Na^+-H^+ Antiport Detected through Hydrogen Ion Currents in Rat Alveolar Epithelial Cells and Human Neutrophils

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ABSTRACT Voltage-activated H^+-selective currents were studied in cultured adult rat alveolar epithelial cells and in human neutrophils using the whole-cell configuration of the patch-clamp technique. The H^+ conductance, g_H, although highly selective for protons, was modulated by monovalent cations. In Na^+ and to a smaller extent in Li^+ solutions, H^+ currents were depressed substantially and the voltage dependence of activation of the g_H shifted to more positive potentials, when compared with the "inert" cation tetramethylammonium (TMA^+). The reversal potential of the g_H, V_{rev}, was more positive in Na^+ solutions than in inert ion solutions. Amiloride at 100 μM inhibited H^+ currents in the presence of all cations studied except Li^+ and Na^+, in which it increased H^+ currents and shifted their voltage-dependence and V_{rev} to more negative potentials. The more specific Na^+-H^+ exchange inhibitor dimethylamiloride (DMA) at 10 μM similarly reversed most of the suppression of the g_H by Na^+ and Li^+. Neither 500 μM amiloride nor 200 μM DMA added internally via the pipette solution were effective. Distinct inhibition of the g_H was observed with 1% $[Na^+]_o$, indicating a mechanism with high sensitivity. Finally, the effects of Na^+ and their reversal by amiloride were large when the proton gradient was outward (pH_o//pH_i 7//5.5), smaller when the proton gradient was abolished (pH 7//7), and absent when the proton gradient was inward (pH 6//7). We propose that the effects of Na^+ and Li^+ are due to their transport by the Na^+-H^+ antiporter, which is present in both cell types studied. Electrically silent H^+ efflux through the antiporter would increase pH_i and possibly decrease local pH_o, both of which modulate the g_H in a similar manner: reducing the H^+ currents at a given potential and shifting their voltage-dependence to more positive potentials. A simple diffusion model suggests that Na^+-H^+ antiport could deplete intracellular protonated buffer to the extent observed. Evidently the Na^+-H^+ antiporter functions in perfused cells, and its operation results in pH changes which can be detected using the g_H as a physiological sensor. Thus, the properties of the g_H can be exploited to study Na^+-H^+ antiport in single cells under controlled conditions.

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INTRODUCTION

A variety of membrane transport systems that affect the intracellular pH, pH_i, of alveolar type II epithelial cells have been described or proposed, including Na^+-H^+ antiport (Nord, Brown, and Crandall, 1987; Sano, Cott, Voelker, and Mason, 1988; Shaw, Steele, Butcher, Ward, and Olver, 1990; Brown, Heming, Benedict, and Bidani, 1991), Cl^-/HCO_3^- exchange (Nord, Brown, and Crandall, 1988), Na^+-HCO_3^- symport (Lubman and Crandall, 1991), H^+-ATPase (Lubman, Danto, and Crandall, 1989), a K^+-H^+-ATPase (Boyd, Kemp, and Roberts, 1990), and a voltage-activated H^+-selective conductance, g_H, (DeCoursey, 1991). These transporters may be active under different conditions. Na^+-H^+ exchange was detected in intact cells only at pH_i 6.8 or lower (Nord et al., 1987). Cl^-/HCO_3^- exchange is activated during recovery from alkaline loads and may help maintain pH_i under normal conditions (Nord et al., 1988). Na^+-HCO_3^- symport may contribute to recovery from acid loads at $pH_i > 7.0$ (Lubman and Crandall, 1991). The H^+-ATPase has been proposed to contribute to maintenance of resting pH_i, recovery from acid loads, and modulation of extracellular alveolar subphase fluid pH (Lubman et al., 1989), although its importance in alveolar epithelium has been questioned in light of evidence that Na^+-H^+ exchange runs down in ATP-depleted cells (Brown et al., 1991). The g_H is activated at low pH_i or high pH_o and at depolarized membrane potentials, and because only outward currents are detectable its activation causes cellular alkalinization (DeCoursey, 1991). Functional interactions between these membrane transporters complicate the elucidation of their involvement in pH_i regulation.

General properties of H^+ currents in alveolar epithelial cells (DeCoursey, 1991) and in human neutrophils have been described (DeCoursey and Cherny, 1993), and are generally similar to those in other cells (Thomas and Meech, 1982; Byerly, Meech, and Moody, 1984; Barish and Baud, 1984; Demaurex, Grinstein, Jaconi, Schlegel, Lew, and Krause, 1993; Kapus, Romanek, Yi, Rotstein, and Grinstein, 1993). The g_H is extremely small at large negative potentials, activates in a time-dependent manner during depolarizing voltage pulses, carries only outward steady-state currents, does not inactivate, is extremely selective for H^+, and is inhibited by Cd^{2+} and Zn^{2+}. The most distinct difference between H^+ currents in different tissues is that activation is faster in snail neurons (Byerly et al., 1984; Mahaut-Smith, 1989*a*) than in other cells. Although the g_H is most likely mediated by ion channels, the unitary conductance is too small to resolve under conditions which have been employed to date (Byerly and Suen, 1989; DeCoursey and Cherny, 1993).

We have noticed that H^+ currents in alveolar epithelial cells are partially inhibited by extracellular Na^+. We explore the possibility that the Na^+-H^+ antiporter might be responsible for this phenomenon. Electrogenic membrane transporters can be detected directly by electrophysiological measurements. Because it is electroneutral, both in alveolar epithelium (Nord et al., 1987; Shaw et al., 1990) and in other eukaryotic cells (Kinsella and Aronson, 1980; Tse, Levine, Yun, Brant, Counillon, Pouyssegur, and Donowitz, 1993), operation of the Na^+-H^+ antiporter is not detectable as net current. However, given concentration gradients favoring Na^+ influx and H^+ efflux, operation of the antiporter would be expected to cause depletion of intracellular protons and accumulation of extracellular protons at least

very close to the membrane, with opposite effects on local Na^+ concentrations. Harvey and Ten Eick (1989) attributed the inhibition of cardiac myocyte inward rectifier K^+ currents upon removal of external Na^+ to a reduction in pH_i due to loss of Na^+-H^+ exchange. There is thus a precedent for detection of the activity of the Na^+-H^+ antiporter in cells studied in the whole-cell configuration of the patch clamp technique. Here we describe effects of external Na^+ on H^+-selective currents, which are most simply attributed to the activity of the Na^+-H^+ antiporter.

Based on the properties of the g_H, one would make three main predictions about the effects of Na^+-H^+ exchange on H^+ currents. The g_H, whether it is due to ion channels or to some other mechanism, is located within the membrane and must be sensitive to local pH. Outward H^+ currents would be expected to be smaller due to the reduced transmembrane concentration gradient for H^+. The second prediction is that depletion of internal protons and accumulation of external protons due to Na^+-H^+ exchange both would tend to shift the voltage dependence of the g_H to more positive potentials. The voltage dependence of the g_H is sensitive both to pH_i and pH_o, such that increased pH_i (Byerly et al., 1984; Mahaut-Smith, 1989*a*; Demaurex et al., 1993; DeCoursey and Cherny, 1993) or decreased pH_o (Byerly et al., 1984; Barish and Baud, 1984; Meech and Thomas, 1987; DeCoursey, 1991; DeCoursey and Cherny, 1993) both shift g_H activation positively along the voltage axis. Third, the reversal potential of the g_H, V_{rev}, ought to be more positive in the presence of high $[Na^+]_o$ owing to depletion of intracellular protons. All of these effects were observed when the cationic composition of the extracellular solution was exchanged to Na^+ from "inert" (i.e., not substrates for the Na^+-H^+ antiporter) cations tetramethylammonium (TMA^+) or *N*-methyl-D-glucamine (NMG^+) (Cala and Hoffmann, 1989). Amiloride or dimethylamiloride (DMA), a more specific inhibitor of the Na^+-H^+ antiporter (Kleyman and Cragoe, 1988), substantially reversed each of the effects of Na^+. The most straightforward interpretation of these data is that the Na^+-H^+ antiporter continues to function in alveolar epithelial cells under whole-cell voltage-clamp conditions, and that its effects on voltage-activated H^+ currents can be detected.

If the effects of Na^+ and amiloride are indeed due to the action of the Na^+-H^+ antiporter, analogous effects ought to occur in other cells which possess both a g_H and Na^+-H^+ antiporter. With this in mind, we studied human neutrophils, which have H^+ currents (DeCoursey and Cherny, 1993) and a Na^+-H^+ antiporter (reviewed by Swallow, Grinstein, and Rotstein, 1990). We observed qualitatively similar phenomena in human neutrophils as in rat alveolar epithelial cells. In conclusion, the Na^+-H^+ antiporter continues to function in perfused, patch-clamped cells, in a manner consistent with its behavior in intact cells.

METHODS

Cells

Type II alveolar epithelial cells were isolated from adult rats using enzyme digestion, lectin agglutination, and differential adherence, as described elsewhere (DeCoursey, Jacobs, and Silver, 1988; DeCoursey, 1990). H^+ currents were studied in cells up to several weeks after isolation. Approximately spherical cells were selected for study.

Neutrophils isolated from normal human blood by density gradient centrifugation (Schmeichel and Thomas, 1987), generously provided by Dr. Larry L. Thomas (Rush Presbyterian St. Luke's Medical Center, Chicago, IL), were kept on ice in RPMI-1640 media for not more than 6 h before use. Immediately before recording, neutrophils were transferred to the glass recording chamber, and superfused with Ringer's solution (Table I). Neutrophils often adhered to the glass and exhibited shape changes and movement, suggesting that some degree of activation may have occurred, although we detected no difference in the g_H between adherent and nonadherent human neutrophils (DeCoursey and Cherny, 1993). In some experiments fresh blood from the authors was studied without purification, and cells presumed to be neutrophils were identified visually by their size (~8 μm in diameter) and granular appear-

TABLE I

Composition of Solutions

Extracellular (bath) solutions							
Name	Na^+	K^+	X^+	Ca^{2+}	$CH_3SO_3^-$	Cl^-	Buffer
				mM			
Ringer's	160	4.5	0	2	0	170.5	5
$NaCH_3SO_3$	150	0	0	2	150	4	20
KCH_3SO_3	0	150	0	2	150	4	20
XCH_3SO_3	0	0	~150	2	~150	4	20

Buffer was HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) for Ringer's solution at pH 7.4, BES (*N,N*-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid) for pH 7 solutions, or MES (2[*N*-morpholino]ethanesulfonic acid) for pH 6 solutions. X^+ includes TMA^+, Cs^+, and Li^+ hydroxides and NMG^+, each of which was neutralized with methanesulfonic acid. Solutions were titrated to the appropriate pH with the hydroxide of the predominant cation, or with NMG^+ in the case of $NMGCH_3SO_3$. Ringer's includes 1 mM Mg^{2+}.

Intracellular (pipette) solutions							
Name (pH)	X^+	Cl^-	$CH_3SO_3^-$	Mg^{2+}	Ca^{2+}	EGTA	Buffer
				mM			
NMG-MES (5.5)	119	1.4	90	0	0.7	3.7	119
TEA-PIPES (7.0)	~101	3.8	73	0	1.9	12	73
K-aspartate (7.2)	179	23.28	0*	2	9.64	10	0

X^+ indicates the predominate cation, i.e., NMG^+, TEA^+, or K^+. Solutions were adjusted to ~300 mosM, so the cation concentrations listed are approximate. EGTA is ethylene glycol bis-(b-aminoethyl ether) *N,N,N',N'*-tetraacetic acid, PIPES is piperazine-*N,N'*-bis[2-ethane-sulfonic acid].
*Includes 140 mM $aspartate^-$.

ance. A g_H similar in magnitude and properties to that in purified neutrophils was observed in these fresh nonpurified cells.

Whole-Cell Recording

Conventional whole-cell patch-clamp technique was 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 (RTD) element (Omega Engineering, Stamford, CT) immersed in the bath solution, and the temperature stored along with each record. When bath solutions were changed, possible temperature effects were avoided by waiting until the bath temperature had equilibrated before recording. 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). Pipettes were coated with Sylgard 184 (Dow Corning Corp., Midland, MI), and heat polished to a tip resistance ranging typically between 2 and 6 MΩ. Electrical contact with the pipette solution was achieved by a thin sintered Ag-AgCl pellet (In Vivo Metric Systems, Healdsburg, CA) attached to a silver wire covered by a Teflon tube. Pipette solutions were filtered at 0.1–0.2 μm (Millipore Corp., Bedford, MA). A reference electrode made from a Ag-AgCl pellet was connected to the bath through an agar bridge made with Ringer's solution. The current signal from the patch clamp (List Electronic, Darmstadt, Germany) was recorded and analyzed using an Indec Laboratory Data Acquisition and Display System (Indec Corp., Sunnyvale, CA).

Solutions

Solutions are described in Table I, and are referred to by their pH and their predominant cation. Except where explicitly stated, all solutions are low in Cl^- with $CH_3SO_3^-$ and buffer as the predominant anions. Internal solutions included high buffer concentrations to minimize the H^+ depletion effects which occur as a result of large outward H^+ currents (DeCoursey, 1991; Kapus et al., 1993). Demaurex et al. (1993) monitored pH_i with a fluorescent pH indicator in cells in the whole-cell configuration, and found that a pipette solution buffered with 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) lowered pH_i to near that in the pipette within 1–2 min. Internal solutions included a small amount of Cl^- in order to avoid electrode polarization. Buffers, amiloride, DMA, and EGTA were purchased from Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI). Amiloride and DMA solutions were prepared daily from 10 mM stock solutions of 30% ethanol in water. Liquid junction potentials, V_{jct}, were corrected off-line according to measured values, including a correction both for the initial pipette solution/bath interface, and for the bath electrode/bath interface. The correction for V_{jct} was large (>5 mV) for NMG^+, K^+, and Cs^+ solutions, but the difference between Na^+ and TMA^+ was <2 mV. Derived data plotted in figures and in tables all have been V_{jct} corrected, but raw current records are plotted without correction except where specified. Data are presented without correction for leak current, except for g_H-V relationships for which a linear leak estimated at subthreshold potentials was subtracted. All experiments were conducted in the nominal absence of HCO_3^- in order to minimize complications due to HCO_3^- related transporters, including Cl^-/HCO_3^- exchange (Nord et al., 1988) and Na^+-HCO_3^- symport (Lubman and Crandall, 1991). The Cl^- concentration was minimized for similar reasons, and also to prevent interference by Cl^- currents which are present in isolated rat alveolar epithelial cells (see Results) and in human neutrophils (Krause and Welsh, 1990; Stoddard, Steinbach, and Simchowitz, 1993).

H^+ Current Saturation, Depletion of Protonated Buffer during Large H^+ Currents, and Related Problems

Several interrelated problems inherent in the study of H^+ currents in small patch-clamped cells must be considered. First, in keeping with its proposed function, activation of the g_H tends to alkalinize the cell. During large long pulses current saturation and "droop" result from depletion of intracellular protons and protonated buffer as a direct result of the massive H^+ efflux which is observable directly as H^+ current. H^+ current droop is reduced but not abolished by using high concentrations of buffers in the pipette solution (DeCoursey, 1991; Kapus et al., 1993), and thus apparently reflects bulk depletion of protonated buffer from the cell. A second complication is that the g_H activates quite slowly in alveolar epithelial cells, and full equilibration is not achieved in many cells even during 8-s pulses, at least for small depolarizations. Therefore the peak H^+ current or the g_H derived from this measurement does

not provide a true steady-state estimate of the g_H attainable at a given potential. Using longer pulses exacerbates the depletion. During small depolarizing pulses, the H^+ current in human neutrophils continued to rise for as long as 3 min before finally reaching an apparent steady-state value (DeCoursey and Cherny, 1993). A third problem, not necessarily related to the other two, is that H^+ currents tended to increase during the first 10 min or so after achieving whole-cell configuration, and also after the first few solution changes. An increase in H^+ current amplitude can be ascribed to pipette buffer diffusing into the cell and lowering the pH_i in those experiments in which lower than physiological pH_i was used. However, H^+ currents also increased after each of the first few changes of the bathing solution. Operationally, we repeatedly changed the bath solution until the current amplitude stabilized, after which it tended to remain fairly constant for the duration of the experiment. Even after these precautions, in some experiments slower changes, usually increases, in the g_H were observed on a time scale approaching hours. Therefore we interspersed "control" measurements, usually in TMA^+ or NMG^+ solutions, between "test" solutions. We also found that the data were most reproducible when families of pulses using identical protocols were applied in each solution, rather than when individual test pulses were applied. Some depletion of protonated buffer occurred during these families because a finite time interval was used between pulses. This systematic error was deemed to be an acceptable compromise when balanced against the already long times required for each family to be recorded, the possibility of slow changes in the g_H in a given experiment, and the finite lifetime of each cell.

Diffusion Model

Changes in pH_i due to net H^+ transport across the membrane (which could occur via the g_H, the Na^+-H^+ antiporter, or any other mechanism) were calculated using a compartmental model similar to that described by Hille (1977). This type of problem has been thoroughly explored by Mathias, Cohen, and Oliva (1990); we used the model to examine the experimental conditions in this study. The model pipette tapered uniformly over 3 mm from a tip diameter of 0.5–1 μm to an inner shank diameter of 1.15 mm. The pipette tip was located at the center of the "cell" to facilitate the calculation by allowing the diffusion shells to be concentric spheres. Shell thickness was 1 μm in the bath, 0.5 μm inside the cell, and increased from 1 to 10, then 100, then 1,000 μm in the pipette, at increasing distance from the tip. The concentration of protonated buffer, [BH], was calculated as diffusion was allowed to occur in the model. The flux, m, from one compartment to the next was calculated in time increments of 70 μs, from: $m_{n,n+1} = (c_n - c_{n+1})DA_n/\Delta x$, where c indicates the concentration (mol/cm^3), D is the buffer diffusion coefficient, A is the area between adjacent compartments (cm^2), and Δx is the thickness of the compartment (cm). D was assumed to be 0.5×10^{-5} cm^2/s based on molecular weight. Initial values of [BH] were calculated from the Henderson-Hasselbalch equation (Henderson, 1908), given the pKa of the buffer and the pH in each compartment: $[BH] = B_T\{10^{(pH-pKa)} + 1\}^{-1}$. Intrinsic cellular buffers were ignored in this calculation, but immobile buffers likely add to the buffering power of the cell, even when the cell is perfused by the pipette solution (DeCoursey, 1991). A fixed membrane proton efflux was specified, which lowers [BH] near the membrane inside the cell, and BH diffuses from the pipette to replace the depleted buffer. The diffusion of unprotonated buffer was not calculated; it was assumed that the total buffer, $B_T = B^- + BH$ remains constant in each compartment (i.e. that B^- and BH have the same diffusion coefficient), and therefore the new local pH can be calculated from: $pH = pKa + \log\{(B_T - BH)/BH\}$. After a given H^+ efflux rate was specified, the changes in local pH were calculated until a quasi-steady-state was achieved. We assume that experimental V_{rev} measurements reflect [BH] in the cellular compartment next to the membrane.

RESULTS

Extracellular Na⁺ Inhibits H⁺ Currents

Fig. 1 illustrates the effects on H^+ currents of replacing TMA^+ with Na^+ in the extracellular solution, with $pH_o//pH_i$ 7.0//5.5 and a pipette solution containing NMG^+. With TMA^+ in the bath, a small, slowly rising outward H^+ current could just be seen (at higher gain) to activate during the pulse to −20 mV. The H^+ current increased more rapidly during progressively larger depolarizing pulses. At large positive potentials the currents appear to reach a steady-state level during these 8-s

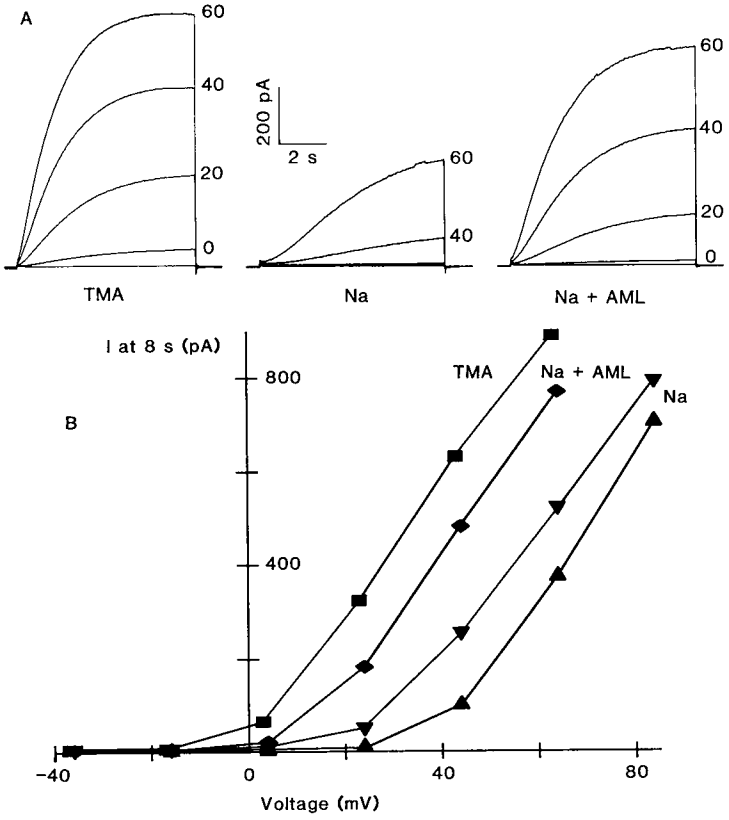

FIGURE 1. (*A*) Families of H^+ currents in an alveolar epithelial cell in the presence of TMA^+, Na^+, or Na^+ with 100 μM amiloride (*AML*). For each family, uncorrected whole-cell currents are superimposed for voltage pulses from V_{hold} of −60 mV applied in 20-mV increments to −20 mV through +60 mV (before V_{jct} correction of +2.8 mV in TMA^+ and +4.1 mV in Na^+ solutions). Pulses were applied in order of increasing amplitude at 44-s intervals. Calibration bars apply to all families. All data are at pH 7.0//5.5, with $CH_3SO_3^-$ as the external anion and NMG-MES as the pipette solution. Filter 200 Hz, ~19.8°C, time in whole-cell configuration 45 min, 52 min, and 66 min at the start of the families in TMA^+, Na^+, and Na^+ plus amiloride, respectively. (*B*) Currents measured at the end of the 8-s pulses in the experiment in *A* are plotted without leak correction, for TMA^+ (■), Na^+ (▲), Na^+ with 100 μM amiloride (◆), and also Na^+ after washout of amiloride (▼).

pulses. When TMA^+ was replaced by Na^+, with all other components of the solutions including pH identical, the H^+ currents were inhibited, as is evident from the second family of currents in Fig. 1 *A* recorded during identical pulses, and the corresponding current-voltage relationships plotted in Fig. 1 *B* (TMA^+ ■, Na^+ ▲). Several changes can be discerned. The currents first activated at a more positive potential in Na^+, at +20 mV in this cell. H^+ currents in Na^+ solutions were obviously smaller and turned on more slowly at any given potential. The kinetics of H^+ current activation also appeared to be more sigmoid in Na^+, i.e., the current increased with a more pronounced delay at the start of the pulse. With the possible exception of this alteration in H^+ current waveform, all of these changes can be summarized as a shift in the voltage dependence of the g_H to more positive potentials, by 30–40 mV in this cell. The effects of replacing TMA^+ with Na^+ required 1–2 min to develop fully and were reversible. During long experiments the differences between the behavior of the g_H in Na^+ and other cation solutions gradually diminished. Even so, a qualitatively similar Na^+ effect persisted in experiments lasting up to 2 h or longer.

We hypothesized that Na^+-H^+ exchange occurring when the bath contained Na^+ might be responsible for some of the observed changes in the behavior of the g_H. Na^+-H^+ exchange in eukaryotes is voltage independent (Aronson, 1985) and therefore would proceed continuously at V_{hold} and during voltage pulses. We examined whether the effects of Na^+ could be reversed by 100 μM amiloride, a well-known inhibitor of Na^+-H^+ antiport (Kleyman and Cragoe, 1988), which at this concentration nearly abolishes Na^+-H^+ antiport in rat alveolar epithelial cells (Nord et al., 1987; Sano et al., 1988). As can be seen in Fig. 1, *A* and *B* (◆), in the continued presence of Na^+, amiloride restored the g_H nearly to its behavior in the presence of TMA^+. Washout of amiloride (▼) resulted in substantial but incomplete recovery toward the position in Na^+ solution.

Quantitation of the Na^+ and Amiloride Effects: g_H–V Relationships

The voltage dependence of macroscopic currents through ion channels is often evaluated by measuring peak conductance–voltage relationships. This approach is convenient, and given knowledge of rectification properties of the channels involved, can indicate the voltage dependence of channel opening. We explored this approach to quantify the effects of Na^+ and amiloride. Fig. 2 *A* illustrates the chord conductance–voltage relationships calculated from the currents in Fig. 1. The best-fitting Boltzmann function is shown for each data set. The data were fitted by nonlinear least-squares to:

$$g_H(V) = g_{H,max}/(1 + \exp[(V - V_{1/2})/k]),$$

allowing the limiting conductance $g_{H,max}$, the half-activation potential $V_{1/2}$, and the slope factor k to vary. The slope factors were approximately constant within each cell analyzed in this way in TMA^+ and Na^+ solutions, consistent with the characterization of the effect as a simple voltage shift. In this cell $V_{1/2}$ was shifted by 39 mV in Na^+ solution and adding 100 μM amiloride reversed the shift by −28 mV, not quite back to its value in TMA^+.

The fitted parameters of the g_H–V relation provide a convenient way of comparing families of currents obtained in a the same cell using identical pulse protocols, but do

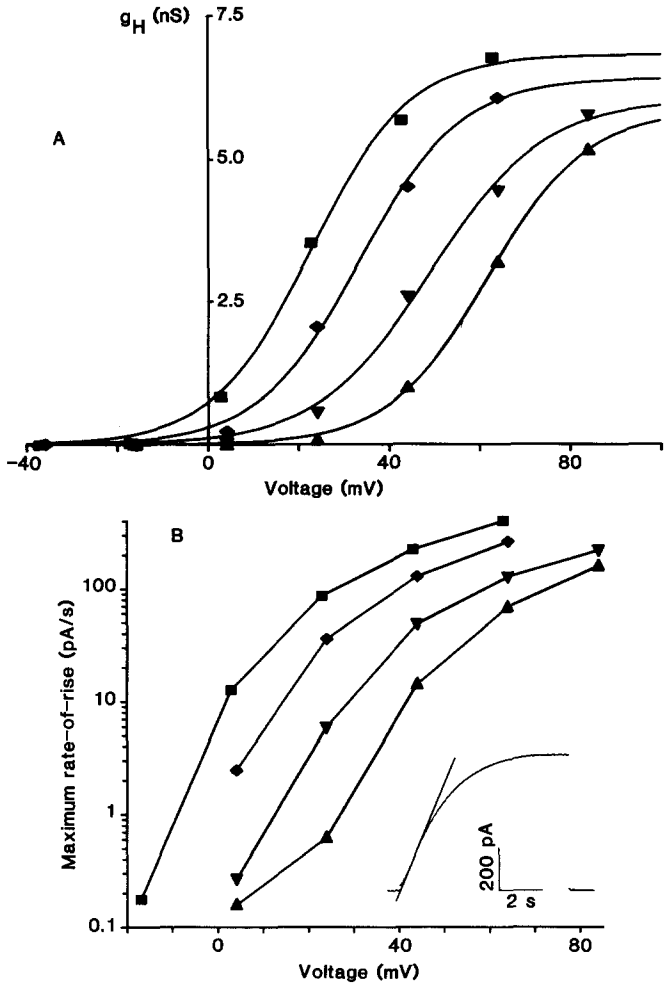

FIGURE 2. (*A*) The relationship between the g_H and voltage is plotted for the currents in Fig. 1 *B*, using the same symbols. The curves illustrate the best-fitting Boltzmann function (see text) to each data set with the fitted parameters, midpoint $V_{1/2}$, slope factor k, and limiting conductance $g_{H,max}$: (■) TMA^+ 22.9 mV, −10.9 mV, 6.83 nS; (▲) Na^+ 61.9 mV, −11.0 mV, 5.84 nS; (◆) Na^+ + 100 μM amiloride 33.6 mV, −11.2 mV, 6.43 nS; (▼) Na^+ post-amiloride 49.2 mV, −12.6 mV, 6.05 nS. g_H was calculated using V_{rev} measured in this experiment of −67 mV for TMA^+, −51 mV for Na^+, and −61 mV for Na^+ with amiloride. (*B*) I'_H plotted against membrane potential for the same experiment using the same symbols. I'_H was extracted by positioning a line on the steepest part of the rising phase of the H^+ current record, as illustrated in the inset. I'_H in this example for a pulse to +40 mV in TMA^+, was 228 pA/s.

not possess absolute significance. For example, k in the same cell was dependent on the test pulse duration, because the H^+ current was far from its limiting value at intermediate potentials during shorter pulses. Although the $g_{H,max}$ values were approximately the same in TMA^+ and Na^+ solutions, the data do not extend positively enough to define this parameter very well. We did not attempt to extend

the voltage range too far in most experiments, because large prolonged depolarizations tended to destroy the cell membrane, and because in some cells we observed distinct droop during extreme pulses, indicating that even 119 mM MES buffer in the pipette solution was inadequate to maintain constant pH_i. For this reason, apparent saturation of the measured g_H with depolarization would not convincingly establish a genuine saturation of the permeability mechanism. The value of $g_{H,max}$ is also compromised because it requires knowledge of V_{rev}, which may change during large H^+ currents. We therefore abandoned the use of $V_{1/2}$ and $g_{H,max}$ values from g_H–V relationships (Fig. 2 *A*). Instead, we estimated shifts of the g_H by considering the entire H^+ current–voltage relationship (Fig. 1 *B*) or the rate-of-rise of H^+ currents.

Maximum Rate-of-Rise of H^+ Currents (I'_H)

Fig. 2 *B* illustrates another method for quantifying the voltage dependence of g_H activation, measuring the maximum rate-of-rise of H^+ current, I'_H, as illustrated in the inset. Because I'_H occurs early in the pulse after a relatively small net H^+ efflux, this value is less subject to distortion than peak g_H. This measurement is not immune from depletion errors, but is less affected, and can be determined in the presence of nonohmic extraneous conductances (e.g., g_K or leak) provided they are time independent. An additional advantage is that I'_H can be determined accurately for depolarizing pulses during which the current does not reach steady-state. I'_H is strongly voltage dependent, varying over three to four orders of magnitude in many experiments. Furthermore, the I'_H vs. voltage relationship appeared to shift along the voltage axis in a parallel manner when the external cation was changed.

Changing the bath from TMA^+ to Na^+ in the experiment in Fig. 2 *B* shifted I'_H 41 mV to more positive potentials, and amiloride produced an opposite shift of −30 mV. Table II summarizes the relative shifts of the voltage dependence of the g_H in a number of experiments. The shifts estimated from H^+ current–voltage and I'_H–voltage relationships in each experiment were similar. The average shifts produced by Na^+ in the I'_H and H^+ -current vs. voltage relationships, respectively, were 33 and 31 mV. The addition of amiloride to Na^+ solutions resulted in a negative shift, i.e., a shift in the opposite direction, of −25 or −23 mV. On average then, addition of amiloride to Na^+ restored 75% of the original voltage dependence of the g_H. The effects of amiloride typically were only partially reversible; washout resulted in a positive shift (Table II) half the size of the negative shift produced by the inhibitor. A subsequent addition of amiloride produced a smaller shift of the g_H parameters than did the first. The incomplete recovery after washout of amiloride was observed consistently. It is possible that amiloride is only slowly reversible, or has an irreversible component, but another possibility is that the activity of the Na^+-H^+ antiporter declined slowly with time during these experiments. Even in cells exposed simply to TMA^+ or Na^+ solutions, the shift of the g_H parameters upon replacement of TMA^+ with Na^+ tended to decrease slowly with time. The reason for this "rundown" is not clear, although the ATP dependence of Na^+-H^+ exchange suggests one intriguing possibility (Brown et al., 1991), which will be tested in future experiments. Even during the longest experiments at pH 7.0//5.5 however, the qualitative effect of Na^+ substitution was observed. The H^+ currents in TMA^+

solution remained fairly stable during long experiments, if anything sometimes slowly increasing, so that the diminution of the Na^+ effect is compatible with a gradual loss of Na^+-H^+ antiport.

Inhibitory Effects of Amiloride on the g_H

Part of the reason that amiloride did not completely restore the voltage-dependence of the g_H in Na^+ solution to that in TMA^+ may be that amiloride inhibited the g_H independent of its effect on the exchanger. Addition of 100 μM amiloride to TMA^+ produced a small inhibition of the g_H, corresponding to a positive shift of 10 mV

TABLE II

Voltage Shifts at pH 7.0//5.5

Initial	Test	n	I'_H	H^+ currents
			mV	
TMA^+	Na^+	26	33.3 ± 11.1	31.0 ± 9.9
TMA^+	Li^+	7	13.9 ± 4.3	13.8 ± 2.3
TMA^+	K^+	4	22.5 ± 6.3	—
TMA^+	Cs^+	3	2.2 ± 2.6	—
TMA^+ (post DMA, AML)	Na^+	9	23.1 ± 2.5	19.8 ± 5.0
Na^+	+AML[1]	4	−25.0 ± 8.0	−23.3 ± 8.8
Na^+ + AML[1]	Na^+	3	12.7 ± 4.9	10.7 ± 5.8
Na^+	+DMA[1]	7	−20.4 ± 6.7	−21.1 ± 6.6
Na^+	+DMA[1], +AML[1]	11	−22.1 ± 7.2	−21.9 ± 7.1
Na^+ +DMA[1], +AML[1]	Na^+	7	11.9 ± 4.0	10.8 ± 4.1
Na^+	+AML[2], +DMA[2]	6	−9.2 ± 3.2	−9.5 ± 3.0
Li^+	+DMA	3	−9.3 ± 8.3	−8.3 ± 4.6
TMA^+	+AML	4	9.8 ± 2.5	9.9 ± 5.1
TMA^+	+DMA	3	2.0 ± 2.0	3.2 ± 1.4

n, number of cells. Values are ± SD. Voltage shifts observed when the bath was changed from "initial" to "test" solutions were determined by plotting maximum H^+ currents or I'_H (maximum rate-of-rise of H^+ currents) vs. voltage for identical families of pulses in each solution, then measuring the distance between the curves at several points. Sometimes the shift was greater at more positive potentials, but generally the effects observed were fairly well described as a simiple shift. A positive value indicates a shift to more positive potentials, i.e., inhibition of the g_H; conversely, a negative shift indicates enhancement of the g_H at a given potential. Superscripts indicate first or second exposure of a cell to drug. Amiloride (AML) was added at 100 μM and DMA at 10 μM. The major anions for all solutions were $CH_3SO_3^-$ and buffer; the pipette solution was NMG-MES. Extraneous nonlinear conductances in K^+ and Cs^+ solutions prevented quantitation of H^+ current shifts.

(Table II). Amiloride also inhibited the g_H in K^+ and NMG^+ solutions (Table III). Amiloride thus inhibited the g_H under conditions in which the Na^+-H^+ antiporter would not be active, but enhanced the g_H under conditions in which Na^+-H^+ antiport would be substantial, namely a large inward Na^+ gradient (high $[Na^+]_o$) and a large outward proton gradient (pH 7.0//5.5).

Amiloride and some of its analogues can act as protonophores, or "uncoupling agents" under certain conditions (Davies and Solioz, 1992). If this mechanism occurred in our experiments, these drugs should have produced H^+ currents at the holding potential, V_{hold}, in a direction predicted by E_H and V_{hold}. The holding current

at −40 mV was <10 pA in 11 cells selected for this measurement. At pH 7.0//7.0 in TMA^+ solution the holding current changed +0.22 ± 0.27 pA (mean ± SD, n = 5) upon addition of 100 μM amiloride, and at pH 7.0//5.5, addition of 100 μM amiloride or 10 μM DMA changed the holding current by +0.04 ± 0.51 pA (n = 6). Evidently, at the concentrations used here amiloride and DMA did not act as protonophores.

Reversal Potential of the g_H

Fig. 3 *A* illustrates the measurement of V_{rev} using "tail" currents in TMA^+, Na^+, and Na^+ with 100 μM amiloride, all in the same cell. The decaying tail current transients were fitted with a single exponential and the amplitude plotted in Fig. 3 *B*. Interpolation gives V_{rev} in this experiment −62 mV in TMA^+ (■), −38 mV in Na^+ (▲), and −49 mV in Na^+ with amiloride (◆). In all experiments V_{rev} averaged −68.0 ± 4.6 mV (mean ± SD, n = 22) in TMA^+, −45.0 ± 9.8 mV (n = 13) in Na^+, and

TABLE III

Voltage Shifts at pH 7.0//7.0

Initial	Test	n	I'_H	H^+ currents
			mV	
TMA^+	Na^+	8	18.3 ± 8.7	19.3 ± 7.7
TMA^+	K^+	5	9.5 ± 4.0	11.7 ± 5.1
TMA^+	NMG^+	3	−3.0 ± 2.6	−4.7 ± 2.5
Na^+	+AML	5	−7.0 ± 3.5	−6.2 ± 2.7
Na^+ + AML	Na^+	3	0	0
K^+	+AML	4	7.3 ± 5.0	6.8 ± 7.4
K^+ + AML	K^+	3	−5.5 ± 4.7	−6.5 ± 5.9
NMG^+	+AML	3	19.0 ± 12.2	18.9 ± 14.5
TMA^+	+AML	4	7.0 ± 2.9	8.2 ± 3.9

Values are ± SD. The pipette solution for these experiments was TEA-PIPES. See Table II legend for details.

−57.7 ± 7.0 mV (n = 6) in Na^+ with amiloride or DMA. Because V_{rev} was measured in cells with large g_H and small leak currents, there may have been some bias in the selection process for these measurements. The more positive V_{rev} in Na^+ could indicate either Na^+ permeability through the g_H mechanism, or increased pH_i due to Na^+-H^+ antiport. The partial recovery of V_{rev} toward its value in TMA^+ by addition of amiloride to Na^+ solution strongly supports the latter interpretation.

Concentration Dependence of the Na^+ Effect

Activity of the Na^+-H^+ antiporter depends on the gradient of both Na^+ and protons (Aronson, 1985). We therefore examined the $[Na^+]_o$ dependence of the effect, using various mole fractions of TMA^+ and Na^+. Fig. 4 illustrates the H^+ current at +40 mV in TMA^+ solution (*dotted record*). After addition of 1% Na^+, 99% TMA^+ to the bath, the H^+ current was substantially reduced. The current during a pulse to +60 mV indicates that the effect of 1% $[Na^+]_o$ is nearly equivalent to a 20-mV shift. In the

experiment illustrated, 100% Na^+ solution shifted the g_H kinetics by 32–33 mV (not shown), suggesting that the K_d for this effect of Na_o^+ was <2 mM. In other experiments a range of Na^+ mole fractions shifted the g_H consistent with a K_d of 1–2 mM Na_o^+. The observed $[Na^+]_o$ dependence is consistent with a Na^+-specific exchange-mediated phenomenon rather than a nonspecific effect of changes in bulk external ionic composition.

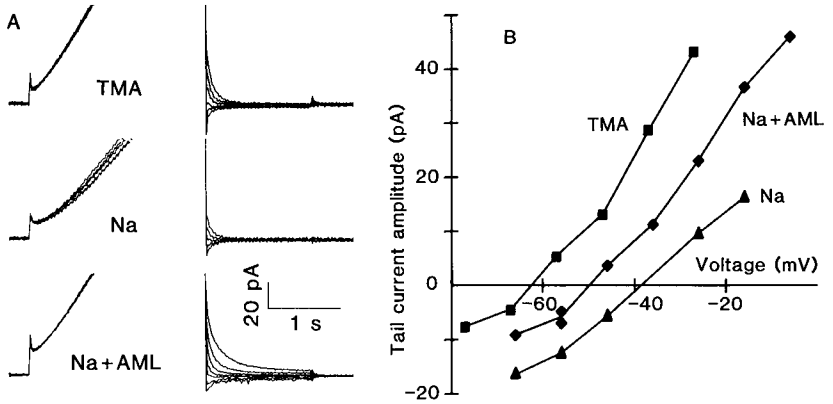

FIGURE 3. (*A*) Reversal potentials of the H^+ current measured in a cell bathed in TMA^+, Na^+, and Na^+ with 100 μM amiloride, all at pH 7.0//5.5, with NMG-MES in the pipette. In each case, a 2.5-s prepulse was applied to activate the g_H, followed by test pulses 1.5 s long to a range of potentials. Shown are test pulses in 10 mV increments with extremes −30 to −80 mV (TMA^+), −20 to −60 mV (Na^+), and −10 to −70 mV (Na^+ with 100 μM amiloride). The current at the end of the prepulse (here truncated) was about 190, 140–160, and 195 pA in the three solutions, respectively. Calibration bars apply to all three sets of measurements. Data shown before V_{jct} correction; derived data in *B* have been corrected. (*B*) The decaying ("tail") current transients during the test pulses were fitted by eye with a single exponential, and the amplitude of this current transient is plotted against voltage for the measurements illustrated in *A*. Although we attempted to use prepulses which activated the g_H to about the same extent, +40 mV in TMA^+ and +60 mV in Na^+ and Na^+ + amiloride, the currents in Na^+ solution were somewhat smaller, and the slope of the "instantaneous" current–voltage relation is consequently shallower. In this cell, the g_H was shifted +28 mV between TMA^+ and Na^+, and −13 mV after addition of 100 μM amiloride.

Substrate Specificity

We explored the effect of several monovalent cations on H^+ currents. Because its well-known effects on pH_i (Roos and Boron, 1981) would have complicated interpretation of the data, NH_4^+ was not tested. Fig. 5 *A* illustrates families of H^+ currents in a cell in TMA^+, Li^+, and Na^+ solutions all at pH 7.0//5.5. The behavior of the g_H in Li^+ solution was intermediate between that in TMA^+ and Na^+ solutions. The voltage shifts produced by Li^+ were about half as large as those in Na^+ solutions, averaging 14 mV (Table II). In alveolar epithelium (Nord et al., 1987) as in other cells (Aronson, 1985), Li^+ is transported by the Na^+-H^+ antiporter but at a lower rate than

Na^+. Other cations which are not substrates for Na^+-H^+ exchange, including Cs^+ and NMG^+, did not significantly alter H^+ currents compared with TMA^+ (Tables II and III). An exception was K^+ which appeared to shift g_H activation to more positive potentials. This effect was rather variable, and was complicated by the presence of large voltage- and time-independent K^+ currents in cells studied at pH 7.0//5.5, attributable to a Ca^{2+}-activated K^+ conductance described below. This g_K was obvious in experiments at pH_i 5.5 but not pH_i 7, perhaps because EGTA is a poor Ca^{2+} buffer at low pH (Martell and Smith, 1974; McGuigan, Lüthi, and Buri, 1991), and perhaps also because the pH_i 7 solution contained a high TEA^+ concentration. For this reason we studied the effects of K^+ at pH 7//7. The inhibition of the g_H by K^+ was not reversed by amiloride (Table III), and thus appears to represent a separate phenomenon.

Effects of DMA

Although amiloride is widely used to inhibit Na^+-H^+ exchange, it is not selective, being a more potent inhibitor of epithelial Na^+ channels (Benos, 1982), and also weakly inhibiting of other transporters such as the Ca^{2+}/H^+ exchanger (Kleyman and

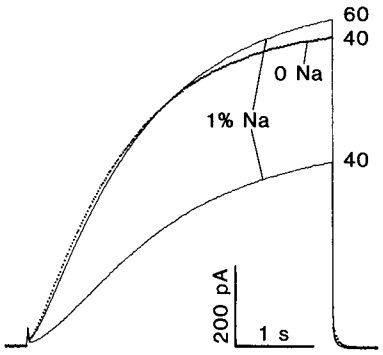

FIGURE 4. $[Na^+]_o$ sensitivity of the g_H at pH 7.0//5.5, with NMG-MES in the pipette. The H^+ current during a pulse to 40 mV in TMA^+ solution is plotted with dark dots ("0 Na"), and currents during pulses to 40 and 60 mV in 1% Na^+, 99% TMA^+ are plotted as light lines.

Cragoe, 1988). We therefore tested DMA, a more potent and selective inhibitor of Na^+-H^+ exchange (Kleyman and Cragoe, 1988). In Fig. 5 *B* I'_H is plotted in a cell bathed in TMA^+ (■), Li^+ (◆) or Na^+ (▲). Li^+ shifted I'_H by 17 mV to more positive potentials than in TMA^+, about half of the 36-mV shift produced by Na^+. Addition of 10 μM DMA (*open symbols*) had almost no effect in TMA^+, but largely reversed the shifts in Li^+ and Na^+ solutions. The inset illustrates the markedly different H^+ currents at +20 mV in the presence of the three cations, and that addition of DMA practically eliminated the differences (*lighter records*). In this experiment the H^+ currents at large positive potentials decayed with time. This decay is the result of depletion of protonated buffer from the cell, rather than a decrease in the level of activation of the g_H, which does not inactivate (Byerly et al., 1984; Meech and Thomas, 1987; Mahaut-Smith, 1989*b*; DeCoursey, 1991; DeCoursey and Cherny, 1993; Kapus et al., 1993). The effects of DMA at 10 μM were virtually identical with

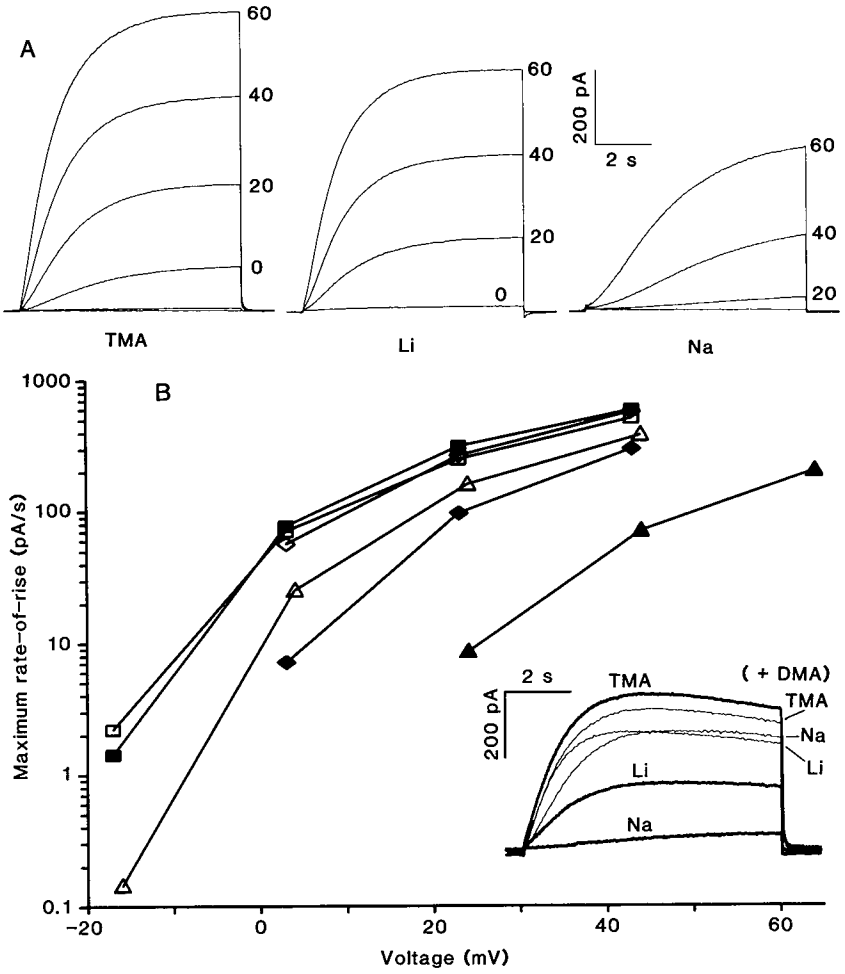

FIGURE 5. (*A*) Families of H^+ currents in a cell in TMA^+, Li^+, or Na^+ solutions, all at pH 7.0//5.5. Currents are superimposed for pulses to −40 through +60 mV in TMA^+ and 0 through +60 mV in Li^+ and Na^+ solutions. In each solution V_{hold} was −60 mV and 8-s pulses were applied at 44-s intervals in 20-mV increments. Calibration bars apply to all families. (*B*) I'_H in TMA^+ (■), Li^+ (◆), or Na^+ (▲) at pH 7.0//5.5 in a different cell from *A*. The open symbols indicate the same measurements and solutions, but with 10 μM DMA added. The inset shows currents recorded in this experiment during identical pulses to +20 mV from V_{hold} of −40 mV, in the three solutions, as indicated. The lighter lines show currents in these solutions with 10 μM DMA. Note that DMA slightly inhibited the H^+ current in TMA^+, but increased the currents both in Li^+ and in Na^+ solutions to nearly that recorded in TMA^+.

those of amiloride at 100 μM (Table II), except that 10 μM DMA only slightly inhibited the g_H in TMA^+ solution (by 2–3 mV), while amiloride produced inhibition equivalent to a 10-mV shift. On average, DMA shifted the g_H by −20 to −21 mV when added to Na^+ solution, and reversed about two-thirds of the shift by Li^+, comparable with the partial reversal by amiloride or DMA of the shift by Na^+.

Amiloride and DMA Do Not Inhibit Na^+-H^+ Antiport from Inside the Cell

High concentrations of amiloride or DMA were added to the pipette solution at pH 7.0//5.5 where the Na^+ effect was large. In four cells studied with 500 μM amiloride or 200 μM DMA in the pipette changing the external cation from TMA^+ to Na^+ shifted the H^+ current–voltage relation 35.7 ± 8.7 mV (mean ± SD), and I'_H–voltage by 38.3 ± 5.3 mV. These shifts are at least as large as observed in experiments without internal blockers in Table II. Furthermore, addition of 5 μM DMA to the external solution in these experiments produced a negative shift of the g_H in Na^+ solutions, of mean amplitude −28 mV, also generally similar to that in Table II. This result confirms that the shift observed when replacing TMA^+ with Na^+ in these cells was due to Na^+-H^+ antiport which was sensitive to DMA, but only from the external solution.

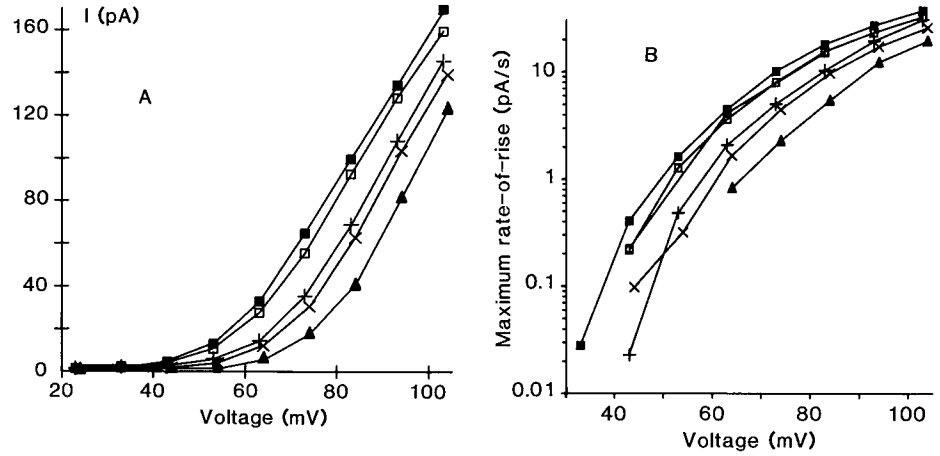

FIGURE 6. The effects of Na^+ and amiloride on H^+ currents at symmetrical pH 7.0//7.0 were similar to those at pH 7.0//5.5, but smaller. (*A*) Whole-cell currents measured at the end of 8-s pulses studied at pH 7//7 in TMA^+ (□), Na^+ (▲), Na^+ plus 100 μM amiloride (×), TMA^+ plus 100 μM amiloride (+), and again in TMA^+ (■), in that order. Pipette contained TEA-PIPES. (*B*) I'_H from the same experiment. The sequence of measurements was: TMA^+ (□), Na^+ (▲), Na^+ plus 100 μM amiloride (×), TMA^+ (□), TMA^+ plus 100 μM amiloride (+), TMA^+ (■).

Experiments at Symmetrical pH 7//7

When pH_i was increased from 5.5 to 7.0, the voltage dependence of activation of the g_H was shifted to more positive potentials, as has been observed in other cells (Byerly et al., 1984; Thomas, 1988; Mahaut-Smith, 1989*a*; Demaurex et al., 1993; DeCoursey and Cherny, 1993). In the experiment illustrated in Fig. 6, the H^+ currents at pH 7//7 were first activated at ~30 mV in TMA^+ solution, compared with ~−20 mV in cells studied at pH_i 5.5. The potential at which H^+ current was first detectable averaged −18.0 ± 9.3 mV (mean ± SD, n = 31) at pH 7//5.5 and +29.2 ± 8.8 mV

(n = 10) at pH 7//7. The "threshold" voltage of the g_H thus shifted ~47 mV when E_H was changed 87 mV.

In general, the effects of cations on the g_H at pH 7//7 were qualitatively like those at pH 7.0//5.5, but smaller. The H^+ currents in the cell illustrated in Fig. 6 *A* and I'_H in Fig. 6 *B* were shifted to more positive potentials by 15–16 mV when TMA^+ (□) was replaced with Na^+ (▲). The average shift at pH 7//7 (Table III) was 18–19 mV, clearly smaller than that observed with pH_i 5.5 (Table II). Addition of 100 μM amiloride partially reversed the inhibition by Na^+ (×, Fig. 6), but inhibited H^+ currents in TMA^+ solution (+). Thus in the presence of amiloride the H^+ currents were practically the same in Na^+ and TMA^+. Table III shows that K^+ had a small inhibitory effect on the g_H, but that addition of amiloride further inhibited the g_H, reversibly shifting the g_H to more positive potentials. That amiloride shifted the g_H in opposite directions in Na^+ and K^+ solutions supports the interpretation that the effect of Na^+ is due to Na^+-H^+ antiport while the effect of K^+ must occur through a different mechanism. With symmetrical pH, H_i^+ efflux must have been driven by the inward Na^+ gradient.

Effects of Low pH_o

Fig. 7 illustrates an experiment comparing the Na^+ effect at two pH_o. A typically large depolarizing shift of I'_H was observed upon replacement of TMA^+ (■) with Na^+ (▲) at pH 7.0//5.5. In the same cell, when pH_o was lowered to 6.0, ~30 mV larger depolarizing pulses were required to activate the g_H in TMA^+ (□). This result illustrates a well-established property of the g_H, that lowering pH_o shifts its voltage dependence to more positive potentials (Byerly et al., 1984; Barish and Baud, 1984; Meech and Thomas, 1987; DeCoursey, 1991; DeCoursey and Cherny, 1993). The reported shift of the g_H is less than the change in E_H when pH_o is lowered, ranging 29–46 mV/unit pH_o (Byerly et al., 1984; Thomas, 1988; Mahaut-Smith, 1989*a*). At pH_o 6, replacing TMA^+ with Na^+ still shifted I'_H to more positive potentials (*open symbols* in Fig. 7), but the shift was much smaller than at pH_o 7. Records superimposed in the inset in Fig. 7 illustrate that the difference in H^+ currents at +40 mV in TMA^+ and Na^+ solutions were large at pH_o 7 (*darker lines*), but much smaller at pH_o 6 (*lighter records*). Evidently H_o^+ inhibited Na^+-H^+ antiport, as has been shown in intact cells (Aronson, 1985).

In the experiment illustrated in Fig. 8 *A* the proton gradient was inward (pH 6//7). Replacing TMA^+ (■) with Na^+ (▲) or addition of 100 μM amiloride to either solution had practically no effect on I'_H. Data summarized in Table IV are consistent with a barely detectable shift by Na^+, and a slight but reversible inhibition by amiloride in both TMA^+ and Na^+ solutions (cf. *insets* to Fig. 8 *A*). All of these effects are quite small. Under these conditions, V_{rev} was independent of external cation (Figs. 8, *B* and *C*). On average V_{rev} was +59.2 ± 2.6 mV (n = 3) in TMA^+ and +59.8 ± 2.5 mV (n = 3) in Na^+ at pH 6//7. With large inward gradients of both Na^+ and H^+, the effects of Na^+-H^+ exchange on the g_H were greatly reduced or eliminated.

The average negative shift produced by amiloride or DMA was no larger than the positive shift produced by Na^+ or Li^+ at all pH_o//pH_i studied (Tables II–IV). This pattern also holds at the level of individual cells. In Fig. 9 the positive voltage shift

when TMA^+ was replaced by Na^+, plotted on the abscissae, ranged from 2 to 51 mV with various pH gradients, with greater than twofold variability for each specific condition. The subsequent negative shift produced by amiloride or DMA, plotted on the ordinates, is correlated with the initial shift, but does not exceed it. If the shift produced by Na^+ were mediated by a mechanism other than Na^+-H^+ exchange, its amplitude would not necessarily be correlated with that of the leftward shift produced by amiloride or DMA, nor would the leftward shift be expected to be consistently less than the rightward shift. Part of the reason that amiloride did not completely restore

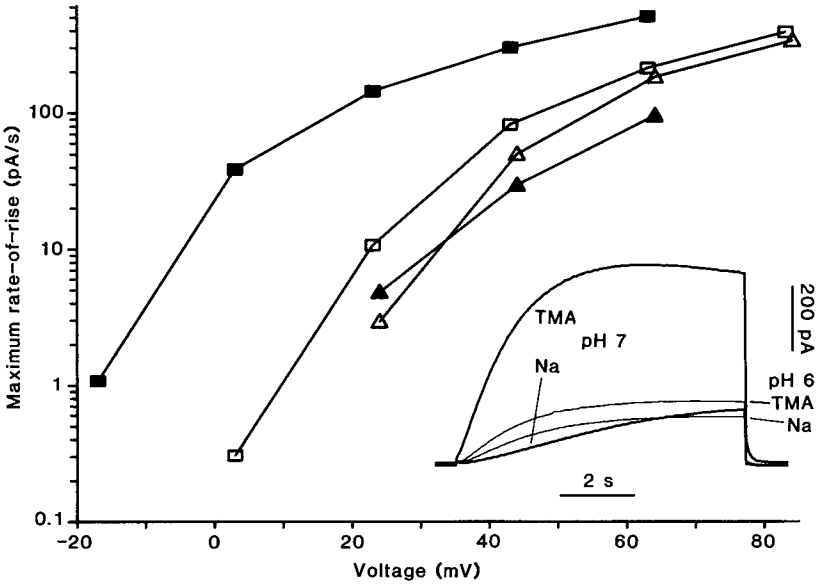

FIGURE 7. pH_o dependence of the Na^+ effect on I'_H in a cell with NMG-MES pH 5.5 in the pipette. Filled symbols indicate pH_o 7.0, open symbols indicate pH_o 6.0, squares indicate TMA^+, triangles indicate Na^+. The sequence of measurements was: TMA^+ pH_o 7 (■), Na^+ pH_o 7 (▲), Na^+ pH_o 6 (△), TMA^+ pH_o 6 (□) Superimposed in the inset are H^+ currents during pulses to +40 mV in TMA^+ and Na^+ at pH 7.0//5.5 (*dark lines*) and at pH 6.0//5.5 (*light lines*) in the same experiment. At both pH_o the current in TMA^+ was larger than in Na^+, but the difference was much greater at pH_o 7. In addition, the more sigmoid activation kinetics in Na^+ solution was obvious only at pH_o 7. V_{hold} was −40 mV for pH_o 7.0 and −20 mV for pH_o 6.0. Upon returning to TMA^+ at pH_o 7 after these measurements, H^+ currents and I'_H were slightly larger at all potentials than in the first set of measurements (data not shown).

the voltage dependence of the g_H in Na^+ to that in TMA^+ was attributed to a nonspecific inhibitory effect on the g_H. The lines extending from some data points in Fig. 9 show the combination of the amiloride shift in Na^+ solution and the inhibitory shift measured in the same cell when amiloride was added to TMA^+ solution. After this correction, the data approach the line which indicates the direct relationship expected if amiloride reversed the entire voltage shift produced by Na^+.

Evidence of Na⁺-H⁺ Antiport in Human Neutrophils

The effects of Na^+ and Li^+ on H^+ currents in human neutrophils were qualitatively similar to those in alveolar epithelial cells. Fig. 10 shows that the H^+ current at +60 mV was distinctly smaller in Na^+ than in TMA^+ solution, and that this effect was partially reversed by 100 μM amiloride. H^+ currents in neutrophils are smaller than in alveolar epithelial cells, being reduced roughly in proportion to the smaller surface area (DeCoursey and Cherny, 1993), and consequently were more variable, with a less favorable signal-to-noise ratio. The average voltage shift in the g_H–V relation by Na^+ was 23–25 mV (Table V), compared with 31–33 mV in alveolar epithelial cells. At pH 6.0//5.5 the shift was much smaller in both types of cells. It was thus possible to detect evidence of Na^+-H^+ antiport in neutrophils.

Possible Effects of Other Ion Channels

Potassium currents. In K^+-containing solutions, inward K^+ currents attributable to two types of K^+ channels were observed. Voltage-gated delayed-rectifier channels could be identified by their rapid activation upon depolarization above −50 mV, inactiva-

TABLE IV

Voltage Shifts at pH 6.0//7.0

Initial	Test	n	I'_H	H^+ currents
			mV	
TMA^+	Na^+	4	3.0 ± 1.3	2.4 ± 1.5
Na^+	+AML	3	1.3 ± 3.2	2.1 ± 1.9
Na^+ + AML	Na^+	3	−2.7 ± 2.3	−1.7 ± 1.6
TMA^+	+AML	3	0.5 ± 2.8	0.2 ± 2.3

The pipette solution for these experiments was TEA-PIPES. See Table II legend for details.

tion at positive potentials with slow recovery, and pharmacological sensitivity (DeCoursey et al., 1988; DeCoursey, 1990; Jacobs and DeCoursey, 1990). V_{hold} was usually positive enough to inactivate these channels. However, a distinct K^+-selective conductance, g_K, was observed in some experiments, which was voltage-insensitive, at least at potentials negative to those at which the delayed rectifier was activated. Because this g_K was observed mainly under conditions where Ca^{2+} buffering by EGTA would be ineffective (pH_i 5.5), we suspected that it was Ca^{2+}-activated. Fig. 11 *A* illustrates currents during voltage ramps in an experiment in which the pipette solution contained K^+ aspartate with 10 μM free Ca^{2+}. In 160 mM K^+ solution the conductance was large, rectified inwardly, and reversed near 0 mV. Reducing $[K^+]_o$ to 4.5 mM shifted V_{rev} to −86 mV and reduced the slope conductance measured at V_{rev} from 13 to 2.5 nS. Removing all external K^+ further reduced the conductance and shifted V_{rev} negative to −110 mV. In alveolar epithelial cells studied previously with low $[Ca^{2+}]_i$ we did not observe a large g_K with these properties (DeCoursey et al., 1988; DeCoursey, 1990), although in some cells there were small K^+-selective "leak" currents perhaps due to this conductance at a low level of activation (unpublished

data). A Ca^{2+}-activated g_K has not been reported in alveolar epithelial cells, but is present in white blood cells (Gallin, 1991). The complicating effect of the Ca^{2+}-activated g_K is illustrated in Fig. 11, *B* and *C*. Time-dependent H^+ currents are evident during identical pulses in both K^+ (*B*) and TMA^+ (*C*) solutions. The initial jump in current during the pulses in K^+ was apparently due to the time-independent g_K, because the subsequent family of H^+ currents in TMA^+ solution exhibited practically no initial jump. The simultaneous presence of K^+ and H^+ currents suggests that they are mediated by different channels.

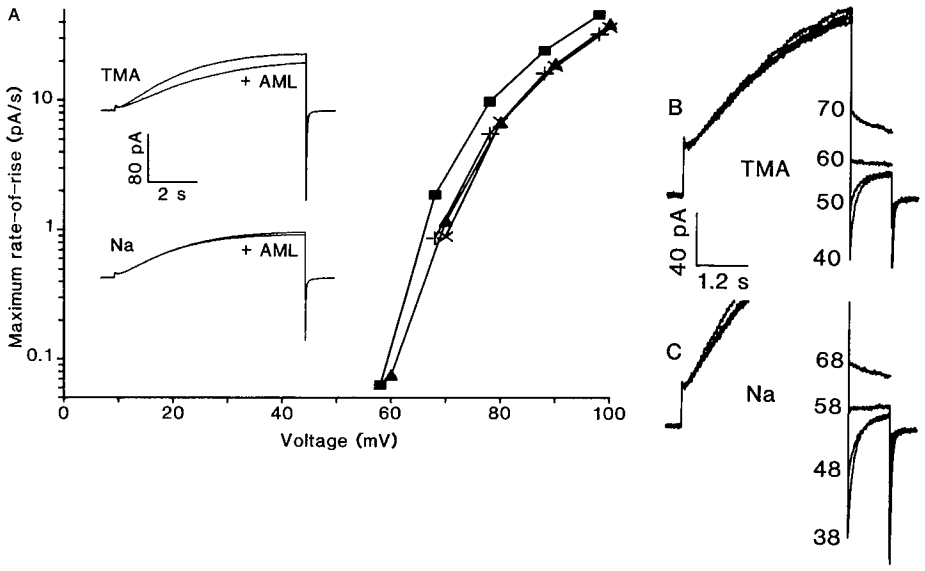

FIGURE 8. H^+ currents in a cell studied at pH 6.0//7.0 were barely affected by changes in external cation species. (*A*) I'_H measured in: TMA^+ (■), TMA^+ plus 100 μM amiloride (+), Na^+ (▲), Na^+ plus 100 μM amiloride (×). Inset shows the currents in this experiment at +90 mV in TMA^+ (*upper*) and in Na^+ (*lower*) with and without 100 μM amiloride, as indicated. Tail currents in a different cell at pH 6.0//7.0 in TMA^+ (*B*) and in Na^+ solutions (*C*). Test pulses are labelled with V_{jct} corrected voltages, and V_{rev} was +59 mV in each solution. Prepulses to 90 mV 3 s long were applied from V_{hold} of −20 mV. Pipette solution TEA-PIPES in all parts.

Cl^- Currents. A Cl^- conductance was observed in most cells when Cl^- was present in the external solution (E. R. Jacobs and T. E. DeCoursey, unpublished data). These outwardly rectifying currents were reduced when Cl^- was replaced by $CH_3SO_3^-$, and further reduced in glutamate$^-$ solution, indicating a conductance sequence: $Cl^- > CH_3SO_3^- >$ glutamate$^-$. The current rectified outwardly even in symmetrical $CH_3SO_3^-$, thus rectification is a property of the conductance mechanism. The amplitude of the g_{Cl} varied with time in whole-cell configuration, being low within seconds after establishing whole-cell configuration, rapidly increasing to a substantial value, and then slowly decreasing. Because the g_{Cl} did not exhibit obvious

time-dependent activation, $CH_3SO_3^-$ current through Cl^- channels may account for the small jump in current at the start of the voltage pulse in some records.

Amiloride-sensitive Na^+ channels. Amiloride-sensitive nonselective cation channels have been reported in fetal rat alveolar epithelial cells (Orser, Bertlik, Fedorko, and O'Brodovich, 1991), and macroscopic Na^+ currents inhibited by amiloride have been reported in rabbit type II alveolar epithelial cells (Matalon, Kirk, Bubien, Oh, Hu, Yue, Shoemaker, Cragoe, and Benos, 1992). Because our pipette solutions were Na^+-free, Na^+ currents would have a large inward driving force at V_{hold} of −40 or −60 mV. At pH 7.0//5.5 in cells with small leak currents, the whole-cell holding current increased by −7.3 ± 5.6 pA (mean ± SD, n = 12) when TMA^+ was replaced by Na^+. Addition of 100 μM amiloride changed the current by +2.8 ± 2.5 pA (n = 3), and 10 μM DMA changed the current by +0.02 ± 0.14 pA (n = 4). In cells studied at pH 7//7, the holding current at −20 or −40 mV changed by −1.1 ± 1.8 pA (n = 5) when TMA^+ was replaced by Na^+. Addition of 100 μM amiloride changed

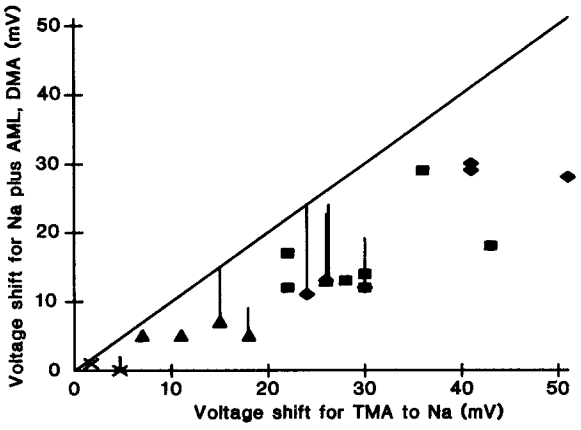

FIGURE 9. Comparison of the amplitude of the voltage shift in I'_H between TMA^+ and Na^+ solutions on the abscissae, with the voltage shift after addition of 100 μM amiloride (◆) or 10 μM DMA (■) to Na^+ solutions on the ordinates. Each symbol represents a measurement in a different cell, with pH indicated 7//5.5 (■, ◆), pH 7//7 (▲), and pH 6//7 (×). At pH 7//7 and 6//7 only amiloride was used. Vertical lines extending from data points indicate the inhibition by amiloride measured in TMA^+ solution in the same cell. The diagonal line indicates a direct correspondence between the two measurements.

the current by 0.15 ± 0.44 pA (n = 5). Amiloride did not change the holding current in TMA^+ solution at either pH_i. Under the conditions of this study amiloride-sensitive and DMA-insensitive Na^+ currents were at most extremely small, which would minimize any influence they might have had. If anything, Na^+ influx would reduce the activity of the antiporter by dissipating the Na^+ gradient, opposite to the effect we observed upon addition of Na^+ to the external solution. Na^+ uptake into rat alveolar epithelial cells is inhibited by 10 μM amiloride, but not by 100 μM DMA (Russo, Lubman, and Crandall, 1992), which suggests that DMA does not block amiloride-sensitive Na^+ channels in this preparation at concentrations 10-fold higher than those we used to inhibit Na^+-H^+ antiport. Amiloride-inhibitable Na^+ channels in taste cells can conduct protons (Gilbertson, Avenet, Kinnamon, and Roper, 1992); however these currents are insensitive to Cd^{2+} and are inhibited by 30 μM amiloride. The voltage-activated g_H studied here is inhibited by Cd^{2+} and augmented by amiloride in Na^+ solutions, and thus clearly occurs via a different mechanism. In

summary, the effects we describe appear to be unrelated to amiloride-sensitive Na^+ channels.

DISCUSSION

Na^+ and to a smaller extent Li^+ inhibited the H^+ currents in rat alveolar epithelial cells and in human neutrophils. This inhibition apparently occurred as a result of Na^+-H^+ exchange, which was enabled by adding substrate (Na^+ or Li^+) to the external solution. To a first approximation, the effects of Na^+ and Li^+ were interpretable as simple voltage shifts, analogous to shifts observed when pH_o or pH_i is changed. Changes in V_{rev} were consistent with the proposed pH changes. The voltage shifts and V_{rev} changes produced by Na^+ were largely reversed by amiloride and DMA at concentrations reported to inhibit Na^+-H^+ antiport. Thus, the inhibition of the g_H by Na^+ is not direct, but rather reflects the extrusion of protons by the exchanger, which evidently takes place at a rate capable of detectably increasing the pH_i in cells perfused by the whole-cell recording configuration of the patch-clamp technique.

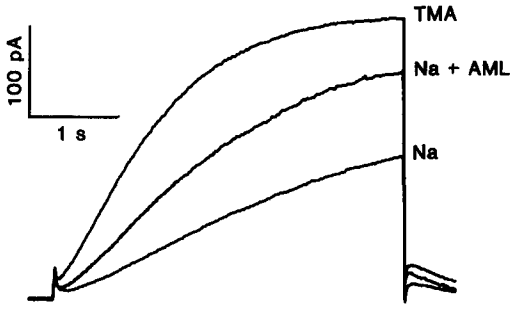

FIGURE 10. Manifestations of Na^+-H^+ antiport in a human neutrophil. Currents during pulses to +60 mV from V_{hold} of −40 mV are superimposed, in a cell at pH 7.0//5.5 perfused with NMG-MES. The sequence was TMA^+, Na^+, then Na^+ with 100 μM amiloride. Washout of the amiloride by Na^+ solution resulted in only slight reversal of the amiloride effect, but a subsequent run in TMA^+ was superimposable on the first control run, assessed both H^+ current–voltage and I'_H–voltage relationships.

A reduction of H^+ currents by extracellular Na^+ might also occur due to Na^+/HCO_3^- symport. Three factors argue against this possibility. First, the solutions used were nominally HCO_3^- free. Atmospheric CO_2 would result in ~66 μM HCO_3^- at pH_o 7.0 and less at lower pH. Secondly, the Na^+/HCO_3^- symporter in alveolar epithelium is insensitive to 1 mM amiloride (Lubman and Crandall, 1991). Since 100 μM amiloride restored more than half of the H^+ current suppressed by Na^+, and the remaining difference between the g_H in the presence of Na^+ and inert cations can be accounted for by a small inhibitory effect of amiloride, the majority of this effect of Na^+ must be due to Na^+-H^+ exchange. Finally, the Na^+/HCO_3^- symporter is electrogenic (Boron, 1986), thus its activity would result in outward net current.

Na^+-Ca^{2+} exchange has been reported in alveolar epithelial cells (Gerboth, Effros, Roman, and Jacobs, 1993). If Na_o^+ activated Na^+-Ca^{2+} exchange, pH_i might decrease

because of the reciprocal relationship between $[H^+]_i$ and $[Ca^{2+}]_i$ (e.g., Thomas, 1989), which would produce effects opposite to those observed.

Selectivity of the Effect

The only monovalent cations other than Na^+ and H^+ which are transported by most Na^+-H^+ antiporters are Li^+ and possibly NH_4^+, with K^+, Rb^+, Cs^+, choline$^+$, NMG$^+$, and TMA$^+$ being inert (Kinsella and Aronson, 1980; Aronson, 1985). The Na^+-H^+ antiporter in alveolar epithelium similarly transports Li^+ only one-fourth as well as Na^+ (estimated from Fig. 4 of Nord et al., 1987), while Rb^+, K^+, Cs^+, and TMA$^+$ were not transported detectably (Nord et al., 1987). Among the small monovalent cations studied here, Na^+ had the largest inhibitory effect on the g_H, Li^+ and K^+ had smaller inhibitory effects, while Cs^+ was essentially identical with TMA$^+$ and NMG$^+$, two larger inert cations (Aronson, 1985; Cala and Hoffmann, 1989). The inhibitory effect of K^+ in some cells might be interpreted to indicate inward K^+ transport through the Na^+-H^+ antiporter. Although there is evidence for weak interaction of

TABLE V

pH Dependence of Voltage Shifts for TMA$^+$ to Na^+

pH_o//pH_i	n	Rat alveolar epithelial cells		n	Human neutrophils	
		I'_H	H^+ currents		I'_H	H^+ currents
		mV			*mV*	
7.0//5.5	26	33.3 ± 11.1	31.0 ± 9.9	4	25.1 ± 6.7	23.3 ± 3.7
7.0//7.0	8	18.3 ± 8.7	19.3 ± 7.7		—	—
6.0//7.0	4	3.0 ± 1.3	2.4 ± 1.5		—	—
6.0//5.5	3	6.7 ± 0.8	3.2 ± 1.2	3	5.7 ± 2.9	3.7 ± 1.5

The differences between the shifts in alveolar epithelial cells and neutrophils are not statistically significant. The mean values for shifts of both I'_H and H^+ currents are significantly smaller at pH 7//7 than at 7//5.5, and both values at pH 6//7 are significantly smaller than those at pH 7//7 ($P < 0.01$ for each). The pipette solution was NMG-MES for all pH_i 5.5 measurements, and TEA-PIPES for all pH_i 7.0 measurements. Some of the epithelial cell data are also given in Tables II–IV.

external K^+, Rb^+, or Cs^+ with the Na^+-H^+ antiporter no detectable transport occurs in most cells (Aronson, 1985). Recently K_o^+ was found to compete with Na_o^+ for a site on expressed rat NHE-1 but not NHE-3 isoforms of the Na^+-H^+ exchanger, but was not detectably transported (Orlowski, 1993). We believe that the effect of K^+ on H^+ currents is not attributable to K^+ transport via the Na^+-H^+ antiporter because amiloride added to K^+ solutions further depressed the g_H, in contrast with its increase of H^+ currents in Na^+ or Li^+ solutions. Alternative pathways could be responsible for the apparent inhibitory effect of K^+, such as amiloride-insensitive K^+/H^+ exchange described in other cells (Cala, 1980; Bonanno, 1991), or a K^+/H^+-ATPase in alveolar epithelial cells (Boyd et al., 1990). Another possibility is that K^+ has a direct inhibitory effect on H^+ currents, or an indirect effect related somehow to the various K^+ currents present.

Quantitation of the Effects of Na^+-H^+ Antiport

If the entire effect of Na^+ were due to exchange for H^+, then the magnitude of the observed voltage shift should be relatable to the resulting increase in pH_i, assuming that pH_o does not significantly change. Increasing pH_i shifts the voltage dependence of the g_H to more positive potentials in all cells in which this has been examined, with

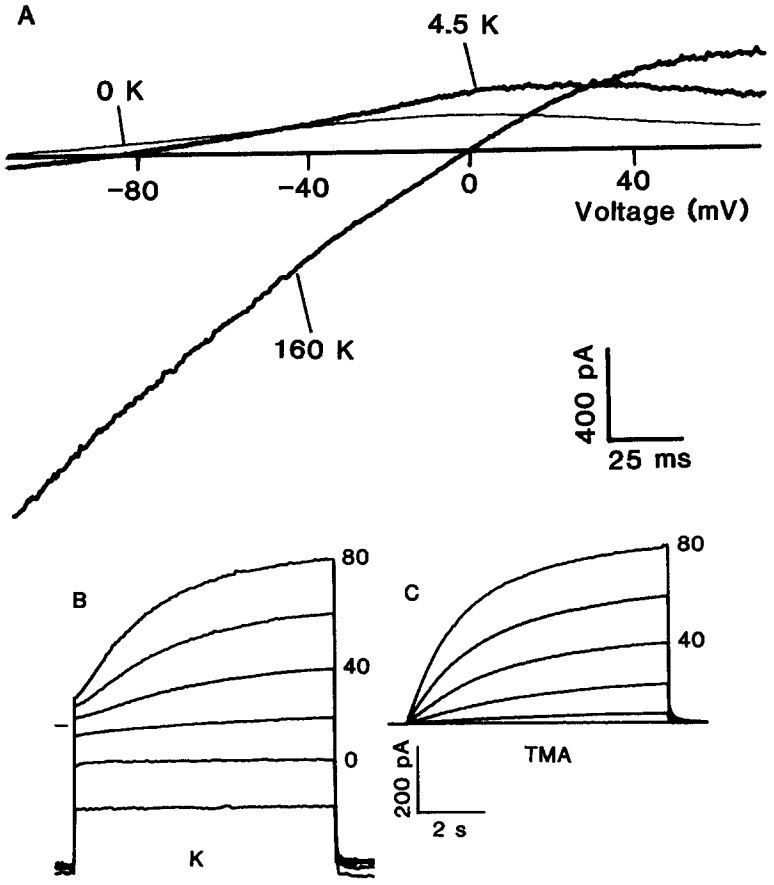

FIGURE 11. (*A*) K^+ currents activated by 10 μM Ca^{2+} in an alveolar epithelial cell with K^+ aspartate in the pipette. The cell was held at −90 mV and voltage ramps applied from −110 mV to +70 mV at 0.72 mV/ms. Currents recorded during individual ramps are illustrated for 160 mM KCl Ringer's (160 K), normal Ringer's (4.5 K), and in K^+-free NaCl Ringer's (0 K). The Na^+ Ringer's solution was identical to Ringer's in Table I with KCl omitted, and 160 K was identical to this solution with KCl substituted for NaCl. (*B*) A family of H^+ currents contaminated by the Ca^{2+}-activated K^+ conductance measured in KCH_3SO_3 solution at pH 7.0//5.5, with NMG-MES in the pipette. The cell membrane was held at −40-mV and pulses applied in 20-mV increments as indicated. The tic-mark on the left indicates the zero current level, which is the same as that in *C* for an identical family of pulses in the same cell in TMA^+ solution after washout of K^+. The large inward K^+ currents at V_{hold} −40 mV apparently changed the intracellular K^+ concentration sufficiently that V_{rev} for the g_K was ~30 mV, corresponding roughly with 40 mM $[K^+]_i$.

the shift ranging 23–40 mV/unit pH (Byerly et al., 1984; Thomas, 1988; Mahaut-Smith, 1989*a*; Demaurex et al., 1993; DeCoursey and Cherny, 1993). The change in V_{rev} when pH_i was altered in these studies was similar or larger, but less than the change in E_H. In the present study, the average "threshold" for g_H activation changed by 47 mV between pH 7//5.5 and 7//7, V_{rev} changed by 73 mV (data not shown), and E_H by 87 mV. In contrast, when TMA^+ was replaced by Na^+ at pH 7.0//5.5 the voltage dependence of the g_H was shifted 31–33 mV, but V_{rev} was only changed 23 mV. There may be pH gradients within the cell such that the pH which determines V_{rev} is not the same as the local pH near the "channel," which presumably controls its voltage dependence. Changing pH_i by dialysis with a buffered pipette solution will be least effective near the membrane to the extent that pH gradients occur; changes in pH_i due to Na^+-H^+ antiport will be maximal near the membrane and any pH gradients will be in the opposite direction. At any rate, the changes which we attribute to Na^+-H^+ exchange correspond with a pH change near the membrane of 0.4–0.5 U if we assume that the voltage dependence of the g_H and V_{rev} change in direct proportion to E_H.

To evaluate whether H^+ efflux via the antiporter could produce this change in pH_i, one must estimate the rate of Na^+-H^+ antiport. The Na^+-H^+ antiporter is electroneutral, and thus should not be affected materially by voltage pulses. Given the large outward proton gradient at pH 7.0//5.5 and large inward Na^+ gradient, the exchanger likely was operating continuously at a fairly high rate under the experimental conditions used here. The rate of Na_o^+ for H_i^+ exchange increases in intact cells when $[Na^+]_i$ is decreased (Aronson, 1985); the nominally Na^+-free pipette solutions in our experiments may have stimulated Na^+-H^+ exchange, in comparison with measurements in intact cells at comparable $pH_o//pH_i$. Na^+-H^+ antiport in alveolar epithelial cells has been studied only at $pH_i > 6.3$ or higher. Restrepo, Cho, and Kron (1990) found that Na^+-dependent H^+ efflux in HL-60 cells saturated just below pH_i 5.5 at 33.8 mmol H^+liter·min, which would correspond in a 12-μm-diam cell to a net H^+ efflux equivalent to 49 pA (balanced by identical Na^+ influx). Since the alveolar epithelial cells we studied were often larger than this, if we scale the H^+ efflux according to membrane area, a 20 μm-diam cell would have the equivalent of 137 pA net H^+ efflux, assuming comparable behavior of the Na^+-H^+ antiporter.

Changes in pH due to net H^+ transport across the membrane were calculated using a compartmental diffusion model similar to that described by Hille (1977). Fig. 12 shows the steady-state pH_i and V_{rev} calculated in a 12-μm-diam spherical cell for a range of continuous H^+ efflux at pH 7//5.5 with buffers used experimentally. It is noteworthy that over the range calculated, the changes in pH_i and V_{rev} are nearly directly proportional to the rate of H^+ efflux. If the same H^+ efflux occurred in a smaller cell, such as an 8-μm-diam neutrophil, then the calculated depletion surprisingly was almost the same. This is so because most of the concentration drop occurs in the pipette tip, as shown by Mathias et al. (1990), so that the same total membrane flux and tip resistance will result in nearly the same local membrane pH independent of cell size. The extent of BH depletion depends mainly on the balance between the total H^+ efflux rate and the diffusional resistance of the pipette tip, thus doubling the tip diameter would quadruple the H^+ efflux required to produce the same depletion. If H^+ efflux is scaled in proportion to membrane surface area, then steady-state depletion will be much greater in larger cells. For example, the

calculated pH_i and V_{rev} were 5.60 and −81.5 mV in an 8-μm-diam cell and 6.29 and −41.2 mV in a 20-μm-diam cell, assuming the same ratio of H^+ efflux/membrane area. Diffusional equilibrium in the model was faster for smaller cells, with a time constant of 2.5 s for 8-μm-diam, 8.7 s for 12-μm diam, and 38 s for a 20-μm-diam cell, consistent with both empirical and theoretical studies (Pusch and Neher, 1988; Mathias et al., 1990). These calculations show that a rate of Na^+-H^+ antiport which is reasonable for these conditions will cause depletion of protonated buffer comparable with that deduced from experimentally observed changes in the g_H.

Properties of H^+ Channels

Does Na^+ permeate H^+ channels? The substantial depolarization of V_{rev} observed in alveolar epithelial cells when Na^+ replaced inert cations in the external solution could indicate a small Na^+ permeability of the g_H. If the entire deviation of V_{rev} from E_H at pH 7//5.5 in Na^+ were due to Na^+ permeability, then the g_H would still be highly selective, with a relative permeability P_H/P_{Na} of 4×10^5. However, amiloride

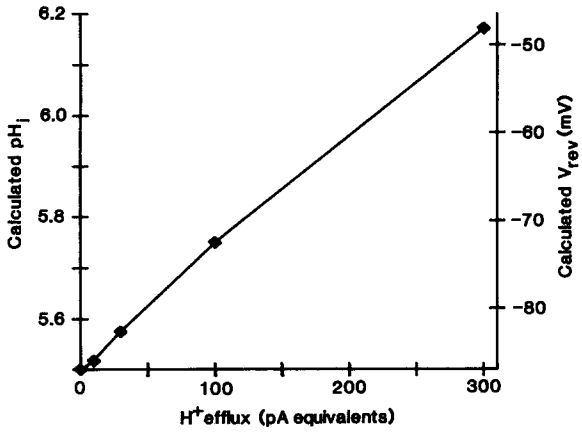

FIGURE 12. Steady-state pH_i and V_{rev} calculated for a 12-μm-diam spherical cell for a range of constant H^+ efflux. The nominal pH is 7.0//5.5, with 119 mM MES buffer in the pipette (pKa 6.1). V_{rev} was calculated from the pH in the compartment just inside the cell membrane, assuming that pH_o is not significantly altered. The nominal E_H for pH 7.0//5.5 is −87 mV. Pipette tip diameter 0.8 μm. See Methods for more details.

or DMA added to Na^+ solutions reduced the change in V_{rev}, which appears to rule out significant Na^+ permeability. In addition, V_{rev} was detectably altered by small mole fractions of Na^+ (1–2%) combined with TMA^+ solutions (data not shown). Finally, at pH 6//7 there was no detectable change in V_{rev} when Na^+ replaced TMA^+ (Fig. 8, *B* and *C*), and V_{rev} (+59.8 mV) was so close to E_H (+58.2 mV) that $P_H/P_{Na} > 10^6$ is indicated. Although each observation might be explained in other ways, the simplest interpretation is that the g_H is not detectably permeable to Na^+ or other ions, and that the change in V_{rev} observed in Na^+ solutions was the result of Na^+-H^+ exchange. Barish and Baud (1984) noted that V_{rev} was 7 mV more positive in Na^+ than TEA^+ solutions in *Ambystoma*, and suggested Na^+-H^+ exchange as one possible explanation.

Effects of amiloride and DMA on H^+ currents. The effects of amiloride on H^+ currents depended on experimental conditions in a manner consistent with two distinct effects: a cation-nonspecific inhibition, and a stimulatory effect which we attribute to inhibition of the Na^+-H^+ antiporter. In the presence of cations which are

not substrates for the antiporter, TMA^+, NMG^+, or K^+ (Tables II and III), amiloride decreased the H^+ current amplitude at a given potential and shifted the voltage dependence of the g_H to more positive potentials. These effects may be explained by the ability of amiloride, a weak base with pKa 8.8 in water (Kleyman and Cragoe, 1988), in its neutral form to permeate cell membranes (Benos, 1982; Kleyman and Cragoe, 1988). Weak acids (Gutknecht and Tosteson, 1973) or bases (Cherny, Simonova, Sokolov, and Markin, 1990) which permeate membranes in uncharged form can significantly alter local pH in unstirred layers near the membrane due to protonation/deprotonation reactions. When uncharged amiloride molecules enter the cell, they are rapidly protonated, thus depleting H_i^+ near the membrane. Depletion of H_i^+ would decrease H^+ currents both by reducing the driving force and by shifting the voltage dependence of the g_H to more positive potentials. Alternatively, amiloride may shift the g_H–V relationship by inducing a more positive surface (zeta) potential of the membrane (see Benos, 1982). On average, the shift of the g_H produced by Na^+ was almost completely offset by the combination of the shifts by amiloride in TMA^+ and Na^+, which suggests that the entire effect of Na^+ can be attributed to Na^+-H^+ exchange. DMA at 10 μM, however, had a smaller inhibitory effect on the g_H in TMA^+ solution but was no more effective than amiloride in reversing the Na^+ shift (Table II). This small discrepancy may be due to progressive loss of activity of the exchanger during the experiment, or to incomplete inhibition of the exchanger by DMA.

Properties of the Na⁺-H⁺ Antiporter in Alveolar Epithelium

Most of the properties of Na^+-H^+ antiport deduced from the present study are consistent with conclusions reached by others. It has been extensively documented that low pH_o inhibits Na^+-H^+ antiport by competing with Na_o^+ (Aronson, 1985; Otsu, Kinsella, Koh, and Froelich, 1992). We observed less evidence of Na^+-H^+ exchange at pH 6.0//5.5 (Table V) than at 7.0//7.0 indicating that the inhibitory effect of H_o^+ on Na^+-H^+ antiport is not simply attributable to the proton gradient. Despite the outward proton gradient at pH 6.0//5.5, imposition of an inward Na^+ gradient resulted in less Na^+-H^+ exchange than at pH 7//7 with no proton gradient.

The main determinant of Na^+-H^+ antiport in intact cells is pH_i (Aronson, 1985), in part because there is nearly always a large inward Na^+ gradient, but also because H_i^+ activates Na^+-H^+ antiport allosterically (Aronson, Nee, and Suhm, 1982). That the effect of Na_o^+ on the g_H was greater at pH 7.0//5.5 than at pH 7.0//7.0 is consistent with the general stimulation of antiport by H_i^+ but we cannot distinguish from our data whether the effect is due to the modulatory action of H_i^+ or simply the result of its substrate role. The low rate of Na^+-H^+ exchange observed at pH 6.0//5.5 in spite of the low pH_i, suggests that inhibition by H_o^+ can override the favorable Na^+ and H^+ gradients and the allosteric enhancement by H_i^+.

Our finding that <2 mM $[Na^+]_o$ activated Na^+-H^+ exchange significantly suggests a higher affinity of Na_o^+ for the external Na^+ transport site than in previous studies of alveolar epithelium in which K_d ranged 16–62 mM (Nord et al., 1987; Sano et al., 1988; Shaw et al., 1990). However, the precise value of K_d depends on experimental conditions (Aronson, 1985), which were quite different here than in studies of Na^+-H^+ antiport in intact cells. Given the absence of Na^+ in our internal (pipette)

solutions, even 2 mM $[Na^+]_o$ would result in an inward Na^+ gradient. In contrast, $[Na^+]_i$ is 51 mM in intact alveolar epithelial cells (Jones, Miles, Lantz, Hinton, and Castranova, 1982), thus at low $[Na^+]_o$ there would be a large outward Na^+ gradient, which would not favor Na^+-H^+ antiport. In membrane vesicle studies in which $[Na^+]_i$ is zero, 1 mM $[Na^+]_o$ activates Na^+-H^+ antiport (e.g., Kinsella and Aronson, 1980). At any rate, quite low $[Na^+]_o$ was sufficient to activate Na^+-H^+ exchange under the conditions employed. In future studies we will explore the role of $[Na^+]_i$ in Na^+-H^+ antiport. The same considerations may explain why we saw evidence of Na^+-H^+ antiport at pH_i 7.0, whereas in intact cells exchange was detected only at pH_i 6.8 or lower (Nord et al., 1987).

The high amiloride and DMA sensitivity of effects we attribute to Na^+-H^+ antiport are more consistent with NHE-1 than NHE-3 being the main isoform (Orlowski, 1993) present in alveolar epithelial cells, although incomplete reversal of Na^+ effects by DMA may reflect minor expression also of a less-sensitive isoform. Indirect evidence suggests that amiloride inhibits Na^+-H^+ antiport at an external site. Amiloride competes with Na_o^+ for the Na^+ transport site, and preincubation of vesicles with amiloride which due to its high lipid permeability would load the vesicles did not detectably alter the sensitivity of Na^+-H^+ antiport to external amiloride (Kinsella and Aronson, 1981). In the present study we demonstrate directly that internally applied amiloride or DMA do not inhibit the Na^+-H^+ antiporter. Because both amiloride and DMA are weak bases that readily permeate membranes in their neutral form (Benos, 1982; Kleyman and Cragoe, 1988), this result cannot be taken at face value to rule out an internal site of action. Several weak base K^+ channel blockers which apparently act at intracellular sites are much less potent when applied in the pipette solution to cells in the whole-cell configuration of the patch clamp technique than when applied to the bathing solution (Jacobs and DeCoursey, 1990). However, amiloride and DMA were added to the pipette solution at pH 5.5, at which most drug molecules would be positively charged, and the concentrations used were large, 500 μM amiloride and 200 μM DMA. It is intriguing that amiloride blocks amiloride-sensitive Na^+ channels only when applied to the external, and not to the internal side of excised membrane patches (Palmer and Frindt, 1986).

Na^+-H^+ antiport has been extensively and intensively studied in a number of cells and also in membrane vesicle preparations. The activity of the exchanger has been deduced from tracer flux and pH measurements, with transmembrane potential sometimes controlled by strategies such as "ionophore voltage-clamping." We demonstrate here that clear evidence of Na^+-H^+ exchange can be observed in single, perfused, voltage-clamped cells. Although this approach, like any other, has certain limitations (for example it cannot detect "futile" modes of operation of the transporter such as Na^+/Na^+ exchange or H^+/H^+ exchange), it provides a novel and highly sensitive means of studying the Na^+-H^+ antiporter.

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Note added in proof: Recent pharmacological evidence corroborates our conclusion that NHE-1 is the predominant isoform in rat alveolar epithelial cells (Lubman, R. L., and E. D. Crandall. 1994. Polarized distribution of Na^+-H^+ antiport activity in rat alveolar epithelial cells. *American Journal of Physiology.* 266:L138–L147).

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