

The Mammalian Na⁺/H⁺ Antiporters NHE-1, NHE-2, and NHE-3 Are Electroneutral and Voltage Independent, But Can Couple to an H⁺ Conductance

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ABSTRACT Na⁺/H⁺ exchange in vertebrates is thought to be electroneutral and insensitive to the membrane voltage. This basic concept has been challenged by recent reports of antiport-associated currents in the turtle colon epithelium (Post and Dawson, 1992, 1994). To determine the electrogenicity of mammalian antiporters, we used the whole-cell patch clamp technique combined with microfluorimetric measurements of intracellular pH (pH_i). In murine macrophages, which were found by RT-PCR to express the NHE-1 isoform of the antiporter, reverse (intracellular Na⁺-driven) Na⁺/H⁺ exchange caused a cytosolic acidification and activated an outward current, whereas forward (extracellular Na⁺-driven) exchange produced a cytosolic alkalization and reduced a basal outward current. The currents mirrored the changes in pH_i, were strictly dependent on the presence of a Na⁺ gradient and were reversibly blocked by amiloride. However, the currents were seemingly not carried by the Na⁺/H⁺ exchanger itself, but were instead due to a shift in the voltage dependence of a preexisting H⁺ conductance. This was supported by measurements of the reversal potential (E_{rev}) of tail currents, which identified H⁺ (equivalents) as the charge carrier. During Na⁺/H⁺ exchange, E_{rev} changed along with the measured changes in pH_i (by 60–69 mV/pH). Moreover, the current and Na⁺/H⁺ exchange could be dissociated. Zn²⁺, which inhibits the H⁺ conductance, reversibly blocked the currents without altering Na⁺/H⁺ exchange. In Chinese hamster ovary (CHO) cells, which lack the H⁺ conductance, Na⁺/H⁺ exchange produced pH_i changes that were not accompanied by transmembrane currents. Similar results were obtained in CHO cells transfected with either the NHE-1, NHE-2, or NHE-3 isoforms of the antiporter, indicating that exchange through these isoforms is electroneutral. In all the isoforms tested, the amplitude and time-course of the antiport-induced pH_i changes were independent of the holding voltage. We conclude that mammalian NHE-1, NHE-2, and NHE-3 are electroneutral and voltage independent. In cells endowed with a pH-sensitive H⁺ conductance, such as macrophages, activation of Na⁺-H⁺

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exchange can modulate a transmembrane H^+ current. The currents reported in turtle colon might be due to a similar "cross-talk" between the antiporter and a H^+ conductance.

INTRODUCTION

Na^+/H^+ antiporters are ubiquitous plasma membrane transport proteins, involved in multiple cellular functions including regulation of intracellular pH (pH_i), control of cell volume, and initiation and/or control of mitogenesis (Grinstein, Rothstein, and Cohen, 1985; Moolenaar, 1986; Grinstein, Rotin, and Mason, 1989). Under physiological conditions, antiporters in eukaryotic cells catalyze the exchange of extracellular Na^+ for intracellular H^+ , a process that is effectively blocked by amiloride and its 5,*N*-alkylated derivatives (see Grinstein et al., 1989; Wakabayashi, Sardet, Fafournoux, Counillon, Pages, and Pouyssegur, 1992, for reviews). Na^+/H^+ exchange has been reported in a wide range of tissues in many animal species. While certain features such as cation specificity are common to all the antiporters studied, the kinetic and pharmacological properties of Na^+/H^+ exchange differ between individual cell types and, in polarized epithelial cells, also between the apical vs basolateral membranes (Haggerty, Agarwal, Reilly, Adelberg, and Slayman, 1988; Fliegel and Frolich, 1993). A structural diversity parallels this functional heterogeneity, as to date, four distinct isoforms of the Na^+/H^+ exchanger (NHE) have been identified in mammalian tissues (Sardet, Franchi, and Pouyssegur, 1989; Orłowski, Kandasamy, and Shull, 1992; Tse, Brant, Walker, Pouyssegur, and Donowitz, 1992; Wang, Orłowski, and Shull, 1993). When expressed heterologously in cells devoid of endogenous Na^+/H^+ exchange activity, three of these isoforms, termed NHE-1 to NHE-3, display unique kinetic and pharmacological profiles that account at least in part for the heterogeneity reported in native tissues (Orłowski, 1993; Tse, Levine, Yun, Montrose, Little, Pouyssegur, and Donowitz, 1993*a*; Levine, Montrose, Tse, and Donowitz, 1993; Yu, Shull and Orłowski, 1993; Kapus, Grinstein, Wasan, Kandasamy, and Orłowski, 1994). The remaining identified isoform, NHE-4 has proven refractory to heterologous expression. Individual NHE isoforms are highly conserved among different species. For example, mammalian NHE-1 homologues have 93–96% amino-acid identity (Tse, Levine, Yun, Brant, Counillon, Pouyssegur, and Donowitz, 1993*b*; Fliegel and Frolich, 1993), and the human NHE-1 shows 64% identity to the trout β -NHE isoform (Borgese, Sardet, Cappadoro, Pouyssegur, and Motais, 1992) and 50% identity to partial clones isolated from turtle (Harris, Richards, Logsdon, Pouyssegur, and Dawson, 1992) and nematodes (Prasad and Bailie, 1989).

Na^+/H^+ exchange is believed to be electroneutral and insensitive to the transmembrane voltage. This central notion is based on the following kinetic and thermodynamic considerations: (*a*) when performed under comparable conditions, measurements of transmembrane fluxes showed that Na^+ uptake and H^+ (equivalent) efflux were nearly identical, consistent with a stoichiometry of 1:1 (Cala, 1980; Boron and Boulpaep, 1983; Grinstein, Cohen, and Rothstein, 1984); (*b*) net flux through the antiporter was negligible when the Na^+ gradient was counterbalanced by an identical H^+ gradient (i.e., $[Na^+]_o/[Na^+]_i = [H^+]_o/[H^+]_i$, where the sub-

scripts *o* and *i* refer to extra- and intracellular, respectively) (Cala, 1980); (c) indirect measurements showed that activation of Na⁺/H⁺ exchange had no effect on the transmembrane potential (V_m) (Kinsella and Aronson, 1980; Aronson, 1985); and (d) conversely, manipulation of V_m with ionophores did not appear to affect the rate of Na⁺/H⁺ exchange (Murer, Hopfer, and Kinne, 1976; Kinsella and Aronson, 1980).

The original information on the electrical correlates of Na⁺/H⁺ exchange relied entirely on indirect isotopic or spectroscopic determinations of V_m , mostly in isolated membrane vesicles (e.g., Murer et al., 1976; Kinsella and Aronson, 1980). Recent studies using direct electrophysiological measurements of the antiporter in vertebrate tissues yielded opposite results. In apically permeabilized layers of turtle colon epithelium, Post and Dawson (1992, 1994) reported the occurrence of well defined antiport-mediated currents. The currents were strictly Na⁺ (or Li⁺) dependent and were sensitive to submillimolar doses of amiloride, hallmarks of the Na⁺/H⁺ antiporter (Post and Dawson, 1992, 1994). These findings have challenged the 20-yr old dictum that the vertebrate antiporter is electroneutral.

Although direct measurements of transepithelial currents sensitive to basolateral amiloride is a convincing criterion for electrogenic Na⁺/H⁺ exchange, identical currents could in principle result from coupling of electroneutral Na⁺/H⁺ exchange to a conductive process. In complex cellular systems endowed with multiple transporters, the ionic concentration changes associated with activation of the Na⁺/H⁺ antiport can conceivably modulate the activity of other, conductive or electrogenic pathways. In fact, such indirect coupling between Na⁺/H⁺ exchange and the electrogenic Na⁺/K⁺ ATPase has been suggested (Rosic, Standaert, and Pollet, 1985; Fehlmann and Freychet, 1981) and coupling with an H⁺ conductive pathway has also been proposed (DeCoursey and Cherny, 1994*a,b*). The latter system, a highly H⁺-selective conductance, is present in invertebrates (Thomas and Meech, 1982; Byerly, Meech, and Moody, 1984; Byerly and Suen, 1989), amphibians (Barish and Baud, 1984; Baud and Barish, 1984) and was recently described in mammalian cells (DeCoursey, 1991; Demaurex, Grinstein, Jaconi, Schlegel, Lew, and Krause, 1993; Kapus, Romanek, Yi, Rothstein, and Grinstein, 1993; DeCoursey and Cherny, 1993). The magnitude of the H⁺ current is exquisitely sensitive to changes in the intra- and extracellular pH (pH_i and pH_o , respectively), not only because H⁺ (equivalents) are the charge-carrying species, but also as a result of direct effects on the conductance activation process (Lukacs, Kapus, Nanda, Romanek, and Grinstein, 1993; DeCoursey and Cherny, 1994*b*). These features may make the H⁺ conductance uniquely sensitive to changes in the activity of the Na⁺/H⁺ antiport. Under conditions when the pH is not stringently controlled, stimulation of Na⁺/H⁺ exchange could conceivably modulate the current flowing through the conductance. The possibility that coupling to a H⁺ conductance contributes to the antiport-mediated currents measured in turtle colon has not been addressed.

As summarized above, the evidence supporting the electroneutrality of the antiporter is partly indirect and was obtained largely in cell-free systems. On the other hand, the findings suggesting electrogenicity were derived from direct electrophysiological determinations, but failed to rule out coupling with other pathways. Moreover, the apparent discrepancies in these reports may be attributed to the involve-

ment of different isoforms. In an attempt to clarify these issues, the present study aimed to determine the electrogenicity of individual isoforms of mammalian (rodent) antiporters, using concurrent measurements of intracellular pH and transmembrane currents. In the course of these studies, we found that, in the whole-cell configuration of the patch clamp technique, the rate of transport of H⁺ by the antiporter outstripped the diffusion of buffers from the pipette, resulting in readily measurable changes of pHi. This enabled us to directly assess the voltage sensitivity of the different isoforms of the antiporter.

METHODS

Materials and Media

Nigericin, 2',7'-bis-(2-carboxyethyl)-5-(and 6) carboxyfluorescein (BCECF) free acid and acetoxy-methyl ester (AM) were purchased from Molecular Probes Inc. (Eugene, OR). Powdered Brewer's thioglycolate was purchased from Difco Laboratories (Detroit, MI). 2-[*N*-morpholino]ethanesulfonic acid (MES) and medium RPMI-1640 were from Sigma Chemical Co. (St. Louis, MO). Tris[hydroxymethyl]aminomethane (TRIS) was obtained from Boehringer-Mannheim Corp. (Indianapolis, IN). All other chemicals were of analytical grade and were from Aldrich Chemical Co. (Milwaukee, WI). Amiloride was a kind gift from Merck Sharp and Dohme (St. Louis, MO). Compound HOE694 was the generous gift of Hoechst.

The composition of the buffers used is shown in Table I. All solutions contained 1 mM MgCl₂ and 1 mM ethylene glycol-bis(β-amino-ethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA); pipette solutions also contained 2 mM MgATP and 200 μM BCECF (free acid). When Zn²⁺ was used, EGTA was omitted and ZnSO₄ was added to a final concentration of 1 mM. The osmolarity was set to 280 ± 5 mosm (pipette) and to 300 ± 5 mosm (bath).

Cells

Peritoneal macrophages were obtained from 6-wk old Swiss Webster mice (Charles River Breeding Laboratories Inc., Wilmington, MA) injected intraperitoneally with 2 ml of thioglycolate 5 d before harvest, as described (Kapus, Romanek, Yi, Rothstein, and Grinstein, 1993). Peritoneal exudate cells were consistently >85% macrophages, as assessed by Wright's stain. The contaminating cells, mostly lymphocytes, are not adherent and were therefore removed during plating. For plat-

TABLE I
Composition of Internal (Pipette) and External (Bath) Solutions

Solution	pH	Buffer	NaOH	CsOH	Aspartate
Ba 1	6.5	100	120	2.5	77.5
Ba 2	6.5	100	—	122.5	77.5
Ba 3	8.0	100	—	85	135
Ba 4	8.0	100	85	—	135
Pi 1	6.5	5	120	30	150
Pi 2	6.0	5	—	150	150

Ba, bath solutions; Pi, pipette solutions. Buffers used were MES (pH 6.0–6.5) or Tris (pH 8.0). Concentrations are in mM. All solutions contained 1 mM MgCl₂ and 1 mM EGTA, and pipette solutions contained in addition 2 mM MgATP and 200 μM BCECF (free acid). The osmolarity was adjusted to 280 ± 5 mosm (pipette) and to 300 ± 5 mosm (bath).

ing, $1-2 \times 10^5$ cells were deposited onto 25-mm diam glass coverslips (Thomas Scientific, Swedesboro, NJ), allowed to adhere for 30 min at 37°C, and washed three times with medium RPMI 1640. Adherent cells were cultured for up to 3 d in a humidified atmosphere of 95% O₂, 5% CO₂ at 37°C. Macrophage viability was >95%, as determined by exclusion of trypan blue.

CHO cells deficient in Na⁺/H⁺ exchange were stably transfected with the full-length cDNAs encoding one of three isoforms (NHE-1, NHE-2, NHE-3) of the rat antiporter, as previously described (Orlowski, 1993; Wang et al., 1993). The transfected cells were grown in α -minimal essential medium (Ontario Cancer Institute) supplemented with 10% fetal calf serum and 1% antibiotic solution (penicillin and streptomycin, GIBCO Laboratories, Grand Island, NY) and passaged twice a week. Cultures were reestablished from frozen stocks and cells from passages 3 to 12 were grown on glass coverslips for 24 to 48 h before the experiments.

Isolation of RNA, reverse transcription and polymerase chain reaction (RT-PCR). Total RNA was isolated from $\approx 10^6$ mouse macrophages by guanidium thiocyanate-phenol-chloroform extraction, based on the method of Chomczynski and Sacchi (1987). Poly(A⁺)-RNA was purified by affinity chromatography with an oligo-dT cellulose column (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). Macrophage mRNA was then reverse transcribed and the complementary DNA amplified by the polymerase chain reaction, using the GeneAmp RNA PCR kit (Perkin-Elmer Corp., Foster City, CA) and a Perkin-Elmer DNA thermal cycler model 480. After completion of the PCR reaction (35 cycles), a 10- μ l sample of the PCR tube was analyzed by electrophoresis on a 0.8% agarose gel prestained with 0.5 μ g/ml ethidium bromide and the gel was photographed under UV illumination. For illustration, the photograph was scanned and labeled using Adobe Photoshop software (Adobe Systems, Inc.). Four isoform-specific sets of primers were used, which hybridized to unique regions of the rat NHE-1, NHE-2, NHE-3 and NHE-4. Primers were as follows: NHE-1, 5' primer: CCT ACG TGG AGG CCA AC, 3' primer: CAG CCA ACA GGT CTA CC, size of the PCR product: 429 bp; NHE-2, 5' primer: GCT GTC TCT GCA GGT GG, 3' primer: CGT TGA GCA GAG ACT CG, size of PCR product: 680 bp; NHE-3, 5' primer: CTT CTT CTA CCT GCT GC, 3' primer: CAA GGA CAG CAT CTC GG, size of PCR product: 574 bp; NHE-4, 5' primer: CTG AGC TCT GTG GCT TC, 3' primer: C GAG GAA ATG CAG CAG C, size of PCR product: 381 bp. All four sets of primers yielded the expected PCR products when linearized pCMV plasmids containing the full-length clone of the corresponding isoform were used as a template, but did not yield discernible products when any of the other isoforms was used as template.

Measurements of intracellular pH. The intracellular pH was measured microfluorimetrically, as described (Kapus et al., 1993). Briefly, cells adhered to coverslips were incubated with 2 μ M of the fluorescent indicator BCECF-AM for 15 min at room temperature and experiments were performed within 1 h. Coverslips were inserted into a Leiden CoverSlip Dish (Medical Systems Corp., Greenvale, NY), which was placed into a holding chamber (Open Perfusion Micro-Incubator; Medical Systems Corp.) attached to the stage of a Nikon Diaphot TMD inverted microscope (Nikon Canada, Toronto). The chamber allowed continuous perfusion of the cells with the indicated media at a rate of ≈ 0.5 ml/min, while maintaining the temperature at 37°C. Solutions could be switched by operating solenoid valves (General Valve, Fairfield, NJ) and total exchange of the bath occurred within ≈ 1 min. The microscope was equipped with a Fluor 40 \times oil-immersion objective, a Hoffman Modulation Contrast video system (Modulation Optics, Greenvale, NY) and an M Series Dual Wavelength Illumination System (Photon Technologies Inc., South Brunswick, NJ). Excitation light provided by a Xenon lamp was directed into 495 \pm 10 nm and 440 \pm 10 nm filters (Omega Optical, Brattleboro, VT) by a chopping mirror rotating at 100 Hz and the alternating light was then reflected at the cells by a 510-nm dichroic mirror. The emitted fluorescence and the red light (>600 nm) used for Hoffman illumination were separated with a 550-nm dichroic mirror. Fluorescence intensity was measured at 535 \pm 25 nm using a photometer, whereas the red light was directed to a video camera. This optical system allowed continuous visualization of the cells without interfering with the fluorescence measurements. Photometric data

were recorded at 2 Hz using a 12-bit A/D board interfaced to a NEC 386 computer. The excitation ratio of the fluorescence was calculated on line using the PTI software. Calibration of fluorescence ratio vs pH was performed on single nonpatched cells, using nigericin (5 μ M final) according to (Thomas, Buchsbaum, Zimniak, and Racker, 1982). One calibration curve was obtained every day by averaging data from 3–6 cells sequentially perfused with KCl media buffered at four different pH values ranging from 6.0 to 7.5. In one series of experiments (Fig. 8 C) macrophage fluorescence was measured in cell suspensions, essentially as described (Swallow, Grinstein, and Rothstein, 1990).

Electrophysiology. The whole-cell configuration of the patch clamp technique was used essentially as described (Kapus et al. 1993). Electrodes made from filament-filled borosilicate glass capillaries (World Precision Instruments, Inc., Sarasota, FL) using a P-87 horizontal puller (Sutter Instrument Co., Novato, CA) were fire polished using a MF-9 microforge (Narishige USA, Greenvale, NY). Pipette resistance ranged from 2 to 10 M Ω and seal resistance from 10 to 50 G Ω . Recordings were made using an Axopatch-1D amplifier (Axon Instruments Inc., Foster City, CA) in the voltage clamp mode. Series resistance varied between 5 and 30 M Ω and cell capacitance ranged between 10 and 70 pF (for macrophages) or between 12 and 34 pF (for CHO cells); both parameters were logged for subsequent use in data analysis. When bath solutions were exchanged, changes in junction potential were <5 mV. Currents were filtered at 50 or 200 Hz and recorded at 0.2 or 1 kHz, respectively, using a 12 bit A/D board (LabMaster, Axon Instruments, Inc.) interfaced to a PC/AT computer. Data acquisition was performed using pClamp (Axon Instruments, Inc.). None of the experiments illustrated were leak subtracted. Data analysis and statistics were carried out using the Origin software (MicroCal Software Inc., Northampton, MA) and are shown as means \pm one standard error (SE) of the number of experiments indicated. All measurements were carried out at 37°C.

Other methods. The preparation and purification of anti-NHE-1 antibodies and the method used for immunoblotting of macrophage membranes have been described in detail elsewhere (Grinstein, Woodside, Sardet, Pouyssegur, and Rotin, 1992).

RESULTS

Antiport-mediated Currents in Murine Macrophages

Fig. 1, A and B, illustrate the experimental paradigms utilized by Post and Dawson (1992, 1994) to elicit Na⁺/H⁺ exchange associated currents. Outward (cell to serosa) current was generated in the turtle colon when the antiport operated in reverse in Na⁺-containing cells bathed in Na⁺-free medium (Fig. 1 A). Conversely, inward current (a decrease in basal outward current) was noted when forward Na⁺/H⁺ exchange was activated by the imposition of an outwardly directed H⁺ gradient.

To define whether antiport-associated currents exist also in mammalian cells, we simultaneously measured transmembrane currents and pH_i in murine macrophages. For this purpose we combined the use of the whole-cell patch clamp technique with microfluorimetric measurements of the emission of the pH-sensitive dye BCECF. The ionic conditions used to elicit reverse Na⁺/H⁺ exchange in turtle colon (Fig. 1 A) were mimicked in macrophages (Fig. 2, *top inset*), using pipette solutions with a low buffering capacity (5 mM MES) to facilitate the detection of antiport-induced pH_i changes. The cells were voltage clamped at -60 mV, which approximates the resting potential of intact macrophages and 1-s depolarizing pulses to 0 mV were applied at 10-s intervals (Fig. 2, *bottom inset*) to reproduce the "short-circuit" conditions utilized in the experiments with colonic epithelia. Ini-

tially, both pipette and bath solutions contained 120 mM Na⁺, pH 6.5 (solutions Ba 1 and Pi 1; see Table I). After establishment of the whole-cell configuration the cytosolic pH, which was initially near neutrality, equilibrated within 3 min at a value similar to that of the pipette solution. At this point, the combined chemical gradient of Na⁺ and H⁺ is near zero and essentially no current was detected at either -60 and 0 mV, the input resistance of the cells being consistently ≥ 25 G Ω in this voltage range. To activate Na⁺/H⁺ exchange, the bath solution was isosmotically replaced with a Na⁺-free solution (solution Ba 2), hence creating an outward (cell-to-medium) Na⁺ gradient. The onset of reverse antiport activity was indicated by a

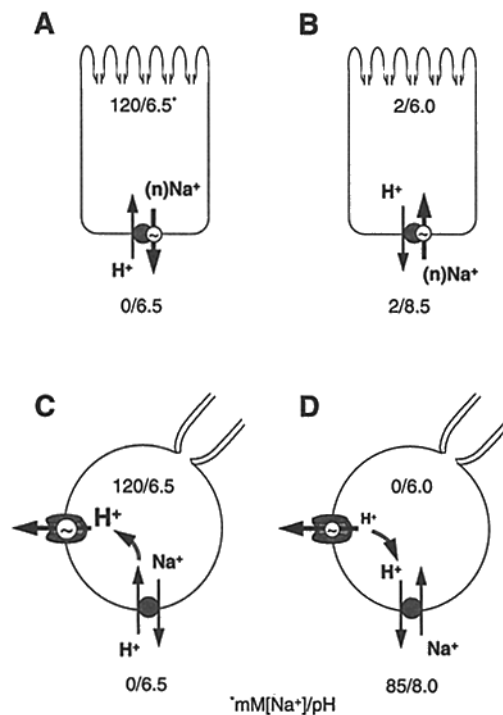


FIGURE 1. Proposed models accounting for Na⁺/H⁺-associated current. (*A* and *B*) The conditions used by Post and Dawson (1994) to detect antiport-associated currents in permeabilized turtle colon epithelium. The Na⁺ concentration (in millimolar) and the pH of the intra- and extracellular solutions are indicated. Currents (*thick arrows*) were observed upon activation of reverse (*A*) or forward (*B*) Na⁺/H⁺ exchange and followed the direction of the Na⁺ fluxes. To account for these observations, the authors proposed a model of Na⁺ to H⁺ stoichiometry >1. (*C* and *D*) The conditions applied in the present whole-cell study of murine macrophages. Basal outward currents (*thick arrows*) through a separate conductive pathway were increased by reverse Na⁺/H⁺ exchange (*C*) and reduced by forward Na⁺/H⁺ exchange (*D*). The basal currents are carried by H⁺ ions and are modulated by changes in intracellular pH. The currents closely mirror the pH changes because H⁺ ions are both substrates and activators of the conductance.

rapid cytosolic acidification, the pH_i dropping by ≈ 0.3 U within 5 min (Fig. 2, *top trace*). The rate of H⁺ translocation by the antiporters thus outstripped the diffusion of H⁺ equivalents and buffers to and from the patch pipette, causing the intracellular pH to deviate from the pipette pH.

The lower panel in Fig. 2 illustrates the current changes associated with activation of the reverse mode of the antiport. The decrease in pH_i was accompanied by an outward current at 0 mV (Fig. 2, *open circles*), whereas the current at -60 mV remained constant (*filled circles*). The current changes recorded at 0 mV closely paralleled the decrease in pH_i, suggesting an association with Na⁺/H⁺ exchange activity.

Accordingly, addition of amiloride (1 mM) to the bath solution reversed both the changes in current and in pH_i , the cytosolic pH alkalizing towards the pH of the pipette. As in other systems, the inhibition of Na^+/H^+ exchange by amiloride was readily reversible, and removal of the drug resulted in a second bout of acidification. The changes in current measured at 0 mV again mirrored the changes in pH_i : the current virtually disappeared in the presence of amiloride but was quickly restored when the inhibitor was removed. The amiloride sensitivity, as well as the

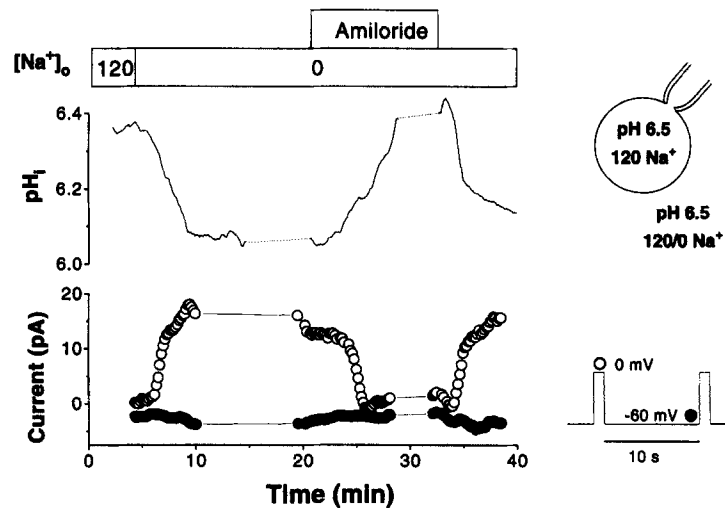


FIGURE 2. Reverse activation of the antiporter, detectable as cytosolic acidification, is associated with an outward current. Simultaneous measurements of intracellular pH (*top*) monitored by microfluorimetry with BCECF and of whole-cell currents (*bottom*) in a mouse peritoneal macrophage. The cell was voltage clamped at -60 mV and 1-s pulses to 0 mV were applied at 10-s intervals (*bottom inset*). The initial bath and pipette solutions (Ba 1, Pi 1 in Table I) were symmetrical and contained 120 mM Na^+ , pH 6.5 (see *top inset and bar*). 5 min after attaining the whole-cell configuration, the external Na^+ was isoosmotically replaced by Cs^+ (solution Ba 2, indicated as 0 $[\text{Na}^+]_o$ in top bar). Where indicated, 1 mM amiloride was applied externally. Current was measured at -60 mV (*filled circles*) and at the end of the 1-s depolarizing pulse to 0 mV (*open circles*). Where indicated by the connecting lines, data acquisition was suspended to perform detailed current-voltage or tail current analysis (e.g., Figs. 4–6). Cell capacitance: 24 pF, access resistance: 9 M Ω . Results are representative of 10 similar experiments.

$[\text{Na}^+]_o$ dependence of the concurrent changes in pH_i and current indicate that both are associated with the antiporter.

We next tested whether forward Na^+/H^+ exchange could induce inward currents through the antiporter, as reported in turtle colon (Post and Dawson, 1994). Using Na^+ -free solutions in bath and pipette (solutions Ba 3 and Pi 2, respectively), a transmembrane pH gradient of 2 U was imposed (Fig. 3, *inset*). Under these conditions, an outward current was present at voltages > -60 mV and large (>400 pA) currents were observed at 0 mV. To minimize changes in the intracellular concen-

tration of ions caused by these large currents, V_m was held at -60 mV and pulses to -50 mV were applied. Imposition of an inward Na^+ gradient by introducing a bath solution containing 85 mM Na^+ (solution Ba 4) produced a brisk cytosolic alkalinization of ≈ 0.5 pH U (Fig. 3, *top trace*). The change in pH_i was accompanied by a reduction of the outward current, measured at -50 mV (*bottom trace*). Again, the changes in current closely mirrored the changes in pH_i , and both were reversibly

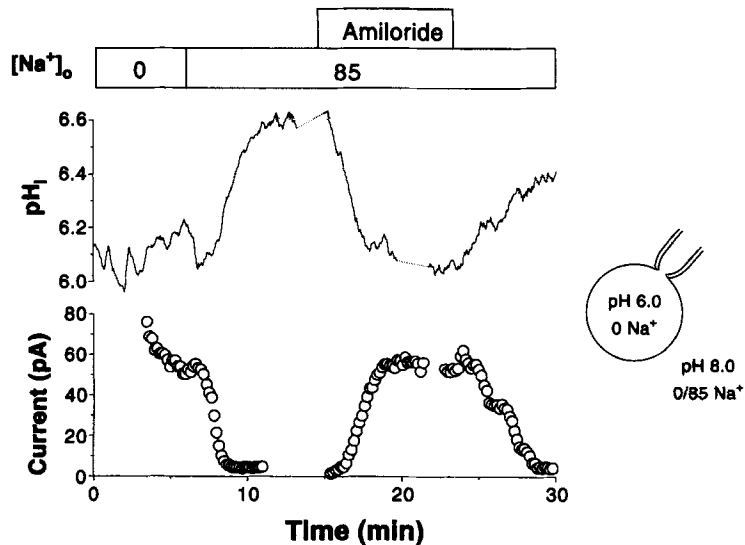


FIGURE 3. Forward activation of the antiporter, detectable as cytosolic alkalinization, is associated with reduced outward current. Concurrent measurements of pH_i (*top panel*) and of whole-cell currents (*bottom panel*) in a mouse peritoneal macrophage. The cell was voltage clamped at -60 mV and 1-s pulses to -50 mV were applied at 10-s intervals. The pipette solution (Pi 2) was Na^+ -free and was titrated to pH 6.0 (*inset*). The initial bath solution was Na^+ -free, pH 8.0 (solution Ba 3). Where noted, the external solution was replaced by an isoosmotic medium containing 85 mM Na^+ (solution Ba 4). Where indicated, 1 mM amiloride was applied externally. The current measured at the end of the depolarizing pulse to -50 mV is shown (*open circles*). Data acquisition was suspended during two periods to perform detailed current-voltage or tail current analysis (e.g., Figs. 4–6). Cell capacitance: 62 pF, access resistance: 7 M Ω . Results are representative of 16 similar experiments.

blocked by amiloride (Fig. 3), indicating involvement of the antiporter. Thus, forward Na^+/H^+ exchange also produced large changes in pH_i in macrophages voltage clamped in the whole-cell configuration. Moreover, activation of the antiporter in the physiological direction is also associated with changes in the transmembrane current.

Voltage Activation of the Currents

These initial observations supported the notion that Na^+/H^+ exchange in mammalian cells can operate in an electrophoretic mode. However, close inspection of the

data revealed important inconsistencies with this interpretation. While an outward current was associated with reverse antiport activity when measured at 0 mV, the current measured at -60 mV remained unchanged (Fig. 2, *filled circles*), despite continued Na^+/H^+ exchange. In addition, forward Na^+/H^+ exchange appeared to suppress a basal outward current rather than to induce an inward current. These findings cannot be readily explained by an electrophoretic exchange mechanism of constant stoichiometry, but rather suggests that a preexisting conductive pathway is modulated by Na^+/H^+ exchange. To test this hypothesis, we have analyzed the kinetics of activation and the voltage dependence of the currents under the dif-

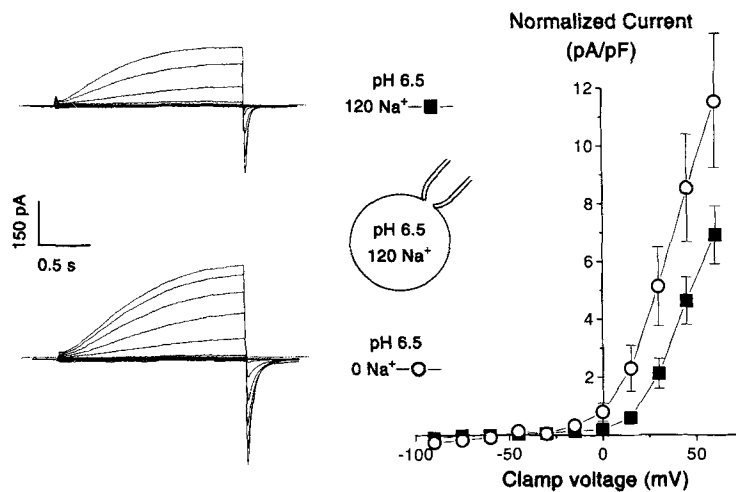


FIGURE 4. Voltage-dependence of the current activated by reverse (Na_i -driven) Na^+/H^+ exchange. A macrophage was voltage-clamped at -60 mV and 2-s pulses to voltages ranging from -90 to $+60$ mV were applied at 10-s intervals in 15-mV increments. Ionic conditions, schematized in inset, were as in Fig. 2. (A) families of currents recorded in the absence (*top*) or presence (*bottom*) of an outward Na^+ gradient. Cell capacitance: 35 pF. (B) Current-voltage relationships obtained in the absence (*filled squares*) and in the presence (*open circles*) of an outward Na^+ gradient. Currents were measured at the end of the voltage pulse and normalized for cell capacitance. Results are means \pm SE of 12 cells for each condition. When not shown, error bars are smaller than symbols.

ferent ionic conditions used in Figs. 2 and 3. In the absence of Na^+ and H^+ gradients (solutions Ba 1, Pi 1), i.e., when Na^+/H^+ exchange is inoperative, the application of 2-s voltage pulses ranging from -90 to $+60$ mV revealed the presence of slowly activating outward currents at voltages >0 mV (Fig. 4, *top left traces, squares*). The currents displayed sharp outward rectification (Fig. 4, *right*) and required ≈ 500 ms for half-maximal activation. These features are characteristic of the H^+ (equivalent) conductance described earlier in phagocytic cells (Demaurex et al., 1993; Kapus et al., 1993; DeCoursey and Cherney, 1993). Because the prevailing ionic conditions used (acidic intracellular solution) activate this pathway, the cur-

rents recorded in the absence of Na⁺/H⁺ exchange most probably flow through the H⁺ conductance of macrophages.

The currents recorded during Na⁺/H⁺ exchange had similar characteristics, i.e., slow voltage activation and sharp outward rectification (Fig. 4, *bottom left traces*). However, the initiation of reverse Na⁺/H⁺ exchange by removal of external Na⁺ (solutions Ba 2, Pi 1) shifted the voltage dependence of the currents by -15 mV (Fig. 4, *bottom left traces, circles*), resulting in a net outward current at 0 mV. Thus, when the cells are clamped at this voltage, the activation of an outward current by

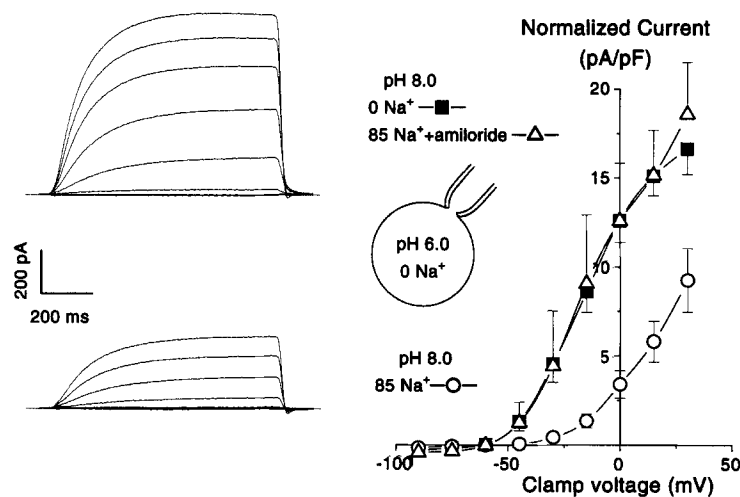


FIGURE 5. Voltage dependence of the currents reduced by forward (Na_o-driven) Na⁺/H⁺ exchange. The voltage protocol was as in Fig. 4 except that 1-s pulses were applied to a maximum voltage of +30 mV. Ionic conditions, schematized in inset, were as in Fig. 3. (A) Families of currents recorded in the absence (*top*) or presence (*bottom*) of an inward Na⁺ gradient. Cell capacitance: 42 pF. (B) current-voltage relationship determined in the absence of Na⁺ (*filled squares*) or in the presence of an inward Na⁺ gradient in media without (*open circles*) or with 1 mM amiloride (*open triangles*). Currents were measured at the end of the voltage pulse and normalized for cell capacitance. Results are means ± SE of 16, 12, and 5 cells for 0 Na_o, 85 Na_o, and 85 Na_o + amiloride, respectively. When not shown, error bars are smaller than symbols.

reverse Na⁺/H⁺ exchange could be explained by a shift in the voltage dependence of a preexisting conductance, likely the H⁺-selective conductance.

The currents associated with forward Na⁺/H⁺ exchange could also be explained by a shift in the voltage dependence of the H⁺ conductance. The basal outward current inhibited by forward Na⁺/H⁺ exchange (e.g., Fig. 3) probably flowed through the H⁺ conductance, as this has been shown to be the predominant conductance in macrophages when a large outward H⁺ gradient is imposed in the absence of other permeant ions (Kapus et al., 1993). Accordingly, under these conditions (solutions Ba 3, Pi 2) large outwardly rectifying and slowly activating currents were observed (Fig. 5, *top left traces*). As expected from the pH_i dependence of the

H⁺ conductance, the basal currents had a threshold voltage for activation around -60 mV (Fig. 5, *filled squares*), consistent with results obtained earlier using similar media (Kapus et al., 1993). The initiation of forward Na⁺/H⁺ exchange by addition of external Na⁺ (solutions Ba 4, Pi 2) shifted the current-voltage relationship by ≥ 25 mV and slowed the kinetics of current activation (Fig. 5, *bottom left traces, open circles*). Both effects were fully reversed by the addition of 1 mM amiloride (Fig. 5, *open triangles*), confirming mediation by the Na⁺/H⁺ exchanger.

Together, the results of Figs. 4 and 5 are consistent with the notion that macrophages possess a H⁺-selective conductance and that the current changes elicited by activation of Na⁺/H⁺ exchange in either the forward or reverse modes can be attributed to modulation of this conductance, likely by the associated changes in pH_i (see Fig. 1, *C and D*).

Ionic Selectivity of the Currents

To determine whether the currents modulated by Na⁺/H⁺ exchange are in fact carried by H⁺ ions, we measured their reversal potential (E_{rev}). E_{rev} cannot be accurately determined from steady state currents because of their outward rectification

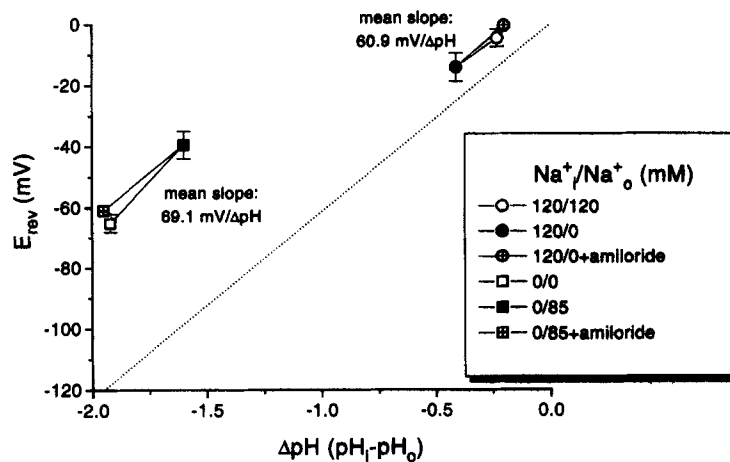


FIGURE 6. Reversal potentials of the currents associated with Na⁺/H⁺ exchange. The reversal potential (E_{rev} ; ordinate, in millivolts) of the tail currents recorded under the conditions specified is plotted as a function of the transmembrane pH gradient (Δ pH; abscissa) defined as the difference between the intracellular pH (pH_i), measured with BCECF, and the pH of the bath solution (pH_o). Initial ionic conditions were as in Fig. 2 (Δ pH = 0), or Fig. 3 (Δ pH = -2) and Na⁺/H⁺ exchange was induced by imposing either an outward or an inward Na⁺ gradient (*filled symbols*). Currents were activated by 1–2-s depolarizing pulses to 0 mV (for Δ pH = -2) or +45 mV (for Δ pH = 0) and tail current amplitude measured 5 ms after stepping back to test potentials ranging from -100 to +20 mV. E_{rev} was calculated by interpolation as the voltage at which tail currents reversed sign. Results are means \pm SE of six to nine determinations, except the two points determined in the presence of 1 mM amiloride (*crossed symbols*) which are the mean of two determinations. The dotted line is the Nernst potential for H⁺ ions at 37°C.

properties. However, inward tail currents can be readily measured, as illustrated in Fig. 4. To determine E_{rev} , tail currents were elicited by applying 1–2-s depolarizing pulses to 0 mV (when an H⁺ gradient was present and currents activated above –60 mV) or +45 mV (when a H⁺ gradient was absent and currents activated >0 mV) and the current amplitude was measured 5 ms after stepping back to test potentials ranging from –100 to +20 mV. The E_{rev} of the tail currents was determined and is plotted in Fig. 6 against the transmembrane pH gradient (ΔpH), taken as the difference between the bath pH (which we assumed constant) and the measured value of the intracellular pH. In the absence of Na⁺ or H⁺ gradients (using solutions Ba 1, Pi 1), E_{rev} averaged –5 mV (Fig. 6, *open circle*). Activation of reverse Na⁺/H⁺ exchange (solutions Ba 2, Pi 1) generated a ΔpH of 0.2 pH U and shifted E_{rev} by –10 mV (*filled circle*). Both the pH and E_{rev} changes were reversed by amiloride (*crossed circle*). In the absence of a Na⁺ gradient but in the presence of a large H⁺ gradient (solutions Ba 3, Pi 2), E_{rev} approximated –60 mV (*open square*). Operation of forward Na⁺/H⁺ exchange (solutions Ba 4, Pi 2) reduced ΔpH by 0.3 pH U and shifted E_{rev} by +25 mV (*filled square*). Both changes were reversed by amiloride (*crossed square*).

The nature of the charge-carrying species can be inferred from comparison of E_{rev} with the equilibrium potentials of the candidate ions. The equilibrium potential for Na⁺ ions, E_{Na} can be determined in the two initial conditions (*open symbols*), assuming that intracellular [Na⁺] equilibrates with the pipette [Na⁺] when Na⁺/H⁺ exchange is inactive. Although E_{Na} is close to 0 mV in both conditions, the measured E_{rev} values differ by 60 mV, suggesting that the basal current is not carried by Na⁺ ions. In contrast, in all conditions studied, E_{rev} varied in parallel with the calculated H⁺ equilibrium potential, E_{H} (Fig. 6, *dotted line*), although the absolute values deviated somewhat from the theoretical predictions, particularly when ΔpH was large. When the relationship between the recorded E_{rev} changes and the ΔpH was estimated, a slope between 60–69 mV/pH was calculated, which compares favorably with the value of 62 mV/pH predicted for a purely H⁺-selective conductance. Thus, the currents modulated by Na⁺/H⁺ exchange are carried mostly, if not exclusively, by H⁺ equivalents.

We next tested the effects of ZnCl₂, which reversibly blocks the H⁺ conductance of phagocytic cells (Demaurex et al., 1993; Kapus et al., 1993; DeCoursey and Cherny, 1993). In macrophages, addition of 2 mM ZnCl₂ greatly inhibits the H⁺ current (>90% block at voltages up to 50 mV above the activation threshold) by shifting the current-voltage relationship by $\approx +60$ mV (Kapus et al., 1993). To test the effects of ZnCl₂, cells were subjected to a H⁺ gradient (solutions Ba 3, Pi 2) and the basal current was activated by 1-s voltage steps from –60 to –10 mV (see left-most segment of Fig. 7). In the cell illustrated, a current of 130 pA was recorded. The bath solution was then exchanged for a Na⁺-free solution containing 2 mM ZnCl₂. Addition of ZnCl₂ (at $t = 7$ min) had no effect on pH_i, which continued to drift downward at a constant rate as the cytosol equilibrated with the pipette, but blocked the basal current by >90% (Fig. 7). The solution was then exchanged ($t = 9$ min) to a Na⁺-containing medium in the continued presence of Zn²⁺ (solution Ba⁴⁺ 2 mM ZnCl₂). This produced an alkalinization of 0.3 pH U, demonstrating that forward Na⁺/H⁺ exchange can still occur in the presence of ZnCl₂. Impor-

tantly, no current changes were observed, despite activation of the antiporter. Removal of Zn^{2+} in the continued presence of Na^+ ($t = 12.5$ min) did not alter the prevailing steady state pH_i , but unmasked outward currents that were smaller (≈ 75 pA) than those recorded in Na^+ -free medium, consistent with the results in Fig. 3. Readdition of $ZnCl_2$ ($t = 15$ min) again blocked the current without changing pH_i , whereas switching to a Na^+ -free solution ($t = 16.5$ min) caused a reacidification that was not accompanied by changes in current. Thus, Na^+/H^+ exchange could be pharmacologically dissociated from its accompanying currents. The currents were reversibly blocked by Zn^{2+} , as expected if carried by the H^+ conductance of macrophages.

It is noteworthy that like the fluxes mediated by Na^+/H^+ exchange, displacement of H^+ by the conductive pathway is expected to alter pH_i . In the experiment of Fig. 7, a cell with a low current density was chosen (3.6 pA/pF at -10 mV) to minimize

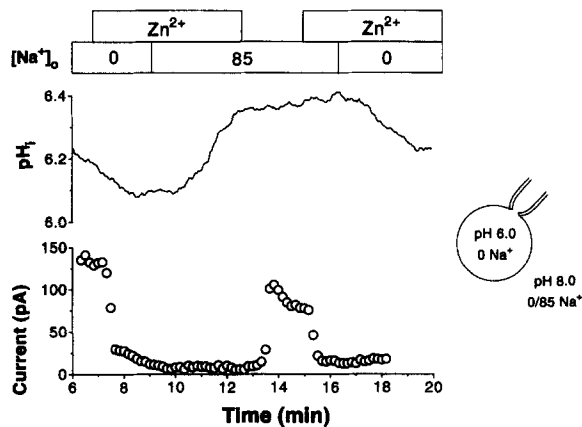


FIGURE 7. Block of antiporter-associated currents by Zn^{2+} does not affect Na^+/H^+ exchange. Ionic conditions (*inset*) and holding voltage were as in Fig. 3. When indicated, the external solution was supplemented with 1 mM $ZnSO_4$. (*Top*) Intracellular pH_i . (*Bottom*) Whole-cell currents elicited by 1-s pulses to -10 mV applied at 10-s intervals. Cell capacitance: 35 pF, access resistance: 13 M Ω . Traces are representative of three similar experiments.

the effects of the H^+ current on pH_i . This has enabled us to reversibly block the conductance without inducing detectable changes in pH_i , and to separate Na^+/H^+ exchange from its accompanying currents. As previously observed (Kapus et al., 1993), however, larger currents generated readily measurable changes of pH_i , confirming that H^+ equivalents mediate the current. Jointly, these experiments suggest that macrophages possess a H^+ conductance that is modulated by the pH_i changes occurring during Na^+/H^+ exchange, whereas the antiporter itself appears to be electroneutral.

Macrophages Express NHE-1, the Ubiquitous Isoform of the Antiporter

Several isoforms of the antiporter exist, which exhibit different kinetic and pharmacological properties. It was thus important to establish which isoform underlies cation exchange in macrophages, because it may differ from that of the basolateral membrane of the turtle colon epithelium. A partial clone isolated from the turtle colon shows $>80\%$ amino-acid identity to the human NHE-1 homologue (Harris et al., 1992), and NHE-1 is believed to predominate in basolateral membranes of

mammalian epithelia. Moreover, the pharmacological profile of Na⁺/H⁺ exchange in the turtle cells is similar to that of NHE-1 (Post and Dawson, 1992). Thus, the currents reported in the basolateral membrane of turtle colon epithelium are probably mediated by an NHE-1-like isoform. To determine which isoform is present in macrophages, we extracted messenger RNA from mouse peritoneal macrophages and assessed the expression of four different isoforms of the antiporter (NHE-1 to 4) by RT-PCR (Fig. 8 A). Isoform-specific primers which hybridized to unique regions of the rat NHE-1, NHE-2, NHE-3 and NHE-4 were used. All four sets of primers yielded the expected PCR products (Fig. 8 A, lanes 1, 4, 7, and 10) when linearized pCMV plasmids containing the full-length cDNA clone of the corresponding isoform were used as template. No discernible products were detected when a specific primer set was used with any of the noncorresponding isoforms as template (not shown). When cDNA obtained by reverse transcription of macrophage mRNA was used as a template, the NHE-1 primers yielded a product of ≈500 bp (Fig. 8 A, lane 2), whereas all the other sets of primers did not yield discernible products (Fig. 8 A, lanes 5, 8, and 11). Omission of reverse transcription prevented appearance of the 500-bp product, ruling out contamination with genomic DNA. Thus, the predominant isoform expressed in macrophages is NHE-1.

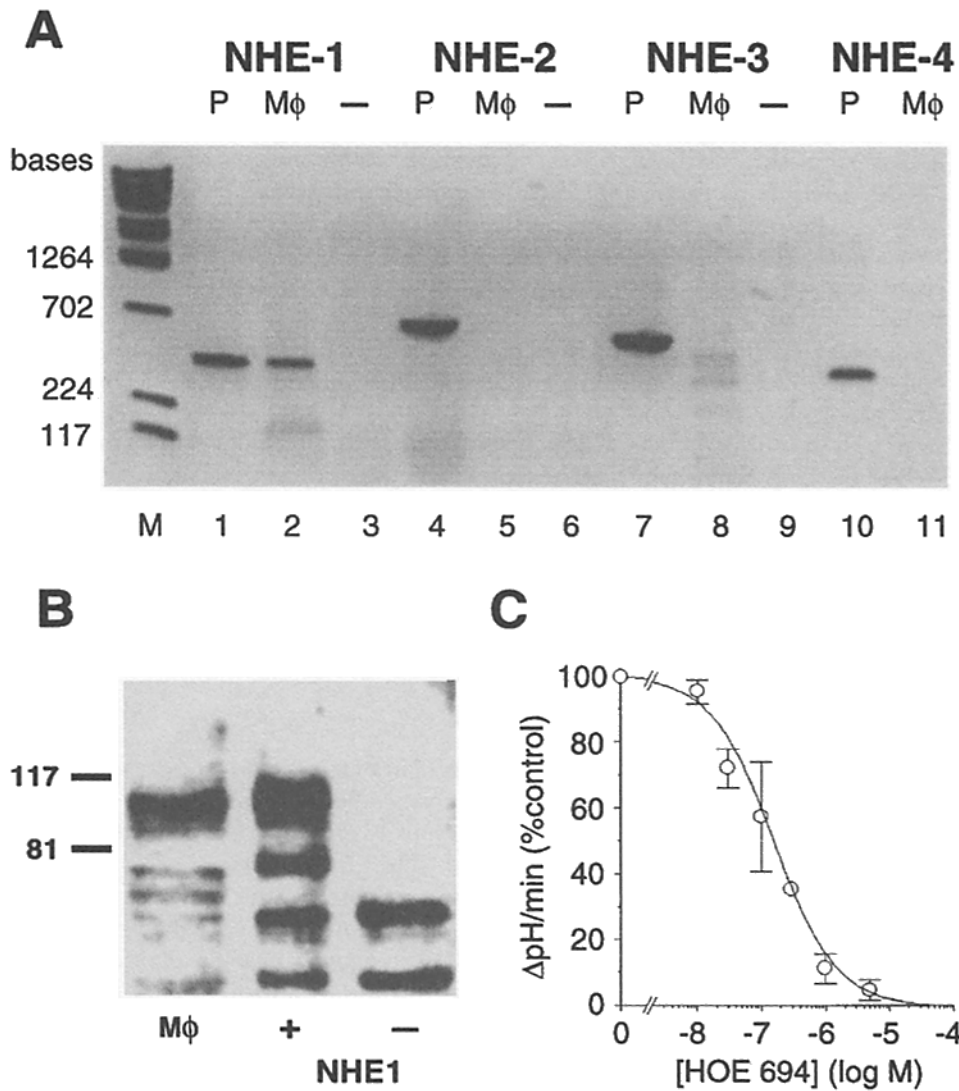
This conclusion was further supported by detection of the NHE-1 protein in a preparation of macrophage microsomes. As shown in Fig. 8 B, a polypeptide of molecular weight ≈115 kD was recognized by an antibody raised against the COOH-terminal 157 amino acids of NHE-1. A polypeptide of similar size was recognized in cells transfected with NHE-1, but not in their untransfected, antiport-deficient counterparts (Fig. 8 B), confirming the specificity of the antibody.

The presence of functional NHE-1 in macrophages could also be demonstrated pharmacologically. As shown in Fig. 8 C, the antiport activity of macrophages, assessed as the Na⁺-dependent recovery from an acid load, could be effectively inhibited by compound HOE694. The concentration of HOE694 required for half-maximal inhibition was 0.15 μM, similar to that reported to inhibit NHE-1 (Counillon, Scholz, Lang, and Pouyssegur, 1993), and much lower than that needed to inhibit either NHE-2 or NHE-3 (*K_i* of 5 and 650 μM, respectively). Together, these findings indicate that NHE-1 is the primary Na⁺/H⁺ antiporter of murine macrophages.

Assessing the Electrogenicity of NHE-1 by Heterologous Transfection

The current associated with activation of NHE-1 in macrophages appeared to result from indirect coupling to a separate, pH-sensitive conductance, whereas the exchanger itself was seemingly electroneutral. This hypothesis could in principle be tested by assessing the electrical properties of NHE-1 in cells devoid of a conductive pathway and lacking any other isoforms of the antiport. To this end, we used CHO cells, which we found not to display detectable H⁺ conductance. That only NHE-1 was expressed in these cells was ensured by heterologous transfection of rat NHE-1 into CHO cells that were previously selected by the H⁺ suicide method, i.e., they lacked functional Na⁺/H⁺ exchange (Orlowski, 1993). The transformants were studied under conditions identical to those used in Fig. 3. The cytosol was acidified through the patch pipette (solutions Ba 3, Pi 2) and an inward Na⁺ gradient was

subsequently imposed (solution Ba 4) to activate forward Na^+/H^+ exchange. Efficient activation of NHE-1 was detectable as a large Na^+ -induced cytosolic alkalinization (Fig. 9, *top*), which exceeded the rate observed in macrophages (cf. Fig. 3). In contrast to macrophages, no basal outward currents were observed in fibroblasts and only small, time-independent currents were elicited by 1-s steps to voltages ranging from -90 to $+60$ mV (Fig. 9, *top inset*). Thus, the voltage-activated H^+ conductance present in macrophages cannot be detected in CHO cells. No changes in current accompanied the pH_i changes induced by introduction of Na^+ (Fig. 9, *bottom trace*) and the whole-cell conductance remained constant across the entire range of voltages tested (Fig. 9, *lower inset*). Addition of amiloride blocked the ex-



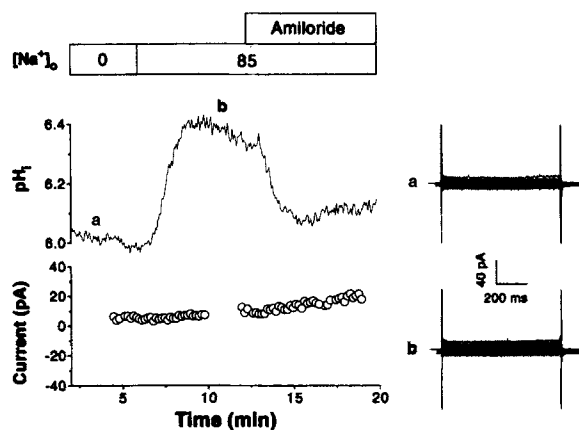


FIGURE 9. NHE-1-mediated Na⁺/H⁺ exchange in fibroblasts is not associated with whole-cell currents. A CHO cell expressing the NHE-1 isoform of the antiporter was subjected to the same protocol as in Fig. 3. (Top) pHi determination. (Bottom) Currents measured at the end of 1-s pulses to 0 mV, applied every 10 s from a holding voltage of -60 mV. At the times indicated by *a* and *b* on the pHi traces, families of currents elicited by 1 s voltage steps ranging from -90 to +60 mV were recorded and are shown in

the right panel. Cell capacitance: 24 pF, access resistance: 5 M Ω . The current scale in *a* and *b* is magnified fivefold compared to that in Fig. 5, which was obtained under comparable conditions in macrophages. Representative of eight experiments.

changer, leading to cellular acidification, but failed to alter the current. Thus, Na⁺/H⁺ exchange through the NHE-1 isoform does not generate detectable currents in CHO cells, consistent with an electroneutral process.

To assess the electrical properties of the other isoforms, the antiport-deficient CHO cells were transfected with either NHE-2 or NHE-3 (attempts to express NHE-4 have thus far been unsuccessful). The results obtained with all of these isoforms are summarized in Fig. 10, together with the results obtained in macrophages. The steady state pHi and the transmembrane conductance were measured concomitantly before, during and after induction of Na⁺/H⁺ exchange. The conductance was derived from currents elicited by voltage pulses of 15 mV (macro-

FIGURE 8. Murine macrophages express the NHE-1 isoform of the antiporter. (A) Messenger RNA was extracted and used as a template for RT-PCR with isoform-specific primers. M: molecular weight markers. P: template was linearized pCMV plasmid containing the full sequence of rat NHE-1 (lane 1), NHE-2 (lane 4), NHE-3 (lane 7), or NHE-4 (lane 10), hybridized with the corresponding primers. M Φ : template was macrophage mRNA, hybridized with primers specific for NHE-1 (lane 2), NHE-2 (lane 5), NHE-3 (lane 8), or NHE-4 (lane 11). Controls used similar template and primers as M Φ , but reverse transcriptase was omitted. Representative of four separate experiments. (B) Microsomal fractions were separated by gel electrophoresis and immunoblotted with a polyclonal antibody specific for the carboxy terminus of the human NHE-1 isoform. (Left lane) Mouse macrophages; (middle lane) CHO cells stably transfected with the NHE-1 isoform; (right lane) untransfected antiport-deficient CHO cells. Representative of two separate experiments. (C) Suspensions of mouse macrophages were loaded with BCECF (2 μ M) and suspended in KCl-free solutions (140 mM NMGC1). Low concentrations of nigericin (0.1 μ g/ml) were used to acidify the cytosol. The rate of fluorescence increase following addition of 50 mM NaCl to the extracellular solution was measured at comparable pHi and is plotted as a function of the concentration of HOE 694. A sigmoidal function of the form: $y = 100 / \{1 + (x/x_0)^n\}$ (line) was fitted to the data ($\chi^2 = 1.01$; $n = 0.89$), and yielded an apparent K_i of 0.15 μ M (dotted line). Data are mean \pm SE of three separate determinations.

phages) or 60 mV (CHO cells) amplitude, and has been normalized per cell capacitance in order to allow comparison between cell types. In all cases, the cytosolic alkalization effected by forward Na^+/H^+ exchange was of comparable amplitude and a secondary acidification was always induced by addition of amiloride, confirming involvement of the antiport. However, a change in transmembrane conductance was observed only in macrophages. This indicates that, under comparable conditions, the three isoforms of the mammalian antiporter behave similarly, i.e., they catalyze electroneutral exchange. Associated currents can only be recorded in cells expressing a H^+ conductance, where pH_i changes resulting from the activity of the antiport seemingly modulate the conductance. Therefore, though the properties of NHE-4 remain to be established, electroneutral exchange appears to be a generic feature of mammalian antiporters.

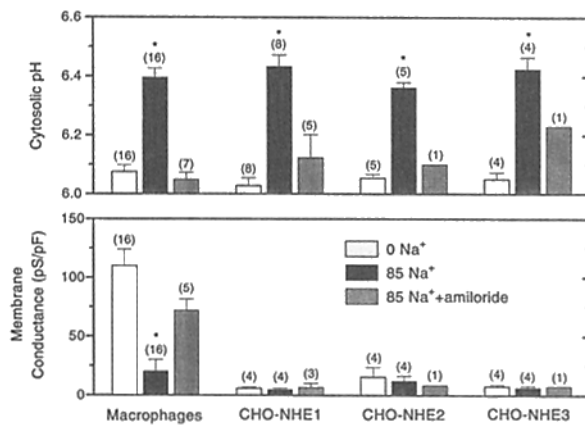


FIGURE 10. Na^+/H^+ exchange in macrophages and in CHO cells transfected with three different isoforms of the antiporter: comparison of effects on pH_i and conductance. Data are from experiments performed with macrophages or with CHO cells transfected with the NHE-1, NHE-2 or NHE-3 isoforms of the antiporter, using a protocol similar to that used for Figs. 3 and 8. (Top) Steady state pH_i , measured before (open bars) and after (filled bars) superfusion with 85 mM Na^+ and after addition of amiloride in the presence of Na^+ (stippled bars). (Bottom) Steady state conductance, normalized for cell capacitance, measured under the same conditions. Conductance was derived from the currents elicited by voltage steps ranging from -50 to 0 mV. Data are means \pm SE of the number of cells indicated in parenthesis. *: $P < 0.0002$ with condition 0 Na^+ (t test).

ride in the presence of Na^+ (stippled bars). (Bottom) Steady state conductance, normalized for cell capacitance, measured under the same conditions. Conductance was derived from the currents elicited by voltage steps ranging from -50 to 0 mV. Data are means \pm SE of the number of cells indicated in parenthesis. *: $P < 0.0002$ with condition 0 Na^+ (t test).

Voltage Sensitivity of the Antiport

Although Na^+/H^+ exchange does not generate detectable currents, the exchange process could nevertheless be affected by changes in V_m . Since our experimental system could readily measure Na^+/H^+ exchange in voltage clamped cells, we were able to directly assess the voltage dependence of the antiporter. Moreover, the three isoforms of the antiporter could be studied independently in the same expression system. We thus performed experiments similar to the one shown in Fig. 9, but held the membrane voltage at different values (-60 , 0 , or $+30$ mV) before initiating forward Na^+/H^+ exchange. Then, the holding voltage was changed during the course of the antiport-induced alkalization. Typical results obtained with this voltage protocol are shown for a NHE-1-transformant (Fig. 11, left panel; dotted line indicates the time of voltage change). Forward Na^+/H^+ exchange occurred

normally at a holding voltage of 0 mV, the alkalization being similar to that observed at -60 mV (see Fig. 9). More importantly, changing the holding voltage to -60 mV did not alter the ongoing alkalization (Fig. 11). The rates of increase of pH_i during the 30 s before or after the change in voltage were compared in six different NHE-1 transformants (Fig. 11, *right*). The absolute rates of pH_i recovery varied from cell to cell, but were in all cases independent of the holding voltage. Similar results were obtained with NHE-2 transformants (four cells) and NHE-3 transformants (three cells; not illustrated). In separate experiments, changing the

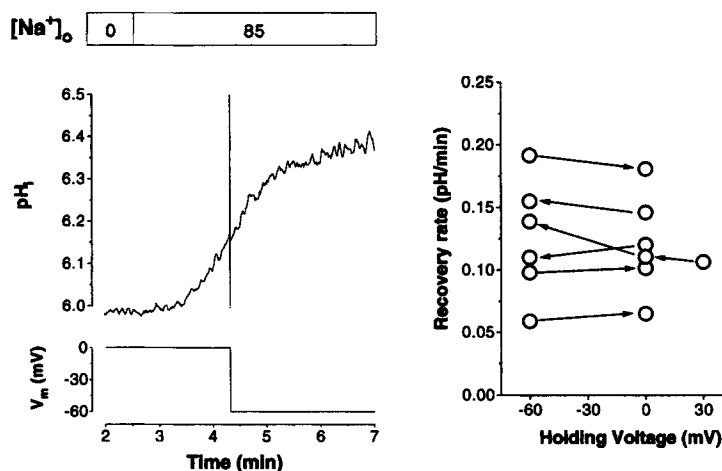


FIGURE 11. Voltage dependence of NHE-1. (*Left*) An NHE-1-transfectant was patch clamped in the whole-cell configuration using a pipette filled with solution Pi 2. The bath initially contained Na⁺-free solution (Ba 3) and, where indicated, was switched to a solution containing 85 mM Na⁺ (Ba 4). The cell was voltage clamped at 0 mV and, when indicated by the dotted line ($t = 4.3$ min), the holding voltage was changed to -60 mV (*bottom*). Cell capacitance: 34 pF, access resistance: 15 M Ω . (*Right*) Voltage dependence of the pH_i recovery rates, derived from experiments similar to the one illustrated in the left. NHE-1 transformants were voltage-clamped at -60, 0, or +30 mV and the holding voltage was stepped during the course of the recovery. The rates of alkalization (in pH/min) during the 30-s intervals before and after the voltage change were compared. The arrows indicate the direction of the voltage changes during the recovery. Results from six different experiments are illustrated.

voltage by ± 60 mV did not alter the steady state pH_i attained during continuous operation of the Na⁺/H⁺ exchanger (not shown). Taken together, these experiments indicate that the activation and sustained function of the antiporter are independent of the transmembrane voltage.

DISCUSSION

The Rodent NHE-1, -2, and -3 Are Electroneutral

The earliest precise determinations of Na⁺/H⁺ exchange, performed in vesicles isolated from brush borders of epithelial cells, suggested a stoichiometry of 1:1 and

failed to reveal associated changes in membrane potential (Murer et al., 1976; Kinsella and Aronson, 1980; Aronson, Nee, and Suhm, 1982). It was therefore generally assumed that Na^+/H^+ exchange in mammalian tissues is electroneutral (Aronson, 1985). Subsequent results obtained with other biological systems were generally consistent with this notion (Cala, 1980; Boron and Boulpaep, 1983; Grinstein et al., 1984). By contrast, recent findings of Post and Dawson (1992, 1994) indicated that a sizable current is associated with the activation of the antiporter of the basolateral membrane of turtle colon cells and were interpreted to mean that Na^+/H^+ exchange is an electrophoretic process. The existence of electrogenic Na^+/H^+ exchange is not unprecedented. Electrogenic, 2 Na^+/H^+ exchange has been detected in a variety of invertebrate species and tissues (Ahearn and Franco, 1990, 1991; Kimura, Ahearn, Busquets-Turner, Haley, Nagao, and DeCouet, 1993). This electrogenic antiporter has been suggested to play a central role in the acidification of the crustacean gut, in Na^+ absorption by crab gills and to indirectly contribute to calcium homeostasis in crustaceans and echinoderms (Ahearn, Zhuang, Duerr, and Pennington, 1994; Grinstein and Wicczorek, 1994).

In view of these apparently incompatible findings, a reassessment of the electrogenicity of the mammalian antiporters seemed warranted. It was particularly important to establish the individual properties of each isoform of the exchanger, inasmuch as differential behavior might account for the apparent inconsistencies described in different biological systems. It is noteworthy that much of the evidence supporting electroneutrality was gathered using vesicles isolated from apical membranes of epithelial cells (Murer et al., 1976; Kinsella and Aronson, 1980; Aronson et al., 1982) and therefore most likely reflected the activity of NHE-3 (Haggerty et al., 1988; Tse et al., 1992; Wang, Orłowski, and Shull, 1993). On the other hand, support for electrogenic behavior was obtained with turtle colon basolateral antiporters. By analogy with mammalian gastrointestinal epithelia (Bookstein, DePaoli, Xie, Niu, Musch, Rao, and Chang, 1994), reptilian cells are likely to express primarily NHE-1 on their basolateral membranes. Indeed, a partial clone isolated from this tissue bears >80% identity with the mammalian NHE-1 homologue (Harris et al., 1992). Moreover, the amiloride sensitivity of Na^+/H^+ exchange activity reported by Post and Dawson (1992) is compatible with that found for the mammalian NHE-1 (Orłowski, 1993).

Using murine macrophages, we were able to reproduce antiport-associated currents that were indistinguishable from those reported by Post and Dawson (1992, 1994). However, close examination of the properties of these currents revealed that they were not an intrinsic property of the Na^+/H^+ exchange process but instead resulted from coupling of the antiporter to a separate endogenous H^+ conductive pathway (see model in Fig. 1, *C* and *D*). The latter conclusion was based on: (a) the similarity between the Na^+ -induced currents and those elicited by comparable changes in pH_i attained by independent means, i.e., without involvement of the antiporter. The voltage dependence and kinetics of the currents were virtually identical in both cases; (b) the reversal potential of the currents was similar to E_{H^+} , consistent with participation of a conductive, H^+ -selective pathway. A different thermodynamic profile would be anticipated if the currents were due to an uneven $\text{Na}^+:\text{H}^+$ stoichiometry; and (c) the current could be dissociated from the activity of

the antiport by means of Zn²⁺, which blocked the endogenous H⁺ conductance without preventing antiport activity, as indicated by the Na⁺-induced and amiloride-sensitive changes in pH_i.

Modulation of H⁺ currents by Na⁺/H⁺ exchange was recently suggested by DeCoursey and Cherny (1994*a*, reviewed in DeCoursey and Cherny, 1994*b*). In human neutrophils and rat alveolar epithelial cells, these authors found that conditions expected to activate forward Na⁺/H⁺ exchange shifted both the current-voltage relationship and the reversal potential of the currents to more positive potentials. To account for these observations, DeCoursey and Cherny (1994*a,b*) speculated that changes in pH_i might occur in perfused cells during Na⁺/H⁺ exchange, and that a 0.4–0.5 pH U alkalization of the submembranous compartment could account for the observed changes in H⁺ current. Our pH_i measurements in macrophages are consistent with these calculations confirming that, in cells endowed with a H⁺ conductance, activation of Na⁺/H⁺ exchange can markedly affect H⁺ currents through changes in pH_i. If a H⁺ conductance were also present in the basolateral membrane of turtle colon epithelium, the currents attributed to electrogenic Na⁺/H⁺ exchange in this system might be due to a similar “cross-talk” mechanism.

Using isoform-specific primers and RT-PCR, macrophages were shown to exhibit only NHE-1. Expression of the NHE-1 isoform was confirmed by immunoblots of macrophage membrane proteins, and the pharmacological profile of Na⁺/H⁺ exchange in macrophages was consistent with NHE-1. Further evidence that this isoform is not electrogenic was obtained using CHO cells transfected with the rat homolog of this isoform. These cells, which lack the endogenous H⁺ conductance found in leukocytes and in some epithelia, displayed robust Na⁺/H⁺ exchange activity without associated current. Because the rate of Na⁺/H⁺ exchange in these cells can be estimated from measurements of pH_i, the magnitude of the current predicted to be associated with a putative electrophoretic exchanger can be calculated. We observed pH_i changes of ≈0.12 pH/min during antiport activation in patch clamped NHE-1 transformants. This value underestimates antiport activity as in intact CHO cells, where determination of ΔpH is not complicated by diffusion of H⁺ equivalents and buffers to and from the patch pipette, the rate of alkalization mediated by NHE-1 under comparable conditions averages 1 pH/min (Kapus et al., 1994). Considering the buffering capacity of the cells in the pH range studied (≈11 and 32 mM/pH for whole-cell patched and intact fibroblasts, respectively), these rates of ΔpH_i are equivalent to 1.2–40 mmol H⁺ equivalents/liter/min. Taking the average volume of CHO cells as 1 pL (measured by electronic sizing) and assuming a 2Na⁺:1H⁺ stoichiometry as suggested by Post and Dawson (1994), the anticipated current would range from 2 to 60 pA (for ΔpH_i of 0.12 or 1 pH/min and buffering powers of 11 and 32 mM/pH, respectively). This value is well within the range measurable by our experimental setup, implying that our failure to detect antiport associated currents is not due to technical limitations.

The absence of complicating H⁺ conductive pathways enabled us to test also the electrical properties of NHE-2 and NHE-3. Like NHE-1, these isoforms were found to be electroneutral. It therefore seems unlikely that differential isoform composition might account for the discrepancies in electrogenicity of the antiporter re-

ported in the literature. Nevertheless, it is important to point out that the behavior of NHE-4 has not been established as it awaits its functional expression. Moreover, the precise isoform content of the turtle colon and possible species differences between NHE-1 homologues remain to be determined.

The Rodent NHE-1, -2, and -3 Are Voltage Insensitive

While electroneutral processes are frequently also voltage-independent, this is not necessarily always the case. Reactions involving displacement of charges or dipoles across the membrane are often part of catalytic cycles that produce no net charge translocation. In the cases where such partial reactions are rate-limiting, the overall yield of the electroneutral process can be altered by the transmembrane potential (Eisner and Lederer, 1985). Therefore, the finding that Na^+/H^+ exchange in mammalian cells is electrically neutral does not rule out the possibility that it might be sensitive to the applied voltage.

In previous reports, the rate of Na^+/H^+ exchange was not affected when the membrane potential was predicted to be altered by manipulation of the ionic environment and/or by addition of conductive ionophores (Cala, 1980; Grinstein et al., 1984). The isoform(s) involved in these studies were not defined, nor was the membrane potential directly measured. To complement existing information, we performed experiments using the transfected CHO cells, which are endowed with a single, well defined isoform of the antiporter. The ability to measure sizable pH_i changes when using the whole-cell configuration of the patch clamp technique allowed us to assess the rate of Na^+/H^+ exchange while accurately controlling the transmembrane potential. Using this experimental paradigm, neither NHE-1, NHE-2 nor NHE-3 displayed detectable voltage sensitivity in the -60 to $+30$ mV range. These findings imply that partial reactions requiring transmembrane displacement of charges or dipoles are not part of the Na^+/H^+ exchange cycle or that, if they are present, such steps are not rate determinant.

The persistence of measurable pH_i changes when using the whole-cell configuration of the clamp, despite the continuity of the cytosolic and pipette solutions, is remarkable. It implies that the rate of transmembrane displacement of H^+ by the exchanger outstrips the diffusion of H^+ equivalents and buffers to and from the pipette. The access resistance in our experiments was not inordinately high ($\approx 5\text{--}30$ M Ω), however, the calculated time constants for H^+ equilibration via a 5-M Ω pipette ranged from 16 to 100 s, in agreement with the value of ≈ 60 s derived from fluorescence measurements (see Appendix for calculation and measurements of the time constants). These slow time constants could be explained by the presence of ≈ 8 mM intracellular "fixed" H^+ buffers (see Appendix) and, combined with the relatively large rates of transmembrane transport, made the detection of pH_i changes possible even in intracellularly perfused cells. It is noteworthy that alkalinization due to Na^+/H^+ exchange was measurable not only in the transformants, but also in macrophages expressing only the endogenous antiporter. These findings should serve as a caution to investigators who, in the course of experiments using the whole-cell configuration, make the assumption that the cytosolic and pipette pH and Na^+ concentration will be identical under all conditions.

Physiological Significance of the Electrical Properties of the Antiporter

Epithelia of invertebrates, where electrophoretic 2Na⁺:1H⁺ exchange has been detected, are exposed to drastic changes in salinity and pH. It has been suggested that in these systems the ionic component of the electrochemical driving force may not always suffice to drive H⁺ in the appropriate direction. Coupling the ionic gradient to the transepithelial electrical potential is regarded as a means to energize the translocation of H⁺ and Na⁺, ensuring the occurrence of fluxes of adequate magnitude and polarity.

The constancy of the extracellular milieu bathing nonepithelial cells as well as the basolateral membrane of epithelia obviates the need for electrical driving force. In mammals, even the mucosal face of epithelia rarely faces the extreme environmental shifts that confront some invertebrates, as in the case of gills of shore crabs. Moreover, in mammalian renal and gastrointestinal epithelia, primary active transport of H⁺ equivalents appears to be the mechanism of choice when translocation of acid-base equivalents against large gradients is required. Therefore, contribution of an electrical component to drive Na⁺/H⁺ exchange is seemingly not essential.

The development of 1:1 Na⁺/H⁺ exchange as a neutral and voltage-insensitive means of transporting H⁺ equivalents probably reflects the need for dissociation of pH regulation from the electrical activity of excitable tissues. Clearly, secondary alterations in membrane potential associated with H⁺ extrusion in metabolically activated nerve, muscle or endocrine cells could result in severe interference with their electrophysiological behavior. Conversely, modulation of the rate of Na⁺/H⁺ exchange by changes in potential during the course of excitable activity is undesirable. The electroneutrality and voltage insensitivity of the antiporters preclude such potentially deleterious interactions.

In summary, three mammalian isoforms of the Na⁺/H⁺ exchanger, NHE-1, -2, and -3, were found to be electrically neutral and insensitive to the applied transmembrane potential. The existence of additional H⁺ transporting systems and the constancy of the transmembrane Na⁺ gradient have enabled mammals to limit the stoichiometry of Na⁺/H⁺ exchange to 1:1, rendering the regulation of cellular pH and volume independent of the electrophysiological activity of the cells.

APPENDIX

Measurement of pHi Transients in Cells Patched in the Whole-Cell Configuration

The activity of the antiporter could be measured readily in cells that were clamped in the whole-cell configuration, despite the continuity of the cytosolic space and the pipette solution. This seemingly unexpected observation can be explained by the comparatively long time required for equilibration of pH buffers between the pipette and the cell. Fluxes to and from the patch pipette follow exponential kinetics of the form: $[x]_i = [x]_p^*(1 - e^{-t/\tau})$, with time constant: $\tau = V/\rho^*R_A/D$, where $[x]_i$ and $[x]_p$ are the intracellular and pipette concentrations of the solute, V is the cell volume, ρ the resistivity of the pipette solution, R_A the access resistance, and D the diffusion constant of x in the cytosol (Pusch and Neher, 1988; Oliva, Cohen,

and Mathias, 1988). Cytosolic diffusion of H^+ equivalents is slower than diffusion in aqueous solutions and depends on the concentration of both fixed and mobile buffers. In whole-cell patch clamp experiments, some fixed buffers such as proteins and lipids on membrane-bound organelles or cytoskeletal proteins remain in the cell, whereas endogenous mobile buffers such as phosphates are dialyzed out and replaced by the pipette buffers. Thus, the apparent diffusion constant for H^+ in the cytosol, D_{Hc} , cannot exceed the diffusion constant of the mobile (i.e., pipette) buffers, D_m , and is further limited by the amount of fixed buffers remaining in the cell, according to: $D_{Hc} = D_m * \beta_m / (\beta_m + \beta_f)$, where β_m and β_f are the buffering powers of the mobile and fixed buffers, respectively (Irving, Maylie, Sizto, and Chandler, 1990). The buffering power of our pipette solutions, β_m , is ≈ 3 mM/pH, most of which is contributed by MES (molecular weight 213), whose diffusion constant in the cytosol should be similar to that of fura-2, i.e., $\approx 3 * 10^{-6}$ cm²/s at 37°C (Neher and Almers, 1986). The buffering power of the fixed buffers remaining in the whole-cell configuration, β_f , has not been measured. However, the total (fixed + mobile) buffering power of intact fibroblasts is ≈ 32 mM/pH, approximately half of which consists of mobile buffers. Thus, in our whole-cell experiments β_f cannot reasonably exceed 16 mM/pH, yielding D_{Hc} values ranging from $5 * 10^{-7}$ cm²/s ($\beta_f = 16$ mM/pH) to $3 * 10^{-6}$ cm²/s ($\beta_f = 0$). Assuming a volume of 1 pI for CHO cells (measured by electronic sizing) and a resistivity of the pipette solution of 100 Ω .cm, for a typical recording such as the one illustrated in Fig. 9 ($R_A = 5$ M Ω), this translates into time constants ranging from 17 s to 100 s, i.e., the presence of intracellular "fixed" buffers might prolong the time required for equilibration of pH_i by a factor of 6. In this cell, as in the ones illustrated in Figs. 2 and 3, the time constant can be estimated from the changes in pH_i that follow inhibition of the antiporter by amiloride. Such changes, which were measured fluorimetrically, essentially reflect the dissipation of the pH gradient by diffusion of buffer from the patch pipette. An exponential fit to the data between $t = 30$ and $t = 240$ s after amiloride addition yielded a τ of 60 s for the cell illustrated in Fig. 9, which, based on the above calculations, corresponds to a "fixed" buffering power of ≈ 8 mM/pH.

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