J. Membrane Biol. 93, 33–42 (1986)

The Journal of
Membrane Biology

Regulation of Intracellular pH in LLC-PK$_1$ Cells by Na^+/H^+ Exchange

Marshall H. Montrose and Heini Murer
Institute of Physiology, University of Zurich, 8057 Zurich, Switzerland

Summary. Suspensions of LLC-PK$_1$ cells (a continuous epitheliod cell line with renal characteristics) are examined for mechanisms of intracellular pH regulation using the fluorescent probe BCECF. Initial experiments determine suitable calibration procedures for use of the BCECF fluorescent signal. They also determine that the cell suspension contains cells which (after 4 hr in suspension) have Na^+ and K^+ gradients comparable to those of cells in monolayer culture. The steady-state intracellular pH (7.05 ± 0.01, $n = 5$) of cells which have recovered in (pH 7.4) Na^+-containing medium is not affected over several minutes by addition of 100 μM amiloride or removal of extracellular Na^+ ($Na_o^+ < 1$ mM). In contrast, when the cells recover from an acid load (caused by NH_4 preincubation and removal), the recovery is largely Na^+ dependent and is sensitive to 100 μM amiloride. These results suggest that with resting pH near neutrality, both Na_o^+/H_i^+ and Na_i^+/H_o^+ exchange reactions are functionally inactive (compared to cellular buffering capacity). In contrast, Na_o^+/H_i^+ exchange is activated by an increased cellular acid load. This activation may be observed directly either as a stimulation of net H^+ efflux or net Na^+ influx with decreasing intracellular pH. The extrapolation of this latter data suggests a "set point" of Na^+/H^+ exchange of approximately pH 7.0, consistent with the observed resting intracellular pH of approximately 7.05.

Key Words Na/H exchange · intracellular pH · renal cell culture · LLC-PK$_1$

Introduction

In many different cell types, Na^+/H^+ exchange is an important element in the control of intracellular pH (pH_i) (Roos & Boron, 1981). In some epithelia (e.g. renal proximal tubule and small intestine) Na^+/H^+ exchange performs a more specialized function of facilitating transepithelial bicarbonate and chloride transport through changes in pH. In mammalian proximal tubular bicarbonate reabsorption, proton extrusion via Na^+/H^+ exchange is a primary event leading to bicarbonate reabsorption as $CO_2 + H_2O$ (Murer & Burckhardt, 1983). Absorption of NaCl by the small intestine is probably mediated by a similar coupling of Na^+/H^+ exchange to Cl^-/HCO_3 exchange (Knickelbein et al., 1985).

Activity of Na^+/H^+ exchange is controlled by several different regulatory pathways. Some mechanisms which have been implicated are allosteric activation by pH_i (Aronson, Nee & Suhm, 1982; Paris & Pouyssegur, 1984; Grinstein et al., 1985*b*; Grinstein, Rothstein & Cohen, 1985*c*), covalent protein modification through kinase C (Grinstein, Cohen, Goetz & Rothstein, 1985*a*), and changes in the amount or turnover of existing transport units (Kinsella, Cujdik & Sacktor, 1984). The allosteric control of Na^+/H^+ exchange by pH_i was first described in a preparation of rat renal brush border membrane vesicles (Aronson et al., 1982). However, studies with isolated membranes provide limited insight into cellular control under physiological conditions of cytosolic metabolism, buffering capacity and Na^+ activity. It is therefore of interest to identify whether Na^+/H^+ exchange is controlled by pH_i in intact renal epithelial cells as observed in membrane vesicles and other cell types. To approach this question we have studied pH_i regulation in LLC-PK$_1$ cells, a continuous cell line derived from pig kidney. Previous work has established that LLC-PK$_1$ expresses metabolic and membrane transport functions characteristic of renal epithelial cells (Dayer et al., 1981; Misfeldt & Sanders, 1981; Rabito, 1981; Biber, Brown & Murer, 1983; Rabito & Karlish, 1983; Strewler, 1984; Gstraunthaler, Pfaller & Kotanko, 1985), including measurable Na^+/H^+ exchange in monolayers of cells and apical membrane vesicles (A. Moran, J. Biber & H. Murer, *submitted*).

The use of a fluorescent probe capable of reporting the activity of intracellular protons is an extremely attractive technique because of the sensitivity and time resolution of such probes (Thomas, Buchsbaum, Zimniak & Racker, 1979). Unfortunately, quantitative work with epitheliod mono-

layers of cells may be complicated by trapped spaces for dye, difficulties in dye calibration, and complicated optical path geometry. To initiate studies with one such probe (BCECF), we have elected to work with cell suspensions. This allows the observation of an average cell population in a simple optical path (a normal fluorometer cuvette).

While the use of LLC-PK$_1$ cells in suspension is one of the simplest systems for the use of optical techniques, it represents a compromise concerning the status of epithelioid cells, which lose the functional polarity produced in monolayer culture. Cell suspensions may still be used, however, to answer questions that do not strictly require a polarized epithelial layer. This paper uses suspended cells of renal origin to evaluate the importance of amiloride-sensitive Na^+ transport for the maintenance of pH_i in the absence and presence of an acid load.

Materials and Methods

Cell Culture and Cell Suspension

LLC-PK$_1$ cells (serial passage 148) were originally purchased from Flow Laboratories (Irvine, Scotland), and experiments were performed using cells between passage 156 and 198. Cells were maintained in culture as previously described (Biber et al., 1983) with the following exceptions: The culture medium was additionally supplemented with 2 mM glutamine and cells were subcultured using 0.1% trypsin in phosphate buffered saline (Dulbecco's formula without Ca and Mg; Flow Laboratories, Irvine, Scotland). For experimental purposes, some cells were subcultured on 35-mm plastic petri dishes (NUNC, Denmark) by seeding with 1.3×10^5 cells in 2 ml of growth medium and grown two days. In order to grow large numbers of cells for creating cell suspensions, 6.5×10^6 cells were seeded in 840 cm^2 roller bottles (Bellco) and grown for 10–13 days prior to use. Cell suspensions were made from cells harvested off the roller bottles by a rubber policeman in 40 ml phosphate-buffered saline. Cells were mechanically dispersed into small groups of cells with a large bore syringe needle and 15 plunges of a syringe. Cells were then centrifuged ($1400 \times g$, 10 sec in a Sorvall RT 6000), and the supernatant was decanted. The pellet was resuspended either in medium resembling growth medium ("normal medium," containing in mM; 152 Na, 5 K, 2 Ca, 1 Mg, 1 PO_4, 1 SO_4, 149 Cl, 25 glucose, 20 HEPES, pH 7.4 at 25°C) or in medium with tetramethylammonium replacing Na^+ ("TMA medium"). Centrifugation was repeated either once (normal medium) or twice (TMA medium) in the desired medium prior to final suspension of the cells (at 1–2 mg protein/ml) in 40 ml of solution in a small volume spinner flask on a microcarrier stirrer (Techne model MCS-104). Aliquots of this material were used for all ensuing work with cell suspensions.

Flame Photometry

Total cell Na^+ and K^+ of cell monolayers or suspensions were determined using a Corning 400 flame photometer. When using cells attached to 35-mM dishes, the extracellular Na^+ and K^+ were removed by washing the dishes four times with ice-cold "stop solution" (containing in mM: 155 TMA, 3 Mg, 1 SO_4, 1 EGTA, 154 Cl, 10 HEPES; pH 7.4 at 25°C). Cells were then lysed by adding 1 ml 10% perchloric acid (PCA). After 5 min, the solution was removed, read directly in the flame photometer, and compared to standards prepared in 10% PCA. To prepare cell suspension samples for flame photometry, 200-μl aliquots were placed on 0.8 or 1.2 μm pore filters (prewashed with 10% PCA; Millipore) and washed twice with 2.5 ml of ice-cold stop solution. Cells on the filter were lysed with 1 ml 10% PCA, and the extract was assayed as described for monolayer samples. With both monolayer and suspension samples, additional washing produced no change in Na^+ values, suggesting that contamination with extracellular medium was minimized. Cell ion content is expressed as moles per mg protein. Protein was determined by the Bradford procedure (Biorad) on cell samples dissolved in concentrated formic acid and compared to γ globulin standards. All the averaged data is presented as mean ± SEM.

Intracellular pH Measurement

Aliquots of suspended cells (2 ml) were placed in Eppendorf microfuge tubes and gently mixed with 2.5 nmol BCECF/AM (Molecular Probes, Junction City, OR) and 0–30 mM NH_4Cl for 45–60 min. After this interval, cells were centrifuged (Eppendorf microfuge, 1 sec), the supernatant was aspirated, and the pellet resuspended in 50 μl of medium without BCECF/AM. 50 μl of this cell suspension was then injected into 1950 μl of medium stirring in a thermostated cuvette (25°C) in the fluorometer (Shimadzu RF-510, light source stabilized). Excitation of the probe was set routinely at 500 nm (3 nm bandwidth) while emission was monitored at 530 nm (10 nm bandwidth). Chart records were taken with a 2-channel LKB recorder. The second pen could be used to keep a simultaneous record of the medium pH as determined by mini pH electrodes (MI-506, -402; Microelectrodes Inc., Londonderry, NJ) immersed within the cuvette solution, but placed out of the light path. Unless mentioned, additions made to the cuvette altered the medium pH by less than 0.01 pH units.

The fluorescence observed from BCECF-loaded cells under these conditions is not reporting exclusively from a compartment which is inaccessible to rapid extracellular pH (pH_o) changes. As shown in Fig. 1, addition of small aliquots of acid cause instantaneous deflections in the signal which suggest 21 ± 1.5% ($n = 10$ separate preparations) of the total dye was immediately accessible to the acid (addition of TMAOH or H_2SO_4 gave identical results). Even if one assumes that all of the 2.5 nmol of BCECF/AM was cleaved to BCECF during the dye-loading interval, this fraction was larger than predicted (<7%) from knowledge of the medium dilution (>150-fold) and percent of total dye loading, and it could not be reduced by additional washing of the cells (*data not shown*). This fraction (referred to as "extracellular probe") is probably bound to the cell surface or is within very leaky cells in the population.

Figure 1 illustrates the calibration protocol followed for every individual loading to estimate the amount of extracellular probe. A comparison is made of the sensitivity of the fluorescent signal to changes in medium pH (pH_o) before and after release of the dye by 50 μM digitonin (Calbiochem). The linear response (fluorescence versus pH, *see* Fig. 2) of the dye from pH 6.5–7.4 is then used to calculate the fluorescence of extracellular probe at appropriate values of pH_o and this background is subtracted from the total signal. The remaining signal is assumed to report

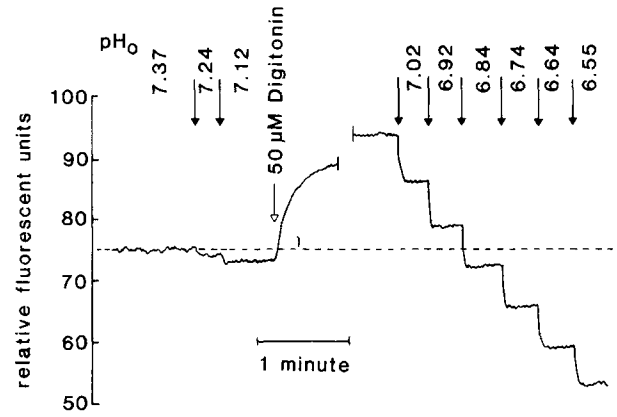

Fig. 1. An example of the calibration protocol used for BCECF loaded LLC-PK$_1$ cells. At the times indicated by the filled arrows, roughly 2 μmol of HNO_3 was added to the cuvette and the new steady state of fluorescence and medium pH was recorded. The rapid sensitivity of the fluorescent signal to changes in medium pH before digitonin addition is assumed to be due exclusively to extracellular dye (intracellular fluorescence is assumed constant). The signal after digitonin addition is assumed to represent total dye (intracellular plus extracellular)

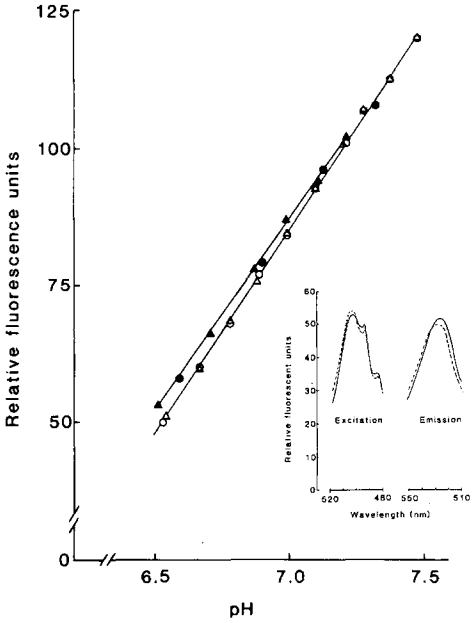

Fig. 2. Standard curves of fluorescent response versus medium pH. In all cases, the pH was defined by mini pH electrodes in the cuvette and curves were normalized to 120 fluorescent units at pH 7.48. Unesterified BCECF was examined using either 100 nM dye in normal medium (○) or 10 μM dye in high K^+ medium (△) (this medium contained in mM; 140 K, 16 Na, 1 Mg, 1 PO_4, 1 SO_4, 20 HEPES, 0.1 μM added Ca, 145 Cl). Dye which was made by cells in situ from BCECF/AM was calibrated either after release of dye by digitonin (▲) or in situ in the presence of 3.5 μM nigericin (●) and 152 mM extracellular K^+ (identical to normal medium except K^+ replaced Na^+). A linear least squares fit of the unesterified BCECF data and the digitonin data is presented. The inset shows the spectral response of cells loaded with dye when pH$_o$ = 7.04 (solid line) and the spectra of digitonin released dye at the same pH (dashed line)

exclusively pH$_i$ values. When the pH$_i$ fluorescence signal was not within the linear range of dye response versus pH (below pH 6.5), pH$_i$ values were calculated by direct comparison to an extended standard curve of unesterified BCECF fluorescence versus pH. These calculations make several assumptions (which have been evaluated) concerning a similar fluorescent response of intracellular dye versus extracellular dye.

Several different experiments contributed to the characterization of the BCECF probe presented in Fig. 2. The sensitivity of unesterified BCECF (molecular probes) to changes in pH is presented twice: once for 100 nM of dye in normal medium and once for 10 μM dye in high K, low Ca^{++} medium. As shown in Fig. 2, the changes in concentration and ion content had no detectable effect on dye response. A pH titration of dye produced by cells from BCECF/AM is also presented twice. When the dye produced in situ is released by (50 μM) digitonin, the sensitivity of this material to changes in medium pH is 93 ± 1% (n = 13 preparations) of the value predicted from standard curves of pure BCECF. A typical example is shown in Fig. 2 (with 92% of predicted slope). Cellular BCECF could also be calibrated in situ by the method of Thomas et al. (1979) using 3.5 μM nigericin (this concentration of nigericin produced a maximal change in the signal). In this experiment, the sensitivity of the dye to changes in medium pH was 94% of the predicted slope (and 97% of the slope of digitonin-released material). The inset shows the excitation and emission spectra of BCECF in situ and the same dye released into the medium with no change in signal gain, when the pH$_o$ is kept at a value near the estimated pH$_i$ (7.05). Under standard conditions of excitation and emission measurement, the signal changed by an amount corresponding to approximately 0.03 pH units. This change is within the experimental error of the measurements and no correction is applied to the data which appears later in the paper. The similarity of the response to pH changes suggests that titration of digitonin-released dye may be used to reliably calibrate intracellular dye (after correction for contamination by extracellular dye).

In the presented experiments, the cells in the cuvette (0.9–1.3 mg protein/ml) were loaded intracellularly with about 8% of the BCECF originally available as BCECF/AM. In one series of preparations, the intracellular concentration of dye was estimated to be 31 ± 6 μM (n = 7, assuming 3 μl cell H_2O/mg protein). Autofluorescence and light scattering typically contributed to less than 2% of the total fluorescent signal, and no correction was applied for this component. Loss of intracellular dye from the cells was approximately 0.5% per minute (25°C) assessed by either (i) measurement of cell supernatant after filtering (Gelman, 0.2 μm filters) or (ii) by increases in the amount of extracellular dye assessed by the fluorescent response to changes in medium pH. No correction was applied for dye loss because the estimation of extracellular probe was routinely made within 2 min of all calibrated points.

Miscellaneous Techniques

Whenever amiloride was used, cells were exposed to 100 μM amiloride at the time when 50 μM of the cell suspension was injected into the cuvette to initiate recording. The changes in pH$_i$ observed in many experiments using Na^+-depleted cells were often correlated with a second experimental measurement (either

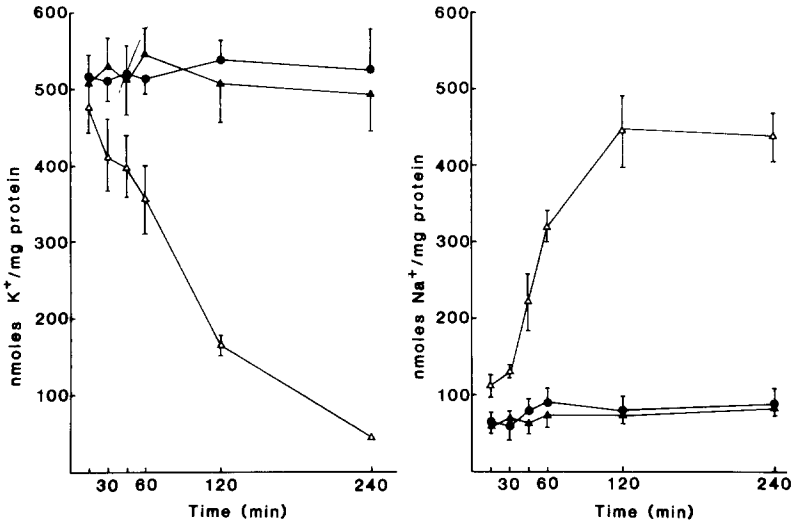

Fig. 3. Total cellular Na^+ and K^+ in monolayers of subconfluent LLC-PK$_1$ cells. At time zero, growth medium was replaced with "normal" medium (*see* Materials and Methods). Cells attached to 35-mm dishes were incubated at either room temperatures (●) or 37°C (▲, △) for the indicated times. Some cells were additionally exposed to 100 μM ouabain at time zero (△). At the indicated times, medium Na^+ and K^+ were removed and extracts of cells were assayed by flame photometry. Means ± SEM are presented for five preparations (n = 4 for ouabain treatment)

H^+ flux or isotopic Na^+ flux). In order to measure ^{22}Na unidirectional influx, a second cell sample was run parallel to the BCECF sample. Aliquots (200 μl) were taken from this parallel cuvette at 5, 20, 40, and 60 sec after addition of 20 mM $^{22}NaCl$ to the suspension, and washed by rapid filtration (0.65 μm Sartorius). In order to simultaneously monitor pH_i and pH_o changes, HEPES (and 10 mM TMA) in the TMA medium was replaced by 30 mM mannitol for the solutions used to resuspend the BCECF-loaded cells. Using the chart recorder at 0.4 pH units full scale, the extracellular pH electrode was used to simultaneously evaluate pH_o changes following the addition of 20 mM NaCl to the medium. Buffering capacities of cell filtrate (from the cuvette) and medium with HEPES removed were identical (*data not shown*). In all cases, at least 2 min (with ^{22}Na experiments, exactly 2 min) were allowed to establish a baseline of pH_o or fluorescence change prior to the addition of 10 μl of 4 M NaCl to initiate Na^+-dependent changes.

Abbreviations

BCECF	—2′,7′-bis-(2-carboxyethyl)-5,6 carboxyfluorescein
BCECF/AM	— acetoxymethyl ester of BCECF
Amil	—amiloride
SITS	—4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate
HEPES	—4-(-2-hydroxyethyl)-1-piperazine ethansulfonate
pH_i (pH_o)	—intra(extra)cellular pH
H_i^+ (H_o^+)	—intra(extra)cellular H^+ concentration
Na_i^+ (Na_o^+)	—intra(extra)cellular Na^+ concentration
VAL	—valinomycin
NMGCl	—*n*-methyl-D-glucamine Cl
TMA^+	—tetramethylammonium
FCCP	—carbonylcyanide *p*-trifluoromethoxyphenylhydrazone
$\Delta\psi$	—membrane potential difference
PCA	—perchloric acid

Results

Prior to work with the fluorescent probe, it was essential to show that the LLC-PK$_1$ cell suspension contains viable cells after they are removed from a monolayer. In particular, the maintenance of a Na^+ gradient is of interest since Na^+-dependent processes are involved in pH_i regulation of most cells examined to date (Roos & Boron, 1981).

For these reasons, flame photometry of total cell Na^+ and K^+ was used to evaluate the condition of the cells. In order to obtain information on minimally perturbed cells, we examined ion levels in subconfluent cells still attached to culture dishes. The results are presented in Fig. 3. The results show that transfer of cells into 37°C "normal medium" (medium containing Na^+, *see* Materials and Methods) causes no detectable change in cellular ion levels. Another series of points in the figure demonstrate that direct inhibition of the (Na^+/K^+)-ATPase with 100 μM ouabain rapidly compromises ion levels and suggests that cells maintain at least a 10-fold K^+ gradient and fivefold Na^+ gradient. In contrast to the effects of ouabain, extended incubation at room temperature produces no significant change in Na^+ or K^+ levels. Thus, at the lower temperature, the ability of the (Na^+/K^+)-ATPase pump to cope with leaks of Na^+ and K^+ is not visibly compromised and physiological ion gradients are maintained. Addition of ouabain to the cell suspensions caused similar shifts in Na^+ and K^+ levels to those observed in cells attached to culture dishes (*data not shown*). For experimental convenience and because of this supporting evidence, experiments with suspended cells were performed at room temperature.

The levels of Na^+ and K^+ observed in suspended cells are shown in Fig. 4. As the cells are left for longer times after creation of the suspension, the ion gradients recover. After 4 hr of recovery, the levels of Na^+ and K^+ are both close to the monolayer values and have reached a steady state (which is maintained for over 12 hr, *data not*

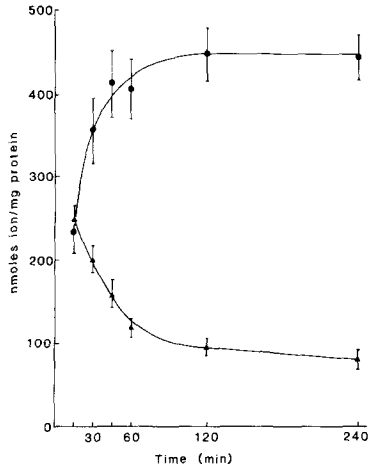

Fig. 4. Total cellular Na^+ and K^+ in suspension of LLC-PK$_1$ cells. At time zero, cells were removed from monolayer and suspended in normal medium at room temperature. At the indicated times, aliquots of cells were washed by rapid filtration and prepared for flame photometry of Na^+ (▲) and K^+ (●). Values are corrected for the amount of protein trapped by the filter. Means ± SEM of four preparations are presented

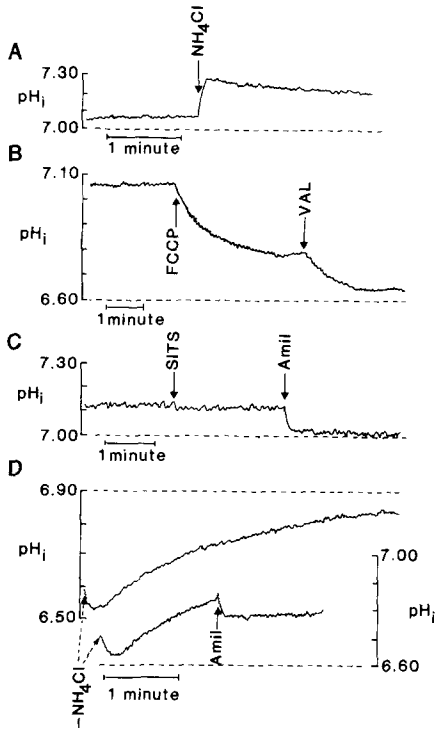

Fig. 5. Properties of the steady-state pH_i of cells which are kept in normal medium. At the times indicated by the solid arrows, the following additions were made to the cuvettes containing cells loaded with BCECF; 20 mM NH_4Cl, 10 μM FCCP, 9 μM valinomycin (*VAL*), 300 μM SITS, 100 μM amiloride (*Amil*). The quench of fluorescence caused by amiloride in *C* and *D* is also produced by amiloride in solutions of unesterified BCECF (*data not shown*). Because of this quench, the scale of pH_i in *C* and *D* accurately corresponds only to the data collected after amiloride addition. In *D*, cells were preincubated with both BCECF/AM and 30 mM NH_4Cl. At the time indicated by the dashed arrow in *D*, 50 μl of cells (washed free of extracellular dye) was injected into the cuvette to initiate recording simultaneously with a 40-fold dilution of the extracellular NH_4Cl. Similar results were obtained in at least three preparations

shown). The recovery of full ion gradients suggests that cells are metabolically capable and that membranes are not irreversibly damaged during cell isolation. Experiments were performed routinely using cells which were loaded with the pH-sensitive dye, BCECF, following 4 hr of recovery after creation of the cell suspension.

Suspended cells at a steady state in normal medium were examined first to characterize the steady-state pH_i maintained by the cells. In five preparations, the resting pH_i was 7.05 ± 0.01, determined after calibration of intracellular BCECF. As shown in Fig. 5*A*, addition of a weak base demonstrates simply that the fluorescence signal (and presumably pH_i) may be manipulated independently of pH_o (implying that the dye is contained within a compartment with a pH which is not rapidly equilibrating with medium pH). In Fig. 5*B*, addition of 10 μM FCCP causes a rapid acidification of pH_i and suggests that in the presence of high proton conductance the pH_i shifts towards its electrochemical equilibrium (in the presence of a negative membrane potential ($\Delta\psi$)). Subsequent addition of valinomycin demonstrates that the cells have high enough H^+ permeability to respond quickly to changes in $\Delta\psi$. It is important to note that the indicated steepening of the H^+ gradient due to FCCP cannot be explained by an increased contribution of the plasma membrane H^+ permeability to $\Delta\psi$. After FCCP addition, any increased contribution of the H^+ permeability to $\Delta\psi$ can only decrease the magnitude of the induced acidification. Thus, estimates of the magnitude of the gradient of H^+ activity compared to electrochemical equilibrium (0.61 ± 0.04-fold below, $n = 4$) are underestimates when FCCP changes $\Delta\psi$ or partially dissipates the plasma membrane H^+ gradient. As shown in Fig. 5*C*, when the known transport inhibitors SITS (300 μM) or amiloride (100 μM) are added, there is no effect other than a slight quenching of the fluorescence signal by amiloride. This implies that any net H^+ flux inhibited by these agents is negligible compared to cellular buffering capacity. Since other evidence suggests the presence of a Na^+/H^+ exchange reaction capable of rapidly altering pH_i, the data in Fig. 5*C* suggest one of three alternative explanations. It is possible that the exchanger operates at "normal" rates but catalyzes no net H^+ flux ($Na_i^+/Na_o^+ = H_i^+/H_o^+$), that the exchanger operates at an undetectable rate, or that amiloride is not effective. In order to show that amiloride is effective with these high concentrations of Na_o^+, cells were acid loaded by preincubation and removal of NH_4. As shown in the two

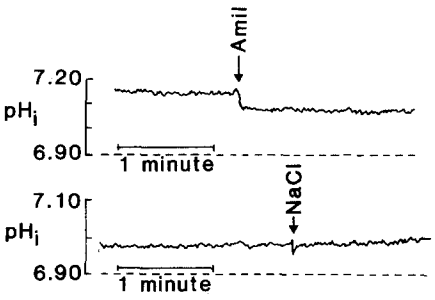

Fig. 6. Properties of pH_i when extracellular Na^+ is rapidly replaced by TMA^+. After cells recover for 4 hr and are loaded with BCECF in normal medium, the centrifugation and resuspension used to remove extracellular dye is also used to replace extracellular Na^+ with TMA^+. Medium Na^+ in the fluorescence cuvette (measured by flame photometry of cell-free filtrates) was reduced to 0.3–0.9 mM in three preparations. In the two experimental runs presented using these cells, either 100 μM amiloride or 20 mM NaCl was added at the indicated time

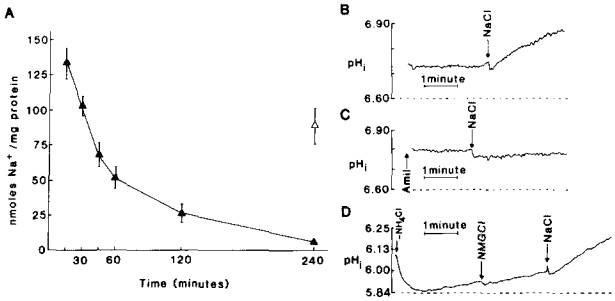

Fig. 7. Na^+ depletion effects on pH_i. (*A*) Flame photometry of suspended cells which were removed from a monolayer at time zero and suspended in TMA medium (▲) or normal medium (△). Means ± SEM for four preparations are presented. In *B–D*, Na^+-depleted cells were loaded with BCECF. Additions of 20 mM NaCl or 20 mM NMGCl were made during recording at the times indicated by the arrows. In *C*, the injection of 50 μl of cells which initiated recording also initiated exposure of the cells to 100 μM amiloride. Similarly in *D*, a 40-fold dilution of (preloaded) 30 mM NH_4Cl was initiated with injection

traces of Fig. 5*D*, pH_i rapidly recovers from the acid load and this recovery is inhibited by 100 μM amiloride. This is consistent with previous work which has suggested a noncompetitive inhibition of Na^+/H^+ exchange by amiloride in the LLC-PK$_1$ cells (A. Moran, J. Biber, H. Murer, *submitted*).

Experiments were then performed to determine the status of the Na^+/H^+ exchange reaction in cells with normal Na_i^+ levels. The question is whether the exchanger is at equilibrium or is operating extremely slowly. After changing the ion gradient for Na^+, an active exchanger in the membrane would be predicted to catalyze a net flux of protons once again. As shown in Fig. 6, when the medium Na^+ is changed drastically (<1 mM Na_o^+), there is still no evidence of an amiloride-sensitive or Na^+-dependent component at pH_i homeostasis (i.e., no change in the slope of the tracings). There is also no detectable change in resting pH_i induced by rapid removal of Na_o^+. This strongly suggests that Na^+/H^+ exchange is functionally inactive at resting pH_i.

In order to characterize more fully the activation of Na^+/H^+ exchange by acid loads, cells were depleted of Na_i^+ over the 4 hr interval normally used to allow ion gradients to recover. As shown in Fig. 7*A*, in the absence of medium Na^+ (TMA^+ replacement), Na_i^+ falls to levels which are 15-fold lower than the steady-state Na_i^+ levels reached by the same population of cells maintained in normal medium containing Na^+. After 4 hr, cellular K^+ levels of Na^+-depleted cells were one half of those observed in cells in Na^+ medium (*data not shown*). The Na^+-depleted cells also have a more acidic pH_i (6.68 ± 0.03, n = 13 preparations), suggesting that Na^+ is essential for maintenance of normal pH_i by the cells. As shown in Fig. 7*B* and *C*, the more acidic pH_i of these cells may now be changed by addition of low (20 mM) concentrations of Na^+. The Na^+-dependent pH_i alkalinization is also amiloride sensitive. Because of the intracellular acidity of the Na^+-depleted cells, modest concentrations of NH_4 could be used to produce large acidifications of the cytosol compared to the medium. An example is shown in Fig. 7*D*. This figure also demonstrates the Na^+ specificity of the recovery from an acid load compared to addition to 20 mM NMG.

In Fig. 7*D*, the apparent increase in pH_i, prior to Na^+ addition is probably not real. With extremely large pH gradients, even very slow leaks of dye out of the cell can cause large changes in the baseline fluorescence (this is because of the large increase in fluorescence which occurs as the dye leaves a very acid compartment and enters an alkaline one). In seven preparations and over a wide range of H^+ gradient conditions (6- to 35-fold H^+ gradients), any drift in signal can be calculated to represent 0.2–1.1% of cellular dye leaving per minute (0.61 ± 0.1, n = 7). This agrees well with our estimates of dye leakage by other means (*see* Materials and Methods).

In Fig. 8, we have examined the capability of the cells to activate 20 mM Na^+ transport when the cellular acid load is increased. In Fig. 8*A*, a single experiment is presented to demonstrate that only the amiloride-sensitive component of Na^+ influx is altered by changes in the acid load (similar results were observed in five preparations). In Fig. 8*B*, the amiloride-sensitive component of Na^+ influx is presented versus the pH_i determined with BCECF in similarly treated samples.

In Fig. 8, the activation of Na^+ transport probably represents a net uptake of Na^+ (versus Na^+/Na^+ exchange) because of the extremely low Na_i^+ in the cells. However, to show that this represents a net Na_i^+/H_o^+ exchange reaction, experiments were per-

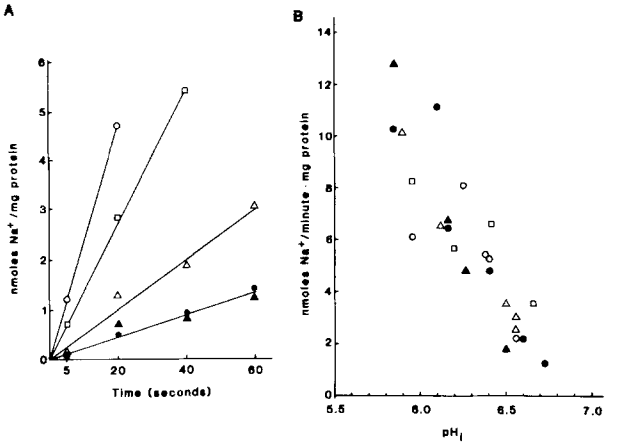

Fig. 8. Effect of an increased acid load on amiloride-sensitive 20 mM Na^+ influx into Na^+-depleted cells. Cells were acid loaded by preloading and removal of 5–40 mM NH_4Cl. (*A*) Representative experiment showing the effects of decreased pH_i on total Na^+ influx. Some cells were preincubated with 100 μM amiloride for 2 min prior to $^{22}NaCl$ addition (▲, ●). Cells which were treated identically except for BCECF loading were used to determine that pH_i was 6.50 (▲, △), 6.17 (□), or 5.85 (●, ○). A *y*-intercept of 1.2 nmol/mg protein was subtracted from each presented value. (*B*) Amiloride (100 μM) sensitive influx of 20 mM Na^+ is plotted versus pH_i as determined by BCECF. Each symbol represents data from a separate preparation

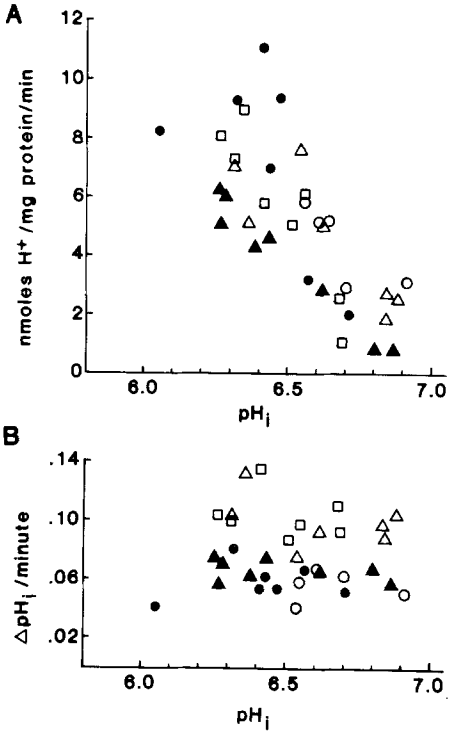

Fig. 9. Simultaneous measurement of changes in pH_i and pH_o after the addition of 20 mM NaCl to Na^+-depleted cells. In both *A* and *B*, the Na^+-dependent component of pH change is presented. Each symbol represents data from a separate preparation. Following dye and (0–35 mM) NH_4Cl loading, cells were resuspended in medium with 30 mM mannitol replacing 20 mM HEPES (and 10 mM TMA^+). The buffering capacity of 2 ml of the low buffer medium was 900 nmol H^+/pH unit over the pH range used in these studies. For these experiments, NH_4Cl was removed from cells at the time of resuspension in 50 μl (prior to injection into the cuvette) to maximize the dilution of NH_4

formed to evaluate rates of net H^+ flux. This was evaluated with simultaneous measurements of H^+ appearance in the medium (changes of pH_o of weakly buffered medium) and pH_i changes (BCECF). As shown in Fig. 9*A*, the (20 mM) Na^+-dependent appearance of protons in the medium is strongly correlated with the previously observed (amiloride-sensitive) Na^+ influx values over a wide range of acid loads. Curiously, there was no corresponding stimulation in the rate of pH_i change (*see* Fig. 9*B*). This could be easily explained by an increase in buffering capacity in the acid range; however, estimates of cellular buffering capacity failed to demonstrate any change in proton buffering capacity between pH 5.8 and 7.0. This data is presented in the Table. Note that a value of about 2.2 μl cell H_2O/mg protein may be used to convert between the two units of buffering capacity.

Discussion

It was first shown that the LLC-PK$_1$ cells in suspension recover Na^+ and K^+ gradients very similar to those of LLC-PK$_1$ attached to culture dishes, implying that after recovery the cells are metabolically active and stable. In addition, experiments using FCCP suggest that the cytosolic activity of protons is below electrochemical equilibrium. This suggests that H^+ conductance is much less important than alternate mechanisms of H^+ transport for defining the steady-state pH_i. It should be noted that the FCCP-induced change in cytosolic pH could not be explained by dissipation of mitochondrial H^+ gradients, but could be explained by dissipation of very large lysosomal H^+ gradients. However, some evidence suggests that K^+ is in electrochemical equilibrium across the lysosomal membrane (Goldman & Rottenberg, 1973; Reijngould & Tager, 1975). So although we cannot disprove an involvement of lysosomal H^+ gradients, the rapid action of FCCP and the effects of valinomycin are encouraging that high plasma membrane H^+ permeability is contributing to the steady-state distribution of protons after FCCP addition.

When the steady-state pH_i of suspended cells in normal medium was examined further, it was found that there is no significant sensitivity of the pH_i to rapid changes in the Na^+ gradient (Na_o = 152, 20 or <1 mM). There is also no sensitivity to addition of 100 μM amiloride. Amiloride at this concentration

Table. Buffering capacity of cell suspensions

pH range	Digitonin-permeabilized cells[a] (nmol H^+/mg protein/pH unit)	NH_4-pulsed cells[b] (mM H^+/pH unit)
7.0 – 6.8	114 ± 14 (5)	54 ± 6 (6)
6.8 – 6.6	111 ± 18 (5)	44 ± 5 (5)
6.6 – 6.4	121 ± 12 (5)	64 ± 4 (8)
6.4 – 6.2	129 ± 13 (5)	57 ± 6 (4)
6.2 – 6.0	139 ± 8 (5)	60 ± 5 (3)
6.0 – 5.8	117 ± 13 (4)	

[a] Cells were suspended (at 20 ± 2 mg protein/ml, 4 experiments; or 2.2 mg/ml, 1 experiment) in normal medium with HEPES replaced by 30 mM mannitol. In one experiment, phosphate was also removed from the medium. Cells were exposed to digitonin for 1 hr (25 nmol/mg cell protein) and then pH was titrated with nitric acid. Cellular buffering capacity was calculated by subtraction of medium buffering capacity. Data are presented as mean ± SEM of (n) experiments.

[b] Cells were loaded with BCECF in the presence of 0–40 mM NH_4Cl and resuspended in TMA medium. The resultant pH_i change after (1.4–10 mM) NH_4Cl injection was the basis for the calculation of buffering capacity (Ross & Boron, 1981). Na^+-depletion had no significant effect on the results, so the mean ± SEM of (n) determinations from 6 experiments (3 Na-depleted, 3 normal Na^+) are presented.

can be shown to be effective for inhibiting the cellular recovery from an acid load in the presence of the same high Na_o^+ (152 mM). These data show that at resting pH_i, Na^+/H^+ exchange (observed as an amiloride-sensitive or Na^+-dependent change in pH_i) is inactivated. As discussed below, this does not preclude a role of Na^+/H^+ exchange in setting the resting pH_i of the cell.

The data suggest a more significant role of Na^+/H^+ exchange when the cell recovers from an acid load (caused by preloading and removal of a weak base). This suggests a change in the capacity of Na^+/H^+ exchange to produce a net H^+ flux. This change in capacity was observed directly with two techniques. Using Na^+-depleted cells (so that net fluxes of Na^+ versus Na^+/Na^+ exchange could be observed), it is found that amiloride-sensitive unidirectional influx of 20 mM Na^+ is greatly stimulated by acid loads. A similar stimulation of isotopic Na^+ influx into cloned LLC-PK$_1$ cells which were treated with NH_4 or nigericin has been observed by others (Haggerty, Cragoe, Slayman & Adelberg, 1985). It is also observed that the Na^+-dependent appearance of protons in the medium is greatly stimulated by acid loads. The absolute values from direct measurements of H^+ efflux and Na^+ influx suggest a 1 : 1 coupling of Na^+/H^+ fluxes over a wide range of pH_i.

Extrapolation of these data suggest that the set point of the exchanger (no net flux) is near 7.0, in close agreement with the steady-state pH_i of 7.05 observed in cells with normal Na_i^+. So the data imply that net flux by the Na^+/H^+ exchanger is responsible for the approach of cellular pH to 7.05, but that the exchanger progressively decreases activity as the cytosol approaches the pH set point. The acidification which occurs when cells are Na^+ depleted probably reflects the inability of cells to extrude metabolically produced acid and later a reversal of the exchange reaction, in the absence of extracellular Na^+, as the exchanger is activated at more acidic pH_i.

In the same experiments where an acid load stimulates the rate of appearance of protons in the medium up to 10-fold over resting levels, the rate of pH_i change stays relatively constant. A couple of possibilities may be eliminated which could produce this effect. Because net H^+ efflux into the medium is measured simultaneously, buffering of the net H^+ flux by transmembrane transport (e.g. via Na^+/HCO_3^- cotransport or Cl^-/OH^- exchange) may be eliminated as a possibility. Similarly, the observed rates of Na^+ flux suggest that changes in cell volume are not likely to contribute to the effect.

One simple explanation is that the estimates of buffering capacity do not accurately represent the cytosolic buffering capacity. In fact, if the buffering capacity measured in digitonin-permeabilized cells is applied to the rates of ΔpH_i/min observed at pH 6.8–6.9, it implies that the flux of nmol H^+/mg protein/min sensed by the extracellular pH electrode is about fourfold too low. Since this difference could be easily detected by the electrode system, it implies that these estimates of buffering capacity must not accurately represent only cytosolic values. Similarly, assuming 2–3 μl cell H_2O/mg protein, the results of NH_4 pulsing of cells imply that the electrode system should have detected at least threefold higher rates at pH 6.8–6.9. We must conclude that either there is an artifact in the BCECF measurements or that we are unable to deliver a defined number of protons to the cell cytosol containing BCECF. One explanation of the latter possibility is that a second intracellular compartment (without BCECF) is permeable to ammonium such that net proton flux from this compartment complicates both buffering capacity measurements and measurements of cytosolic pH change. If the measured buffering capacity is indeed in error, a prediction from the combination of the two diagrams in Fig. 9 would be an increased cytosolic buffering capacity at more acidic pH_i values. This would be useful for the cells as an additional mechanism to protect the cell from acidic pH_i excursions and has been observed in a variety of cell types (Roos & Boron, 1981).

The data presented concerning a "set point" of

Na^+/H^+ exchange is not uncommon in a pH-regulatory system. Similar behavior has been demonstrated in pH regulation of lymphocytes (Grinstein et al., 1985*a* and *b*), barnacle muscle fibers (Roos & Boron, 1981) and fibroblasts (Paris & Pouyssegur, 1984). In membrane vesicles from the renal tubular epithelium, Aronson et al. (1982) have been able to demonstrate a similar change in capability of Na^+/H^+ exchange modulated by intravesicular pH. These authors have proposed the existence of an intracellular allosteric site for H^+, which acts independently of the substrate site to activate Na^+/H^+ exchange. Our data is consistent with this hypothesis.

This data is interesting in comparison to the component of acid secretion in the renal proximal tubule, which is Na^+-dependent (and amiloride-sensitive; Howlin, Alpern & Rector, 1985; Chan & Giebisch, 1982). In particular, if the cell has an inactive exchanger at resting pH_i, what does this imply concerning the mechanism of (Na^+-dependent) acid secretion? Assuming that Na^+/H^+ exchange is involved in this process (Aronson, 1981), one must postulate that either the pH_i or the set point is different when Na^+-dependent acid secretion occurs. Boron and Boulpaep (1983) have suggested that acid loads due to HCO_3^- flux are responsible for activating Na^+/H^+ exchange and therefore proximal tubular acid secretion in the salamander. In other cellular systems, it has been shown that when lymphocytes are stimulated to volume regulate or when growth factors activate fibroblasts, activation of Na^+/H^+ exchange can occur through a change in the apparent affinity for H_i^+ (Paris & Pouyssegur, 1984; Grinstein et al., 1985*b*,*c*). Changes in the rate of Na^+/H^+ exchange have also been observed at the membrane level in renal brush border vesicles in response to glucocorticoid treatment (Freiberg, Kinsella & Sacktor, 1982), parathyroid hormone or dibutyryl cAMP treatment (Kahn et al., 1985), or metabolic acidosis (Kinsella et al., 1984). The last reference cited examined the change kinetically and attributed it to a change in V_{max} and not a change in affinity for intravesicular H^+. The interactions of Na^+-dependent pH_i regulation by LLC-PK$_1$ cells with the functioning of the cell within an epitheliod monolayer remain to be examined.

We wish to thank Corinne Burlet for excellent secretarial help. This work was supported by funds from the Swiss Nationalfonds (Grant No. 3.881.085 and 3.881.185). M.H.M. was supported by a grant from the Roche Research Foundation and a Fogarty International Postdoctoral Fellowship.

References

Aronson, P.S. 1981. Identifying secondary active solute transport in epithelia. *Am. J. Physiol.* **240:***F1–F11*

Aronson, P.S., Nee, J., Suhm, M.A. 1982. Modifier role of internal H^+ in activating the Na^+-H^+ exchanger in renal microvillus membrane vesicles. *Nature (London)* **299:**161–163

Biber, J., Brown, C.D.A., Murer, H. 1983. Sodium-dependent transport of phosphate in LLC-PK$_1$ cells. *Biochim. Biophys. Acta* **735:**325–330

Boron, W.A., Boulpaep, E.L. 1983. Intracellular pH regulation in the renal proximal tubule of the salamander. Basolateral HCO_3 transport. *J. Gen. Physiol.* **81:**53–94

Dayer, J.-M., Vassalli, J.-D., Bobbitt, J.L., Hull, N., Reich, E., Krane, S.M. 1981. Calcitonin stimulates plasminogen activator in porcine renal tubular cells: LLC-PK$_1$. *J. Cell Biol.* **91:**195–200

Freiberg, J.M., Kinsella, J., Sacktor, B. 1982. Glucocorticoids increase the Na^+-H^+ exchange and decrease the Na^+ gradient-dependent phosphate-uptake systems in renal brush border membrane vesicles. *Proc. Natl. Acad. Sci. USA* **79:**4932–4936

Goldman, R., Rottenberg, H. 1973. Ion distribution in lysosomal suspensions. *FEBS Lett.* **33:**233–238

Grinstein, S., Cohen, S., Goetz, J.D., Rothstein, A. 1985*a*. Osmotic and phorbol ester-induced activation of Na^+/H^+ exchange: Possible role of protein phosphorylation in lymphocyte volume regulation. *J. Cell Biol.* **101:**269–276

Grinstein, S., Cohen, S., Goetz, J.D., Rothstein, A., Gelfand, E.W. 1985*b*. Characterization of the activation of Na^+/H^+ exchange in lymphocytes by phorbol esters: Change in cytoplasmic pH dependence of the antiport. *Proc. Natl. Acad. Sci. USA* **82:**1429–1433

Grinstein, S., Cohen, S., Rothstein, A. 1984. Cytoplasmic pH regulation in thymic lymphocytes by an amiloride-sensitive Na^+/H^+ antiport. *J. Gen. Physiol.* **83:**341–369

Grinstein, S., Rothstein, A., Cohen, S. 1985*c*. Mechanism of osmotic activation of Na^+/H^+ exchange in rat thymic lymphocytes. *J. Gen. Physiol.* **85:**765–787

Gstraunthaler, G., Pfaller, W., Kotanko, P. 1985. Biochemical characterization of renal epithelial cell cultures (LLC-PK$_1$ and MDCK). *Am. J. Physiol.* **248:***F536–F544*

Haggerty, J.G., Cragoe, E.J., Jr., Slayman, C.W., Adelberg, E.A. 1985. Na^+/H^+ exchanger activity in the pig kidney epithelial cell line, LLC-PK$_1$: inhibition by amiloride and its derivatives. *Biochem. Biophys. Res. Commun.* **127:**759–767

Howlin, K.J., Alpern, R.J., Rector, F.C., Jr. 1985. Amiloride inhibition of proximal tubular acidification. *Am. J. Physiol.* **248:***F773–F778*

Kahn, A.M., Dolson, G.M., Hise, M.K., Bennett, S.C., Weinman, E.J. 1985. Parathyroid hormone and dibutyryl cAMP inhibit Na^+/H^+ exchange in renal brush border vesicles. *Am. J. Physiol.* **248:***F212–F218*

Kinsella, J., Cujdik, T., Sacktor, B. 1984. Na^+-H^+ exchange in isolated renal brush border membrane vesicles in response to metabolic acidosis. *J. Biol. Chem.* **259:**13224–13227

Knickelbein, R., Aronson, P.S., Schron, C.M., Seifter, J., Dobbins, J.W. 1985. Sodium and chloride transport across rabbit ileal brush border. II. Evidence for Cl-HCO_3 exchange and mechanism of coupling. *Am. J. Physiol.* **249:***G236–G245*

Misfeldt, D.S., Sanders, M.J. 1981. Transepithelial transport in cell culture: D-glucose transport by a pig kidney cell line (LLC-PK$_1$). *J. Membrane Biol.* **59:**13–18

Murer, H., Burckhardt, G. 1983. Membrane transport of anions across epithelial of mammalian small intestine and kidney proximal tubule. *Rev. Physiol. Biochem. Pharmacol.* **96:**1–51

Paris, S., Pouyssegur, J., 1984. Growth factors activate the Na^+/H^+ antiporter in quiescent fibroblasts by increasing its affinity for intracellular H^+. *J. Biol. Chem.* **259:**10989–10994

Rabito, C. A. 1981. Localization of the Na^+-sugar cotransport system in a kidney epithelial cell line (LLC-PK_1). *Biochim. Biophys. Acta* **649:**286–296

Rabito, C.A., Karlish, M.V. 1983. Polarized amino acid transport by an epithelial cell line of renal origin (LLC-PK_1). *J. Biol. Chem.* **258:**2543–2547

Reijngould, D.-J., Tager, J.M. 1975. Effect of ionophores and temperature on intralysosomal pH. *FEBS Lett.* **54:**76–79

Roos, A., Boron, W.F. 1981. Intracellular pH. *Physiol. Rev.* **61:**296–434

Strewler, G.J. 1984. Release of cAMP from a renal epithelial cell line. *Am. J. Physiol.* **246:***C*224–*C*230

Thomas, J.A., Buchsbaum, R.N., Zimniak, A., Racker, E. 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry* **18:**2210–2218

Received 11 February 1986; revised 28 May 1986