Characterization of Na^+-H^+ antiporter activity associated with human cheek epithelial cells

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McMurchie, Edward J., Sharon L. Burnard, Glen S. Patten, Emma J. Lee, Roger A. King, and Richard J. Head. Characterization of Na^+-H^+ antiporter activity associated with human cheek epithelial cells. *Am. J. Physiol.* 267 (*Cell Physiol.* 36): C84–C93, 1994.—Na^+ transport activity was characterized in human cheek epithelial cells obtained from normotensive adult subjects. The cells were isolated using a mouth-wash procedure and assayed for Na^+ uptake using a radioactive ($^{22}Na^+$) rapid filtration assay. Cheek cells displayed proton-dependent Na^+ uptake activity that was dependent on the magnitude of the externally directed proton gradient measured using the fluorescent probe 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein to determine intracellular pH. Amiloride, ethylisopropylamiloride (EIPA), 5-(*N*,*N*-dimethyl)-amiloride, 5-(*N*-methyl-*N*-isobutyl)-amiloride (MIA), and 5-(*N*,*N*-hexamethylene)-amiloride (NNHA) all inhibited proton-dependent Na^+ uptake, with MIA, EIPA, and NNHA being the most potent. The Michaelis constant (K_m) for extracellular Na^+ was 5.7 mM, while the maximum velocity for Na^+-H^+ antiporter activity was 4.3 nmol $Na^+ \cdot$mg protein$^{-1} \cdot 30\ s^{-1}$. The K_m for intracellular H^+ was 0.17 μM, with a Hill coefficient of 0.7. Stimulation by ouabain and inhibition by bumetanide of cheek cell proton-dependent Na^+ uptake indicated only relatively low activities of Na^+-K^+-ATPase and Na^+-K^+-$2Cl^-$ cotransport, respectively. These results are consistent with the presence of Na^+-H^+ antiporter activity in cheek cells. Cheek cells therefore provide a convenient, relatively noninvasive source of tissue for examining Na^+-H^+ antiporter activity in human subjects.

amiloride; sodium-hydrogen antiporter; sodium transport; 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein

THE SODIUM-HYDROGEN antiporter (exchanger) associated with the plasma membrane has been extensively studied in many cell types in which it has been shown to play an important role in the regulation of intracellular pH and cell volume, in the influence of growth and proliferation of cells, and in Na^+ reabsorption in the kidney (6). In response to an externally directed proton gradient, the Na^+-H^+ antiporter exchanges intracellular H^+ for extracellular Na^+, and this activity is regulated by various hormones, growth factors, and other biologically active compounds. It has been suggested that the Na^+-H^+ antiporter may be a ubiquitous component of all eukaryotic cells (6). A recent review of the pharmacological, kinetic, and regulatory properties of the Na^+-H^+ antiporter in various systems has highlighted many of the properties of this transport system, its regulators, as well as the variety of cell types in which its activity has been examined (6).

Depending on the cell type and the plasma membrane location of the Na^+-H^+ antiporter (e.g., brush border, basolateral), this transport system shows differential sensitivity to amiloride and its congeners (6, 12). This, together with the multifunctional role of the Na^+-H^+ antiporter, has led to the suggestion that there may be more than one form of the Na^+-H^+ antiporter and that these forms may coexist in certain cells (6, 12). This is reinforced by the differential effects of protein kinases and other modulators on the activity of the Na^+-H^+ antiporter from various cell types and plasma membrane locations (21, 36) and by studies on the control and the cloning of Na^+-H^+ antiporter genes (35).

Disorders in cell membrane function and particularly alterations to various membrane-associated ion-transporting systems have been linked to both human essential hypertension and experimental animal models of hypertension (2, 7, 15). Altered activity of monovalent cation transport systems, most notably the Na^+-H^+ and the Na^+-Li^+ antiporter/exchanger systems, has been reported in human essential hypertension (4, 21). Alterations in the activity of the Na^+-H^+ antiporter in red blood cells, platelets, and various types of white blood cells in hypertension may reflect alterations in the activity of the Na^+-H^+ antiporter in renal proximal tubule epithelial cells and in vascular smooth muscle cells within resistance beds. Changes resulting from abnormal Na^+-H^+ antiporter activity, such as the rate of Na^+ retention in the kidneys, or the associated level of Ca^{2+}-dependent contractility in vascular smooth muscle cells, are important determinants of blood pressure and may be involved in the development and/or maintenance of the hypertensive state, thereby implicating a role for the Na^+-H^+ antiporter in human essential hypertension (2, 15).

Sampling of blood for determining cation transport activity in subjects at increased risk of developing hypertension is invasive as a screening procedure, particularly in the young. Furthermore, blood-borne cells may not reflect the status of systemic tissues with regard to disorders such as essential hypertension. As an alternative, we have utilized cheek cells, which can be obtained in a relatively noninvasive manner, as a source of material for biochemical analysis. In this study we show that human cheek epithelial (buccal mucosal) cells exhibit proton-dependent, amiloride-sensitive, Na^+ uptake consistent with the presence of Na^+-H^+ antiporter activity. We report some of the kinetic properties of the cheek cell-associated Na^+-H^+ antiporter.

EXPERIMENTAL PROCEDURES

Cheek cell isolation. Cheek cells were obtained from healthy adult volunteers on the morning of assay by washing the mouth. Subjects swirled distilled water around their mouths

for a short period of time in conjunction with a gentle molar scraping action. The expectorate containing cheek cells was collected several times. Samples were filtered through three layers of 250-μm nylon mesh and centrifuged at 3,250 *g* for 10 min at 20°C. The cheek cell pellet was gently resuspended (by vortexing and aspirating using a plastic Pasteur pipette) in loading buffer (defined below) to a final volume of ~15 ml. Cheek cells were also resuspended in distilled water for loading with 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-acetoxymethyl ester (AM), as described below. Na^+-H^+ antiporter activity was assayed on the day of cheek cell isolation.

Measurement of Na^+-H^+ antiporter activity. $^{22}Na^+$ uptake was measured after cheek cells were equilibrated in a proton-loading buffer (usually pH 5.5, osmotic strength 320 mosM) for 3 h at 20°C followed by a 10-fold dilution of the sample into a second buffer of higher pH to establish an externally directed transmembrane H^+ gradient, essentially as described by Seiler et al. (32). The magnitude of the externally directed proton gradient was determined from the measurement of intracellular pH (pH_i) using the pH-sensitive fluorescent probe BCECF. The second buffer (uptake buffer) contained $^{22}Na^+$, and uptake of isotope into cells was measured by a rapid filtration technique. The final pH of the various assay solutions was achieved by titrating a pH 5.0 buffer containing (in mM) 230 mannitol, 55 2-(*N*-morpholino)ethanesulfonic acid (MES), 5 *N*-methyl-D-glucamine (NMG), and 1 ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), with a pH 8.6 buffer containing (in mM) 230 mannitol, 33.6 glycylglycine, 26.4 NMG, and 1 EGTA. After equilibration, cheek cells were centrifuged at 3,250 *g* for 10 min at 20°C and resuspended in a small volume of buffer (pH as indicated), in the presence or absence of 1 mM ouabain and/or 1 mM amiloride (or other inhibitors as indicated), and preincubated for 15 min at 25°C. The timed, proton-dependent $^{22}Na^+$ uptake was then initiated at 25°C by a 10-fold dilution of extracellular pH (pH_o) 5.5 loaded cheek cells (15–30 μl) into uptake buffer (150–300 μl final volume) to give an externally directed transmembrane proton gradient as indicated. Standard conditions for determination of Na^+ uptake were as follows: cells initially equilibrated in pH_o 5.5 buffer routinely had a pH_i of 5.69 (see Fig. 3).

Equilibrated cells were transferred as a 10-fold dilution to pH_o 7.86 buffer to give a final pH_o of 7.5. This generated a nominal externally directed pH gradient of 100:1, but an actual pH gradient of 65:1. For experiments in which the proton gradient was varied, cheek cells in pH_o 5.5 buffer were added as a 1:10 dilution to buffers of varying pH to produce final pH_o values and proton gradients of pH 6.63 (9:1), pH 6.83 (14:1), pH 7.0 (21:1), pH 7.33 (44:1), and pH 5.5 (65:1). The non-proton-dependent $^{22}Na^+$ uptake condition (no externally directed pH gradient) was measured by adding cheek cells in pH_o 5.5 buffer into uptake buffer at pH_o 5.5 (or cheek cells in pH_o 7.8 into pH_o 7.8 uptake buffer). Uptake buffer for the standard proton gradient assay (65:1) contained a final concentration of (in mM) 230 mannitol, 20.4 NMG, 15.5 MES, 24.1 glycylglycine, 1 EGTA, and 1 Na^+ (gluconate), unless otherwise indicated, as well as $^{22}Na^+$ (Cl^-), with activity of 2×10^7 counts·min^{-1}·ml^{-1} (237 kBq/ml), 500–2,000 μg/ml cheek cell protein, plus or minus ouabain and other additions as indicated. The final osmotic strength of the uptake media was 288 mosM. The reaction was terminated by the rapid addition of 25-μl aliquots of the final reaction mixture to 4 ml of ice-cold wash buffer containing (in mM) 100 mannitol, 100 $MgCl_2$, 8 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 4 tris(hydroxymethyl)aminomethane (Tris), and 1 NaCl, pH 7.2 (final osmotic strength 270 mosM), followed by rapid filtration through 0.45-μm Millipore filters; these buffers were essentially those described by Seiler et al. (32). Triplicate 25-μl aliquots were sampled for all assays. Filters were washed twice with 4 ml of the above medium. Filters were dried, covered with 3 ml of Econofluor scintillant, and counted in a beta liquid-scintillation counter.

Measurement of pH_i. pH_i was measured using the pH-sensitive fluorescent probe BCECF. Fluorescence was measured using a Hitachi model 650–10S spectrofluorometer equipped with a magnetic stirrer, a thermostatically warmed cuvette holder, and 3-ml cuvettes. pH_i was determined by measuring fluorescence at excitation wavelengths of 490 nm (pH sensitive) and 439 nm (pH insensitive; isosbectic point), with slit width of 5 nm, and an emission wavelength of 530 nm, with slit width of 5 nm.

Calibration was performed using the method of James-Kracke (16), in which the ratio of the fluorescence intensities for BCECF was converted to pH values between the range of 4 and 9 using *Eq. 2* of James-Kracke (16), given below. For converting the fluorescence of BCECF in cheek cells to pH_i, the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added, and the cells were titrated to pH_i 4 with acid, then to pH 9 with base. The gradual change in fluorescence was observed as it asymptotically reached its limiting minimum (acid) and maximum (alkaline) values. This procedure was chosen for two reasons. *1)* Calibration methods based on the high-K^+ buffer, nigericin technique as described by Thomas et al. (33) are used widely for cell types in which intracellular K^+ would be expected to be ~140 mM. Because we have found that the intracellular K^+ concentration in isolated exfoliated cheek cells is well below this value (20a), the nigericin high-K^+ buffer calibration method may not be the most appropriate method for use with these cells. *2)* The standard conditions we have chosen for establishing the externally directed proton gradient of 100:1 (nominal value) for the Na^+-H^+ antiporter assay were to initially acidify cheek cells by suspension for at least 1 h in the mannitol, MES/glycylglycine, NMG, EGTA buffer adjusted to pH 5.5, as described in EXPERIMENTAL PROCEDURES. Because the pK_a of BCECF is 6.97 (30), the (nominal) pH_i value of 5.5 would have been considerably lower than the probe pK_a value and likely outside its linear response range using the nigericin high-K^+ buffer calibration method (33). Although pH_i has been successfully studied using BCECF in the nonlinear range from pH 5.0 to 6.0 (29), most calibrations have been done over the linear range of pH values from ~6.0 to 8.0. However, with the use of the method and the equations of James-Kracke (16), it is possible to accurately measure pH_i using BCECF down to at least pH 5.0, as indicated from the results of that study and in the studies cited therein.

A calibration curve of BCECF (free acid) was first constructed by measurement of the fluorescence ratio when BCECF (0.10 μM final concentration) was added to the mannitol, MES/glycylglycine, NMG, EGTA buffer adjusted to pH 7.2. Figure 1 shows a sigmoidal relationship for the fluorescence ratio of 490 to 439 nm as a function of the buffer pH measured with a microelectrode sensitive to 0.01 pH units, after the addition of acid or base. The buffer pH was first reduced by successive additions of small amounts of 1 M HCl to pH 4.0 (fluorescence minimum), then the pH was raised by successive additions of 1 M NaOH to pH 9.0 (fluorescence maximum). This fluorescence ratio is pH sensitive, but independent of dye concentration or photobleaching of the dye. For cells loaded with BCECF, the fluorescence ratio is also independent of cell density (10). The pK_a for BCECF in the above buffer was 6.94 ± 0.02 (n = 4 determinations). The correlation of pH measured by the pH electrode to the pH of BCECF was

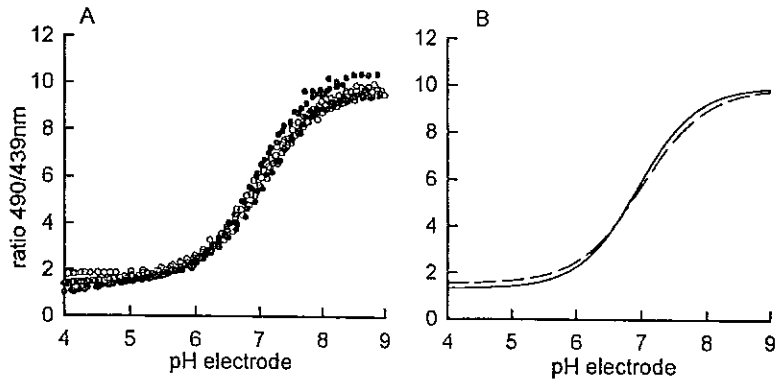

Fig. 1. pH dependence of 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) fluorescence in buffer and human cheek cells. Change in fluorescence ratio (R; 490/439 nm, excitation) for BCECF in mannitol buffer and in human cheek cells as a function of pH. *A*: ●, data points for value of R for BCECF (free acid) in mannitol buffer described in EXPERIMENTAL PROCEDURES. Points were derived from 4 separate determinations, each with 41 data points; pK_a = 6.94 ± 0.02. ○, Data points obtained for BCECF-loaded cheek cells in presence of 5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Points were derived from 4 separate determinations, each with 49 data points; pK_a = 7.00 ± 0.01. *B*: solid line, line of best fit for data shown in *A* for BCECF fluorescence in mannitol buffer; dotted line, line of best fit for data shown in *A* for BCECF fluorescence in human cheek cells.

calculated from *Eq. 2* of James-Kracke (16)

$$pH_i = pK_a - \log\left[\frac{(R_{max} - R)}{(R - R_{min})} \times \frac{F_{base\,439}}{F_{acid\,439}}\right]$$

where the pK_a for BCECF is 6.97 (30), R is the ratio of the fluorescence obtained when BCECF is excited at 490 and 439 nm when emission is measured at 530 nm, R_{max} is the ratio of these fluorescences when the fluorescence is at its maximum under alkaline conditions, R_{min} is the ratio of the fluorescence at pH 4.0, and $F_{base\,439}/F_{acid\,439}$ is the ratio of the fluorescences at 439 nm under the basic and acidic conditions used to obtain R_{max} and R_{min}, respectively. This correlation is shown in Fig. 2, where R_{max} is 9.92 ± 0.39, R_{min} is 1.06 ± 0.07, and $F_{base\,439}/F_{acid\,439}$ is 1.06 ± 0.03 (n = 4 determinations). The calibration of BCECF (free acid) was also tested in the presence of (non-BCECF loaded) human cheek cells. The calibration curve and the value of the parameters associated with it were not altered after subtraction of the background fluorescence obtained with cheek cells alone.

An in situ calibration curve for BCECF-loaded cheek cells was performed as follows. Human cheek cells (~10^6 cells/ml) suspended in distilled water were incubated with 10 μM (final concentration) BCECF-AM for 60 min at 25°C in the dark. This was sufficient incubation time to ensure adequate loading of cheek cells with the dye as determined from the time course for the increase in fluorescence due to deesterification of BCECF-AM during the loading procedure. We noted that BCECF-AM in the mannitol buffer exhibited a steady increase in fluorescence due to liberation of the free acid form of BCECF. [BCECF (free acid) in mannitol buffer gave a stable fluorescence reading over many hours.] After the loading step, cells were washed twice by centrifugation in distilled water to remove excess dye, then were resuspended in various buffers for immediate measurement of pH_i, as indicated in RESULTS. Thereafter, a calibration curve was done on each separate preparation of dye-loaded cheek cells. All fluorometric measurements were conducted from a stock of dye-loaded cells that were maintained in the dark in buffer, with each batch being washed by rapid centrifugation to remove any extracellular BCECF immediately before fluorescence measurements. Before fluorescence measurements for in situ pH calibration, FCCP at a final concentration of 5 μM was added to cells, and successive additions of acid to pH 4.0, followed by successive additions of base to pH 9.0, were carried out as described for the calibration curve of BCECF in buffer alone. The autofluorescence (including scatter) of the cells was subtracted before ratios were calculated. Leakage of dye under these conditions was ~7.5% over 60 min. Calibration curves took ~35 min to perform. The conversion of the fluorescence to pH units over the pH range from 4.0 to 9.0 was achieved using *Eq. 2* of James-Kracke (16) described above. Figure 1 shows the typical sigmoidal relationship for the fluorescence ratio of 490 to 439 nm as a function of the buffer pH after the addition of acid or base in BCECF-loaded cells in the presence of FCCP. The pK_a for BCECF-loaded cheek cells was 7.00 ± 0.01 (n = 4 determinations). Figure 2 shows a typical pH_i calibration curve for dye-loaded cells in the presence of FCCP. Values for the computational parameters for Fig. 2 are as follows: R_{max} equals 9.81 ± 0.07; R_{min} equals 1.56 ± 0.05; $F_{base\,439}/F_{acid\,439}$ equals 1.09 ± 0.03 (n = 4 determinations).

Protein. Protein was measured by the method of Lowry et al. (22) using bovine serum albumin as standard.

Measurement of osmotic strength of buffers. The osmolarity of various buffers was measured using a Knauer vapor pressure osmometer, which was calibrated with urea standards.

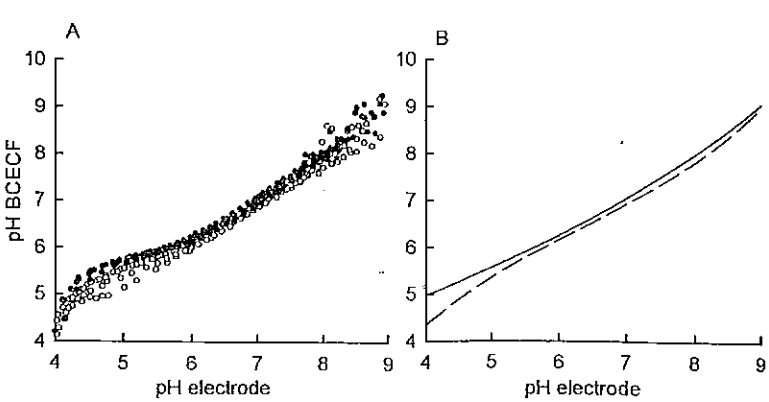

Fig. 2. pH calibration of BCECF fluorescence in buffer and human cheek cells. pH calibration procedure for BCECF involved conversion of fluorescence parameters minimum ratio (R_{min}), maximum ratio (R_{max}), and fluorescence ratio at 439 nm ($F_{base\,439}/F_{acid\,439}$) to pH units, using procedures and equation outlined in EXPERIMENTAL PROCEDURES. Data shown are derived from Fig. 1 for BCECF in mannitol buffer alone (●) or in BCECF-loaded cheek cells in presence of 5 μM FCCP (○). Individual data points are shown in *A*, while lines of best fit for BCECF in mannitol buffer (solid line) and for BCECF-loaded cheek cells in presence of 5 μM FCCP (dotted) are shown in *B*. Mean values for R_{min}, R_{max}, and $F_{base\,439}/F_{acid\,439}$ are given in EXPERIMENTAL PROCEDURES.

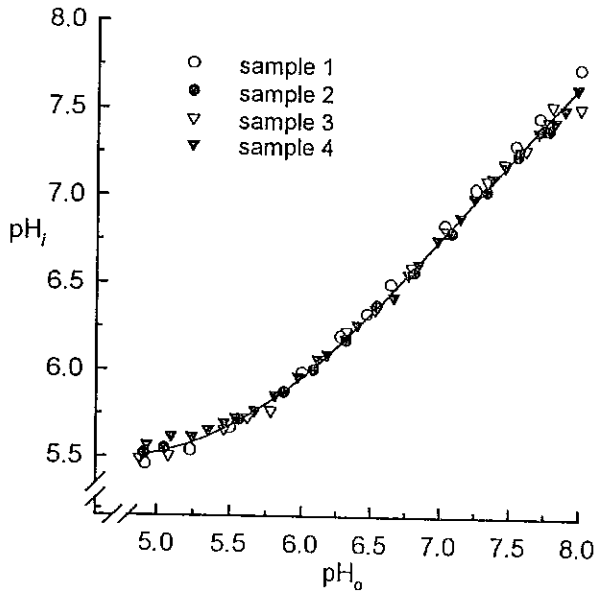

Fig. 3. Relationship between intracellular pH (pH_i) and extracellular pH (pH_o) of cheek cells. Human cheek cells were loaded with pH-sensitive fluorescent probe BCECF acetoxymethyl ester, and pH_i values were determined using fluorescence parameters and equation described in EXPERIMENTAL PROCEDURES. After measurements of fluorescence at different pH_o values, calibration of BCECF in cheek cells was performed using the protonophore FCCP according to method of James-Kracke (16), with representative curves being shown in Figs. 1 and 2. Shown are data from 4 separate cheek cell preparations in which pH_i was measured using BCECF after various adjustments to mannitol buffer pH value (pH_o) were made as described in EXPERIMENTAL PROCEDURES.

Materials. Carrier-free $^{22}NaCl$ and Econofluor scintillant were obtained from New England Nuclear. The amiloride derivatives ethylisopropylamiloride and 5-(*N*,*N*-hexamethylene)-amiloride were generous gifts of Merck Sharp & Dohme (Australia). Other amiloride derivatives, 5-(*N*,*N*-dimethyl)-amiloride and 5-(*N*-methyl-*N*-methylisobutyl)-amiloride, were purchased from Research Biochemicals. Amiloride and buffering agents were purchased from Sigma or were of the highest commercial quality available. BCECF and BCECF-AM were from Molecular Probes (Eugene, OR).

Statistical analysis. For all individual experiments, measurements were made in triplicate, and results were represented as means ± SE.

RESULTS

Cheek cells isolated by the methods described had little or no bacterial contamination (as determined by microscopic examination) and did not swell or lyse in distilled water (20a). The yield of cheek cells was 8.9 ± 1.3 × 10^6 (SE) cells (n = 18 adult subjects). Cheek cells maintained a value of between 72 and 96% for trypan blue exclusion from cells for all subjects examined. Their histological appearance in distilled water was identical to that in sucrose- or mannitol-containing buffers. From the methods described in the companion paper (20a), measurement of cellular Na^+ and K^+ concentrations on a per cell basis, together with measurement of cell volume, allowed an estimate of the approximate range of cellular Na^+ and K^+ concentrations at the time cells were examined for Na^+-H^+ antiporter activity. These were 0.3–0.5 mM for Na^+ and 7–13 mM for K^+.

The relationship between pH_o and pH_i for human cheek cells measured using BCECF is shown in Fig. 3. This relationship was independent of the manner in which the pH of the mannitol, MES, NMG/glycylglycine buffer was adjusted, i.e., the results were identical if acid or alkali was used to alter buffer pH or if the buffer was adjusted by titrating the pH 5.0 with the pH 8.6 mannitol buffer to the required pH, as detailed in EXPERIMENTAL PROCEDURES. The results in Fig. 3 were obtained within ~35 min after termination of the dye-loading step to minimize leakage of the deesterified fluorescent dye. At a pH_o of 5.5, we routinely obtained a pH_i for cheek cells of 5.69, indicating that significant intracellular acidification was achieved by the acid-loading step. Under the conditions employed in this study, this would give an externally directed proton gradient value of at least 65:1. To determine the effect of intracellular acidification on the activation of Na^+ uptake (as shown in Fig. 8), cheek cells were suspended in buffers of varying pH between 5.5 and 7.8, and respective pH_i values were determined using BCECF fluorescence.

Na^+ uptake in cheek cells. Uptake of $^{22}Na^+$ into cheek cells was stimulated by an externally directed proton gradient that contrasted with the lower Na^+ uptake observed in the absence of an externally directed proton gradient (Fig. 4). Na^+ uptake was rapid at the early time points, plateaued after 5 min, and was inhibited by 1 mM amiloride. After 30 min, the total (proton-dependent) Na^+ uptake approximated that observed for the control (non-proton-dependent) Na^+ uptake. Proton-dependent amiloride-sensitive Na^+ uptake was indistinguishable when cells were isolated from the mouth using a 250 mM sucrose buffer or distilled water. The

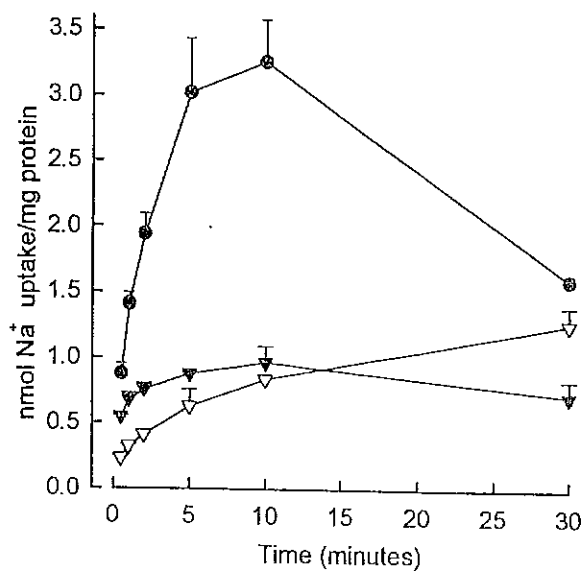

Fig. 4. Na^+ uptake in cheek cells. Time course for proton-dependent $^{22}Na^+$ uptake was determined at a final proton gradient value (inside: outside) of 65:1 (pH_i 5.69 → pH_o 7.8; final pH_o 7.5) in absence (●) or presence (▼) of 1 mM amiloride. Control non-proton-dependent $^{22}Na^+$ uptake was determined in absence of an externally directed proton gradient (pH_o 5.5 → pH_o 5.5) (▽). All experiments were performed with 1 mM ouabain and 1 mM Na^+ ($^{22}Na^+$). Results shown are means ± SE for 3 adult subjects.

inclusion of 10 mM glucose or 1 mM ATP did not affect the Na^+ uptake profile (data not shown).

The initial rate of proton-dependent Na^+ uptake into cheek cells was determined at 10-s intervals over the first 2 min of the time course (Fig. 5). The initial rate was linear up to 30 s, after which time the rate of Na^+ uptake decreased. Therefore, the 30-s time point was used thereafter for the purposes of determining kinetic parameters associated with the initial rate of the transport reaction, while the 5-min time point was used for determining parameters associated with the maximum amount of Na^+ taken up by cheek cells.

Data for Na^+ uptake activity in cheek cells obtained from 18 normotensive adult subjects are shown in Table 1 for the 30-s and 5-min time points. Compared with the total (proton-dependent) uptake, 1 mM ouabain, an inhibitor of Na^+-K^+-ATPase activity, stimulated Na^+ uptake by ~6% at both time points. Due to this consistent stimulation by ouabain on the rate of Na^+ uptake, which was presumably due to inhibition of efflux of (transported) Na^+ from the cheek cells via the Na^+-K^+-ATPase, ouabain was therefore routinely included in all assays. The effect of bumetanide, an inhibitor of Na^+-K^+-$2Cl^-$ cotransport activity, when tested in combination with ouabain and compared with the rate of Na^+ uptake measured in the presence of ouabain, resulted in an inhibition of 7.6 and 8.9% at the 30-s and 5-min time points, respectively. However, in the absence of ouabain, there was a 12% stimulation at the 30-s time point and a 3% inhibition at the 5-min time point. This would indicate that if present in cheek cells, the Na^+-K^+-$2Cl^-$ cotransport system would only play a minor role in the uptake of Na^+. Amiloride, an inhibitor of Na^+-H^+ antiporter activity, inhibited proton-dependent Na^+ uptake in cheek cells by ~36 and 53% at the 30-s and 5-min time points, respectively, resulting in amiloride-sensitive rates of Na^+ uptake of 0.34 and 1.01 nmol Na^+/mg protein at these two time points. Non-proton-dependent

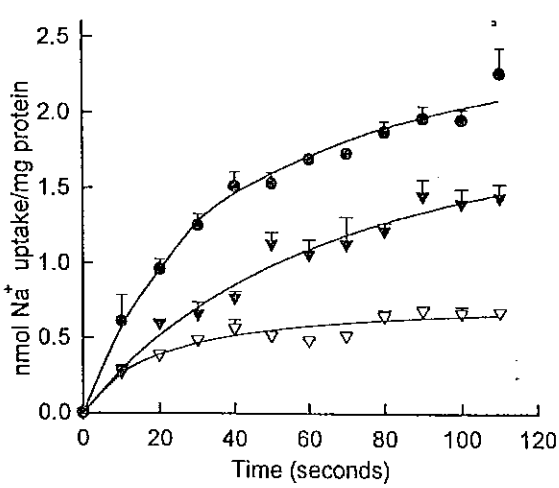

Fig. 5. Initial time course of Na^+ uptake in cheek cells. A sample was taken from assay every 10 s for initial 2 min. Proton-dependent $^{22}Na^+$ uptake (pH_i 5.69 → pH_o 7.8; final pH_o 7.5) was determined at a proton gradient value of 65:1 (inside:outside). ●, Total; ▼, in presence of 1 mM amiloride; ▽, in absence of an externally directed proton gradient. Results shown are means ± SE for 3 adult subjects.

Table 1. *Na^+ uptake in human cheek cells*

Condition	Na^+ Uptake, nmol Na^+/mg protein	
	30 s	5 min
Total (65:1 proton gradient)	1.06 ± 0.02	1.80 ± 0.29
Total + ouabain	1.12 ± 0.14	1.91 ± 0.22
Total + bumetanide	1.19 ± 0.12	1.74 ± 0.28
Total + bumetanide + ouabain	1.03 ± 0.12	1.74 ± 0.25
Total + ouabain + amiloride	0.72 ± 0.07	0.90 ± 0.12
Amiloride sensitive [(total + ouabain) − (total + ouabain + amiloride)]	0.34 ± 0.06	1.01 ± 0.13
Control (pH_o 5.5) + ouabain	0.38 ± 0.05	0.49 ± 0.05
Control (pH_o 7.86) + ouabain	0.51 ± 0.07	0.67 ± 0.08
Net [total + ouabain − control (pH_o 5.5) + ouabain]	0.74 ± 0.11	1.42 ± 0.21
Net [total + ouabain − control (pH_o 7.86) + ouabain]	0.61 ± 0.12	1.24 ± 0.24

Values are means ± SE of Na^+ uptake at 1 mM Na^+ at 30 s and 5 min and 25°C. Results are shown for 18 adult subjects, each assayed on 2 occasions and averaged. Total activity was determined at a 65:1 (inside:outside) proton gradient value [extracellular pH (pH_o) 5.5; intracellular pH (pH_i), 5.69 → pH_o 7.86; final pH_o 7.5] as described in EXPERIMENTAL PROCEDURES. Control is non-proton-dependent activity, i.e., in absence of an externally directed proton gradient (pH_o 5.5 → pH_o 5.5, or pH_o 7.86 → pH_o 7.86). Net proton-dependent activity is total activity minus the activity in the absence of a proton gradient (at pH_o 5.5 or pH_o 7.86). Amiloride-sensitive Na^+ uptake is total activity minus activity in presence of amiloride. When added, as indicated, ouabain and amiloride were 1 mM and bumetanide was 0.1 mM.

Na^+ uptake into cheek cells, indicated by the rate of Na^+ uptake in the absence of an outwardly directed proton gradient (the control rate), was determined at pH_o values of 5.5 and 7.86. For the control condition in which there was an absence of an externally directed proton gradient, the reduction in Na^+ uptake was greatest with the pH_o 5.5 control condition. For both pH controls, the reduction in Na^+ uptake was greater at the 5-min time point in comparison with the 30-s time point. As a result, the net rate of uptake (total minus control condition) was slightly higher for the first condition, i.e., with the pH_o 5.5 control. [The pH_o 5.5 control condition (pH_o 5.5 = pH_i 5.69 → pH_o 5.5) would actually result in a small internally directed proton gradient of 1.5:1. The pH_o 7.86 control condition (pH_o 7.86 = pH_i 7.5 → pH_o 7.86) would result in a small externally directed proton gradient of 2.3:1. These differences may account for the lower value observed for the pH_o 5.5 control condition (higher net Na^+ uptake) compared with the pH_o 7.86 control condition.]

Effect of proton gradient on Na^+ uptake in cheek cells. The rate of Na^+ uptake into cheek cells was dependent on the magnitude of the externally directed proton gradient at both the 30-s and 5-min time points (Fig. 6). Rates of Na^+ uptake were determined for cheek cells loaded in pH_o 5.5 buffer and incubated in uptake media containing 1 mM $^{22}Na^+$ (gluconate) at a final pH_o (as described in EXPERIMENTAL PROCEDURES) to produce actual proton gradient values of 9, 14, 21, 44, and 65:1 (inside:outside). For both assay times, there was an apparent biphasic response to the increasing proton gradient with a rapid increase in cheek cell Na^+ uptake

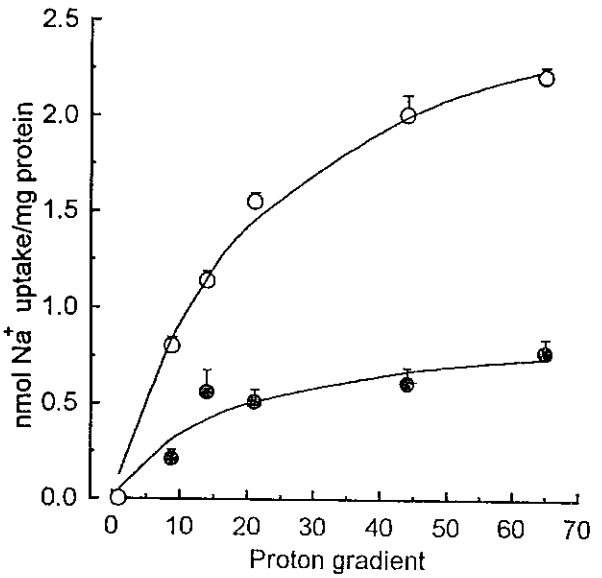

Fig. 6. Effect of altered proton gradient value on cheek cell Na^+ uptake. Values for proton-dependent uptake of $^{22}Na^+$ into cheek cells of 3 adult subjects are shown as means ± SE for proton gradient values (inside:outside) of 9:1, 14:1, 21:1, 44:1, and 65:1 after 30 s (●) and 5 min (○). Net uptake is proton-dependent $^{22}Na^+$ uptake minus non-proton-dependent value (pH_o 5.5 → pH_o 5.5).

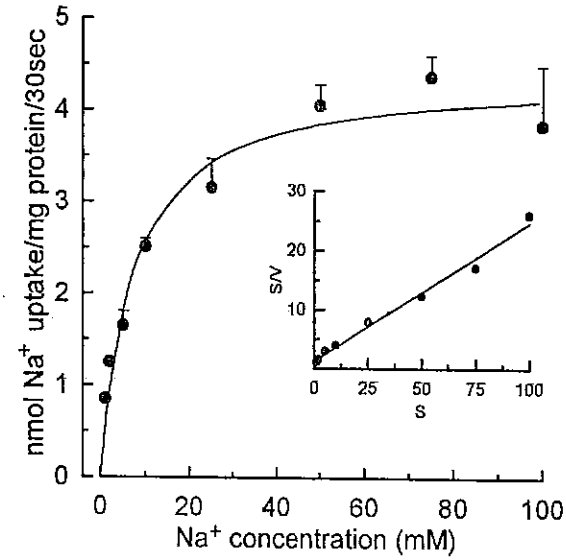

Fig. 7. Effect of extracellular Na^+ concentration ($[Na^+]_o$) on proton-dependent Na^+ uptake in cheek cells. Proton-dependent $^{22}Na^+$ uptake [pH_i 5.69 → pH_o 7.8; final pH_o 7.5; 65:1 (inside:outside) proton gradient] minus non-proton-dependent value (pH_o 5.5 → pH_o 5.5) was determined for $[Na^+]_o$ values of 1–100 mM in presence of 1 mM ouabain after 30 s. Osmolarity of medium was maintained at 288 mosM by adjusting mannitol concentration as appropriate. *Inset*: Hanes plot of S/V vs. S ($r^2 = 0.99$) [where S is $[Na^+]_o$ in mM and V is rate of Na^+ uptake in nmol Na^+ (uptake)·mg protein^{-1}· 30 s^{-1}] gave a Michaelis constant (K_m) for $[Na^+]$ of 5.7 mM and a maximum velocity value (V_{max}) of 4.3 nmol Na^+·mg protein^{-1}·30 s^{-1}. Data are means ± SE of 3 separate experiments.

to proton gradient values up to ~21:1. Thereafter, the magnitude of the response decreased. Acid loading of cheek cells in pH_o 5.5 buffer was routinely performed over a period of 3 h as described in EXPERIMENTAL PROCEDURES. Acid loading of cells for periods of between 1 and 3 h made no difference in Na^+ uptake (data not shown). The effect on proton-dependent Na^+ uptake of acid loading for a period of < 1 h was not tested.

Effect of storage and temperature on Na^+ uptake. Cheek cell proton-dependent Na^+ uptake was routinely assayed in freshly isolated cheek cells. Storage of cheek cells at 4°C for 24 h reduced activity by ~13% at the 5-min assay time point. Storage of cheek cells at −80°C for 5 wk resulted in a 44% reduction in activity at the same assay time point (data not shown).

Effect of Na^+ concentration on Na^+ uptake. Proton-dependent Na^+ uptake in cheek cells was routinely assayed at a Na^+ concentration of 1 mM due to limitations imposed by isotopic dilution of $^{22}Na^+$ by higher concentrations of nonradioactive Na^+. To determine the Michaelis constant (K_m) and maximum velocity (V_{max}) for cheek cell-associated Na^+-H^+ antiporter activity, proton-dependent Na^+ uptake was determined over a range of Na^+ concentrations up to 100 mM (Fig. 7). Due to isotopic dilution, 30 s was the earliest time point that could be measured with accuracy when examining Na^+ dependence of cheek cell Na^+ uptake over the concentration range. The 30-s time point was in the linear phase for Na^+ uptake (Fig. 5). Analysis of the data using a Hanes plot yielded a K_m value of 5.7 mM and a V_{max} of 4.3 nmol Na^+·mg protein^{-1}·30 s^{-1}.

Effect of cellular acidification on Na^+ uptake. The activation of cheek cell Na^+ uptake by cellular acidification is shown in Fig. 8. Cheek cells were preloaded with buffers of pH_o value ranging from 5.5 to 7.8 to give the

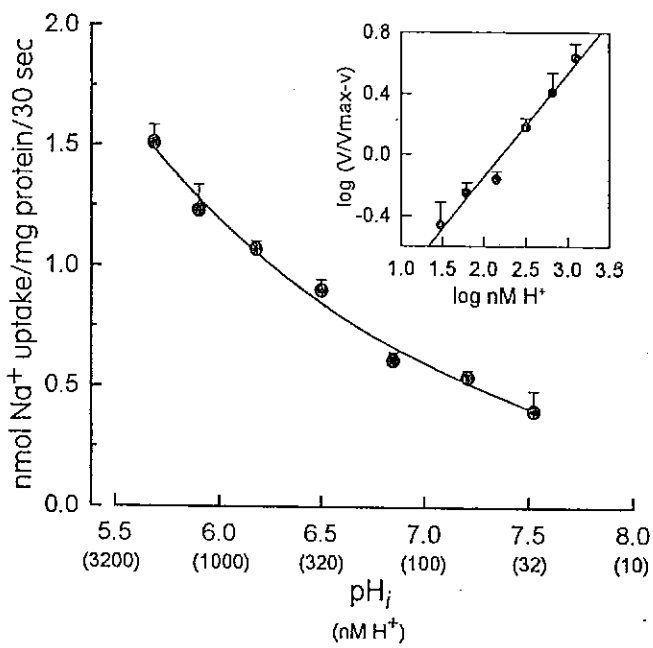

Fig. 8. Effect of internal proton concentration (pH_i) on cheek cell Na^+ uptake. Cheek cells were incubated for 3 h in buffers of varying pH_i (between pH_i 5.69 and 7.5), as determined using BCECF fluorescent probe method, and $^{22}Na^+$ uptake was measured after 30 s following transfer of cells into uptake medium of pH_o 7.8. Data are shown as net rate at each pH_i value (total proton-dependent Na^+ uptake minus non-proton-dependent Na^+ uptake; pH_o 5.5 → pH_o 5.5). *Inset*: Hill plot for effect of internal proton concentration on $^{22}Na^+$ uptake. K_m for $[H^+]$ was 0.17 μM, and Hill coefficient (n_{app}) was 0.7 as calculated according to Ref. 5. Data are means ± SE for 3 separate experiments.

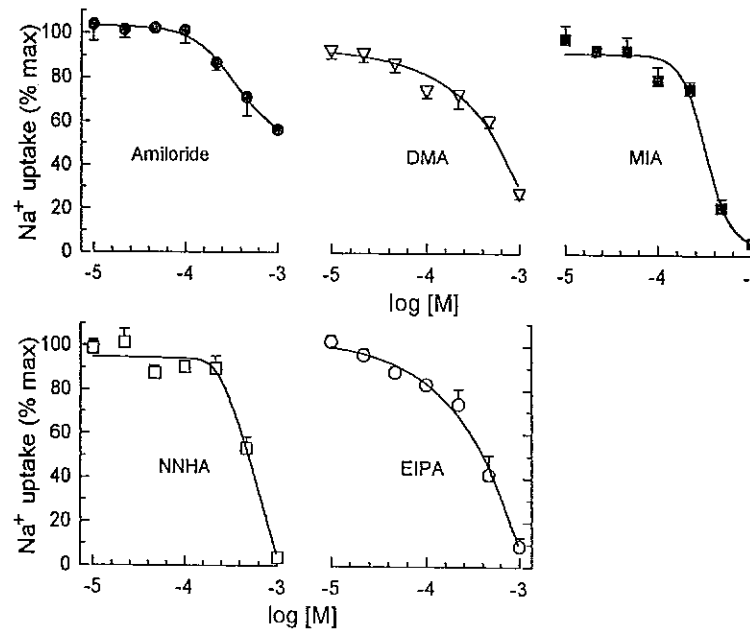

Fig. 9. Effect of amiloride and amiloride congeners on proton-dependent Na^+ uptake in cheek cells. Data are shown as net proton-dependent $^{22}Na^+$ uptake (total: pH_i 5.69 → pH_o 7.8, final pH_o 7.5; minus control: pH_o 5.5 → pH_o 5.5) for each inhibitor. Data are shown as means ± SE for 3 separate experiments and are expressed as a percentage of net rate of proton-dependent Na^+ uptake in absence of an inhibitor. DMA, 5-(*N*,*N*-dimethyl)-amiloride; MIA, 5-(*N*-methyl-*N*-isobutyl)-amiloride; NHHA, 5-(*N*,*N*-hexamethylene)-amiloride; EIPA, ethylisopropylamiloride.

range of pH_i values indicated in Fig. 8. Proton-dependent uptake of $^{22}Na^+$ was then determined after transfer to pH_o 7.8 uptake medium containing 1 mM Na^+ ($^{22}Na^+$). An increase in the rate of Na^+ uptake was apparent with decreasing pH_i values. The dependence of the Na^+-H^+ antiporter on cellular [H^+] and the value of n_{app}, the number of internal H^+-binding sites, was analyzed using the Hill equation as outlined by Canessa et al. (5). A plot of log V/V_{max}-V vs. log pH_i for V_{max} estimated from the maximum rate of Na^+ uptake from the pH_i activation curve at an external Na^+ concentration of 1 mM, with V being the rate of Na^+ uptake at the various internal pH values, is shown in Fig. 8, *inset*. Application of the Hill equation (5) yielded a Hill coefficient of 0.7 and a K_m for H^+ of 0.17 μM for cheek cells.

Effect of inhibitors on Na^+ uptake in cheek cells. Amiloride and amiloride congeners have been shown to inhibit Na^+-H^+ antiporter/exchanger activity in a variety of tissues, although some tissues are reported to be insensitive to amiloride, and this may reflect the presence of different subtypes of the Na^+-H^+ antiporter (6). Cheek cell proton-dependent Na^+ uptake was sensitive to amiloride (Figs. 4 and 5 and Table 1). Dose-response curves for amiloride and several amiloride congeners are shown in Fig. 9 for cheek cell proton-dependent Na^+ uptake measured at the 30-s time point. The rank order for inhibition along with 50% inhibitory dosage (ID_{50}) values were as follows: 5-(*N*-methyl-*N*-isobutyl)-amiloride (MIA), 0.31 mM; ethylisopropylamiloride (EIPA), 0.40 mM; 5-(*N*,*N*-hexamethylene)-amiloride (NNHA), 0.5 mM; 5-(*N*,*N*-dimethyl)-amiloride (DMA), 0.64 mM, and amiloride, >1 mM. At the highest concentrations tested, both amiloride and DMA did not result in 100% inhibition of proton-dependent Na^+ uptake.

Effect of cations on Na^+ uptake in cheek cells. The effect of cations on cheek cell proton-dependent Na^+ uptake is shown in Fig. 10. The control value was taken as the amount of radioactivity ($^{22}Na^+$) associated with cheek cells when proton-dependent Na^+ uptake was terminated after 30 s in the presence of 0.01 mM Na^+ ($^{22}Na^+$) gluconate, as described in EXPERIMENTAL PROCEDURES. Increasing the external Na^+ ($^{22}Na^+$) gluconate concentration to 1 mM reduced proton-dependent $^{22}Na^+$ uptake by 61% compared with the control (Fig. 10).

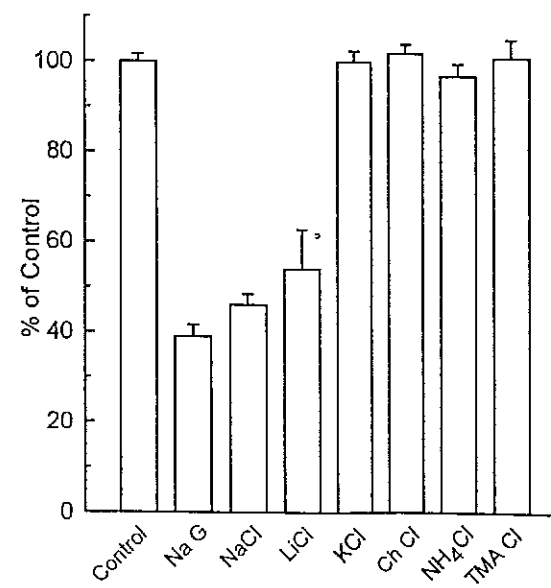

Fig. 10. Effect of extracellular cations on proton-dependent Na^+ uptake in human cheek cells. Net Na^+ uptake was measured at a proton gradient value of 65:1 (inside:outside) (pH_i 5.69 → pH_o 7.8; final pH_o 7.5) minus non-proton gradient control (pH_o 5.5 → pH_o 5.5) in presence of indicated salts at 1 mM final concentration after 30 s. Proton-dependent Na^+ uptake in presence of various cations was normalized to control value that was net $^{22}Na^+$ uptake in presence of 0.01 mM Na^+ (gluconate), which was assigned a value of 100%. Data shown are means ± SE for 3 separate experiments. Na G, sodium gluconate; Ch Cl, choline chloride; TMA Cl, tetramethylammonium chloride.

Addition of 1 mM NaCl to the uptake medium reduced uptake by 53%, indicating that the form of the anion was not a major factor influencing Na^+ uptake. Addition of 1 mM LiCl to the uptake medium reduced uptake by 46% compared with control, indicating that Li^+ was competing with, or inhibiting, the proton-dependent uptake of Na^+ into cheek cells. KCl, choline chloride, NH_4Cl, and tetramethylammonium chloride did not compete with or inhibit proton-dependent Na^+ uptake (Fig. 10).

A preliminary examination of the subcellular distribution of proton-dependent Na^+ uptake was made after disruption of cheek cells by passage through a French pressure cell (FPC) followed by differential centrifugation to isolate membrane fractions. Cheek cells could not be disrupted by procedures such as osmotic stress or mechanical homogenization. FPC treatment resulted in the complete disruption of all cheek cells, which was verified by morphological examination using phase-contrast microscopy. Of the relatively small amount of proton-dependent Na^+ uptake that was recovered following FPC treatment, most activity was confined to the fraction sedimenting between 600 and 5,000 *g* in which a 1.9-fold enrichment but only a 4% recovery over that initially present in whole cells was evident for proton-dependent Na^+ uptake. This fraction also contained the greatest enrichment of 5′-nucleotidase activity (~5.7-fold over that initially present in whole cells). However, for this plasma membrane marker, ~57% of the initial activity was recovered (data not shown).

DISCUSSION

With the use of a radiometric, rapid filtration assay, this study showed isolated human cheek epithelial cells exhibit proton-dependent amiloride-sensitive Na^+ uptake activity, which is consistent with the presence of Na^+-H^+ antiporter activity in these cells. The assay conditions, which involved an initial acid-loading step to establish an externally directed proton gradient, were defined by measurement of the cheek cell pH_i using the pH-sensitive fluorescent probe BCECF. This study also indicated that the Na^+-K^+-ATPase and the Na^+-K^+-$2Cl^-$ cotransport systems, while present in cheek cells, did not significantly contribute to the overall transmembrane movement of Na^+ in these cells. The extent of passive permeability of cheek cells to Na^+ was well below the Na^+ uptake achieved in the presence of an externally directed proton gradient at the early time points. (It is significant to note that the intracellular concentration of Na^+ in isolated cheek cells is very low and in the order of 0.3–0.5 mM.) At later time points in the assay, proton-dependent Na^+ uptake declined and often approached a level achievable by passive influx processes alone. This decline has been observed for Na^+-H^+ antiporter activity in a number of other cell types and membrane systems (9). All of the above give strong support to the existence of Na^+-H^+ antiporter activity in cheek cells.

Proton-dependent Na^+ uptake in cheek cells exhibited saturation kinetics with respect to Na^+ with a K_m of 5.7 mM. This compares with reported K_m values for Na^+ of between ~5 and 30 mM in brush-border membrane vesicles (BBMVs) from kidney and intestine (18, 31, 34) and ~5–18 mM in basolateral membrane vesicle (BLMV) preparations from intestine, parotid, and liver (3, 23, 26). In an extensive review of Na^+-H^+ antiporter properties in over 30 different systems, including cultured cell lines (6), K_m values for Na^+ were reported to range from 5 to 63 mM. It should be noted that cheek epithelial cells before isolation are bathed in saliva, which has a Na^+ concentration considerably lower than that of plasma, which constitutes the extracellular medium of many of the above-mentioned cell types.

In many cell types, amiloride-sensitive Na^+-H^+ antiporter activity has been shown to be activated by reduction of pH_i, thereby implicating an important role for the Na^+-H^+ antiporter in pH_i homeostasis (11, 26). Regulation of Na^+-H^+ antiporter activity by pH_i can occur by positive cooperativity due to H^+ binding at an intracellular regulatory/binding site (1, 6). The nature of the relationship between Na^+-H^+ antiporter activity and pH_i with regard to the steepness of the activation profile or the presence of a sigmoidal relationship between these parameters can indicate positive cooperativity (5). Although activated by decreasing pH_i values, cheek cell Na^+ uptake did not appear to exhibit positive cooperativity based on the Hill coefficient (n_{app}) value of 0.7 with an apparent K_m for intracellular H^+ concentration ($[H^+]_i$) of 0.17 μM when assayed at an extracellular Na^+ concentration ($[Na^+]_o$) of 1 mM. The lack of sigmoidal effects of $[H^+]_i$ on Na^+-H^+ antiporter activity reported for rabbit ileum villus cell BBMV and BLMV has been cited as evidence against the presence of an internal modifier site in the above preparations (20). In the above-cited preparations, the apparent K_m for $[H^+]_i$ was 0.47 μM (BLMV) and 0.23 μM (BBMV). Evidence of positive cooperativity of Na^+-H^+ antiporter activity by internal protons was also not observed in rat colonic apical membrane vesicles, which displayed an apparent K_m for $[H^+]_i$ of 2.8 μM measured in the presence of 0.1 mM $[Na^+]_o$ (28). In addition to the observations of positive cooperativity of Na^+-H^+ antiporter activity associated with renal proximal tubules (1), the human erythrocyte Na^+-H^+ antiporter has been reported to exhibit positive cooperativity with a Hill coefficient varying between 1.7 and 2.6 (assayed in the presence of 150 mM $[Na^+]_o$); the Hill coefficient was reduced in subjects with essential hypertension (5). Finally, the Hill coefficient has been shown to exhibit quite complex behavior for the Na^+-H^+ antiporter activity associated with the HL-60 cell line, where its value appears dependent on the pH_i value and varies between 0.5 and 1.5 (29). For the HL-60 system, the apparent K_m for $[H^+]_i$ was 1.35 μM when measured in the presence of 129 mM $[Na^+]_o$. In addition to probable intrinsic differences in the Hill coefficient for the Na^+-H^+ antiporter in different systems, differences in the assay conditions employed make direct comparisons of these particular kinetic parameters associated with the cheek cell Na^+-H^+ antiporter with those of other cell types and membrane preparations difficult.

The Na^+-H^+ antiporter associated with cheek cells exhibited sensitivity to amiloride, although greater inhi-

bition and considerably lower ID_{50} values were seen with MIA, EIPA, and NNHA. Each of these latter compounds was able to reduce proton-dependent Na^+ uptake to the level observed for cheek cells assayed in the absence of an externally directed proton gradient. The most potent inhibition was MIA, with an ID_{50} of ~313 μM. This compares with reported values for amiloride inhibition of 425 μM for the rat colonic epithelial Na^+-H^+ antiporter (8) and 230 μM for the rabbit ileal epithelial Na^+-H^+ antiporter (19). However, other epithelial cell preparations do exhibit comparatively greater sensitivity to amiloride and its congeners (in the order of 20–50 μM amiloride) (6) than is the case for cheek cells and the examples cited above. It is significant that the cheek cell Na^+-H^+ antiporter exhibits considerable sensitivity to the amiloride congener EIPA. In other tissues, EIPA has been shown to be a potent and specific inhibitor of electroneutral Na^+-H^+ antiporter activity (13).

In this study we have surveyed a number of properties related to the uptake of Na^+ in cheek cells. In addition to the kinetic parameters of Na^+ uptake, which strongly implicate the presence of a proton-dependent amiloride-sensitive Na^+-H^+ antiporter in cheek cells, a number of other properties of this system should be mentioned. Proton-dependent Na^+ uptake in cheek cells was minimally affected by transmembrane potential as revealed by K^+ and its ionophore valinomycin, suggesting that the Na^+ transport system under investigation was operating in an electroneutral manner rather than by electrodiffusional coupling (data not shown). Monensin (Na^+-H^+ ionophore) and nigericin (K^+-H^+ ionophore), which facilitate Na^+-H^+ exchange across the cell membrane, markedly stimulated proton-dependent Na^+ uptake into cheek cells particularly early in the time course (data not shown). A similar effect of these monovalent cation ionophores has been observed for Na^+ uptake catalyzed by the Na^+-H^+ antiporter associated with rat jejunum BLMVs (27). Finally, disruption of cheek cells by the FPC technique and subsequent isolation of membrane fractions by differential centrifugation revealed that Na^+ uptake cosedimented with 5′-nucleotidase activity, implicating a plasma membrane location for the Na^+-H^+ antiporter in cheek cells (data not shown), as has been reported for the Na^+-H^+ antiporter in most other cell types (6).

The results with cheek cells with respect to the effects of other cations indicate that Li^+, but not K^+, choline, NH_4^+, or tetramethylammonium, can effectively compete with Na^+ in the exchange process, which is in accordance with other studies showing that Li^+ has affinity for the Na^+-H^+ antiporter (14). These results suggest that cheek cells may also possess Na^+-Li^+ antiporter (exchange) activity. Considerable debate exists over the role of the Na^+-Li^+ exchanger and its uniqueness from the Na^+-H^+ antiporter, with similarities and differences being noted between Na^+-H^+ and Na^+-Li^+ antiporter/exchangers depending on the cell and membrane system under investigation (17).

Cheek cells offer a convenient, relatively noninvasive method of obtaining tissue for biochemical analysis. With the use of a simple mouth wash technique, an average of 9 million cells can easily be collected from any one subject. Morning collections before the subject had breakfast resulted in the greatest yield of cheek cells. The technique provides sufficient cells to allow a range of parameters related to Na^+-H^+ antiporter activity to be assayed. The $^{22}Na^+$ rapid filtration assay used to determine transport activity also allows activity profiles from a number of subjects, including children, to be measured each day. Furthermore, because Na^+ transport is linear over a wide range of cheek cell protein concentrations, from 5 to 50 μg protein/25-μl filter sample (data not shown), direct comparisons can be made where the yield of cheek cells from subject groups may be variable. The activity of monovalent cation transport systems, particularly Na^+-H^+ and Na^+-Li^+ antiporters present in cells from blood, has been reported to be altered in essential hypertension (2, 4, 15, 21). Owing to the relatively noninvasive technique used to isolate cheek cells, a new opportunity therefore exists to examine both the relationship of monovalent cation transport systems to essential hypertension and their use as predictive markers for hypertension in a wide range of community groups. In this regard we have recently observed that cheek cell proton-dependent Na^+ uptake is significantly reduced in untreated essential hypertensive subjects when compared with normotensive control subjects (24). Furthermore, we have also shown that cheek cell proton-dependent Na^+ uptake is reduced in adolescents who for reasons of both a high blood pressure tracking characteristic and a positive family history of hypertension would be considered at increased risk of later development of hypertension (25).

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