

Cytosolic pH Regulation in Osteoblasts

Interaction of Na⁺ and H⁺ with the Extracellular and Intracellular Faces of the Na⁺/H⁺ Exchanger

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ABSTRACT The interaction of Na and H ions with the extracellular and intracellular sites of the Na⁺/H⁺ exchanger of the osteosarcoma cell line UMR-106 was investigated. Na ions interact with a single, saturable extracellular transport site. H⁺ and amiloride appear to compete with Na⁺ for binding to this site. The apparent affinity for extracellular Na⁺ (Na_o⁺) and amiloride was independent of intracellular H⁺ (H_i⁺), Na_i⁺, or an outwardly directed H⁺ gradient. The interaction of H⁺ with the intracellular face of the exchanger had a sigmoidal characteristic with a Hill coefficient of ~2. The apparent affinity for H_i⁺ was independent of Na_o⁺ between 25 and 140 mM. The apparent affinity for H_i⁺, but not the number of intracellular sites, increased with the increase in the outwardly directed H⁺ gradient across the membrane. Na_i⁺/H_o⁺ exchange (reverse mode) is an electroneutral process with a Na⁺/H⁺ stoichiometry of 1. The dependence of Na_i⁺/H_o⁺ exchange on Na_i⁺ was sigmoidal, with a Hill coefficient of 2.16. Na_i⁺ competes with H_i⁺ for binding to at least the transport site. The apparent affinity for Na_i⁺ decreased with the increase in the outwardly directed H⁺ gradient. High H_o⁺ inhibited exchange activity in the reverse mode. We conclude that intracellular Na⁺ and H⁺ can activate the exchanger. The exchanger has two separate and asymmetric extracellular and intracellular transport sites. The relative apparent affinities of the internal transport site for Na⁺ and H⁺ are determined by the direction and magnitude of the H⁺ gradient across the membrane. Kinetic characterization of the exchanger suggests that Na⁺/H⁺ exchange is compatible with a simultaneous transport model, although a ping-pong transport model could not be excluded.

INTRODUCTION

An electroneutral Na⁺/H⁺ exchange mechanism has been demonstrated in a variety of epithelial and nonepithelial cell types (Roos and Boron, 1981; Seifter and Aron-

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son, 1986). The exchanger contributes to (Grinstein et al., 1984; Muallem et al., 1985), and in some cells dominates (Paris and Pouyssegur, 1983; Paradiso et al., 1987), acid extrusion from the cytosol and Na^+ influx during volume-regulatory increase (Eveloff and Warnock, 1987). The exchanger can be activated by growth factors (Moolenaar et al., 1983; Macara, 1985) and hormones (Smith and Brock, 1983; Garrison et al., 1984).

Understanding the mechanism of exchanger activation requires the knowledge of its basic kinetic behavior. The initial kinetic characterizations of the exchange process were done in microvillus membrane vesicles from kidney cortex (Murer et al., 1976; Kinsella and Aronson, 1980) and were later confirmed in intact cells of different types (Aronson, 1985; Seifter and Aronson, 1986). Na^+ ions bind to a single, saturable extracellular site (Aronson, 1983). Both H^+ and the diuretic amiloride appear to compete with extracellular Na^+ for binding to this site (Kinsella and Aronson, 1981). It was suggested that on the cytoplasmic face of the exchanger, there are two ion-binding sites, a transport and a regulatory site (Aronson et al., 1982). Binding of H^+ to the regulatory site activates the exchanger. Although other ions can bind to the cytoplasmic face of the exchanger (Burnham et al., 1982; Aronson et al., 1983), it is not clear whether they interact with both sites or only the transport site.

In the present study, we investigate the kinetic properties of the Na^+/H^+ exchanger in an osteoblast-like cell line. Cytosolic pH regulation by the osteoblasts is of particular interest since these cells are likely to maintain the alkaline pH of the bone extracellular fluid during bone formation (Parfitt and Kleerekoper, 1980) and are exposed to an acidic environment during bone resorption (Baron et al., 1985). These cells therefore have to remove acid from the cytosol to the plasma during both bone formation and resorption. To study the mechanism of acid extrusion by the osteoblast, we used the osteosarcoma cell line UMR-106, which possesses many osteoblastic characteristics, including a cyclic AMP response to parathyroid hormone but not to calcitonin (Partridge et al., 1983), high alkaline phosphatase activity (Partridge et al., 1981), synthesis of type I collagen (bone specific), insulin sensitivity, and the ability to form bone in vivo (Martin et al., 1976).

The present study demonstrates the presence of Na^+/H^+ exchange in UMR-106 cells. The interactions of Na^+ and H^+ with the intracellular and extracellular faces of the exchanger are analyzed.

MATERIALS AND METHODS

Reagents

Nigericin and monensin were purchased from Sigma Chemical Co., St. Louis, MO. (2', 7')-bis(carboxyethyl)-(5, 6)-carboxyfluorescein acetoxymethyl-ester (BCECF-AM) was obtained from Molecular Probes, Inc., Eugene, OR. Amiloride was from Merck, Sharp & Dohme, West Point, PA. The UMR-106 clonal cell line was the generous gift of Dr. T. J. Martin, University of Melbourne, Melbourne, Australia.

Solutions

The following solutions were used during the experiments. Solution A contained 140 mM NaCl, 1 mM MgCl_2 , 10 mM HEPES/Tris, 5 mM glucose. Solutions B (KCl) and C (TMA-Cl)

were prepared by iso-osmotic replacement of NaCl by KCl or TMA-Cl, respectively, but were otherwise identical. The pH of each solution was adjusted as specified in the text.

Culture Conditions

UMR-106 cells were used between passages 8–9 and subpassages 3–14. Cells were seeded at a density of 4×10^5 cells/cm² in 75-cm²-area flasks and grown at 37°C in a humidified 95% air/5% CO₂ atmosphere in Ham's F12 (Dulbecco's modified Eagle's media [1:1], supplemented with 14.3 mM NaHCO₃, 1.2 mM L-glutamine, 7% fetal bovine serum, 0.1 mg/ml streptomycin, and 100 U/ml penicillin). The cells reached confluence within 5–6 d in culture and were used on day 6–12 of growth.

Measurement of Intracellular pH

Measurements of intracellular pH (pH_i) were made by incorporating the pH-sensitive fluorescent dye BCECF into UMR-106 cells that had been released from tissue culture flasks by trypsin/EDTA (0.25%/0.2%). Trypsin/EDTA treatment took place for 3–5 min at 37°C, followed by a rapid 25-fold dilution with a solution containing (millimolar): 140 NaCl, 5 KCl, 1.0 MgCl₂, 0.5 CaCl₂, 10 HEPES/Tris, pH 7.4, 5 glucose, 0.1% bovine serum albumin (BSA). The cells were then washed twice by centrifugation at 600 *g* for 3 min at 25°C, resuspended in the same solution, and incubated with 2 μM BCECF-AM in a shaking water bath at 37°C for 20 min. Next, the cells were washed with medium of desired composition and treated with ionophores to modify pH_i, Na⁺, and K⁺, as outlined below. Approximately 10⁶ cells/ml were used in each set of experiments. Fluorescence was measured in a fluorescence spectrophotometer (650-40, Perkin-Elmer Corp., Norwalk, CT), with excitation and emission wavelengths of 500 and 530 nm and slits of 3 and 12 nm. The dye signal was calibrated in intact cells by suspending Na⁺-loaded cells in solution A, pH 7.1, or K⁺-loaded cells in solution B, pH 7.1. The cells suspended in Na⁺-containing medium were exposed to 2 μM monensin, while cells suspended in high-K⁺ solution were exposed to 0.5 μM nigericin. The pH of the extracellular medium was varied between 6.1 and 8.2 and the fluorescence signal was measured. Calibration of the dye signal was also done by releasing the dye from the same cells with 50 μM digitonin and measuring the fluorescence at pH 6.1–8.2. A linear response was obtained in the pH range 6.3–8.0. The difference between dye calibration in the medium and cells was 0.11 ± 0.01 ($n = 14$) pH units. In all experiments shown, the indicated pH was corrected by this factor.

Acidification of the Cytosol

Cells loaded with BCECF were washed twice and resuspended in 2 ml solution B. The pH of the medium was adjusted to the level desired in the cytosol (6.0–7.4) and the cells were incubated with 0.5 μM nigericin and 2 μM monensin for 5 min at 37°C. Monensin was included to deplete the cells completely of Na⁺ (see below).

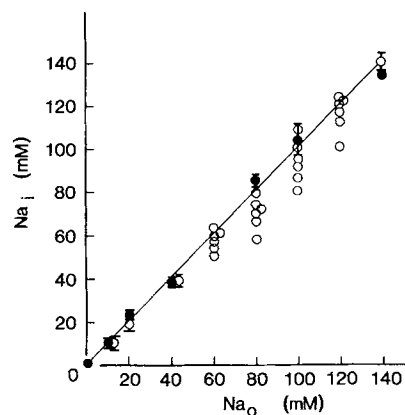
The cells were then washed twice and resuspended in solution B of the same pH but containing 10 mg/ml BSA to scavenge the ionophores. The Na⁺-depleted, acidified cells were kept at room temperature until used. The initial pH, K⁺ content of the cells, and Na⁺/H⁺ exchange activity were stable for at least 2 h when pH_i was clamped at 6.0 and for longer times at higher values.

Loading the Cells with Na⁺

For studying the interaction of Na⁺ with the intracellular face of the exchanger, it was necessary to develop a rapid and reproducible technique to load the cells with Na⁺. To do so, we took advantage of the ability of monensin and nigericin to rapidly equilibrate internal and

external ionic content. BCECF-loaded cells were washed twice with solution A, pH 7.1, or a combination of solutions A and B to yield the desired Na^+ concentration. The cells were incubated with $0.5 \mu\text{M}$ nigericin, $2 \mu\text{M}$ monensin, 0.5 mM ouabain, and 0.2 mM amiloride for 5 min at 37°C . The cells were then washed once with ice-cold solution containing the same Na^+ and K^+ concentrations, 10 mg/ml BSA, and 0.2 mM amiloride, and once with the same solution but without amiloride. Finally, the cells were suspended in $\sim 0.5 \text{ ml}$ of a solution of the same Na^+ and K^+ concentrations with 10 mg/ml BSA and 0.5 mM ouabain and kept in an ice-cold water bath until used. For chemical measurements of Na^+ content, cell volume was determined at the end of the clamping procedure. Cells ($7\text{--}10 \text{ mg}$ dry wt) were suspended in the corresponding Na^+ and K^+ solutions, which also contained [^{14}C]inulin, and were incu-

FIGURE 1. The efficiency of the ionophore technique to clamp intracellular Na^+ . Filled symbols: chemical measurement of Na^+ . Cells were suspended in medium containing 0.2 mM amiloride, 0.5 mM ouabain, and the indicated extracellular concentration of NaCl , at pH 7.1. NaCl was replaced isoosmotically by KCl . Intracellular Na^+ was clamped by the ionophore treatment, as described in the Methods. The cells were then washed once with solution C (TMA-Cl), pH 7.1, containing 0.2 mM



amiloride and 0.5 mM ouabain by a 10-s centrifugation. The pellets were used to determine cell water and Na^+ content as described in the Methods. The figure shows the mean \pm SD of three determinations. Open symbols: null-point titration of Na_i^+ . BCECF-labeled cells were loaded with Na^+ as described above, except that amiloride was omitted from the wash solutions after the ionophore treatment and the cells were suspended in the corresponding NaCl concentrations and kept at 0°C until used. Samples of cells were then added to 2 ml warm medium of the same NaCl and KCl content, and the effect of $2 \mu\text{M}$ monensin on pH_i was measured. If alkalization occurred, the same samples of cells were suspended in a medium of decreasing $[\text{NaCl}]$ and increasing $[\text{KCl}]$ until monensin had no effect on pH_i . This value was taken as the Na_i^+ . The open symbols shown are from 6 of 14 similar experiments. At 10, 20, 40, and 140 mM NaCl , the means \pm SD are given. At 60, 80, 100, and 120 mM NaCl , individual symbols represent individual experiments. They are given to show the variation between experiments when Na_i^+ was measured after different times of maintaining the cells at 0°C .

bated for 5 min at 37°C . Wet weight was determined after the cells were collected by a 10-s centrifugation. The pellets were dried by vacuum and the dry weight was determined; the pellets were then dissolved in 1 ml of 2 M HNO_3 . Samples ($100 \mu\text{l}$) were used to determine the [^{14}C]inulin and Na^+ content by flame photometry. Cell water of the same samples was calculated by subtracting the inulin space from the total volume, which was obtained as the difference between the wet and dry weights. Cell water was independent of the Na^+ content of the cells, probably since internal Na^+ was replaced with K^+ . Cell water was $2.94 \pm 0.17 \mu\text{l/mg}$ dry wt, not significantly different from that of untreated cells, which was $3.1 \pm 0.22 \mu\text{l/mg}$ dry wt. The former value was used to calculate the intracellular Na^+ concentration (Na_i^+). $20\text{--}40 \mu\text{l}$ of cell suspension was added to 2 ml of prewarmed solution (37°C) for measurement of Na^+/H^+ exchange activity. Fig. 1 shows the efficiency of this procedure to clamp

Na_i^+ . Measurement of Na^+ content of the cells by flame photometry immediately at the end of the clamping procedure shows that cells could be loaded with the desired $[\text{Na}^+]$ by varying the medium $[\text{Na}^+]$ during the clamping procedure.

An independent verification of $[\text{Na}^+]$, $[\text{K}^+]$, and $[\text{H}^+]$ in the cells was made by null-point titration. First, pH_i was measured from the BCECF signal. Then the cells were added to solutions containing different Na^+ concentrations at an extracellular pH (pH_o) identical to pH_i (7.1), and the effect of monensin on pH_i was measured. The open symbols in Fig. 1 represent separate experiments in which Na_i^+ was clamped and then measured by null-point titration. We found that immediately after the clamping procedure, Na_i^+ was close to the desired value, and the chemical and null-point titration measurements of Na_i^+ agree very well. Using null-point titration, we found that Na^+ content remained constant in cells loaded with 0–40 and 140 mM Na^+ , whereas cells loaded with intermediate Na^+ concentrations gradually lost Na^+ despite the fact that ouabain was present in the medium and the temperature was maintained at 0°C . The cells lost more Na^+ when ouabain was omitted or when the cells were kept at room temperature. Dye leakage was also reduced at the low temperature. Therefore, the cells were kept at 0°C until use.

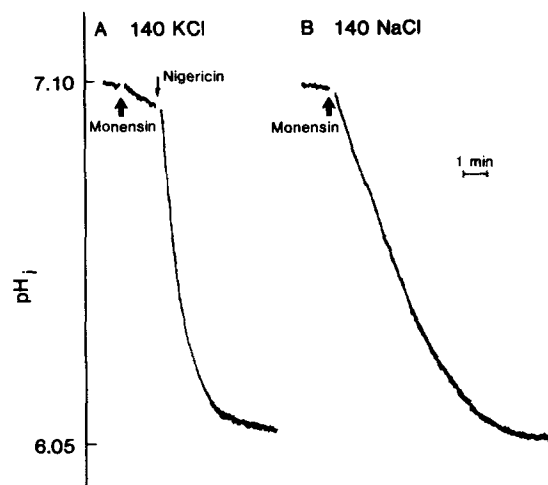


FIGURE 2. Selectivity of monensin for Na^+ over K^+ . Cells were loaded with BCECF and then either depleted of Na^+ (A) or loaded with 140 mM Na^+ (B) as described in the Methods. The cells were then added to 2 ml of prewarmed BSA-free solution C (TMA), containing 0.2 mM amiloride. Ionophores were added as indicated in the figure and BCECF fluorescence was measured and calibrated as described. This experiment represents four others with similar results.

Measurement of Buffer Capacity

The buffer capacity of the cells was estimated from the effect of 10 mM NH_4Cl on the pH_i of untreated and ionophore-treated cells as described previously (Roos and Boron, 1981). Using this technique, the buffering power was found to be $89.7 \pm 6.4 \text{ mmol} \cdot \text{liter cell water}^{-1} \cdot \text{pH unit}^{-1}$ ($n = 5$). This value is considerably higher than that reported for other cells (Roos and Boron, 1981). An alternative way of measuring the buffering power, which is also useful in estimating Na_i^+ , is the determination of the effect of ionophores on pH_i (see Figs. 2, 5, and 11). To be able to use monensin to estimate buffering capacity, the internal ion concentration and the selectivity of the ionophore must be known. Fig. 2 shows the effect of monensin on the pH_i of cells loaded with 140 mM KCl or NaCl. Cells loaded with 140 mM KCl or NaCl, as described before, were added to 2 ml of solution C (TMA-Cl), pH 7.4, containing 0.2 mM amiloride. In the presence of amiloride, the pH_i of Na^+ - or K^+ -loaded cells remained stable at 7.1. Monensin induced only a small acidification when added to K^+ -loaded cells and acidified the cytosol of Na^+ -loaded cells to 6.05. Monensin, therefore, has the required selectivity for Na^+ over K^+ . Nigericin was less selective and was able to partially acidify Na^+ -loaded cells

(not shown). For buffer capacity measurements, we used Na^+ -loaded cells at pH 7.1. First, the initial Na_i^+ was measured by null-point titration. Then the cells were diluted into solution C (TMA-Cl), pH 7.4, containing 0.2 mM amiloride. After addition of monensin, pH_i stabilized when $\text{Na}_i^+/\text{Na}_o^+ = \text{H}_i^+/\text{H}_o^+$. Since Na_o^+ , H_o^+ , and H_i^+ are known, Na_i^+ after monensin acidification is complete can be calculated. Since monensin has a Na^+/H^+ stoichiometry of 1, the buffering capacity can be calculated from the changes in pH_i and Na_i^+ . Using cells loaded with Na^+ concentrations of 40–140 mM, the buffering power was found to be 92.9 ± 2.3 mmol · liter cell water⁻¹ · pH unit⁻¹ ($n = 11$). This value is in very good agreement with that measured using NH_4Cl . The agreement between the two values of measured buffer capacity also indicates that in amiloride-treated UMR-106 cells, the net monensin-induced Na^+ and H^+ fluxes are higher than the fluxes of these ions by other Na^+ - and H^+ -transporting proteins. Thus, monensin-induced Na^+ -dependent pH_i changes can be used to determine buffering power and Na_i^+ by null-point titration. The measured buffering power was used to calculate the rate of H^+ fluxes (J_{H^+}) and the Na^+/H^+ stoichiometry.

²²Na⁺ Uptake

UMR-106 cells loaded with different concentrations of Na^+ were collected by brief centrifugation. The medium was removed and the cells were resuspended in 175 μl solution A (NaCl), pH 7.8, containing 0.5 μCi ²²Na⁺, with or without 0.2 mM amiloride. 50- μl samples were transferred to cation-exchange columns (Dowex 50 W, 100–200 mesh, Sigma Chemical Co.) to remove extracellular radioactivity as described before (Muallem et al., 1985). 1.5-cm columns were prepared in Pasteur pipettes and washed with 0.5 ml of cold solution containing 300 mM sucrose, 5 mM HEPES/Tris, pH 7.4, 25 mg/ml BSA, and 0.2 mM amiloride. The cells were eluted from the columns with two portions of 0.5 ml of the same solution, but without albumin. Amiloride-sensitive ²²Na⁺ uptake was determined from ²²Na⁺ uptake in the presence and absence of amiloride.

RESULTS

Extracellular Na⁺

To study the effect of extracellular Na^+ on H^+ efflux, UMR-106 cells were depleted of Na^+ , loaded with K^+ , acidified to pH_i 6.3, and then added to solution C (TMA-Cl). The cytosol remains acidified despite the pH and K^+ gradients across the plasma membrane (Fig. 3, left). This indicates that both ionophores were effectively extracted from the plasma membrane. When 140 mM NaCl was added to the medium, the cytosol alkalinized to pH_i 7.2. The effect of Na^+ could be blocked by 0.2 mM amiloride. The same behavior was observed when the experiment was repeated with solution B (KCl). The membrane potential in these cells is close to the K^+ diffusion potential (Ferrier et al., 1987), which suggests that the extracellular Na^+ -dependent H^+ efflux was independent of the membrane potential. This assay of Na^+/H^+ exchange has been used to study the dependence of H^+ efflux on extracellular Na^+ (Fig. 4). Increasing the medium $[\text{Na}^+]$ was followed by an increased rate of H^+ efflux. Analysis of the results by a double-reciprocal plot shows an apparent affinity for Na^+ ($K_{0.5}$) of 69 ± 7 mM, with a calculated Hill coefficient of 1.02.

Effect of Amiloride

Fig. 5 shows that addition of 0.2 mM amiloride to the medium completely blocked Na_o^+ -dependent alkalinization. That the effect of amiloride was specific is shown by

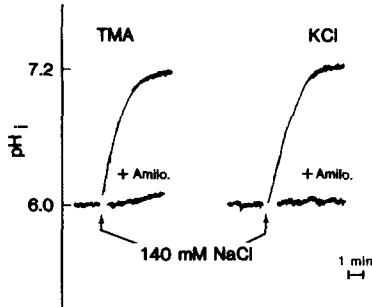


FIGURE 3. Effect of the membrane potential on Na^+/H^+ exchange. BCECF-loaded cells were depleted of Na^+ , loaded with 140 mM KCl, and acidified to pH_i 6.0 as described in the Methods. The cells were then added to 2 ml of solution C (TMA) or 2 ml of solution B (KCl). Separate samples of cells were added to the same media also containing 0.2 mM amiloride. Where indicated, 140 mM NaCl was added to the medium and the change in fluorescence was measured and calibrated to estimate pH_i .

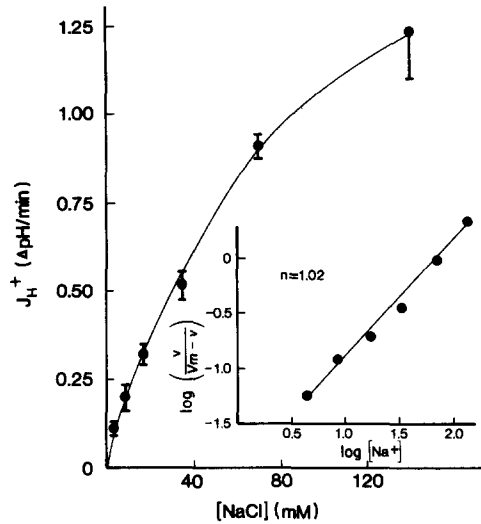


FIGURE 4. The dependence of Na^+/H^+ exchange on Na^+ . Cells loaded with BCECF were depleted of Na^+ and acidified to pH_i 6.3. The cells were added to 2 ml of solution A containing the indicated NaCl concentrations, pH 7.4. Osmolarity was maintained with TMA-Cl (solution C). The rate of amiloride-sensitive pH_i change was recorded and plotted against Na^+ . The data were also used to derive the Hill coefficient (inset), which was calculated by linear regression. The figure shows the mean \pm SD of three experiments using the same subpassage of cells.

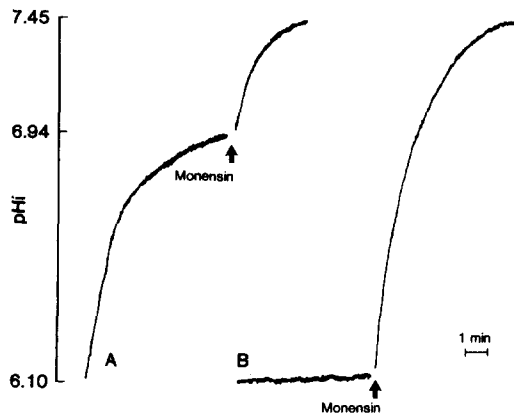


FIGURE 5. Effect of monensin on pH_i of Na^+ -depleted cells. BCECF-labeled cells were acidified to pH_i 6.1 and added to 2 ml of BSA-free solution A (NaCl), pH 7.4, and then exposed to 2 μM monensin (A). The cells were also added to the same medium containing 0.2 mM amiloride before the exposure to monensin (B). The pH_i was estimated from the change in dye fluorescence. This experiment is one of three similar experiments.

the ability of monensin to increase the pH of amiloride-treated cells. Monensin also increased the pH of cells already alkalinized by the Na^+/H^+ exchanger, which indicates that the exchanger becomes quiescent before the driving force for Na^+/H^+ exchange became zero. The finding that monensin alkalinizes control and amiloride-treated cells to the same level suggests that the stoichiometry of Na_o^+ -dependent H^+ efflux is different from 0.5 or 2 and is likely to be 1. Since monensin has a Na^+/H^+ stoichiometry of 1, then if the exchanger operated at the other stoichiometries, monensin would not alkalinize the cytosol to the same levels when added to control and to amiloride-treated cells.

Fig. 6 shows the effect of different concentrations of amiloride on Na_o^+ -dependent H^+ efflux from Na^+ -depleted cells acidified to different pH_i values. The $K_{0.5}$ for amiloride at 140 mM Na_o^+ and pH_o 7.4 was $\sim 6.7 \mu\text{M}$. The same $K_{0.5}$ was found with or without preincubation of the cells with amiloride before the start of the experiment (not shown). When extracellular Na^+ and H^+ were kept constant at 140

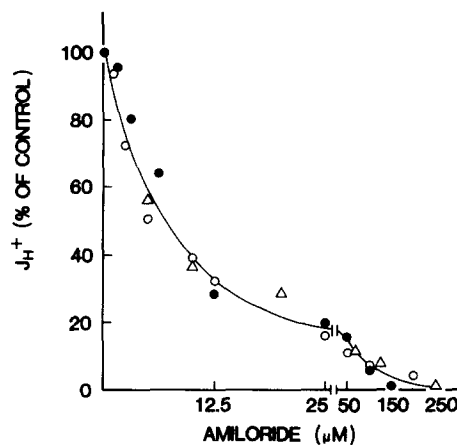


FIGURE 6. Effect of pH_i on the $K_{0.5}$ for amiloride. The cells were depleted of Na^+ and acidified to pH_i 6.2 (filled circles), 6.56 (open circles), and 6.80 (triangles) as described in the Methods. The cells were then added to 2 ml of solution A (NaCl), pH 7.4, containing the indicated concentrations of amiloride. The initial rate of change in pH_i ($\Delta\text{pH}_i/\text{min}$) was measured and plotted against the amiloride concentration. The same results were obtained when the extent of alkalinization was plotted against the concentration of amiloride.

mM and 40 nM, respectively, varying the ΔpH across the membrane between 0.6 and 1.2 pH units by reducing pH_i had no measurable effect on the $K_{0.5}$.

The Relationship between Extracellular Amiloride, H^+ , and Na^+

The apparent affinity of the exchanger for extracellular Na^+ at two extracellular pH values and in the presence of $5 \mu\text{M}$ amiloride is shown in Fig. 7. At pH 7.4, the $K_{0.5}$ for Na^+ was $\sim 62 \text{ mM}$. Reducing pH_o to 7.0 had no effect on V_{max} but increased the $K_{0.5}$ for Na^+ to $\sim 280 \text{ mM}$. From the equation $K_{0.5} = K_m \{1 + [\text{H}^+]/K_m(\text{H}^+)\}$, it is possible to calculate an affinity (K_m) for Na_o^+ of 28.3 mM and a K_m for H_o^+ of 35.5 nM. Fig. 7 also shows that amiloride competes for binding to the extracellular site. $5 \mu\text{M}$ amiloride increased the $K_{0.5\text{Na}}$ to 340 mM. Using the above equation, we calculated a K_i for amiloride of $1.07 \mu\text{M}$.

Fig. 8 shows the effect of increasing pH_o on Na^+/H^+ exchange activity at pH_i values of 6.3 and 7.0. It can be seen that, independently of the initial pH_i ,

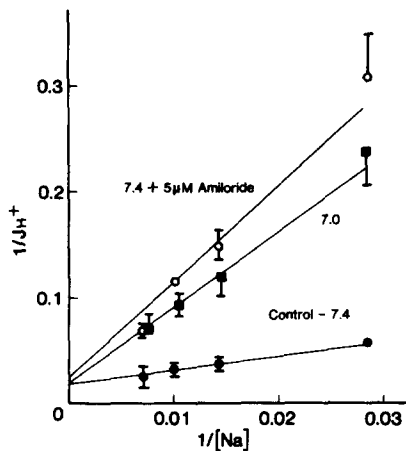


FIGURE 7. The relationship between Na_o^+ , H_o^+ , and amiloride. Cells were depleted of Na^+ and acidified to pH_i 6.0. The cells were added to solutions containing 40–140 mM NaCl at pH_o 7.4 (filled circles), pH_o 7.0 (filled squares), or pH_o 7.4 and 5 μM amiloride (open circles). The rate of H^+ efflux at 0 mM NaCl was subtracted from the individual H^+ fluxes. The results of the three experiments shown are expressed as double-reciprocal plots and the slopes were calculated by linear regression. The calculated kinetic parameters from the curves are given in the text.

exchanger activity could not be detected when pH_o was close to pH_i despite the presence of a large Na^+ gradient.

The thermodynamic driving force (DF) for exchange is directly proportional to the term $\log(\text{Na}_o^+/\text{Na}_i^+) - (\text{pH}_i - \text{pH}_o)$ and, therefore, Na^+/H^+ exchange should proceed until $(\text{Na}_o^+/\text{Na}_i^+) = (\text{H}_o^+/\text{H}_i^+)$, i.e., $\text{DF} = 0$ (Aronson, 1981). Even when we assume that Na^+ -depleted cells contain 1.4 mM Na^+ , then at 140 mM Na_o^+ , pH_i 6.3, and pH_o 6.2, the term $\log(\text{Na}_o^+/\text{Na}_i^+) - (\text{pH}_i - \text{pH}_o)$ is 1.9, while that at a similar Na^+ gradient and at pH_i 7.0 and pH_o 6.6 is 1.6. Thus, an exchange of Na_o^+ for H_i^+ by the exchanger was too small to be detected in the presence of a large inwardly directed Na^+ gradient but in the absence of an outwardly directed H^+ gradient. At both pH_i values, increasing pH_o was followed by almost maximal activation of the exchanger. Further, exchanger activity increased above that predicted from the

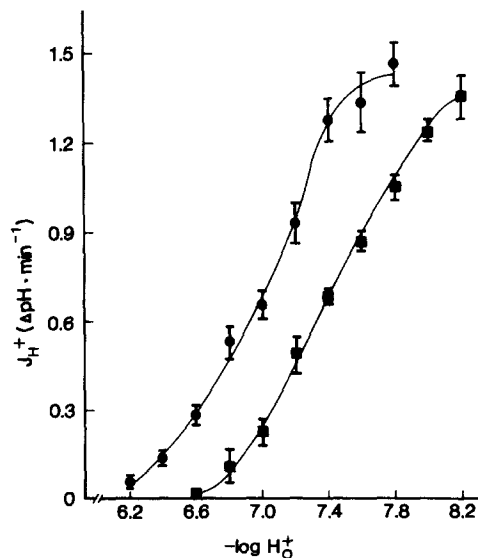


FIGURE 8. Effect of pH_o on $\text{Na}_o^+/\text{H}_i^+$ at two values of pH_i . BCECF-labeled cells were Na^+ depleted and acidified to pH_i 6.3 or 7.0. The cells were then added to 2 ml of solution A (NaCl), at a pH ranging between 6.2 and 8.2. The rates of alkalinization at each pH_o value were measured. The figure shows the mean \pm SD of four experiments.

increase in the DF or the reduced competition between Na_o^+ and H_o^+ . For example, at pH_i 6.3, increasing pH_o from 6.2 to 7.0 increased the exchange rate from ~ 0.05 to $0.67 \Delta\text{pH}/\text{min}$. Using the K_m for Na_o^+ and H_o^+ (Fig. 7), it is possible to calculate that the reduced competition between Na_o^+ and H_o^+ can account for an increased rate of $\text{Na}_o^+/\text{H}_i^+$ exchange to $0.13 \Delta\text{pH}/\text{min}$. The increased H^+ gradient further increased the $\text{Na}_o^+/\text{H}_i^+$ exchange rate by $(2.4/1.6)$ 1.5-fold to $0.195 \Delta\text{pH}/\text{min}$. Thus, the $\text{Na}_o^+/\text{H}_i^+$ exchange rate measured at pH_i 6.2 and pH_o 7.0 was ~ 3.4 -fold higher than that predicted from the reduced competition between Na_o^+ and H_o^+ and the increased DF. It is also clear from Fig. 8 that, in Na^+ -depleted cells and at pH_i 7.0, high rates of $\text{Na}_o^+/\text{H}_i^+$ exchange can be observed at high pH_o .

Intracellular H^+

The apparent affinity of the exchanger for intracellular H^+ was measured with Na^+ -depleted cells acidified to different values of pH_i . The cells were added to solution A

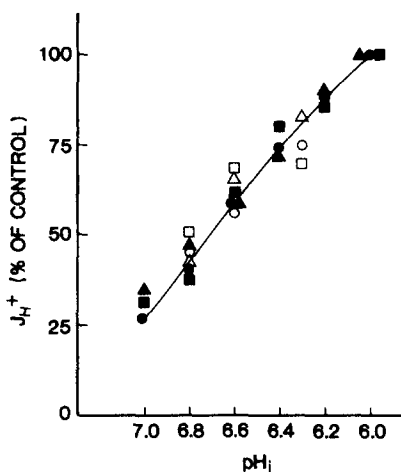


FIGURE 9. The pH_i dependence of $\text{Na}_o^+/\text{H}_i^+$ exchange at three Na_o^+ values. Cells were acidified to pH_i between 7.0 and 6.0 as described in the Methods. The cells were added to solution A, pH 7.4, containing 25, 50, or 140 mM NaCl . TMA-Cl was used for isosmotic replacement of NaCl . The rates of alkalization of cells acidified to pH_i 6.0 at 25, 50, and 140 mM Na_o^+ were ~ 0.48 , 0.73 , and $1.30 \Delta\text{pH}/\text{min}$, respectively, and were taken as 100% control to calculate the relative rates at the other values of pH_i . The figure shows the results of two experiments (filled and open symbols).

containing 25, 50, or 140 mM NaCl , pH_o 7.4, and the rate of amiloride-sensitive H^+ efflux was measured (Fig. 9). Varying Na_o^+ between 25 and 140 mM had no effect on the apparent affinity for intracellular H^+ .

The results in Fig. 8 suggest that the apparent affinity for H_i^+ might have been affected by the H^+ gradient across the membrane. To test the effect of the H^+ gradient on the apparent affinity for intracellular H^+ , the intracellular pH dependence of the exchanger was measured at three values of pH_o (Fig. 10). Reducing pH_o from 7.4 to 6.8 decreases the apparent affinity for H_i^+ by ~ 0.62 pH units and increasing pH_o to 8.0 increased the apparent affinity for H_i^+ by ~ 0.55 pH units (Fig. 10 A). However, calculation of the Hill coefficient shows that changes in pH_o had little, if any, effect on the Hill coefficient, which was 1.77 at pH_o 7.4, 1.98 at pH_o 6.8, and 1.80 at pH_o 8.0 (Fig. 10 B). Therefore, it appears that pH_o affects only the apparent affinity for H^+ but does not prevent the activation of the exchanger by H_i^+ .

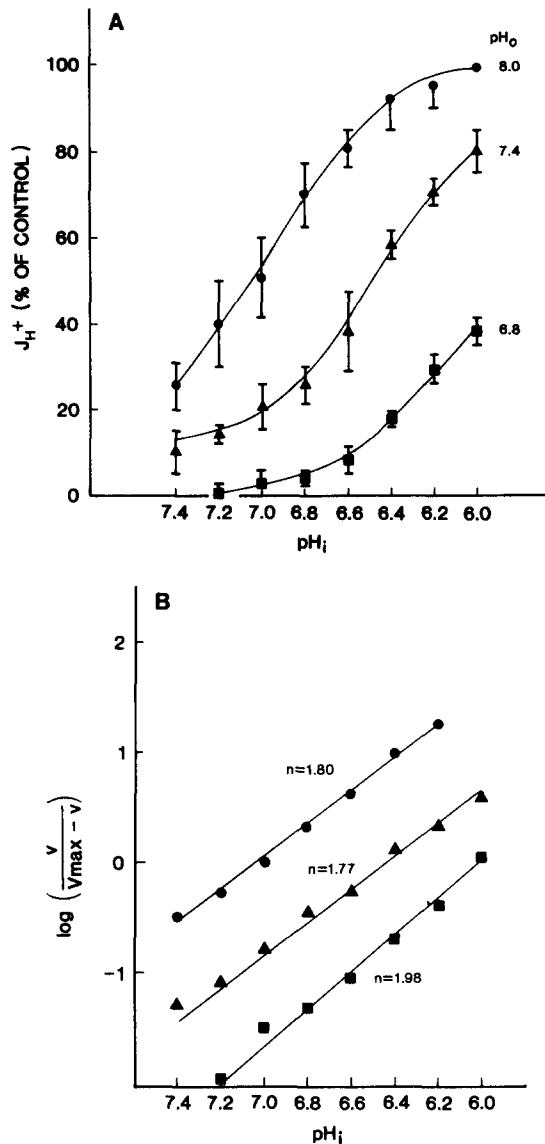


FIGURE 10. Effect of pH_o on the pH_i dependence of Na_o^+/H_i^+ exchange. BCECF-loaded cells were acidified to the indicated pH_i . The cells were then added to 2 ml solution A (NaCl) at a pH_o of 6.8, 7.4, or 8.0 and the rate of amiloride-sensitive pH_i change was measured. The rate of alkalinization of cells acidified to pH 6.0 and added to pH_o 8.0 was taken as 100% control. The rate of pH_i change was measured and plotted against pH_i (A). The figure shows the mean \pm SD of five experiments. The data in A were also expressed as a Hill plot (B) and the slopes in B were calculated by linear regression.

Na_i^+ -dependent H^+ Influx

Na_i^+ -dependent H^+ influx was studied by preloading the cells with 140 mM Na^+ , pH_i 7.1. Dilution of the cells into solution C (TMA) at pH 7.4 (Fig. 11, left) was followed by acidification of the cytosol to pH_i 6.42. When 140 mM NaCl was added to the medium, the cytosol alkalinized to near the original pH_i . The acidification could be blocked by amiloride. Addition of monensin to the amiloride-treated cells was followed by an acidification to a pH_i of \sim 6.06. Subsequent addition of 15 mM

NaCl to the medium increased the pH_i to ~ 6.42 . The same results were obtained when the cells were diluted into solution B (KCl) (Fig. 11, right), which suggests that Na_i^+/H_o^+ exchange is independent of the membrane potential and is therefore an electroneutral process. An additional important observation in this experiment is that, in the presence of an outwardly directed ion gradient, high rates of exchange can be measured when the term $\log(Na_o^+/Na_i^+) - (pH_i - pH_o)$ is ~ 1.7 . Exchanger activity stopped when this term was 0.8.

The protocol shown in Fig. 11 was also used to estimate the Na^+/H^+ stoichiometry of the exchange process as follows. The initial and final pH_i owing to Na^+/H^+ exchange were estimated from the fluorescence signals. The cytosolic H^+ buffering capacity was measured by NH_4Cl or monensin-induced pH_i changes as explained in the Methods. The initial Na_i^+ is measured chemically or by null-point titration. To

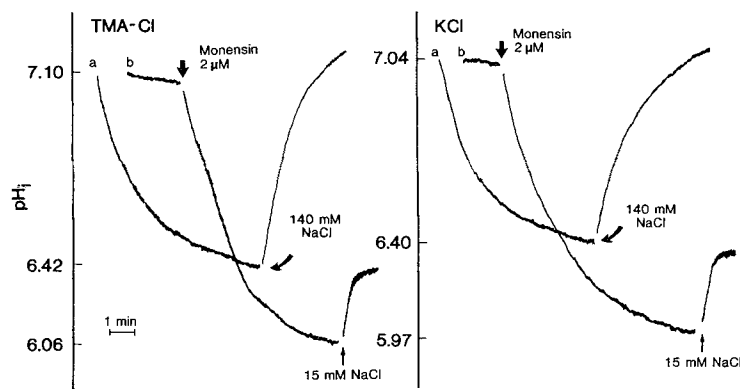


FIGURE 11. Effect of the membrane potential on Na_i^+/H_o^+ exchange. BCECF-labeled cells were depleted of K^+ , loaded with 140 mM NaCl, and clamped at pH_i 7.1 as described in the Methods. (Left) Samples of cells were added to 2 ml of solution C (TMA, *a*) or solution C containing 0.2 mM amiloride (TMA, *b*). (Right) The cells were added to 2 ml solution B (KCl, *a*) or solution B containing 0.2 mM amiloride (KCl, *b*). Where indicated, monensin or NaCl was added to the medium. In other experiments, monensin and then NaCl were added to control cells and the same results were obtained. The figure shows one of four similar experiments.

measure the amount of Na^+ transported by the exchanger, Na_i^+ at the end of the exchange process is measured by addition of monensin to the cells. Monensin further acidifies the cells' cytosol until $Na_i^+/Na_o^+ = H_i^+/H_o^+$. Na^+ is then gradually added to the medium until the pH_i is equal to that induced by the exchanger (6.38 in Fig. 11). At that point, Na_i^+ can be calculated from $Na_i^+ = Na_o^+(H_i^+/H_o^+)$. From this value and the initial Na_i^+ , the amount of Na^+ transported by the exchanger can be calculated. From the change in pH_i and the H^+ buffering capacity, the amount of H^+ transported by the exchanger can be calculated and therefore the stoichiometry of the exchanger can be determined. Table I depicts the measurement of Na^+/H^+ stoichiometry of the exchanger at different initial Na_i^+ values. The stoichiometry was independent of the initial Na_i^+ and was 1.01 ± 0.04 ($n = 12$).

TABLE I
The Stoichiometry of $\text{Na}_i^+/\text{H}_o^+$ Exchange Transport

Intracellular Na^+ <i>mM</i>	Na^+/H^+ stoichiometry
15	0.87
30	1.02
60	1.25, 0.94
80	0.78, 1.12
100	1.15
120	0.94
140	1.08, 0.91, 1.21, 0.88
Overall	1.01 ± 0.04

Cells were loaded with the indicated concentrations of Na^+ as described in the Methods. The cells were then added to Na^+ -free medium and the change in pH_i was measured as in Fig. 11 (left). After ~ 3 min of incubation at 37°C , the buffer capacity and $[\text{Na}^+]_i$ were measured by the monensin titration technique as explained in the text. These values were used to calculate the Na^+/H^+ stoichiometry of the exchanger for the indicated initial values of $[\text{Na}^+]_i$.

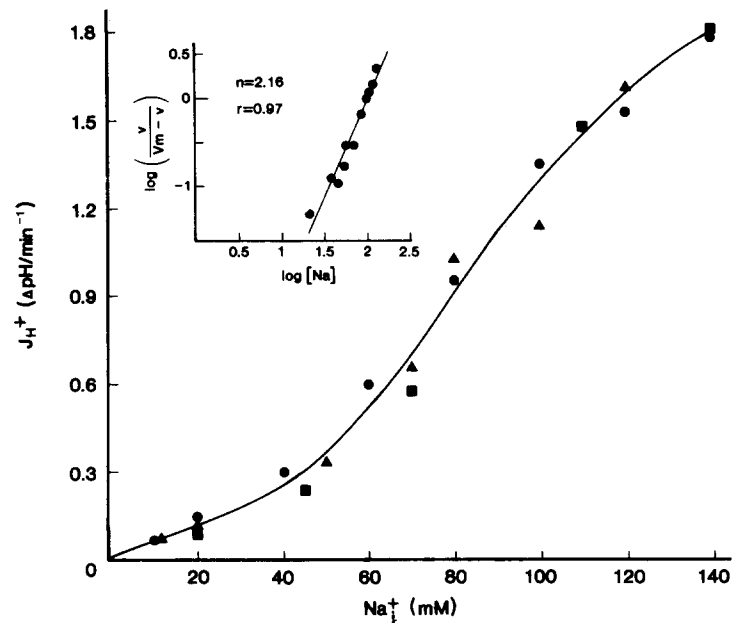


FIGURE 12. The dependence of $\text{Na}_i^+/\text{H}_o^+$ exchange on Na_i^+ . Cells were loaded with the indicated NaCl concentrations and clamped at $\text{pH } 7.1$ as described in the Methods. The Na_i^+ was measured by null-point titration, and then cells were added to solution C (TMA-Cl), $\text{pH } 7.4$, and the rate of pH_i change was measured at each Na_i^+ . The three different symbols represent separate experiments. The data in the main panel were also plotted as a Hill plot (inset), and the slope was calculated by linear regression.

Apparent Affinities for Na_i^+ and H_o^+

The intracellular Na^+ dependence of H^+ influx was measured by loading the cells with different Na^+ concentrations at pH_i 7.1 (see Methods) and diluting them into solution C (TMA) at pH_o 7.1. Fig. 12 shows that the intracellular Na^+ dependence of H^+ influx was sigmoidal. The calculated Hill coefficient for the three experiments shown was 2.16 (inset).

To further investigate the kinetic properties of $\text{Na}_i^+/\text{H}_o^+$ exchange, we measured the dependence of this exchange (reverse mode) on H_o^+ (Fig. 13). The rate of H^+ influx increased with the increase in H_o^+ and followed simple saturation until H_o^+ was ~ 40 nM. The calculated Hill coefficient of this part of the curve was 1.06 (inset). However, when H_o^+ was increased further, the H_o^+ dependence deviated from simple saturation, which was reflected by a gradual reduction of the Hill coefficient. This occurred despite an increase in the driving force and in the substrate concentration.

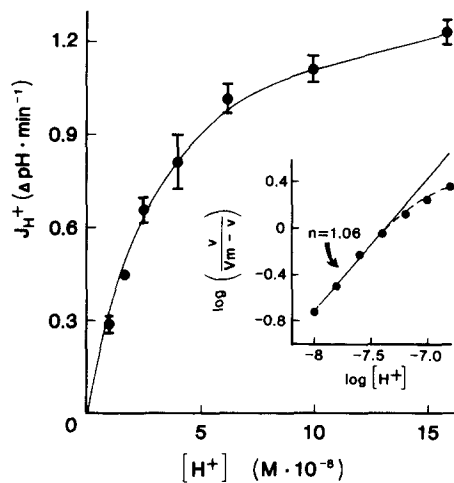


FIGURE 13. The dependence of $\text{Na}_i^+/\text{H}_o^+$ exchange on H_o^+ . Cells were depleted of K^+ , loaded with 140 mM NaCl , and clamped at pH 7.1 as described in the Methods. The cells were suspended in solution A, pH 7.1, with 0.5 mM ouabain and kept in an ice-cold bath until use. Samples (20–25 μl) of cells were then added to 2 ml of prewarmed (37°C) solution C (TMA), where the values of pH ranged between 6.8 and 8.0. The rate of amiloride-sensitive acidification was measured. The figure shows the mean \pm SD of three experiments. The inset shows a Hill plot of the same results. The slope was calculated from the rates measured at H_o^+ up to 39.8 nM (pH 7.4).

Competition between Na_i^+ and H_i^+

Since Na_i^+ can be transported by the exchanger similar to H_i^+ , it was of interest to study the relationship between Na^+ and H^+ during $\text{Na}_o^+/\text{H}_i^+$ (forward) exchange. To examine this relationship, cells were loaded with 0–40 mM Na^+ while the initial pH_i was set at 6.3 or 7.1. Because of the relatively low apparent affinity for Na_i^+ at pH_i 7.1 (Fig. 12), at Na_i^+ between 0.0 and 40.0 mM, mostly one site is occupied with Na^+ . Hence, the relationship between Na^+ and H^+ at this site can be studied. Increasing Na_i^+ inhibited exchanger activity at both values of pH_i (Fig. 14 A). Inhibition of $\text{Na}_o^+/\text{H}_i^+$ exchange by Na_i^+ can result from inhibition of H^+ efflux or stimulation of H^+ influx due to $\text{Na}_i^+/\text{H}_o^+$ exchange. However, using the calculated K_m

values for Na_o^+ , H_o^+ (Fig. 7), Na_i^+ , and H_i^+ (see below) and the equation $V = V_{\max} \cdot S / K_m(1 + I/K_i)$, it is possible to calculate that under the conditions of the experiments in Fig. 14 and at 40 mM Na^+ , the contribution of $\text{Na}_i^+/\text{H}_o^+$ exchange to net H^+ fluxes is small. Therefore, it is likely that Na_i^+ inhibits $\text{Na}_o^+/\text{H}_i^+$ exchange. Na_i^+ inhibited $\text{Na}_o^+/\text{H}_i^+$ exchange more effectively at pH_i 7.1 when pH_o was set at 8.1. Further, inhibition by Na_i^+ was a function of the H_o^+ . At the same pH_i of 7.1, Na_i^+ inhibited exchanger activity more effectively at a pH_o of 7.3 compared with Na_i^+ inhibition of the exchanger at pH_o 8.1. The apparent affinity for Na_i^+ was therefore affected by H_o^+ . The relationship between Na_i^+ and H_i^+ is apparent from expression of the mea-

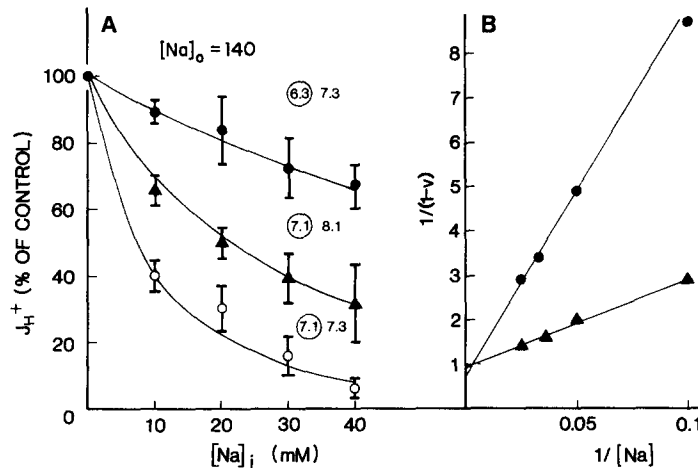


FIGURE 14. Inhibition of $\text{Na}_o^+/\text{H}_i^+$ exchange by Na_i^+ . (A) Cells were loaded with 0–40 mM NaCl and acidified to pH_i 6.3 or 7.1. The cells, were then added to solution A (NaCl), pH 7.3, and those acidified to pH 7.1 were added to solution A, pH 8.1 or 7.3. The rate of H^+ efflux from Na^+ -depleted cells acidified to pH_i 6.3 was ~ 0.93 $\Delta\text{pH}/\text{min}$. The rate of H^+ efflux from Na^+ -depleted cells acidified to pH_i 7.1 and added to medium of pH 8.1 was 0.845 $\Delta\text{pH}/\text{min}$ and that of similar cells added to medium of pH 7.3 was ~ 0.27 $\Delta\text{pH}/\text{min}$. In each set of experiments, these rates of alkalization were taken as the respective 100% control and used to calculate the relative rates of alkalization at the different values of Na_i^+ . The figure shows the mean \pm SD of three experiments. The results were also plotted as $1/(1 - V)$ against $1/\text{Na}^+$ (B) and the slopes were calculated by linear regression.

sured effect of Na_i^+ at identical ΔpH as $1/(1 - V)$ vs. $1/\text{Na}^+$ (Fig. 14 B). It appears that Na_i^+ inhibits $\text{Na}_o^+/\text{H}_i^+$ exchange activity by competing with H_i^+ . From the measured $K_{0.5}$ values for Na^+ , 20.6 mM (pH_i 7.1) and 122 mM (pH_i 6.3), it is possible to calculate the K_m for the ions as explained before. The K_m for Na_i^+ is 1.58 mM and that for H_i^+ is 6.6 nM. The more reliable of these two values is the K_m for Na^+ , while the K_m for H^+ should be measured at pH_i above 7.4 and pH_o above 8.4, which is precluded due to the relative insensitivity of the dye to H^+ at high pH_i values, and the effect of pH_o above 8.4 on exchanger activity (Aronson et al., 1983).

The curves in Fig. 14 could be used to show the relationship between Na_i^+ and H_i^+

TABLE II
Effect of Intracellular Na⁺ and H⁺ on the Apparent Affinity for Extracellular Na⁺

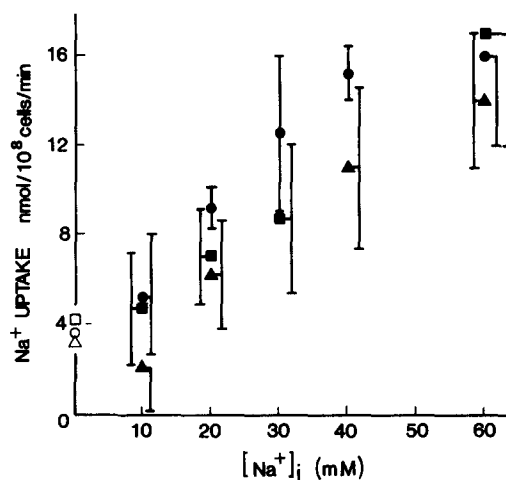
Conditions		$K_{0.5}$ for Na ⁺ <i>mM</i>
pH _i	[Na ⁺] _i	
6.3	0	62 ± 11
6.3	40	61 ± 15
6.8	0	66 ± 8
Overall mean		63 ± 10

UMR-106 cells were loaded with H⁺ and Na⁺ as described in the Methods. The cells were then added to solutions containing NaCl concentrations between 35 and 140 mM and the rate of H⁺ extrusion was measured. The accumulated results were analyzed by Lineweaver-Burke plots and the K_m values were calculated from the corresponding curves by linear regression. The results shown are the mean ± SD of three separate experiments.

only if the intracellular content of the cells does not affect the interaction of the ions with the extracellular face of the exchanger. Table II shows that intracellular Na⁺ or H⁺ has no effect on the apparent affinity for extracellular Na⁺.

To further elucidate the nature of the inhibition by Na_i⁺, we measured the effect of Na_i⁺ on amiloride-sensitive ²²Na⁺ uptake. Fig. 15 shows that as Na_i⁺ is increased, the rate of amiloride-sensitive ²²Na⁺ uptake increased, although the rate of Na⁺/H⁺ exchange under similar conditions decreased (Fig. 14). The large scatter in these

FIGURE 15. Effect of Na_i⁺ on the rate of Na⁺/Na⁺ exchange. Cells loaded with the indicated NaCl concentrations, pH_i 7.1, were resuspended in the respective NaCl solutions and kept in an ice-cold water bath until used (10–20 min). Samples of $\sim 5 \times 10^8$ cells were centrifuged for 3 s at 12,000 *g*. The medium was removed and ²²Na⁺ uptake was initiated by suspending the cells in 175 μ l of solution A (140 mM NaCl), pH 7.8, containing ²²Na⁺ with or without 0.2 mM amiloride. After 20 s (0–20 mM NaCl) or 15 s (30–60 mM NaCl) of incubation at room temperature, 50- μ l samples were removed to Dowex 50 columns to stop the reaction and to remove external ²²Na⁺ as described in the Methods. Amiloride-sensitive ²²Na⁺ uptake into Na⁺-depleted cells (open symbols) was taken as Na⁺ uptake owing to Na⁺/H⁺ exchange. The contribution of Na⁺/H⁺ exchange to ²²Na⁺ uptake at the different Na_i⁺ values was calculated from Fig. 14. The filled symbols show the amiloride-sensitive ²²Na⁺ uptake owing to Na⁺/Na⁺ exchange, which was obtained by subtracting ²²Na⁺ uptake owing to Na⁺/H⁺ exchange from total ²²Na⁺ uptake. The figure shows the results of three experiments and the symbols show the mean ± SD of triplicate measurements.



experiments is due to the presence of 140 mM Na_o^+ during $^{22}\text{Na}^+$ uptake. It can be seen that Na^+/Na^+ exchange could be measured at ~ 60 mM Na_i^+ , which is the concentration of Na_i^+ required to maximally inhibit $\text{Na}_o^+/\text{H}_i^+$ exchange under similar conditions. The increase in $^{22}\text{Na}^+$ uptake rate despite the inhibition of Na^+/H^+ exchange is compatible with previous reports that Na^+/Na^+ exchange is faster than Na^+/H^+ countertransport (Aronson et al., 1983).

DISCUSSION

The present study shows that UMR-106 cells possess the Na^+/H^+ exchanger. These cells demonstrate many features characteristic of normal osteoblasts (Martin et al., 1976; Partridge et al., 1981, 1983), so it is conceivable that the exchanger exists also in normal osteoblasts. Since alkalization of the cells after cytosolic acidification was Na^+ dependent and amiloride sensitive, we conclude that the Na^+/H^+ exchanger is the major mechanism used by these cells to extrude acid. The Na^+/H^+ exchanger may therefore play a role in the acid-base regulation of the bone extracellular fluid during bone formation and resorption (Parfitt and Kleerekoper, 1980; Baron et al., 1985).

In the present report, we used UMR-106 cells in an attempt to study the interaction of H and Na ions with the extracellular and intracellular faces of the Na^+/H^+ exchanger and the influence of outwardly directed H^+ and Na^+ gradients on exchanger activity. The Na^+/H^+ exchanger exhibits a single external transport site that accepts Na^+ , H^+ , or amiloride. On the internal face of the exchanger, transport and regulatory sites can be detected. Both internal sites can bind H^+ and Na^+ ; therefore, Na_i^+ , like H_i^+ , is able to activate the exchanger. Na^+ and H^+ compete for binding to the internal transport site and this competition appears to govern the rate of net H^+ transport by the exchanger. Measurement of the K_m for Na^+ and H^+ of the internal and external transport sites reveals that the sites are asymmetric. The kinetic characterization of the exchanger suggests that the Na^+/H^+ exchanger is compatible with a simultaneous model. Our study also shows that an outwardly directed H^+ gradient is essential to observe exchanger activation by Na_i^+ and H_i^+ . We will now discuss the evidence for these findings.

Extracellular Site

The interaction of Na ions with the external site obeys simple saturation kinetics with a calculated affinity (K_m) for Na^+ of ~ 28 mM. From the effect of H_o^+ on the apparent affinity ($K_{0.5}$) of Na_o^+ , as with other cells (Grinstein et al., 1984), H_o^+ competes with Na_o^+ for binding to the extracellular transport site. Further, H_o^+ dependence of $\text{Na}_i^+/\text{H}_o^+$ (reverse) exchange, down to pH_o 7.4, also follows simple saturation kinetics. The calculated K_m for H_o^+ from the two experimental protocols were 35.5 and 39 nM, respectively, which are in good agreement. This indicates that with both protocols we measured the K_m for H^+ of the same site. A similar K_m for H_o^+ has been reported for the proximal tubule Na^+/H^+ exchanger (Aronson et al., 1983).

Amiloride inhibited exchanger activity by binding to the same external site; the calculated K_i for amiloride was 1.07 μM . These studies indicate that the exchanger has a single extracellular transport site that accepts Na^+ , H^+ , or amiloride.

Intracellular Sites

The pH_i dependence of Na^+/H^+ exchange at 140 mM Na_o^+ and any pH_o did not follow simple saturation kinetics. This phenomenon was first reported for the Na^+/H^+ exchange of microvillus membrane vesicles from kidney cortex (Aronson et al., 1982) and was subsequently confirmed in other cell types (Grinstein et al., 1984; Boron and Boulpaep, 1983). Taken together with stoichiometry measurements of Na^+/H^+ exchange and the kinetics of Na^+ interaction with the extracellular site, it was concluded that H_i^+ , apart from being transported, also activates the exchanger. In the present study, we demonstrate that Na ions can also activate the exchanger. This is concluded from the following: the stoichiometry of $\text{Na}_i^+/\text{H}_o^+$ exchange (reverse) is close to 1 at 15–140 mM Na_i^+ . As expected from this stoichiometry, $\text{Na}_i^+/\text{H}_o^+$ exchange is independent of the membrane potential. These two findings indicate that Na^+/H^+ exchange in the reverse mode is an electroneutral transport process. This mode of transport shows simple saturation kinetics with respect to H_o^+ . However, the Na_i^+ dependence of the process is along a sigmoidal curve with a calculated Hill coefficient of ~ 2 . These findings, when taken together, suggest that Na ions can bind to both intracellular transport and regulatory sites and activate the exchanger. It is likely that activation of the exchanger by Na_i^+ is a general property of the exchanger. This is suggested from measurements of the rate of $\text{Na}_i^+/\text{H}_o^+$ exchange in Na^+ -loaded cells (Grinstein et al., 1984; Vigne et al., 1984; Cassel et al., 1986) at a pH_i of ~ 7.1 and a pH_o of 7.4. These rates are similar to $\text{Na}_o^+/\text{H}_i^+$ exchange (forward) into acidified cells in which the exchanger is activated by H_i^+ .

Since activation of the exchanger and therefore the apparent affinity of the regulatory site for the ions were affected by the gradients of the ions across the membrane, it was impossible to determine the K_m for this site. However, under physiological conditions of pH_o 7.4 and 140 mM Na_o^+ , the $K_{0.5}$ for H_i^+ is $\sim 0.35 \mu\text{M}$. Half-maximal activation of the exchanger by Na_i^+ was obtained at ~ 100 mM. These relatively low apparent affinities allow us to study the interaction of the ions with the transport site by studying the effect of Na_i^+ on $\text{Na}_o^+/\text{H}_i^+$ and Na^+/Na^+ exchange. The finding that both the dependence of Na^+/Na^+ exchange on Na_i^+ and the inhibition of $\text{Na}_o^+/\text{H}_i^+$ exchange by Na_i^+ obey simple saturation kinetics indicates that Na_i^+ and H_i^+ compete for binding to the same site. In these experiments, Na_i^+ probably competes with H_i^+ for binding to only one of the two intracellular binding sites. This appears to be the transport site since, at the indicated Na_i^+ and pH_i , the regulatory site is not occupied by Na_i^+ (Fig. 12). It has been shown that Na_i^+ inhibits $\text{Na}_o^+/\text{H}_i^+$ exchange in other cell types (Grinstein et al., 1984; Grinstein and Furuya, 1986). It is likely that this inhibition also reflects the competition between Na_i^+ and H_i^+ for binding to the transport site.

The calculated K_m values for H^+ and Na^+ of the internal and external transport sites are summarized in Table III. The K_m 's were derived from experiments in which $\text{Na}_o^+/\text{H}_i^+$ exchange was activated by an outwardly directed pH gradient of at least 1 unit. The K_m values for H_i^+ and Na_i^+ were different from those for H_o^+ and Na_o^+ . Thus, there is asymmetry of the transport sites in terms of their affinities for the transported ions. This is in addition to the asymmetry in terms of the activation of the exchanger by intracellular ions that was described before for H^+ (Aronson et al., 1982) and extended in the present study for Na^+ .

The competition between Na_i^+ and H_i^+ seems to govern exchange activity. When exchanger activity was measured in the forward or reverse direction, it always stopped before $\text{Na}_i^+/\text{Na}_o^+ = \text{H}_i^+/\text{H}_o^+$, i.e., before thermodynamic equilibrium had been achieved. On the other hand, when Na^+ or H^+ were not allowed to vary considerably during exchange measurements by using low $[\text{Na}^+]$, it was found that Na^+/H^+ exchange proceeded as expected from the applied ionic gradients (Kinsella and Aronson, 1982). The observed competition between Na_i^+ and H_i^+ suggests that modification of pH_i from normal resting levels can be achieved either by the addition of H^+ or the removal of Na^+ from the cytosol. Alternatively, the values of K_m for H_i^+ and Na_i^+ can be altered during stimulation of the exchanger. It has been shown previously that exchanger stimulation by hormones (Moolenaar et al., 1983), second messengers (Grinstein et al., 1985a), or osmotic shrinkage (Grinstein et al., 1985b) modifies the pH_i dependence of the exchanger. It remains to be determined whether the change in apparent affinity for H_i^+ reflects a change in the K_m of either the transport or the regulatory site, and whether the K_m for Na_i^+ is also modified.

TABLE III
K_m Values for H⁺ and Na⁺ of the Internal and External Transport Sites

	<i>K_m values</i>	
	<i>Na⁺</i>	<i>H⁺</i>
	<i>mM</i>	<i>nM</i>
External transport site	28	39
Internal transport site	1.6	6.6

The K_m values for the external transport site were calculated from Fig. 7 and those for the internal transport site were calculated from Fig. 14.

Effect of Ionic Gradients

Several lines of evidence in the present study suggest that an outwardly directed H^+ gradient is essential to observe exchanger activation. (a) An increase of pH_o allows almost similar activation of the exchanger at two different pH_i values of 6.3 and 7.0. Further, with the increase in pH_o , exchanger activity increased beyond that expected from the contribution of the increased DF, and reduced competition between Na_o^+ and H_o^+ (Fig. 8). This phenomenon was also observed in synaptosomes (Jean et al., 1985). (b) A different pH_i dependence was found at pH_o of 8.0, 7.4, and 6.8, while the Hill coefficient remained at ~ 2 . Thus, activation of the exchanger was possible at each pH_o , but a ΔpH of ~ 1.5 was required for maximal exchanger activation. (c) The apparent affinity for Na_i^+ was a function of pH_i , but was also affected by pH_o (Fig. 14). (d) Forward exchanger activity was minimal or absent in the presence of a large DF with no outwardly directed H^+ gradient (Figs. 8 and 10). On the other hand, a high rate of exchange was measured at a smaller DF when the Na^+ gradients were outwardly directed (Figs. 11 and 12). This indicates that a rate-limiting step prevents exchange activity from proceeding according to the applied DF. Application of an outwardly directed H^+ or Na^+ gradient accelerates the rate of this step. Taken together, this evidence suggests that an outwardly directed H^+ or Na^+ gradient is essential to observe exchanger activation and that an increase in H_i^+ is not sufficient, by itself, to activate the exchanger.

An alternative way of stating the requirement for an outwardly directed gradient for activation of the exchanger is that H_o^+ inhibits the exchanger beyond that expected from competition with Na_o^+ . This might be due to the effect of H_o^+ on the relative apparent affinities for H_i^+ and Na_i^+ . Increasing H_o^+ decreases the apparent affinity for H_i^+ (Fig. 10) and increases the apparent affinity for Na_i^+ (Fig. 14). Hence, binding of H^+ to an inhibitory extracellular site appears to modify the relative affinities of the internal transport site to Na^+ and H^+ , but in the opposite direction. Since Na_o^+ up to 140 mM has no effect on pH_i dependence, the proposed external inhibitory site might accept only H^+ , or the affinity of this site for Na^+ may be very low. This implies that the affinity of the intracellular site is not constant, but varies according to the magnitude of the outwardly directed H^+ gradients. Since Na_i^+ competes with H_i^+ , it is not yet clear whether the affinities for H_i^+ or Na_i^+ or both are affected by H_o^+ .

The above proposed effect of H_o^+ on the apparent affinities for Na_i^+ and H_i^+ cannot, however, explain the effect of high H_o^+ on Na_i^+/H_o^+ exchange. The H_o^+ dependence of the Na_i^+/H_o^+ exchange (reverse) followed simple saturation kinetics only up to ~ 40 nM H_o^+ . With a further increase in H_o^+ , the Hill coefficient gradually deviated from 1. In dog red blood cells (Parker, 1986), increasing H_o^+ from 40 nM to 1 μ M completely inhibited exchanger activity in the reverse mode. This occurred despite the increase in substrate concentration and the DF for exchange activity. One possible explanation for this phenomenon is that the additional extracellular binding site for H^+ has an apparent negative cooperativity effect on the external transport site. Another possibility is that there exists an additional effect of H_o^+ on a rate-limiting step in the turnover cycle of Na^+/H^+ exchange. Previous studies (Aronson et al., 1983) and the present report demonstrate that Na_o^+/Na_i^+ exchange is faster than Na_o^+/H_i^+ exchange. This excludes the binding, transport, and release of Na^+ to the cytosol as a rate-limiting step. Thus, the rate-limiting step may be the release of the transported ion, either Na^+ or H^+ , to the medium. H_o^+ , but not Na_o^+ up to 140 mM, inhibits this step.

Mode of Transport

Two substrate transporters can operate in a simultaneous or ping-pong mode (Segel, 1975; Sachs, 1977). In the simultaneous model, both substrates must be bound simultaneously to the transporter for transport to occur. In the ping-pong model, one substrate is bound, transported, and released, and only then the other substrate is bound, transported, and released by the transporter. The reversibility of the exchanger and the observed competition between Na^+ and H^+ for binding to the transport site on both sides of the membrane might suggest that the Na^+/H^+ exchanger has a single transport site and therefore operates according to the ping-pong model. However, which mode of transport applies to the exchanger can be tested by measuring the effect of one substrate on the V_{max} and the K_m for the second substrate (Sachs, 1977). Only for simultaneous models will the K_m remain unaltered (Segel, 1975). We found that the apparent affinity for extracellular Na^+ and amiloride was independent of $[Na]_i$ and pH_i between 6.3 and 6.8 and the apparent affinity for intracellular H^+ was independent of Na_o^+ between 25 and 140 mM. Thus, the apparent affinities of the extracellular and intracellular sites were not

affected by the specific ionic composition or the transmembrane ionic gradients tested in the present studies (Table II; Figs. 6 and 9).

Finally, activation of the exchanger by agonists or osmotic shrinkage modifies the apparent affinity for H_i^+ (Moolenaar et al., 1983; Grinstein et al., 1985*a, b*; Green et al., 1988) and Na_i^+ (Green et al., 1988) without a concomitant change in the apparent affinities for extracellular H^+ and Na^+ (Grinstein et al., 1985*b*; Green et al., 1988). These findings argue in favor of the simultaneous model. It must be realized, however, that in the case of the Na^+/H^+ exchanger, the kinetic approach for distinguishing between the two modes of transport is limited because of the low K_m for H_i^+ . The effect of one substrate on the K_m of the second substrate needs to be measured at nonsaturating substrate concentrations. With a K_m for H_i^+ of ~ 6.6 nM, the effect of H_i^+ on the K_m for Na_o^+ and the K_i for amiloride should be measured at pH_i between 7.8 and 8.2. However, this is precluded because of the insensitivity of the dye to pH change at these high pH values and the inhibitory effect of high pH on Na^+/H^+ exchange. Therefore, the obtainable kinetic data are not sufficiently strong to distinguish between the two modes of transport. Thus, although some of the present and previous (Green et al., 1988) findings are compatible with the simultaneous model, the evidence to support this mode of transport by the Na^+/H^+ exchanger is limited and the ping-pong model was not experimentally ruled out.

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