidently is not rate limiting.

As discussed above, we imagine that the H⁺ channel is not a water-filled pore but is most likely composed of some combination of amino acid side groups and water molecules linked together in a membrane-spanning hydrogen-bonded chain. Proton conduction is believed to occur by a Grothuss or proton wire mechanism, which requires both hopping and reorientation steps (see INTRODUCTION; Nagle and Morowitz, 1978; Nagle and Tristram-Nagle, 1983). By analogy with ice, the mobility of the H⁺ “ionic defect” is 6.4 × 10⁻³ cm² V⁻¹ s⁻¹ (at −5°C, Kunst and Warman, 1980), about an order of magnitude greater than the Bjerrum L defect mobility, 5 × 10⁻⁴ cm² V⁻¹ s⁻¹ (at 0°C, Camplin et al., 1978), suggesting that the turning step may be rate determining. However, proton transfer may be slower when it occurs between two dissimilar elements of the hydrogen-bonded chain. For example, proton transfer is slowed in mixed solvents because protons become effectively trapped by the solvent molecule with higher H⁺ affinity (Lengyel and Conway, 1983). It is intriguing that the mobility of H⁺ in ice exhibits a large isotope effect, 2.7 for H⁺/D⁺ at −5°C (Kunst and Warman, 1980). Furthermore, the reorientation of hydrogen bonds during proton transport in ice exhibits a H₂O/D₂O ratio of ~1.6 (at −10°C, Eigen et al., 1964), suggesting by analogy that the turning step for water which is constrained in a channel pore may exhibit a larger isotope effect than water in free solution. Although the rate-limiting step in H⁺ permeation appears to occur within the conduction pathway, we cannot resolve whether the hopping or turning step is rate determining.

Are H⁺ channels really ion channels? In Table IV deuterium isotope effects on various membrane transporters other than channels are listed. The precise values depend strongly on the conditions of the measurement, but in general it appears that more complex transport mechanisms exhibit stronger isotope effects on transport rates, >1.7, compared with <1.5 for ion channel permeation (Table III). This result strengthens the conclusion that the H⁺ channel is not a simple water-filled pore, which was based on its high H⁺ selectivity and nearly pH independent conductance. If voltage-gated H⁺ channels are not water-filled pores, should they be considered ion channels at all? H⁺ current does not require ATP or any counter-ion, so the only possibly more accurate term would be a carrier. The essential difference between a carrier and a channel is that each ion transported through a carrier requires a conformational change in the molecule which changes the accessibility of the ion from one side of the membrane to the other, whereas an open channel conducts ions without obligatory conformational changes. (Of course, there are significant interactions between conducted ions and the channel pore.) Biological channels also exhibit gating, without which they would simply be holes in the membrane. The voltage-gated H⁺ channel exhibits well-defined time-, voltage-, and pH-dependent gating. That the conduction process involves protons hopping across a hydrogen-bonded chain seems a minor distinction. The two-stage hop-turn mechanism of the proton-wire (Nagle and Morowitz, 1978) could perhaps be described technically as alternating-access, in that the hydrogen-bonded chain must re-load after each H⁺ conduction event. However, a hop-turn mechanism is also believed to occur when H⁺ are conducted through gramicidin, in which the proton wire is composed entirely of water molecules, and there seems to be consensus that gramicidin is an ion channel, not a carrier. On balance, we prefer the term channel, but recognize that H⁺ conduction by a proton wire (hydrogen-bonded chain) mechanism may bear some similarities to the alternating access mechanism which defines carriers and that H⁺ channels may be unique among ion channels in not having a water-filled pore.

Deuterium Isotope Effects on Gating

Regulation of H⁺ channel gating by pH. The rates of H⁺ channel opening (activation) and closing (deactivation) are voltage dependent, both processes becoming
faster at large voltages. Byerly et al. (1984) found that increasing $pH_1$ or lowering $pH_o$ shifted the voltage dependence of activation kinetics of $H^+$ currents in snail neurons to more positive potentials but that lowering $pH_1$ slowed activation more than could be explained by a simple voltage shift. Subsequent studies in a variety of cells leave the impression that both low $pH_o$ and high $pH$ slow the opening rate of channels. In THP-1 monocytes change of $pH_o$ by 20 mV/U change in $pH$ on deactivation are substantially weaker than on activation. 

Deuterium isotope effects on $H^+$ channels. The opening rate of $H^+$ channels was 3.2, 3.1, and 2.2 times slower in $D_2O$ at pH 8, pH 7, and pH 6, respectively. In contrast, the closing rate was slowed only 1.5-fold or less. In the model proposed to account for the regulation of the voltage dependence of gating by pH, the first step in channel opening is deprotonation at an externally accessible site on the channel, and the first step in channel closing is deprotonation at an internally accessible site (Cherny et al., 1995). If deprotonation at the external site were the rate-determining step in channel opening, then the observed slowing of $\tau_{act}$ could reflect an increase in the $pK_a$ of this site in $D_2O$ by 0.34–0.51 U. We give more weight to the larger $D_2O$ effects, because factors such as $H_2O$ contamination and the possibility that other deuterium-insensitive steps in gating may contribute to the observed kinetics would tend to diminish the size of the observed effect. We conclude that the $pK_a$ of the external site most likely increases by $\sim$0.5 U in $D_2O$. The $pK_a$ of simple carboxylic and ammonium acids increases in $D_2O$ by $\sim$0.5–0.6 U, whereas the $pK_a$ of sulfhydryl acids increases only 0.1–0.3 U (Schowen, 1977). The observed slowing of $\tau_{act}$ thus speaks against cysteine as the amino acid comprising the hypothetical site. We conclude that the modulatory site that governs the opening of $H^+$ channels is most likely a histidine, lysine, or tyrosine residue. The stronger $D_2O$ isotope effect on activation than deactivation suggests that either the external and internal regulatory sites are chemically different, or the first step in channel closing occurs before deprotonation at the internal site.

One remarkable aspect of the data in Fig. 11 is that there is no suggestion of saturation of the relationship between $V_{act}$ and $V_{threshold}$. We previously reported saturation of the shift in the position of the $g_H-V$ relationship above $pH_o$ 8, with only a 10–20-mV shift between $pH_o$ 8 and $pH_o$ 9 (Cherny et al., 1995). In the present study, similar apparent saturation was observed, and extending the measurement to $pH_o$ 10 resulted in no further shift relative to $pH_o$ 9. However, we found that at

Table IV

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Rate (H$_2$O/D$_2$O)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$^+$-ATPase (E$_o$ component)</td>
<td>1.7</td>
<td>Althoff et al., 1989</td>
</tr>
<tr>
<td>H$^+$-ATPase (intact) at 30°C</td>
<td>1.7–5.6</td>
<td>Kotyk and Dvorakova, 1992</td>
</tr>
<tr>
<td>Bacteriorhodopsin DBS5e mutant</td>
<td>2.1–4.7, 6.2, 2.8</td>
<td>Cao et al., 1995</td>
</tr>
<tr>
<td>Na$^+$/H$^+$ antiport at 37°C</td>
<td>1.5$^*$</td>
<td>Elsing et al., 1995</td>
</tr>
<tr>
<td>Bilayer permeability</td>
<td>$\sim$1</td>
<td>Perkins and Cafiso, 1986; Deamer, 1987; Gutknecht, 1987</td>
</tr>
<tr>
<td>Voltage-gated H$^+$ current</td>
<td>1.7–1.9</td>
<td>this study</td>
</tr>
</tbody>
</table>

Except where noted, measurements were done at room temperature (~20°C). The bacteriorhodopsin values reflect the range of inverse time constant ratios of several kinetic components of the photocycle, and the inverse of the time constants of H$^+$ release and uptake, respectively. $^*$Measured in human leukocytes or rat hepatocytes; in neither case was the D$_2O$ effect significant.
high pH_0, V_rev deviated substantially from E_H. In the previous study we felt that we could not resolve V_rev at pH_0, 9 due to the rapid kinetics. Although tail currents at pH_0, 9 or pH_0, 10 were resolved less well than at lower pH_0, when we plot V_threshold against the best estimate of V_rev (Fig. 11), the data fall on the linear relationship consistent with the other, better determined data points. It appears that there is an anomalous loss of control over pHi at very high pH_0. It is difficult to imagine that pHi_0 is not well established by 100 mM buffer in the bath, and, assuming that V_rev reflects the true ΔpH, pH must increase a full unit when pH_0 is changed from 9 to 10. One possibility is that some additional pH-regulating membrane transport process is working under these conditions. For example, a recently described Cl−/OH− exchanger (Sun et al., 1996) working “backwards” might exchange external OH− for internal Cl−, in spite of the rather low (4 mM) Cl− concentration in the pipette solutions. Although we cannot explain the mechanism, the phenomenon merits further study.

The lack of saturation complicates estimation of the pK_2 of the putative regulatory protonation sites on H+ channels.

Predicting the voltage dependence of the g_H in intact cells. The definition of V_threshold is certainly arbitrary, because by using longer pulses, heavier filtering, and higher gain, it is possible to detect smaller and smaller currents, and ultimately V_threshold has no precise theoretical meaning. Nevertheless, predicting the circumstances under which the g_H might be activated in vivo is facilitated by some estimate of V_threshold. The slope of the line in Fig. 11 for the H_2O data corresponds with a 40.0-mV shift/U change in ΔpH, if V_rev changes by 52.4 mV/U ΔpH, as reported previously (Cherny et al., 1995), or a 44.4 mV/U shift if V_rev changed according to E_H. The slope in D_2O was virtually identical. Thus the previous conclusion that the voltage-activation curve is shifted by ~40 mV/U change in ΔpH is in excellent agreement with the present data both in H_2O and in D_2O. We previously proposed that V_threshold in intact cells could be predicted from the empirical relationship:

$$V_{\text{threshold}} = V_0 - 40 \cdot (\text{pH}_0 - \text{pH}_i) \, \text{mV},$$

(6)

where V_0 was typically 20 mV, but varied substantially from cell to cell (Cherny et al., 1995). This relationship is based on the nominal ΔpH. Considering the remarkably linear relationship in Fig. 11 between V_threshold and V_rev, we suggest that a more accurate prediction can be based of the true ΔpH, which we feel is reflected more closely by the observed V_rev than by the applied ΔpH. The new, improved relationship (in H_2O) is:

$$V_{\text{threshold}} = 0.76 \cdot V_{\text{rev}} + 18 \, \text{mV},$$

(7)

This relationship is very similar to that described by Eq. 6, in predicting a ~40-mV shift in V_threshold/U change in ΔpH, and V_threshold near +20 mV at symmetrical pH (ΔpH = 0), but emphasizes the use of V_rev as the ultimate indication of the true ΔpH. The dotted reference line in Fig. 11 illustrates that V_threshold is positive to V_rev over the entire voltage range studied. The regulation of the voltage-activation curve by ΔpH thus results in only steady-state outward currents throughout the physiological range.

We are grateful for constructive comments on the manuscript by Peter S. Pennefather, Duan Pin Chen, the reviewers, and Noam Agmon, who also generously provided preprints. The authors appreciate the excellent technical assistance of Donald R. Anderson, and thank Charles Butler for some determinations of the pK_2 of buffers in normal and heavy water.

This study was supported by a Grant-in-Aid from the American Heart Association and by National Institutes of Health Research Grant HL-52671 to T. DeCoursey.

Original version received 27 November 1996 and accepted version received 27 January 1997.

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