Effects of Buffer Concentration on Voltage-Gated H^+ Currents: Does Diffusion Limit the Conductance?

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ABSTRACT The single-channel proton conductance of the voltage-gated H^+-selective channel, like that of the F_0 component of the H^+-ATPase, is nearly constant over a wide range of pH encompassing the physiological range. To examine the possible contributions of buffer diffusion and buffer-channel proton transfer reactions to this phenomenon, the effects of buffer concentration on voltage-activated H^+ currents were explored in voltage-clamped rat alveolar epithelial cells. Changes in the external buffer concentration ($[B]_o$), evaluated using the whole-cell configuration, had only small effects on H^+ currents (I_H). Lowering $[B]_o$ from 100 to 1 mM did not alter the voltage-activation curve or reversal potential (V_{rev}) but reduced I_H, typically by 10–30%. Changes in internal buffer concentration ($[B]_i$), examined in inside-out patches, usually altered I_H more distinctly and subtly changed the kinetics. Overall, the effects of changing buffer concentration were small and subtle. The maximum attenuation of the single-channel H^+ current at 1 mM buffer was estimated to be ~20% at either mouth of the H^+ channel. Therefore, the rate-determining step in H^+ permeation is neither deprotonation of buffer at the inner mouth of the channel nor protonation of buffer at the external surface. Evidently the rate of H^+ permeation through the channel is itself small enough that diffusion of buffer in bulk solution does not directly limit the conductance significantly.

INTRODUCTION

A surprising property of voltage-activated H^+ channels is that the single-channel conductance, deduced from macroscopic measurements, both in whole-cell (Byerly et al., 1984; Demaurex et al., 1993; Cherny et al., 1995) and excised patch configurations (DeCoursey and Cherny, 1995), appears to be practically independent of the pH on either side of the membrane. Decreasing intracellular pH (pH_i) in individual inside-out patches increased the H^+ conductance (g_H) by a factor of 1.7/unit between pH_i 7.5 and 5.5 (DeCoursey and Cherny, 1995); macroscopic studies, which rely on comparisons between groups of cells, are consistent with this value. [No single-channel H^+ currents have been reported, and the single-channel conductance has been estimated from stationary current variance to be <100 fS at pH_i 5.5–6.0 (Byerly and Suen, 1989; DeCoursey and Cherny, 1993; Bernheim et al., 1993). Because the single-channel H^+ conductance has not been estimated at different pH, an alternative interpretation of the macroscopic data is that lowering pH decreases the number of functioning H^+ channels, so that the macroscopic conductance stays nearly constant, even though the unitary conductance increases (discussed in Cherny et al., 1995). Although this interpretation cannot be ruled out, it seems rather contrived and improbable.]

In sharp contrast, H^+ current through water-filled ion channels like gramicidin is directly proportional to the H^+ concentration, increasing by a factor of 10/unit over the range pH 4 to pH 0 (references cited in DeCoursey and Cherny, 1994b). This and other properties have led to the conclusion that the rate-determining step in H^+ permeation through gramicidin at physiological pH is diffusion limitation in bulk solution (Decker and Levitt, 1988; Akeson and Deamer, 1991). One possible explanation for the surprising behavior of voltage-gated H^+ channels is that the rate-determining step in H^+ conduction is a diffusion-limited proton transfer reaction (cf. Eigen, 1964; Bell, 1973) between buffer and the channel mouth. At the inner mouth the H^+ transfer rate could be approximately constant at its diffusion limit if the pK_a of the intracellular buffer were lower than the pK_a of the channel entrance. Conversely, the H^+ exit rate could be nearly constant if the pK_a of the extracellular buffer were higher than that of the outer mouth of the channel. If direct proton transfer between buffer and channel were the rate-determining step in H^+ conduction, then the H^+ current amplitude should be directly proportional to the buffer concentration. In previous studies of the effects of pH_i on H^+ currents, the internal buffer concentration was not systematically studied (Byerly et al., 1984; Demaurex et al., 1993; Kapus et al., 1993; Cherny et al., 1995; DeCoursey and Cherny, 1995); here we explore the possibility that the near pH_i independence of the g_H is simply the result of the use of constant buffer concentrations. The buffer concentration on both sides of the membrane was varied over a wide range, with small but reproducible effects on H^+ currents.

The main function proposed for voltage-activated H^+-selective channels is H^+ extrusion from cells during acute acid loading (reviewed by DeCoursey and Cherny, 1994b). The pH_i increases substantially during maximum activation of the g_H. In large cells such as snail neurons, pH_i increases over several minutes (Thomas and Meech,

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1982). In small cells activation of the g_H can increase pH_i by one unit within a few seconds (Kapus et al., 1993). One consequence of the efficiency of proton extrusion by H^+ channels is that interpretation of voltage-clamp data is complicated by uncertainties about the extent to which pH_i changes may alter the recorded H^+ currents. Most investigators use high buffer concentrations (e.g., 100 mM), but the extent to which buffer concentration may influence H^+ currents has not been explored systematically. Byerly et al. (1984) found that increasing $[B]_o$ from 65 mM to 172 mM did not affect V_{rev}. Thomas (1988) showed generally that lowering $[B]_o$ accentuated the drop in surface pH and attenuated the increase in pH_i during prolonged depolarization-activated H^+ currents. Another goal of the present study is to clarify the extent to which H^+ currents recorded in whole-cell and excised patch configurations are affected by buffer concentration.

MATERIALS AND METHODS

Alveolar epithelial cells

Type II alveolar epithelial cells were isolated from adult male Sprague-Dawley rats 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 gauze. Lectin agglutinization 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 those of type I cells. These experiments were undertaken with the purpose of advancing knowledge. Before any invasive procedures were initiated, the rats were deeply anesthetized with sodium pentobarbital. The rats were treated humanely, experienced no pain, and were sacrificed under deep anesthesia, in compliance with law and with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. H^+ currents were studied in approximately spherical cells up to several weeks after isolation.

Electrophysiology

Conventional whole-cell or excised, inside-out patch voltage-clamp techniques were used. Experiments were done at 20°C, with the bath temperature controlled by Peltier devices and monitored continuously by a thin-film platinum 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 between 3 and 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 with an Indec Laboratory Data Acquisition and Display System (Indec Corporation, Sunnyvale, CA). Data acquisition and analysis programs were written in BASIC-23 or FORTRAN.

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. Inside-out patches were formed by lifting the pipette into the air briefly. To quantitate H^+ current and g_H amplitudes, a usually small linear leak conductance was subtracted based on currents during subthreshold pulses. No other time-dependent conductances were observed consistently under the ionic conditions employed.

Solutions

Most solutions contained 1 mM EGTA, 2 mM $MgCl_2$, various concentrations of buffer, and tetramethylammonium methanesulfonate (TMA-$MeSO_3$), added to bring the osmolarity to ~300 mOsm and titrated to the desired pH with tetramethylammonium hydroxide (TMAOH) or methanesulfonic acid (solutions using BisTris as a buffer). A stock solution of $TMAMeSO_3$ was made by neutralizing TMAOH with methanesulfonic acid. Some external solutions included 3 mM $CaCl_2$ instead of $MgCl_2$; we could not detect any difference in the behavior of H^+ currents, whether Ca^{2+} or Mg^{2+} was present externally (Cherny et al., 1995). Buffers (Sigma Chemical Co., St. Louis, MO), which were used near their pK, were: pH 5.5–6.0, MES 2-(*N*-morpholino)ethanesulfonic acid; pH 6.5, Bis-Tris (bis[2-Hydroxyethyl]imino-tris[hydroxymethyl]methane); pH 7.0, BES *N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid; pH 7.5, HEPES. The pH of all solutions, especially those with low buffer concentration, was checked frequently using a Radiometer Ion83 Ion meter (Radiometer, Copenhagen, Denmark). The osmolarity of solutions was measured with a Wescor 5500 Vapor Pressure Osmometer (Wescor, Logan, UT).

Conventions

We refer to the pH in the format $pH_o//pH_i$. In the inside-out patch configuration the solution in the pipette determines the pH_o, which is defined as the pH of the solution bathing the original extracellular surface of the membrane, and the bath solution determines the 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.

RESULTS

General premise

Two main classes of diffusion-related phenomena are relevant to this study, which differ drastically in their temporal and spatial scales. H^+ flux through a channel will result in highly localized H^+ depletion or accumulation near the mouth of the pore. This highly localized depletion is established extremely rapidly (Läuger, 1976; Barry and Diamond, 1984) with a time constant of 37 ps, treating free H^+ as a cation and assuming a capture radius of 1 nm. The entire "reaction layer" near the membrane (assuming non-localized channels), within which protons are not at equilibrium with buffer during H^+ currents (Delahay, 1954; Neher, 1986; Mathias et al., 1990), develops rapidly with a characteristic time of 13 μs at 1 mM buffer and 0.13 μs at 100 mM buffer. For all conditions studied here, H^+ current-induced pH gradients localized in the "reaction layer" near the membrane are established practically instantaneously, i.e., with a time constant of <20 μs and a space constant of <400 nm. On a much slower time scale are "diffusion polarization" or "transport number effects" (Barry and Diamond, 1984): progressive changes in pH resulting from the accumulation/depletion of (de)protonated buffer during sus-

tained H^+ flux. A salient feature of this process is that it is enhanced greatly if diffusion is restricted. As might be predicted, bulk diffusion limitation was more obvious in whole-cell measurements, but in some cases could also be detected in excised patches. In this study we attempt to distinguish between diffusion limitation occurring locally near H^+ channels and changes in bulk pH due to H^+ current flow.

Effects of extracellular buffer concentration, $[B]_o$, on H^+ currents

When careful comparisons were made in the same cell, a small effect of $[B]_o$ on H^+ current, I_H, was usually detectable. The I_H elicited by identical depolarizing pulses (Fig. 1) was barely smaller in 10 mM than in 100 mM $[B]_o$, but was clearly smaller at 1 mM $[B]_o$. Our impression was that reducing $[B]_o$ from 100 to 10 mM had a smaller effect than from 10 to 1 mM, but the small magnitude of these effects and the numerous sources of variability do not permit us to draw quantitative conclusions. The effects of $[B]_o$ on H^+ currents were subtle. Small differences in the pH of solutions produced effects as large or larger than some effects of changes in $[B]_o$. For example, the voltage-activation curve was detectably shifted when the pH_o of two solutions differed by <0.1 unit, clearly changing I_H for small depolarizations. Another confounding phenomenon was increased pH_i resulting from depletion of protonated buffer from the cell during large H^+ currents. It takes several minutes for full recovery of pH_i in small cells (Demaurex et al., 1993; Kapus et al., 1993). Given these factors, we hesitate to attribute much significance to small changes, except those that were reversible in a given cell and reproducible from one cell to another.

Families of H^+ currents are compared in the same cell at 1 mM or 100 mM BisTris buffer in Fig. 2. The H^+ currents were distinctly reduced in 1 mM buffer at all potentials at which the g_H was activated (+40, +60, +80 mV). Although the reduction appears greater for pulses eliciting larger I_H, some effect was usually seen during small pulses as well, and attempts to demonstrate voltage or current dependence of the $[B]_o$ effects were not convincing.

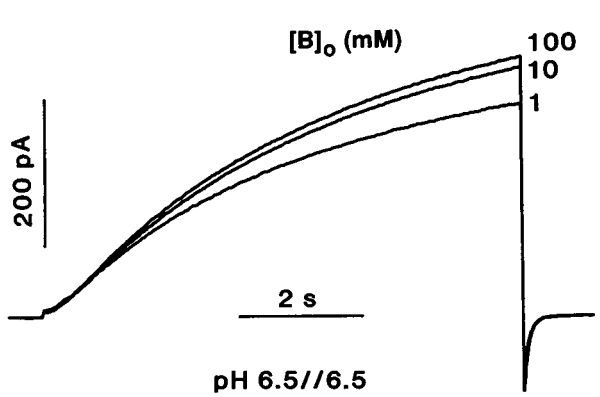

FIGURE 1 Effect of $[B]_o$ on H^+ currents in the whole-cell configuration at pH 6.5//6.5. Identical pulses to +80 mV from V_{hold} = −20 mV in 1 mM, 10 mM, or 100 mM BisTris solutions. Filter 200 Hz, $[B]_i$ = 100 mM, all currents recorded 49–54 min after establishing whole-cell configuration, capacity 48 pF.

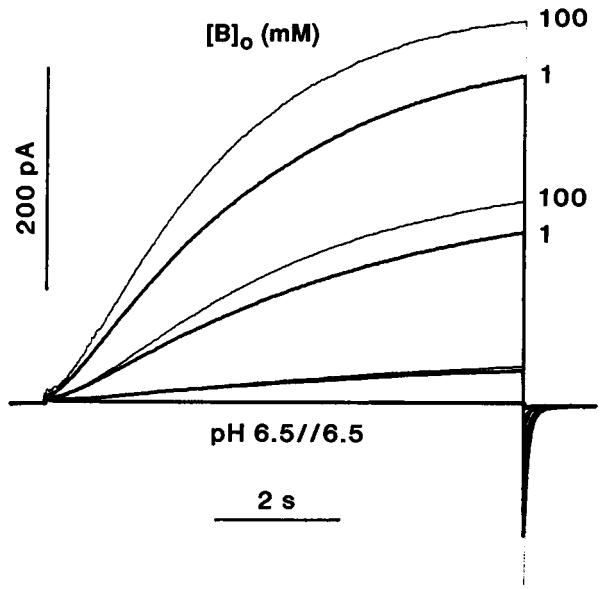

FIGURE 2 Families of whole-cell H^+ currents in a cell bathed in 1 mM (*thicker lines*) or 100 mM (*thinner lines*) $[B]_o$. Pulses are in 20-mV increments from 0 mV through +80 mV. V_{hold} = −20 mV, filter 100 Hz, capacity 57 pF.

$[B]_o$ does not affect $V_{threshold}$

The position of the voltage-activation relationship of the g_H is extremely sensitive to pH_o and pH_i. In the experiment illustrated in Fig. 3, pulses were applied in 5-mV increments to test whether the threshold for activating detectable H^+ current, $V_{threshold}$, occurred at the same potential in low or high $[B]_o$. At both $[B]_o$ studied, there was detectable, slowly activating H^+ current at +25 mV but not at +20 mV. Within the error of the measurement, no difference in $V_{threshold}$ could be detected at any $[B]_o$ in this or in most other experiments. In experiments in which a small difference in $V_{threshold}$ was observed, rechecking the pH of the solutions usually revealed a small difference from nominal values in the direction to account for the discrepancy. The lack of effect of $[B]_o$ on $V_{threshold}$ indicates that the sensitivity of H^+ channels to the pH gradient is established independently of $[B]_o$, and that any local concentration of H^+ near the membrane surface must be equally insensitive to buffer.

If the diffusion of external buffer were significantly rate-limiting, then the effect should be greater during large pulses (cf. Läuger, 1976). The chord conductance measured at the end of the pulses in the experiment in Figs. 2 and 3 is plotted in Fig. 4. The g_H was smaller at all potentials in lower $[B]_o$, although there is a suggestion that the reduction was greater at large potentials. In some

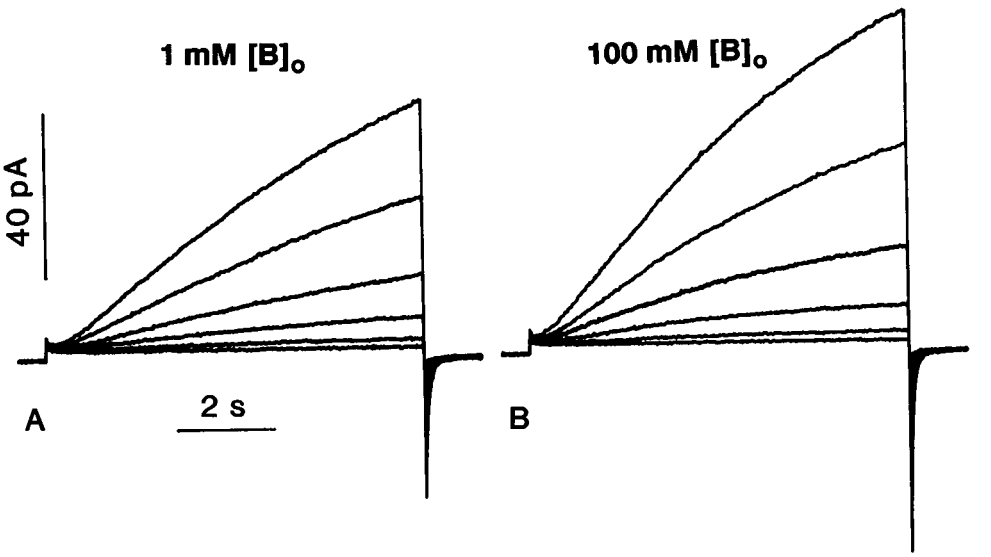

FIGURE 3 H^+ currents near $V_{threshold}$ at 1 mM $[B]_o$ (*A*) or 100 mM $[B]_o$ (*B*) at pH 6.5//6.5 in the same cell as in Fig. 2, but at higher gain. From V_{hold} = −40 mV, pulses were applied in 5-mV increments from +20 mV through +45 mV. Although I_H was smaller at low $[B]_o$, $V_{threshold}$ was the same. Families at low or high $[B]_o$ were recorded 59 min or 70 min, respectively, after establishing whole-cell configuration.

experiments there was a greater reduction during small pulses. In summary, we were unable to demonstrate a clear, consistent correlation between I_H amplitude and the effects of $[B]_o$.

Implications for H^+ channel gating

The limiting slope of the conductance-voltage relationship gives an indication of the quantity of charge movement that occurs during channel gating. The limiting slope in the experiment in Fig. 4 was 4.1–4.2 mV/e-fold change in g_H at either $[B]_o$. In other experiments this slope ranged from 3.1 mV to ~5 mV. This result indicates that the equivalent of six to eight electronic charges moves through the membrane electric field during H^+ channel opening. A higher value might result if P_{open} could be estimated over more than the two decades that we felt were reliable (cf. Zagotta et al., 1994).

$[B]_o$ does not affect V_{rev}

Measurement of V_{rev} by standard tail current protocols requires activation of the g_H followed by repolarization to determine the test potential at which the tail current relaxation is neither inward nor outward. Because the g_H activates slowly, substantial H^+ efflux occurs during the prepulse, and hence depletion/accumulation might occur, which would be reflected in the observed V_{rev}. The experiment in Fig. 5 tests whether the observed value of V_{rev} depends on $[B]_o$. With the prepulse duration adjusted to keep the total H^+ efflux during the prepulse constant, the observed V_{rev} was not detectably different in 100 mM $[B]_o$ (*thin lines*) than in 1 mM $[B]_o$ (*thicker lines*). In both cases, V_{rev} was within 1 mV of E_H = 0 mV at pH 6.5//6.5.

Effects of intracellular buffer concentration, $[B]_i$, on H^+ currents

Effects of $[B]_i$ in whole-cell configuration

We use the term $[B]_i$ here to indicate the concentration of buffer in the pipette solution, recognizing that fixed and

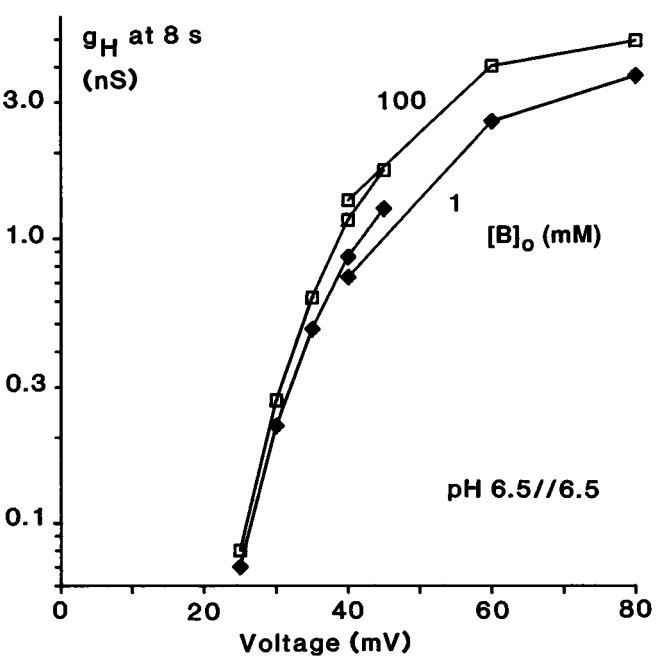

FIGURE 4 H^+ chord conductance in the same cell as in Figs. 2 and 3 at pH 6.5//6.5, with 1 mM BisTris buffer (◆) or 100 mM BisTris buffer (□). H^+ currents were measured at the end of 8-s pulses, the leak current was subtracted, and g_H was calculated using the observed V_{rev}, which was 0 mV in both solutions. The pH checked on the day of this experiment was found to be identical in the two solutions. For the family of small pulses in 5-mV increments near $V_{threshold}$, leak current was measured at the start of each pulse; for the larger pulses at 20-mV increments, the leak was extrapolated linearly from the current at subthreshold potentials.

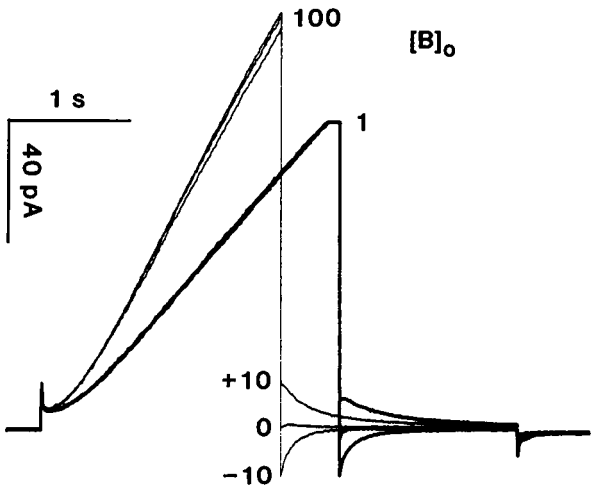

FIGURE 5 The tail current reversal potential, V_{rev}, was not affected by $[B]_o$. At pH 6.5//6.5, in either 100 mM (*thin curves*) or 1 mM (*thick lines*) BisTris, tail currents were elicited by a depolarizing the membrane to +70 mV, and then repolarizing to test potentials of −10, 0, and +10 mV. The prepulse was 2 s or 2.5 s long in high or low $[B]_o$, respectively, so that the leak-corrected integral of the prepulse current, i.e., the total H^+ efflux during the prepulse, was nearly constant at 112–118 pC for each pulse. The currents at low $[B]_o$ were off-scale at the end of the prepulse. The pipette solution contained 100 mM BisTris, V_{hold} was −40 mV, filter 100 Hz, same cell as in Figs. 2–4.

slowly diffusing buffers intrinsic to the cell likely increase the total buffering capacity (see also Discussion). We previously compared H^+ currents in alveolar epithelial cells with low (5 mM) or high (119 mM) $[B]_i$ (DeCoursey, 1991). At low $[B]_i$ V_{rev} was substantially more positive than E_H at all pH_o, suggesting that pH_i was not well controlled, and there was a greater tendency for H^+ currents to "droop" with time during long pulses. Droop of H^+ current during prolonged depolarizing pulses is attributable to the H^+ efflux-induced increase in pH_i, which progressively decreases the driving force during the pulse (DeCoursey, 1991). These conclusions are consistent with studies using simultaneous measurement of I_H and pH_i with intracellular pH electrodes (Thomas and Meech, 1982; Byerly and Moody, 1986; Meech and Thomas, 1987) or fluorescent dyes (Demaurex et al., 1993; Kapus et al., 1993).

To supplement these observations, we did several experiments with 1 mM buffer in the pipette solution (data not shown). When the pipette solution was pH 5.5 and the bath was pH 7.0, the g_H was first activated at a threshold potential, $V_{threshold}$, of +40 mV. In contrast, the $V_{threshold}$ predicted for a 1.5-unit pH gradient is −40 mV, according to an empirical relationship established for these cells in bilateral 100 mM buffer: $V_{threshold} = 20-40\ (\Delta_{pH})$ mV (Cherny et al., 1995), where $\Delta_{pH} = pH_o - pH_i$. This 80-mV shift in $V_{threshold}$ is consistent with a pH_i about 2 units higher than its nominal value. Recovery from a single large depolarization required >10 min to restore the I_H observed during a test pulse to its previous value. A greatly slowed rate of equilibration between the pH in the pipette and that in the cell is to be expected from this situation, in which fixed buffers in the cell greatly outnumber mobile buffer molecules in the pipette solution (Junge and McLaughlin, 1987). Finally, when V_{rev} was estimated by linear interpolation of the current at the end of a 4-s pulse and the instantaneous tail current immediately afterward (*see* Humez et al., 1995, for a description of this technique), V_{rev} was +9 mV after small pulses and as large as +46 mV after a pulse to +140 mV. The actual pH_i indicated by these measurements is 7.15–7.79, about 2 units higher than the nominal pH_i of 5.5. Clearly, 1 mM buffer in the pipette is not adequate to control pH_i, which adopts a value in the normal physiological range, presumably because of intrinsic buffers, and can be considered to be effectively unbuffered. The manifestations of low $[B]_i$ in the whole-cell configuration are V_{rev} positive to E_H, "droop" of I_H during long pulses, and slow recovery from depletion due to previous pulses.

Effects of $[B]_i$ in inside-out patches

Fig. 6 illustrates families of H^+ currents in an inside-out patch with 100 mM, 10 mM, and 1 mM $[B]_i$ (Fig. 6, *A*, *B*, *C*, respectively). This patch was selected for illustration because the H^+ currents were large and the data extensive; however, the effects of $[B]_i$ were smaller in patches with smaller I_H (see Discussion). There were two main effects of reducing $[B]_i$: I_H was smaller at all potentials, and the apparent activation kinetics were altered. The position of the voltage-activation curve was about the same, because in each case H^+ current is just apparent at 0 mV. In contrast to the effects of $[B]_o$, I_H was distinctly smaller at 10 mM than at 100 mM $[B]_i$ in most patches, with a further reduction when $[B]_i$ was 1 mM. Thus, I_H apparently is more sensitive to changes in $[B]_i$ than $[B]_o$. Also evident in Fig. 6, the H^+ current continued to increase throughout each pulse at high $[B]_i$, whereas I_H reached a pseudo-steady state before the end of most pulses at low $[B]_i$. This behavior is more apparent in Fig. 6 *D*, in which the currents at +60 mV at all three $[B]_i$ are superimposed. Initially I_H rises at a similar rate at all $[B]_i$, but then progressively deviates at low $[B]_i$, appearing to saturate sooner. Thus, low $[B]_i$ reduces both I_H and the apparent time constant of I_H activation. The effects of $[B]_i$ seemed generally similar at pH_i 5.5 or 7.0 to what they were at pH_i 6.5.

In several experiments the rising phase of H^+ currents was fitted by a single exponential after a delay. The observed activation time constant, τ_{act}, was about the same at 100 mM or 10 mM $[B]_i$, but was distinctly faster at 1 mM $[B]_i$, confirming the impression gained by simple inspection of the currents. Similar results were obtained when activation was quantitated as the maximum rate of rise of the H^+ current (cf. DeCoursey and Cherny, 1994a). For both parameters the effect was observed at all potentials—that is, there was no clear correlation with the amplitude of the current.

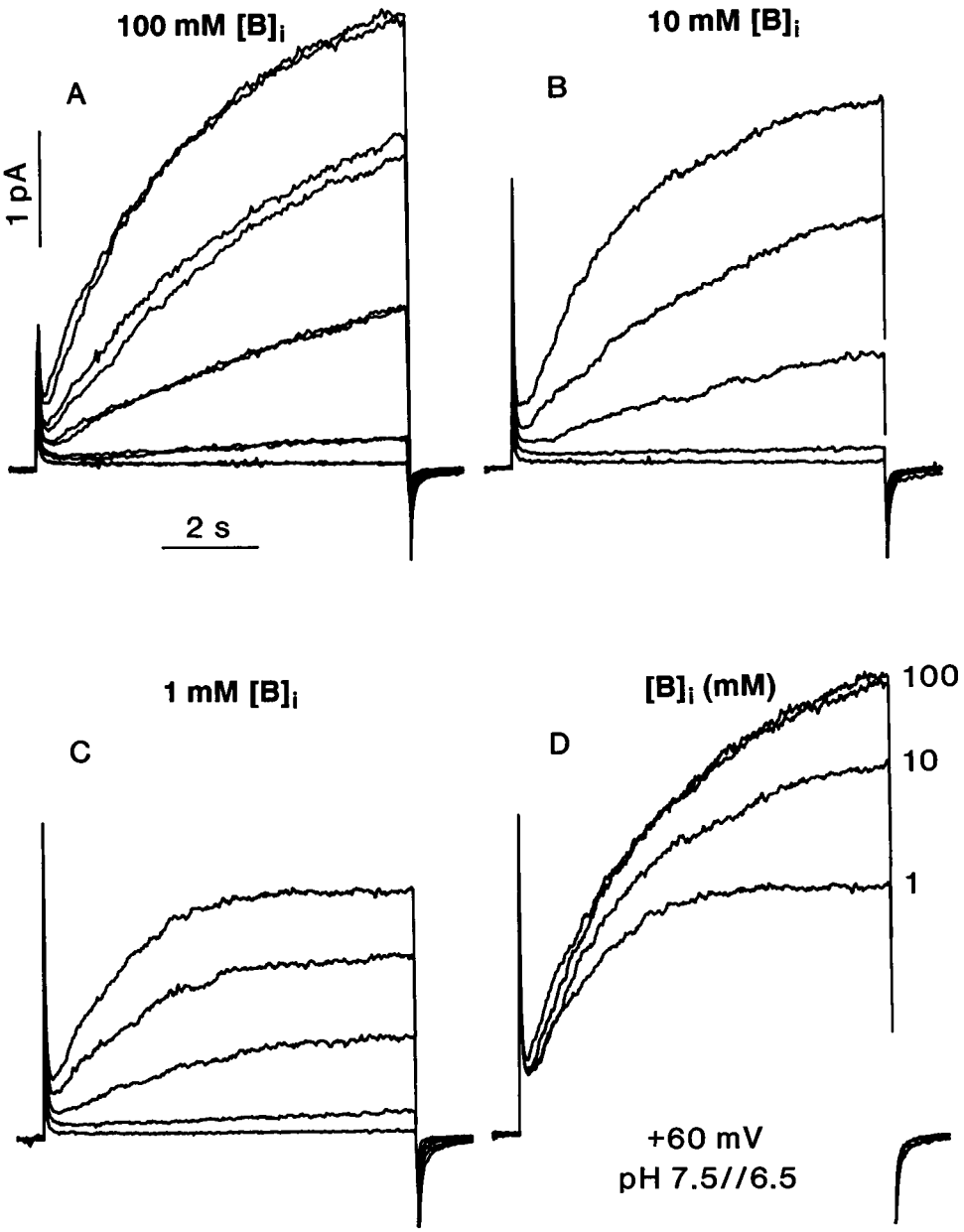

FIGURE 6 H^+ current families in an inside-out patch in 100 mM (*A*), 10 mM (*B*), or 1 mM (*C*) $[B]_i$ at pH 7.5//6.5. In each part, V_{hold} was −40 mV, and pulses were applied in 20-mV increments at 30-s intervals to −20 mV through +60 mV. The sequence was 100, then 10, then 1, then 100 mM BisTris, and the two families at 100 mM $[B]_i$ are superimposed. In *D* the currents at +60 mV from each family are superimposed. Calibration bars apply to all parts. Filter 20 Hz.

Repetitive pulses: premise

Because H^+ currents in mammalian cells activate very slowly, it is evident that before the maximum I_H is achieved, depletion of protonated buffer, BH, might be sufficient to increase pH_i significantly. Even in excised patches, an omega-shaped membrane might enclose sufficient volume of the pipette tip to allow analogous depletion. Without foreknowledge of the ideal behavior, it is impossible to know the extent to which the H^+ current observed during a single pulse may deviate from this ideal. To assess the extent of this type of bulk change in pH, we gave repetitive identical pulses. If significant depletion occurred during the first pulse, then because of the relatively slow rate of diffusion of buffer from one compartment to another (pipette to cell in whole-cell configuration, or bath to pipette tip in patches), the current during the second pulse might exhibit the residual effects of this depletion.

Repetitive pulses in excised patches

Fig. 7 illustrates an experiment using an inside-out patch at pH_o 7.5//pH_i 6.5 with 1 mM or 100 mM BisTris in the bath, i.e., varying $[B]_i$. In each solution, five identical pulses to +20 mV were applied, with an interval of 1.8 s between them. At high $[B]_i$, the H^+ current increased slowly throughout the 8-s pulse. Surprisingly, the rate of rise increased during subsequent pulses, although I_H measured at the end of the pulse increased only slightly. This behavior was surprising because depletion would increase pH_i, which would result in smaller, more slowly

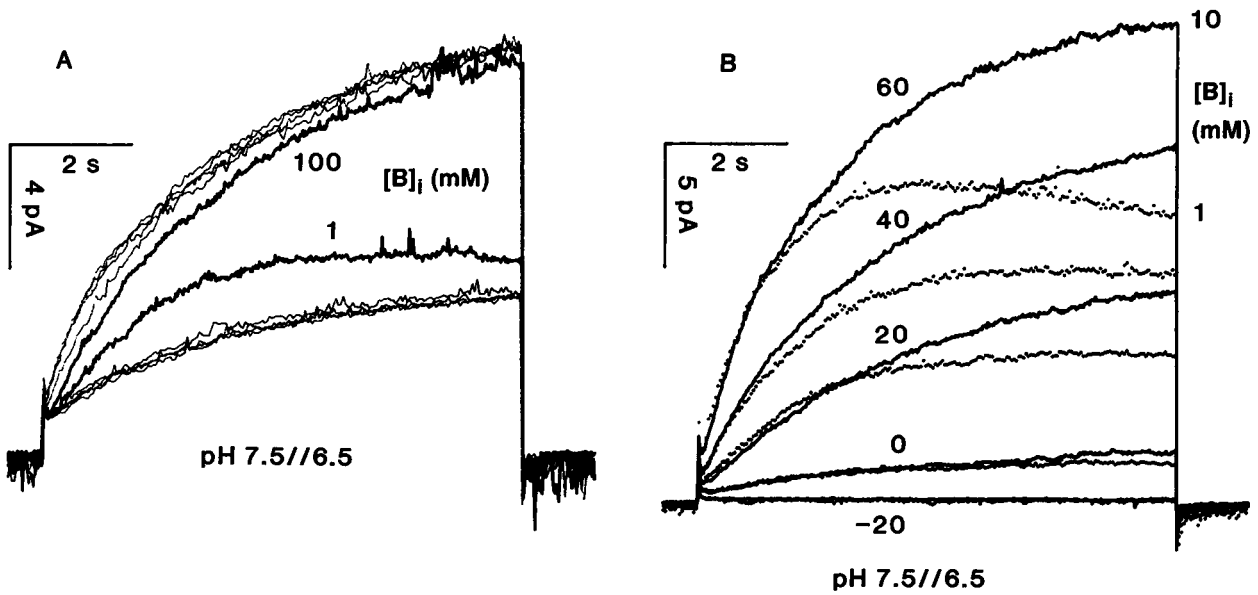

FIGURE 7 (*A*) H^+ currents in an inside-out patch during a series of five identical pulses repeated with an interval of 1.8 s between pulses, in 1 mM or 100 mM BisTris buffer. The first pulse (after resting at V_{hold} for >1 min), is shown with a darker line. Note that the I_H and rate of rise increased with pulsing at high buffer, but decreased at low buffer. V_{hold} was −40 mV, pulses were 8 s to +20 mV, filter 100 Hz. Pipette pH 7.5//pH_i 6.5. (*B*) Families of H^+ currents in the same inside-out patch as in *A*, at 10 mM BisTris (*solid curves*) or 1 mM BisTris (*dotted curves*). V_{hold} was −40 mV with pulses to −20 mV through +60 mV in 20-mV increments.

activating H^+ currents. We attribute the paradoxical enhancement of H^+ current to the existence of a slow deactivation process. H^+ channels open by traversing a pathway comprising at least two closed states preceding the open state (DeCoursey and Cherny, 1994b). Presumably the 1.8-s interval was not long enough for all of the H^+ channels in Fig. 7 *A* to reach the original "deep" closed state(s), so that during a subsequent pulse those channels in "shallow" closed states opened with a more rapid time course. This hypothesis is supported by the observation that when the interpulse interval was increased to allow further recovery (not shown), less enhancement was observed. In addition, when V_{hold} was set at a more negative value during the interpulse interval, less enhancement was seen for a given interval, consistent with the more rapid rate of I_H deactivation at more negative potentials (Cherny et al., 1995). The slower of the two kinetic components of deactivation of alveolar epithelial cell H^+ currents (Cherny et al., 1995) likely accounts for this slow recovery phenomenon. In many patches at high $[B]_i$, we detected no residual depletion at any interpulse interval, and a small enhancement for short intervals (as in Fig. 7 *A*). Therefore, there is no evidence that the H^+ current waveform is altered by bulk depletion effects under these conditions. In a few patches with very large currents (tens of pA), presumably indicating a large enclosed volume at the tip of the pipette, I_H decreased during successive repeated pulses. Increasing the interpulse interval to 20–40 s eliminated this effect, consistent with an enclosed volume that was significant but smaller than in the whole-cell configuration, in which full recovery required several minutes. Qualitatively similar results were obtained when pH_i was 5.5 instead of 6.5 (not shown).

When $[B]_i$ was reduced to 1 mM in the same patch (Fig. 7 *A*), I_H appeared to saturate about half-way through the pulse. In addition, a different pattern emerged during repetitive pulses. The second and all subsequent pulses elicited smaller H^+ currents with slower kinetics; both effects are attributable to an increase in pH_i, presumably in the small conical space enclosed within the tip of the pipette up to the membrane, which did not fully recover in the 1.8-s interval between pulses. The slower rate of rise strongly suggests that pH_i was higher during subsequent pulses, because increased pH_i is known to slow H^+ channel activation (Byerly et al., 1984; Kapus et al., 1993; Cherny et al., 1995; DeCoursey and Cherny, 1995). In conclusion, the H^+ current waveform was distorted by depletion at 1 mM $[B]_i$ but not at 100 mM $[B]_i$.

The effects of bulk depletion from a volume with restricted diffusion should be related directly to the current density. Fig. 7 *B* shows families of H^+ currents in the same patch as in Fig. 7 *A* with $[B]_i$ = 10 mM (*solid lines*) and with $[B]_i$ = 1 mM (*dotted curves*). There is a suggestion of depletion at 1 mM $[B]_i$ already during the pulse to 0 mV, and the currents diverge more during larger depolarizations, as I_H increases. Furthermore, the currents during each pulse nearly superimpose at first but then progressively diverge, presumably because of depletion. During the largest pulse in 1 mM $[B]_i$ the H^+ current droops, strong evidence that the pH gradient across the membrane patch had decreased significantly during the pulse.

Dependence on I_H

The effects of $[B]_i$ in most patches were greater than those of $[B]_o$ in whole-cell experiments, but the magnitude of the effects of $[B]_i$ varied from patch to patch. The effects of changes in $[B]_i$ tended to be greater in patches with large currents. In five patches studied at pH 7.5//6.5 in which I_H at +40 mV at the end of an 8-s pulse was <1 pA, the reduction in I_H when $[B]_i$ was reduced from 100 mM to 1 mM ranged from 12% to 21%. In four patches in which I_H under the same conditions was larger (1–4 pA), the reduction at lower $[B]_i$ was 30–52%. Thus there was a general correlation between the size of the patch currents and the amount of the decrement.

Some of the effect of $[B]_i$ therefore appears to be attributable to restricted diffusion of BH in the enclosed volume at the tip of the pipette (see Discussion). However, in some patches, repeated pulses at 10 mM $[B]_i$ resulted in behavior like that in Fig. 7 *A* at 100 mM $[B]_i$, namely slight enhancement of I_H with no evidence of depletion. The I_H at the end of these pulses was nevertheless clearly smaller than at 100 mM $[B]_i$ in the same patches. Apparently I_H is more sensitive to changes in $[B]_i$ than to $[B]_o$.

Repetitive pulses in whole-cell configuration

In whole-cell experiments repetitive pulse experiments produced results qualitatively like those in patches. During rapidly repeated pulses the initial rise of I_H was sometimes faster, but there was usually a clear and progressive decrease in the current later in the pulse, which would tend to obscure effects on the rising phase. Recovery was slower than in excised patch studies.

DISCUSSION

What fraction of the single-channel H^+ conductance is attributable to diffusion limitation and what fraction to the permeation pathway itself?

That there was a detectable effect of $[B]_o$ on H^+ currents suggests that even in this situation, in which there is no geometrical basis for a restricted diffusion space, a finite contribution to the resistance of the H^+ channel occurs in the diffusional approach. The diffusional approach to the pore mouths (entrance and exit) and the pore itself can be considered three resistors in series, which simply add together. When $[B]_o$ was reduced from 100 mM to 10 mM, the reduction in I_H was barely detectable, suggesting that I_H is not limited by buffer diffusion at 100 mM $[B]_o$ and is only slightly limited at 10 mM $[B]_o$. When $[B]_o$ was further reduced to 1 mM, the attenuation was typically 10–30%. Thus ~20% of the total resistance of the channel when $[B]_o$ = 1 mM may be attributable to the diffusion of external buffer.

We usually observed somewhat larger effects of changes in $[B]_i$ than in $[B]_o$. I_H was reduced by up to 50% at 1 mM and up to 20% at 10 mM $[B]_i$, compared with 100 mM $[B]_i$. However, if we distinguish between the ability of buffer to maintain pH and its ability to supply sufficient protons to sustain H^+ efflux through each H^+ channel, then it seems likely that the larger effect of $[B]_i$ than of $[B]_o$ may be exaggerated. Inspection of Fig. 6 *D* and especially Fig. 7 *B* reveals that the fractional reduction of I_H at lower $[B]_i$ becomes greater at longer times after the start of the pulse. This behavior strongly suggests that the pH gradient, Δ_{pH}, is progressively altered by H^+ efflux.

In both whole-cell and inside-out patch configurations, there may be significant geometrical barriers to diffusion. Obviously depletion of protonated buffer, BH, in the cytoplasmic compartment can occur during I_H, as has been demonstrated experimentally (DeCoursey, 1991; Kapus et al., 1993; DeCoursey and Cherny, 1994a,b). In inside-out patches, if the membrane spans the pipette some distance away from the tip, it will enclose a volume in which there will be restricted diffusion, where the pipette tip is the point of maximum constriction. If we assume a constant H^+ channel density, then the patch membrane area will be directly proportional to I_H. For a ~100 mV depolarization above V_{rev} at pH_i 6.5, I_H in inside-out patches ranged from 0.2 to 40 pA. Assuming the macroscopic value 20 pA/pF for these conditions (Cherny et al., 1995) and a 12° semicone angle at the tip of the pipette gives a range of ~1–200 fl enclosed volume at the tip. The enclosed volume may thus come within an order of magnitude of that in a whole cell, e.g., 905 fl for a 12-μm-diameter spherical cell. For example, I_H at +60 mV in Fig. 6 *C* is consistent with ~20 μm^2 membrane area, and an enclosed volume of 77 fl. The H^+ currents at 1, 10, and 100 mM $[B]_i$ reflect the extrusion of 10, 15, and 18 pC of H^+, respectively. At $[B]_i$ = 10 mM, the average pH in this volume would increase from 6.5 to 6.87 (neglecting replenishment by diffusion through the pipette tip), thus decreasing the driving potential ($V - E_H$) by 20%, comparable with the observed 21% attenuation of I_H. The 10 pC of H^+ extruded at 1 mM $[B]_i$ would deprotonate 1.35 mM buffer, greater than the nominal amount present. One way to account for this discrepancy is that the membrane itself may act as a buffer (e.g., Haines, 1983; Grzesiek and Dencher, 1986). Assuming that the membrane in this experiment contributed the equivalent of 3 mM buffer can completely account for the fractional reduction of I_H at 10 and 1 mM $[B]_i$. Of course, significant amounts of buffer diffuse into the pipette during the pulse. These calculations indicate that "bulk" pH changes in the pipette tip are of the right magnitude to produce the observed attenuation of H^+ current at different $[B]_i$.

The time constant of diffusional equilibrium between pipette solution and cytoplasm in the whole-cell configuration is directly proportional to cell volume (Pusch and Neher, 1988; Oliva et al., 1988). This may explain why residual depletion effects were not seen during repetitive pulses in patches with small I_H but were sometimes ob-

served in patches with large I_H. There was a correlation between the size of the patch currents and the decrement in I_H when $[B]_i$ was reduced from 100 mM to 1 mM. In patches with small H^+ currents (ergo minimal enclosed volumes) the effect of $[B]_i$ was roughly the same as observed for $[B]_o$ in whole-cell experiments. Therefore, the larger effect of $[B]_i$ than of $[B]_o$ may be due at least in part to restricted diffusion in the patch measurements, and a quantitatively similar diffusional resistance may occur at both sides of the membrane. In conclusion, the maximum contribution of buffer diffusion to the resistance of the H^+ channel may be ~20% from either side at 1 mM buffer, and becomes practically negligible as the buffer concentration is increased above 10 mM. These estimates represent an upper limit for the effects of buffer concentration on H^+ channels in the absence of bulk pH changes, and significant bulk pH changes evidently can occur, even in excised patches.

Influence of fixed buffers

Another factor that may enter into the interpretation of the effects of $[B]_i$ is the existence of intrinsic fixed buffers in the cell. Total intracellular buffering power in mammalian cells ranges from 18 to 77 mM/pH (Roos and Boron, 1981). Fixed or slowly diffusing intracellular buffers ought to persist in dialyzed cells. The effective diffusion coefficient of H^+, $D_{H,eff}$, measured in cytoplasm was five times slower than that of mobile buffers (Al-Baldawi and Abercrombie, 1992). $D_{H,eff}$ can be slowed greatly (by several orders of magnitude) by fixed buffers, but this slowing is counterbalanced by the addition of mobile buffers (Junge and McLaughlin, 1987). At low exogenous buffer concentration, e.g., $[B]_i$ = 1 mM, the behavior of whole-cell H^+ currents was consistent with the idea that intrinsic buffers were the predominant factor determining pH_i. The extent to which intrinsic intracellular buffers might persist in excised patches is not clear, although membrane constituents can act as buffers, increasing the effective buffering capacity in inside-out patch experiments. $D_{H,eff}$ may be lower in a living cell than in a dialyzed cell because of fixed buffers. However, if the source of H^+ is located in the membrane, such as Ca^{2+}-H^+ exchange (Meech and Thomas, 1987; Schwiening et al., 1993) or NADPH oxidase (Henderson et al., 1987), then a lower $D_{H,eff}$ would tend to localize acidification near the membrane and would thereby enhance the activity of H^+ channels.

How do protons cross the membrane-solution interface?

Kasianowicz et al. (1987) carefully considered three possible mechanisms by which H^+ might reach the membrane from bulk solution. H^+ can arrive as a free proton (protolysis mechanism), by hydrolysis of water, or by direct proton transfer from protonated buffer. At pH 8.3 Kasianowicz et al. observed a current density of 300 $\mu A/cm^2$. The largest H^+ currents in alveolar epithelial cells are about one order of magnitude smaller, ~25 pA/pF at pH_i 5.5 at ~100 mV positive to V_{rev} (Cherny et al., 1995), or 25 $\mu A/cm^2$ if the membrane capacitance is 1 $\mu F/cm^2$. The H^+ current density can reach 133 $\mu A/cm^2$ in HL-60 granulocytes (Demaurex et al., 1993) and >100 $\mu A/cm^2$ in human neutrophils (DeCoursey and Cherny, 1994b). Thus the largest voltage-gated H^+ currents are smaller than the H^+ flux carried by the S-13 protonophore (Kasianowicz et al., 1987). However, based on indirect estimates of unitary H^+ channel currents, the density of H^+ channels is ~100/μm^2 or less (DeCoursey and Cherny, 1994b), much smaller than 36,000 carriers/μm^2 in the study by Kasianowicz et al. (1987). The local current density and therefore local depletion is more acute for H^+ channels, whereas global depletion may be more acute for carriers.

If H^+ enters the channel directly from the intracellular solution (i.e., the "protolysis" mechanism), then the protonation rate k_r can be estimated from the channel density, {channels}:

$$k_r[H^+]_i\{\text{channels}\} > I_{H,max}/F, \quad (1)$$

which is equation A1 of Kasianowicz et al. (1987). Inserting values appropriate to alveolar epithelium at pH_i 5.5, namely {channels} = 1.66×10^{-14} mol/cm^2, $[H^+]_i = 3.16 \times 10^{-6}$ M, and $I_{H,max}$ = 25 $\mu A/cm^2$, we obtain $k_r > 4.9 \times 10^9$ M^{-1} s^{-1}. At pH_i 7.5, where g_H is reduced 2.5-fold (DeCoursey and Cherny, 1995), $k_r > 2.0 \times 10^{11}$ M^{-1} s^{-1}. Thus the apparent direct protonation rate of H^+ channels is in the vicinity of the fastest reaction occurring in free solution, the recombination of H^+ with OH^- at $1.3–1.4 \times 10^{11}$ M^{-1} s^{-1} (Bell, 1973; Eigen, 1964). A slightly higher rate of protonation has been reported for reactions taking place on the surface of a membrane, 2×10^{11} M^{-1} s^{-1} (Nachliel and Gutman, 1984) or a protein, the Ca^{2+} channel, 4.1×10^{11} M^{-1} s^{-1} (Prod'hom et al., 1987). The latter result was explained by the lower dielectric of the protein upon which this reaction takes place, which would funnel the electric field lines into the channel, enhancing the rate above that occurring in free solution (Prod'hom et al., 1987). Although the plausibility of the protolysis mechanism appears to be stretched to its limit, unlike Kasianowicz et al. (1987) we cannot on this basis rule out protolysis as the predominant mechanism supplying protons to H^+ channels. However, the relative pH independence of the g_H indicates that the entry of free protons into the H^+ channel cannot be the rate-determining step.

What is the capture radius?

Another way to approach the question of how protons arrive at the channel is to calculate a priori the rate at which H^+ can diffuse toward a channel. Several authors have considered how the maximum current through an ion channel is limited by the rate that permeating ions diffuse to the mouth of the pore (Hille, 1970; Läuger, 1976; Andersen, 1983a;

Barry and Diamond, 1984; Nunogaki and Kasai, 1988), which is given by

$$i_{max} = 2\pi F r_o D c \quad (2)$$

for a hemispherical approach, where F is Faraday's constant, r_o is the capture radius, D is the diffusion constant, and c is the concentration of permeating ion. Two complications arise in applying Eq. 2 to H^+ channels: uncertainty as to the appropriate value of r_o and the effects of buffer. On a macroscopic scale, r_o is defined as the difference between the radius of the (spherical) permeating ion and the radius of the (cylindrical) pore; i.e., the probability of permeation is assumed to be unity when the entire molecule enters the pore without hitting the edges, but is presumed to be zero when any part of the molecule collides with the pore mouth (Ferry, 1936). However, on the molecular scale of a single channel, r_o becomes an operationally defined parameter (Andersen, 1983b; Hladky, 1984) that may be effectively increased by various mechanisms. For example, a negatively charged vestibule (Andersen, 1983b; Dani, 1986; Jordan, 1987) would tend to funnel H^+ toward the channel mouth. If H^+ conduction at the surface of the membrane were significantly faster than in bulk solution, r_o would effectively increase (Läuger, 1976). In the context of the operation of the H^+-ATPase the question of rapid surface H^+ conduction has been debated vigorously (Haines, 1983; Nachliel and Gutman, 1984; Prats et al., 1987; Kasianowicz et al., 1987; Morgan et al., 1991; Heberle et al., 1994). Because of the special mechanism of H^+ movement through water, its effective reaction distance is large (Eigen, 1964); thus a large r_o might be reasonable. In fact, Decker and Levitt (1988) found that for H^+ permeation of gramicidin (at pH 3.75, where 95% of the total resistance of the channel is attributable to diffusion in bulk solution), r_o was 0.87 Å in the absence of weak acids (acting as buffers), increasing to 2.3–2.7 Å in the presence of weak acids (Levitt and Decker, 1988). These estimates are almost an order of magnitude larger than 0.33 Å for a series of "ordinary" monovalent cations in gramicidin (Andersen, 1983a,b).

If we assume that r_o is 2.5 Å as in gramicidin (Levitt and Decker, 1988), then the diffusion-limited single-channel H^+ current given by Eq. 2 is 4.1 fA at pH 5.5 and only 0.041 fA at pH 7.5, taking $D_H = 8.66 \times 10^{-5}$ cm^2/s (at 20°C, interpolated from Landolt-Börnstein, 1960). Arbitrarily increasing r_o to 10 Å gives 17 fA and 0.17 fA, respectively. If we incorporate the effects of 100 mM buffer according to the model of Nunogaki and Kasai (1988), these estimates increase slightly to 21 fA and 0.23 fA, respectively, for MES and HEPES buffers. Given the ~10 fS estimated unitary g_H at pH_i 6.0 in human neutrophils (DeCoursey and Cherny, 1993), and considering the observed 1.7-fold/unit decrease in g_H between pH_i 5.5 and 7.5 (Cherny et al., 1995; DeCoursey and Cherny, 1995), the estimated i_H at pH_i 7.5 at E_H + 100 mV is ~0.5 fA, somewhat greater than the calculated diffusion limit. In the end, this approach leaves the impression that diffusion of free protons to the channel is not quite adequate to sustain H^+ currents, but the discrepancy is not large. A significant contribution of protons derived from hydrolysis (Kasianowicz et al., 1987) may be involved.

What is the rate-limiting step in H^+ permeation?

Although we are hard pressed to account for the supply of protons to the channel at pH_i 7.5, we can conclude that H^+ diffusion is not rate determining, because g_H increases far less than in proportion to $[H^+]$ between pH_i 7.5 and pH_i 4.0 (Byerly et al., 1984; Demaurex et al., 1993; Cherny et al., 1995; DeCoursey and Cherny, 1995, 1996). The possibility that direct proton transfer from buffer to the channel entrance or from the channel exit to buffer might be rate limiting can be ruled out because 100-fold changes in $[B]_o$ or $[B]_i$ changed I_H by no more than about twofold. Therefore a diffusion-limited proton transfer reaction cannot account for the near pH independence of the g_H. The rate-limiting step in H^+ permeation must be localized to the channel. H^+ entry could be limited by the pH-independent breaking of hydrogen bonds in water near the pore entrance (Nagle, 1987), or some other step in permeation might limit the conductance.

H^+ channels as local pH meters

Szundi and Stoeckenius (1987) used the color change of bacteriorhodopsin upon protonation as a local pH meter to show that the pH near the purple membrane was significantly lower than bulk pH. The voltage-gated H^+ channel also can be used as a local pH meter to detect the pH gradient, Δ_{pH}, across the membrane (DeCoursey and Cherny, 1994a,b): V_{rev} indicates Δ_{pH} between the two bulk phases, and $V_{threshold}$ reflects the local pH near the putative protonation sites responsible for setting the voltage dependence of H^+ channel gating (Cherny et al., 1995). In the whole-cell configuration, V_{rev} may be much more positive than E_H at low $[B]_i$, but this is largely or entirely attributable to H^+ efflux-induced increases in pH_i (DeCoursey, 1991; Kapus et al., 1993; present data) and the persistence of intrinsic buffers (cf. Byerly and Moody, 1986). The threshold potential for activating detectable H^+ current, $V_{threshold}$, shifts ~40 mV/unit change in Δ_{pH}, somewhat less than the change in E_H or V_{rev} (Thomas, 1988; Cherny et al., 1995). Activation of Na^+-H^+ antiport in the whole-cell configuration increased pH_i, shifting both V_{rev} and $V_{threshold}$ to more positive potentials, but the shift in $V_{threshold}$ was greater, suggesting that these parameters may reflect a different local pH (DeCoursey and Cherny, 1994a). If the pH near the channel deviated significantly from its bulk value, then this deviation might be minimized at high buffer concentration. Thomas (1988) showed that the pH measured at the outer surface of the membrane in snail neurones falls less during sustained voltage-activated H^+ currents at high than at low $[B]_o$. However, under conditions where V_{rev} was not

changed (excised patches for $[B]_i$ measurements), we could detect no difference in $V_{threshold}$ at 1 mM or 100 mM $[B]_o$ or $[B]_i$. By its nature, the measurement of $V_{threshold}$ involves only minimal H^+ flux and thus closely approaches the resting state of the membrane (i.e., without complications due to H^+ flux and attendant depletion/accumulation). In summary, no evidence was found that the local pH near the pH-sensing sites on H^+ channels that regulate the voltage dependence of gating differs from that in bulk solution in the absence of H^+ fluxes. Our data suggest that as near the membrane as buffer molecules have access, the pH is determined by the bulk pH.

Practical consequences

A fairly obvious conclusion of this study is that the higher the buffer concentration in all solutions, the better is the control of the pH, and the more faithfully the observed H^+ currents will reflect the fundamental behavior of the system. Beyond this trivial statement, it can be said that H^+ currents were only subtly affected when $[B]_o$ was reduced from 100 mM to 10 mM in alveolar epithelial cells. The observed τ_{act} might be slightly reduced at 10 mM $[B]_o$, but the I_H amplitude was barely reduced. In cells with a higher I_H density, the I_H waveform would be compromised more by diffusion limitation. On the other hand, $[B]_i$ is more critical, because 1) its effects in most experiments were somewhat larger than those of $[B]_o$, 2) intrinsic buffers must be taken into consideration, and 3) bulk depletion of BH can be a serious limitation, especially in whole-cell studies.

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