Acute phosphate depletion inhibits the Na^+/H^+ antiporter in a cultured renal cell line

JACOB GREEN, OLIVER FOELLMER, CHARLES R. KLEEMAN, AND MIRIAM M. BASIC
Laboratory of Membrane Biology, Division of Nephrology and Department of Medicine, Research Institute, Cedars-Sinai Medical Center, University of California, School of Medicine, Los Angeles, California 90048

Green, Jacob, Oliver Foellmer, Charles R. Kleeman, and Miriam M. Basic. Acute phosphate depletion inhibits the Na^+/H^+ antiporter in a cultured renal cell line. *Am. J. Physiol.* 265 (*Renal Fluid Electrolyte Physiol.* 34): F440–F448, 1993. —We studied the effect of acute P_i depletion on the regulation of intracellular pH (pH_i) in the OK opossum kidney cell line by using the pH-sensitive dye 2′7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF). Cell recovery from an NH_4Cl acid load in HCO_3-free buffer disclosed an Na^+-dependent component blocked by amiloride and a smaller Na^+-independent component that increased on exposure of the cells to a high-K^+ buffer. After 24-h incubation of the cells in phosphate-free medium, pH_i recovery by the Na^+/H^+ exchanger was markedly inhibited, whereas the Na^+-independent pH_i recovery was not affected. The inhibition of Na^+/H^+ exchange was reversible on correction of cellular P_i deficit. A similar phenomenon was observed when cellular P_i depletion was induced by acute exposure (min) to fructose. P_i depletion shifted the pH_i dependence of the exchanger and also reduced its maximal activity. Time-course studies revealed that the effect of P_i depletion could not be attributed to attenuation of Na^+-K^+-adenosinetriphosphatase activity and resultant diminution of the transmembrane gradient for the Na^+ influx. We conclude that acute P_i depletion in cultured proximal tubular cells leads to reversible inhibition of the Na^+/H^+ exchanger. This in vitro finding may relate to the in vivo observation of impaired HCO_3 reabsorption and bicarbonaturia in acute P_i depletion.

OK opossum kidney cell; sodium/hydrogen exchange; sodium-potassium-adenosinetriphosphatase; intracellular pH

THE CLINICAL SYNDROME of P_i depletion results in severe biochemical disturbances that ultimately lead to multisystemic dysfunction (18). The major clinical manifestations of this condition include severe impairment of cardiac and respiratory muscles, rhabdomyolysis, hemolytic anemia, impaired leukocyte function, severe bone disease, and acid-base disturbances.

One of the major acid-base disorders associated with P_i depletion is defective tubular reabsorption of bicarbonate leading to bicarbonaturia (9, 10). Studies performed on dogs and rats have demonstrated that this abnormality can be seen relatively early (within days) following the induction of P_i depletion (9, 10). Notwithstanding the defect in renal bicarbonate reabsorption, there are no changes in plasma pH or HCO_3 level during the early period of P_i depletion (9). Similarly, in patients suffering from P_i depletion, one cannot identify overt metabolic acidosis. This phenomenon has been ascribed to the coexisting severe bone resorption induced by P_i depletion. Mobilization of alkali from the skeleton can therefore offset the consequences of urinary wasting of bicarbonate during the early stages of P_i depletion. When colchicine, an inhibitor of bone resorption, was given to rats during the first month of phosphate deprivation, a pronounced metabolic acidosis developed along with continued urinary excretion of bicarbonate (9). Thus by eliminating the bone contribution of alkali to the extracellular compartment, one can unmask the defect in bicarbonate reabsorption during P_i depletion. The major focus of this paper is to identify a possible cellular basis for the impaired bicarbonate reabsorption in P_i depletion. Specifically, we sought to determine the activity of the amiloride-sensitive electroneutral Na^+/H^+ antiporter in proximal tubular cells exposed to low-phosphate environment. Several microperfusion studies have shown that the Na^+/H^+ antiporter is the major mechanism for apical proton secretion and $NaHCO_3$ reabsorption in the proximal tubule (5, 26). We therefore reasoned that the in vivo observation of bicarbonaturia in P_i depletion may be related to inhibited action of the Na^+/H^+ exchange under these conditions.

For the purpose of our studies, we used the OK opossum kidney cell line which is derived from the renal cortex of the American opossum (19). These cells manifest classic polarized transport characteristics and retain many of the other differential features of the proximal nephron, making it an interesting model system in which to examine proximal transport pathways. Recently, the mechanisms of cytosolic pH (pH_i) regulation in the OK cell have been characterized. The cell expresses a Na^+/H^+ exchanger that is predominantly segregated in the apical cell membrane and is modulated by a variety of hormones and second messengers (12, 15, 23). Steady-state pH_i in this cell is determined by the exchanger as well as by a K^+-dependent H^+ transport (11, 12).

In this study we address the following three questions: *1*) Is there an effect of acute phosphate deprivation on the Na^+/H^+ exchanger of cultured proximal tubule cells? *2*) If there is any effect on the exchanger, then is it a primary effect or is it secondary to altered activity of the Na^+-K^+-adenosinetriphosphatase (Na^+-K^+-ATPase) during P_i depletion? *3*) Are the changes in the activity of Na^+/H^+ exchange reversible on correction of P_i depletion? Our data show that acute phosphate deprivation results in a reversible inhibition of the Na^+/H^+ exchanger independent of decreased activity of Na^+-K^+-ATPase. This could provide a cellular basis for bicarbonaturia seen in P_i depletion.

METHODS

Cell culture. OK cells were obtained as a gift from Dr. D. Warnock from Birmingham, AL, and were used at passages 30–55. The cells were grown on 24 × 8-mm glass cover slips lying on the bottom of 35-mm Falcon culture dishes with the use of Ham's F-12 + Dulbecco's modified Eagle's medium (DMEM)

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(1:1) supplemented with 14.3 mM $NaHCO_3$, 1.2 mM L-glutamine, 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cultures were maintained at 37°C in 5% CO_2 and were confluent at 5–7 days. Cells were subcultured weekly using 0.1% trypsin and were plated at a cell density of 5×10^4 cells/cm^2. For experiments studying the effect of P_i depletion on regulation of pH_i, cells were incubated for 24 h before the experiment in phosphate-free medium supplemented with 0.1% bovine serum albumin (BSA). This medium, commercially available from GIBCO Laboratories (Grand Island, NY), is otherwise identical in its supplements and composition to the regular DMEM as described above. Control cells were kept for the same time period in serum-free medium containing 1 mM P_i.

pH_i measurements. At *day 7* of culture, the cover slips were removed from medium, washed twice, and incubated at 37°C for 45 min in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer. This buffer was then removed and replaced with fresh HEPES buffer containing 15 μM 2′,7′-bis-(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) for 15 min at 37°C. Cells were then washed four times with prewarmed dye-free HEPES buffer, mounted diagonally in a 1 × 1-cm acrylic fluorometric cuvette, and were placed in the sample compartment of a Perkin-Elmer LS-5 spectrofluorometer. The cuvette volume of 1.5 ml was constantly stirred and was perfused at 3.5 ml/min with HEPES buffer prewarmed at 37°C. After 10 min, fluorescence was measured every minute alternating between 450-nm and 490-nm excitation (5-nm slit size, 4-s integration time/measurement) at 540-nm emission (5-nm slit size). At the completion of each experiment, the fluorescence ratio was calibrated with the nigericin-high K^+ equilibration technique described by Thomas et al. (30). Cells were perfused at pH 7.5 for 15 min and then at pH 6.5 for 15 min with a buffer containing 10 μM nigericin and high concentrations of K^+. We then calculated pH_i values from the 490/450-nm ratio values, assuming a linear response between the two calibration points.

Calculation of H^+ flux and buffering capacity. Proton flux (J_{H^+}) was calculated from the fluorescence measurements of the pH_i recovery rate after acid loading using the NH_4Cl pulse technique (27). The J_{H^+} (in mmol·l^{-1}·min^{-1}) was calculated as the product of buffering capacity (β) and the rate of pH_i recovery

$$J_{H^+} = (dpH/dt) \times \beta$$

where dpH/dt (in pH units/min) is the largest change in cell pH within the first 3 min of the recovery period. Buffering capacity, β (mmol·l^{-1}·pH unit^{-1}) was calculated as the amount of acid load divided by the observed change of pH_i produced by this load. The acid load was estimated as the intracellular NH_4^+ assuming that all NH_4^+ exits the cell as NH_3, giving up H^+ in the process. We calculated NH_4^+ from the last pH_i value in the presence of NH_4Cl (pH_{pre}), assuming a pK of 9.0 and complete equilibration of intra- and extracellular NH_3 at extracellular pH (pH_o) 7.4. The change of pH_i (ΔpH_i) was the difference between pH_{pre} and the nadir value after NH_4Cl withdrawal (pH_{post})

$$\begin{aligned}\beta &= \Delta(NH_4^+)_{in}/\Delta pH_i \\ &= [(NH_3)_{out} \times 10^{pK-pH_{pre}}]/\Delta pH_i \\ &= 0.49 \times 10^{9-pH_{pre}}/(pH_{pre} - pH_{post})\end{aligned}$$

One assumption in measuring β is that there are no acid-base transport systems operative during the time β is being measured. In other words, is a true nadir pH_i actually reached before pH_i starts to recover? This type of error can be magnified in slow perfusion systems because the NH_4Cl concentration outside the cell is not instantaneously reduced to zero. The error in β would be small in the absence of Na^+ because the only intact acid-base transport pathway is the passive H^+ permeability. In the presence of Na^+, the magnitude of the error could be substantially larger. The error can be estimated by extrapolating the pH_i recovery curve backward to obtain a predicted pH_i value at the instant of NH_4Cl withdrawal. In our data, such an estimate indicates that the measured value of pH_i may be underestimated by as much as 40% in the presence of Na^+ and by 5% in the absence of Na^+.

$^{22}Na^+$ uptake experiments. Cells grown in 24-well plates, were equilibrated for 15 min at 37°C in a Na^+-free (0-Na), 145 mM K^+ solution containing 1 μM nigericin. The pH of the buffer was adjusted to equal the desired pH_i. Under these conditions, pH_i will finally equilibrate with pH_o. Cells were then switched to the uptake medium containing (in mM) 140 tetramethylammonium (TMA) chloride, 2 NaCl, 5 KCl, 10 HEPES, 1 $MgCl_2$, and 1.5 $CaCl_2$, pH 7.4, supplemented with 5 mg/ml BSA, 1 mM ouabain, and 2 μCi/ml $^{22}Na^+$ in the absence or the presence of 1 mM amiloride. ^{22}Na uptake under these conditions was linear for 4 min; therefore, in most studies measuring Na^+/H^+ antiporter activity as a function of pH_i, ^{22}Na uptake was measured over 2 min. All uptake studies were done at 37°C. At the end of the uptake period, cells were washed with three rapid washes of 1 ml of ice-cold 100 mM $MgCl_2$ containing 0.1 mM amiloride and 0.1 mM HEPES adjusted to pH 7.4. The radioactivity that had been incorporated into the cells was determined by liquid scintillation counting after digestion of the cells in 1 N NaOH. The rate of amiloride-sensitive $^{22}Na^+$ uptake was defined as the difference in the rate of $^{22}Na^+$ uptake measured in the absence and the presence of 1 mM amiloride.

Na^+-K^+-ATPase activity (enzymatic assay). The Na^+-K^+-ATPase was determined by a modification of the method of Schoner et al. (29) in which the resynthesis of the ATP split by the ATPase is coupled via the pyruvate kinase and lactic dehydrogenase reaction to NADH oxidation. In a cuvette, 50 μl of cell homogenate were mixed with 1 ml of a solution containing 5×10^{-3} M $MgCl_2$, 1×10^{-1} M NH_4Cl, 1.5×10^{-1} M NaCl, and 7.5×10^{-1} M imidazole buffer (pH 7.3), with or without 2.4×10^{-3} M ouabain. After preincubation at 37°C for 20 min the reaction was started by addition of 150 μl of a solution containing 3.5×10^{-4} M NADH, 2.5×10^{-3} M sodium ATP, 5×10^{-4} M phospho*enol*pyruvate (PEP), 4.5 U pyruvate kinase, and 3.6 U lactic dehydrogenase, as well as $MgCl_2$, NH_4Cl, NaCl, and imidazole buffer in one-third of the concentration described above. The Na^+-K^+-ATPase activity was calculated as the ouabain-sensitive part of the total ATPase activity. The cell protein was determined after precipitation with ice-cold 10% trichloroacetic acid (TCA) by the method described by Lowry et al. (21) with BSA as standard.

^{86}Rb uptake. Cells grown to confluence in 24-well plates were rinsed twice with serum-free culture medium. The medium was aspirated and replaced with fresh medium containing ^{86}Rb (1 μCi/ml) with or without 0.5 mM ouabain. After 15 min, the reaction was stopped by washing with ice-cold (4°C) solution containing 0.1 M $MgCl_2$. The incorporated radioactivity was measured by scintillation counting.

Chemical determination of intracellular P_i concentration ($[P_i]_i$). Cells grown to confluence were released from tissue culture plates by trypsin-EDTA, then washed two to three times with NaCl-HEPES buffer, pH 7.4. Phosphate concentration in the solution, in the form of KH_2PO_4, was adjusted to correspond to the phosphate concentration in the culture medium during the preincubation period. The cells were incubated for 30 min at 37°C in the NaCl-HEPES solution containing 2 μCi/ml [^{14}C]inulin (13). The wet weight was then determined after the cells were collected by a 10-s centrifugation. The pellets were

dried by vacuum, and the dry weight was determined. The pellets were then dissolved in 1 ml of 10% perchloric acid at 0°C. The ice-cold temperature was necessary to ensure minimal breakdown of organic phosphate compounds during the dissolution procedure. The denatured protein was precipitated by rapid centrifugation, and samples of 100 μl were used to determine the radioactive counts of the remaining [^{14}C]inulin. The supernatant was then neutralized by careful dropwise addition of 3 M K_2CO_3 in 0.5 M triethanolamine. P_i was measured in the deproteinized neutralized extracts using a selective colorimetric assay (4).

Cell water of each sample was calculated by substracting the inulin space from the total water content which was obtained as the difference between the wet and dry weights. Cell water was 3.15 ± 0.19 μl/mg dry weight, and this value was used to calculate the $[P_i]_i$ (in mM). All determinations were performed in quadruplicates, and the experiment was carried out seven times.

Chemical determination of intracellular Na^+ concentration ($[Na^+]_i$). Cells preincubated in different concentrations of P_i were released from tissue culture plates by trypsin-EDTA and then washed two to three times with NaCl-HEPES buffer pH 7.4, which contained the corresponding P_i concentration. Wet and dry weights of the pellets as well as [^{14}C]inulin space were determined as described in the previous section. Dry pellets were dissolved in 1 ml of 2 M HNO_3. Samples (100 μl) were used to determine the Na^+ and K^+ content by flame photometer (Instrumentation Laboratory model 943). The extracellular Na^+ and K^+ concentrations were subtracted from the total content of the electrolytes in the samples. This fraction representing intracellular Na^+ and K^+ was divided by the value of cell water (calculated as described above) to yield values for $[Na^+]_i$ and $[K^+]_i$ (in mM).

ATP content of cells. The ATP content of the cells was measured by the methods of Ashcroft et al. (2a) and Lundin et al. (22). Cells were released from tissue culture flasks by trypsin-EDTA and then placed in Krebs-Ringer-bicarbonate (KRB) incubation medium for 30 min. At the end of the incubation, 200 μl of the medium containing the cells were mixed with TCA with a final concentration of 2.5%. The tube containing the mixture was immersed in liquid nitrogen and was stored at −70°C. On the day of the assay, the mixture was thawed and neutralized with 2 N $KHCO_3$. Samples of 50 μl of the mixture were assayed for ATP. The ATP measurements were made by the firefly luminescence assay with the LAD 535 Luminometer (Turner Design, Sunnyvale, CA). ATP standards were prepared with KRB medium and contained the same amount of TCA and bicarbonate as did the cell samples.

Buffers. The buffers used are listed in Table 1. BCECF-AM and valinomycin were added from 1 mM stock solutions in dimethyl sulfoxide (DMSO), resulting in final DMSO concentrations of 1% and 0.1%, respectively. NaCl-HEPES solutions contained P_i in the form of 1 mM Na_2HPO_4 while 0-Na HEPES solutions contained 1 mM KH_2HPO_4. In experiments where P_i-depleted cells were studied, no phosphate was added to the solution. pH of the solutions was adjusted with 1 N NaOH except for 0-Na solution, in which case 1 N KOH was used.

Table 1. *Buffers*

	HEPES	0-Na, HEPES	0-Na, high K, HEPES	0-Na, NH_4Cl	Calibration
NaCl	135				15
KCl	5	5	50	5	125
HEPES acid	10	5	5	5	10
$CaCl_2$	1	1	1	1	1
$MgCl_2$	0.5	0.5	0.5	0.5	0.5
TMA-Cl		145	95	125	
NH_4Cl				20	
Glucose	10	10	10	10	10

All concn are in mM. 0-Na, zero Na^+ concentration.

Chemicals. Culture supplies were obtained from GIBCO Laboratories. Nigericin, 4,4′-diisothiocyanostilbene-2,2′-ethanesulfonic acid (DIDS), and ouabain were purchased from Sigma Chemical (St. Louis, MO). BCECF was obtained from Molecular Probes (Eugene, OR) and amiloride was from Merck Sharp & Dohme (West Point, PA). $^{22}Na^+$ and ^{86}Rb were purchased from Amersham International (Arlington Heights, IL), and tritiated alanine was obtained from New England Nuclear (Boston, MA). All reagents were of the highest purity commercially available.

Statistical analysis. Results are means ± SD. Comparisons were performed by analysis of variance. Interactions of treatments were analyzed by two-factor variance analysis. $P < 0.05$ was considered significant.

RESULTS

The present study deals with the acute effect of different concentrations of extracellular P_i on regulation of pH_i in the OK cell line. As an initial step of our study, we had to address the following two questions: *1*) Can these cells become P_i depleted by modulating P_i concentration in the medium? *2*) Can P_i-depleted cells maintain viability, and is the P_i depletion state reversible?

Table 2 shows time-sequential changes in $[P_i]_i$ and in ATP level when the cells were incubated for 24 h either in phosphate-free medium or in 1 mM P_i. Both $[P_i]_i$ and ATP levels dropped significantly already after 2 h of incubation in phosphate-free medium. After 24 h in 0 mM P_i, ATP level in the cells reached ~15% of the level observed in control cells (incubated in 1 mM P_i).

The P_i extraction and assay procedures as used in our study were shown to be highly selective for P_i and do not hydrolyze major organic compounds such as ATP (4).

Table 2. *Influence of different concentrations of P_i in culture media on $[P_i]_i$ ATP content and cell proliferation*

	Medium $[P_i]$	
	1 mM	0 mM
$[P_i]_i$, mM		
2 h	1.27±0.05	0.74±0.06*
4 h		0.71±0.05*
24 h	1.24±0.06	0.63±0.04*
ATP, μg/mg protein		
2 h	2.69±0.08	1.17±0.13*
4 h	2.68±0.14	1.40±0.143*
16 h	2.9±0.1	1.01±0.06†
24 h	2.85±0.15	0.41±0.01†
Cell protein, mg/well	0.51±0.05	0.48±0.012
Cell number, ×10^6/well	1.92±0.07	1.85±0.05

Values are means ± SD (n = 4) from 7 independent experiments. Confluent OK cells were preincubated in a medium containing either 1 mM P_i or 0 mM P_i for the indicated time periods. Intracellular P_i concentration ($[P_i]_i$) and ATP levels were then measured as described in METHODS. For measurement of cell protein, cells were grown in 6-well plates (1 well = 9.6 cm^2) and incubated for 24 h either in 1 mM P_i or 0 mM P_i. Protein content was determined following the procedure described by Lowry et al. (18), and were P_i deprived for 24 h before experiment. For the purpose of counting cells under the same conditions, cells were grown in 24-well plates (1 well = 2 cm^2), and were under P_i-deprived conditions for 24 h before experiment. * $P < 0.05$ compared with control (1 mM P_i). † $P < 0.01$ compared with control.

Furthermore, we ascertained the validity of this procedure by using another control experiment. OK cells preincubated for 24 h in 1 mM P_i were incubated for 30 min with [^{14}C]inulin (see METHODS). During the 30-min incubation period, the cells were exposed to 5 mM fructose replacing 5 mM glucose in the solution. (At all times, the solutions contained 1 mM P_i). It has been clearly established that, during very short exposure to fructose, there is a rapid depletion of ATP and a fall in cellular P_i. This results from accumulation of fructose 1-phosphate and reduced synthesis of ATP from ADP in mitochondria (24). Our studies showed that acute (30 min) exposure of the cells to fructose lowered $[P_i]_i$ from a control value of 1.25 ± 0.06 to 0.53 ± 0.02 mM ($P < 0.01$). This remarkable reduction in $[P_i]_i$, which is expected from the known property of fructose, is another indication for the validity of our procedure.

When cells incubated for 24 h in phosphate-free medium were switched to a medium containing 1 mM P_i, $[P_i]_i$ showed a remarkable recovery. Thus, after 1 h of reinstituting normal P_i (1 mM) in the incubation medium, $[P_i]_i$ rose from 0.66 ± 0.06 mM (the level observed after 24 h exposure to phosphate-free conditions) to 1.15 ± 0.04 mM ($P < 0.05$). This fast correction of $[P_i]_i$ can be ascribed to a marked increase in the activity of the Na^+-dependent phosphate transport system in these cells (i.e., adaptive response to phosphate deprivation) (8). It appears, therefore, that the state of P_i depletion achieved by incubating the cells for 24 h in P_i-free medium is reversible. Moreover, viability of the cells while being exposed for 24 h to phosphate-free conditions was higher than 95% as judged by trypan blue exclusion. Also, the data presented in Table 2, which show that cell protein and cell number in phosphate-free medium were the same as in controls, argue against major cytotoxicity of the phosphate-free conditions.

Steady-state pH_i and buffering power. Resting pH_i in P_i-depleted cells (for 24 h) did not differ from pH_i in control cells (incubated in 1 mM P_i). pH_i in HEPES medium (pH 7.4) was 7.15 ± 0.21 and 7.22 ± 0.26 in control and P_i-depleted cells, respectively [P = not significant (NS); $n = 7$]. The use of inhibitors of anion transport was not associated with any changes in basal pH_i either in the control or in P_i-depleted cells. Exposure of control cells for 10 min to 1 mM DIDS resulted in pH_i values of 7.15 ± 0.21 (pre) and 7.05 ± 0.12 (post) (P = NS). P_i-depleted cells exposed for 10 min to the same inhibitor showed pH_i values of 7.24 ± 0.17 (pre) and 7.18 ± 0.12 (post) (P = NS). These results are in accord with data from other investigators showing that in OK cells HCO_3-transporting mechanisms do not play a role in the determination of resting pH_i (12, 17).

P_i-depleted cells (24 h) manifested a significant reduction in buffering power compared with control cells ($\beta = 26.8 \pm 1.12$ vs. 18.7 ± 0.64 mmol·l^{-1}·pH unit^{-1} in control and P_i depletion, respectively; $P < 0.01$). This finding is not unexpected considering the role played by intracellular phosphate compounds in titrating acid loads.

Studies on recovery from acid loads in control and P_i-depleted cells. To study the activity of the Na^+/H^+ exchanger in the OK cells, we measured the recovery from an acid load using the NH_4Cl pulse technique (Fig. 1). Cells were exposed to Na^+-free NH_4Cl solution (see Table 1). After 15 min, the medium was replaced by a Na^+-free solution without NH_4Cl. The removal of NH_4Cl resulted in a rapid acidification of the cells. In the 0-Na conditions minimal recovery from acid load was observed. However, on addition of NaCl medium, rapid cell alkalinization took place that was almost completely abolished by amiloride (Fig. 1*A*). This rapid recovery from acid load, which is Na^+ dependent and amiloride sensitive, is therefore caused by the Na^+/H^+ exchange. The rate of pH_i recovery during the first 30 s was determined as dpH_i/dt. In cells incubated for 24 h in control conditions (1 mM P_i) dpH_i/dt in NaCl medium was 0.25 ± 0.02 ΔpH/min ($n = 6$) and was close to 90% inhibitable by 0.1 mM amiloride (Fig. 1*A*). In cells incubated for 24 h in phosphate-free conditions (Fig. 1*B*) dpH_i/dt in NaCl medium was substantially reduced ($dpH_i/dt = 0.08 \pm 0.003$ ΔpH/min; $P < 0.05$ vs. control cells, $n = 6$). The alkalinization under these conditions was also almost completely abolished by amiloride (not shown). The different pH_i recovery rates between control and P_i-depleted cells could not be ascribed to differences in pH_i at the start of the recovery period, because nadir pH_i after withdrawal of NH_4Cl was not significantly different between the two conditions (6.38 ± 0.05 vs. 6.41 ± 0.07 in control and P_i-depleted cells, respectively; P = NS).

Because the buffering power, β, is markedly reduced in P_i-depleted cells, J_{H^+} ($\beta \times dpH_i/dt$) averaged 1.48 ± 0.01 mmol·l^{-1}·min^{-1} in P_i depletion compared with J_{H^+} of 6.7 ± 0.04 mmol·l^{-1}·min^{-1} in control cells ($P < 0.05$, $n = 6$). Thus by expressing Na^+/H^+ exchange activity as J_{H^+}, the difference between control and P_i-depleted cells becomes

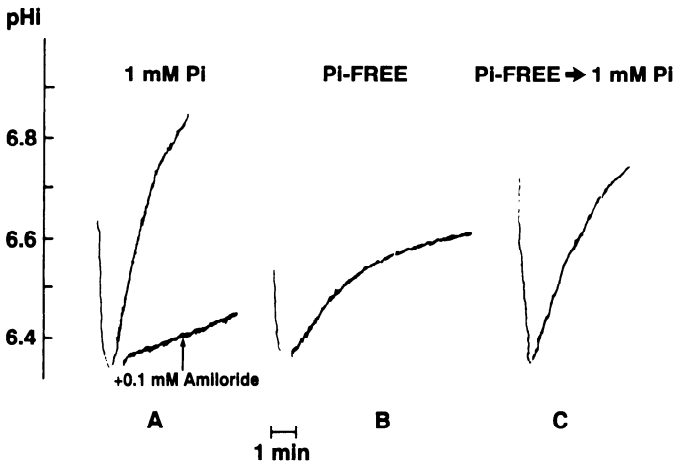

Fig. 1. Acute P_i depletion leads to impaired activity of Na^+/H^+ exchange in OK cells; the effect is reversible. OK opossum kidney cells were preincubated for 24 h in serum-free medium containing 1 mM P_i (*A*) or zero P_i (*B*). In a third group (*C*), cells were reincubated in 1 mM P_i for 1 h following 24 h incubation in phosphate-free medium. On the day of experiment, cells were loaded with 2′,5′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and then acidified by exposure to zero Na^+ (0-Na) NH_4Cl solution followed by removal of this medium and addition of 0-Na HEPES solution (NH_3 prepulse technique). After pH_i reached its nadir, high-NaCl solution (140 mM) was added (HEPES). Change in fluorescence was measured and calibrated to estimate intracellular pH (pH_i). Separate samples of cells were added to NaCl medium also containing 0.1 mM amiloride. This experiment represents 1 of 6 experiments.

even more evident. Interestingly, in cells becoming P_i depleted after 24-h incubation in phosphate-free media and which were subsequently switched to a medium containing 1 mM P_i for 1 h, there was almost complete recovery of the activity of Na^+/H^+ exchange (Fig. 1*C*). Under these conditions, dpH_i/dt was 0.21 ± 0.03 ΔpH/min, and J_{H^+} was 5.62 ± 0.05 mmol·l^{-1}·min^{-1} (n = 6).

Figure 2 describes changes in Na^+/H^+ exchanger activity during P_i depletion achieved by acute exposure of the cells to fructose. This effective maneuver for acute depletion of $[P_i]_i$ in kidney and liver cells (24) has been shown to cause a significant drop in $[P_i]_i$ in our cells (see above). When cells were incubated for 30 min in a medium containing 1 mM P_i and 5 mM fructose (substituted for glucose) there was ~90% inhibition of exchanger activity as assessed by recovery of pH_i in Na^+-containing medium (dpH_i/dt = 0.27 ± 0.02 vs. 0.04 ± 0.003 ΔpH/min in control conditions and cells exposed to fructose, respectively: n = 4, $P < 0.05$). When fructose was combined with P_i surfeit (Fig. 2*C*), the inhibitory effect of fructose on the exchanger activity was almost completely abrogated (dpH_i/dt = 0.19 ± 0.03 ΔpH/min).

Intracellular protons play an important regulatory role in activating the Na^+/H^+ exchanger (2). Our finding, that acute P_i depletion virtually abolishes the ability of the exchanger to be activated by acid pH_i, led us to examine its effect on intracellular proton sensitivity and the maximal activity of the exchanger. In these experiments, described in Fig. 3, we compared the effects of normal P_i conditions (1 mM) and phosphate-free conditions on the ability of the cells to activate the exchanger in response to graded reductions in pH_i. Reductions in pH_i were induced by preincubating the cells with increasing concentrations of NH_4Cl (up to 40 mM) for 15 min prior to its rapid removal. The initial rate of pH_i recovery was measured in NaCl-HEPES medium following withdrawal of NH_4Cl. As expected, pH_i recovery and J_{H^+} increased with increasing acid loads. In control cells, half-maximal effect on

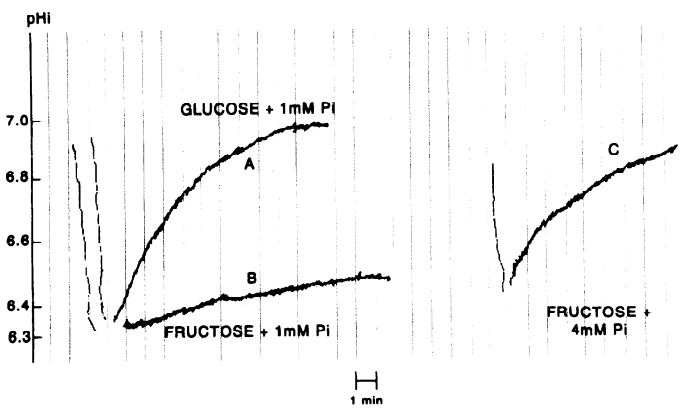

Fig. 2. OK cells were preincubated for 24 h in serum-free medium containing 1 mM P_i. On the day of the experiment, incubation medium was aspirated and replaced with identical medium containing one of the following: *1*) 1 mM P_i and 5 mM glucose (*A*), *2*) 1 mM P_i and 5 mM fructose replacing glucose (*B*), *3*) 4 mM P_i and 5 mM fructose (*C*). Cells were incubated under these conditions for 15 min and then loaded with BCECF and washed with 0-Na NH_4Cl buffer. Solutions used for these procedures contained the same P_i and glucose or fructose concentrations as described above. Recovery of pH_i in NaCl medium following NH_4Cl withdrawal was measured as described in Fig. 1. This experiment represents 1 of 4 similar experiments.

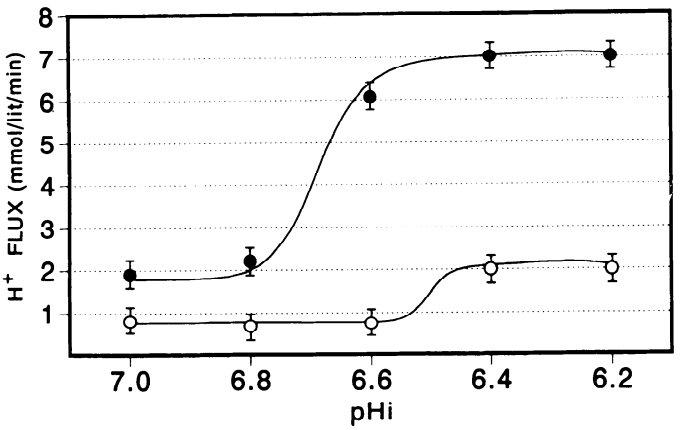

Fig. 3. OK cells were preincubated for 24 h in serum-free medium containing 1 mM P_i (solid circles) or 0 mM P_i (open circles). On the day of experiment, cells were loaded with BCECF and then preincubated at 37°C for 15 min with increasing concentrations (0–40 mM) of NH_4Cl (in Na^+-free solution). Cells were acidified by rapid removal of NH_4Cl solution and rinsing cells with Na^+-free NH_4Cl-free solution. After pH_i reached its nadir, NaCl-HEPES solution was added, and initial rate of pH_i recovery (dpH_i/dt) was determined. Proton flux (J_{H^+}) was calculated as product of dpH_i/dt and buffering capacity and then plotted against pH_i. Data are means ± SD (n = 3) and are typical of 3 similar experiments.

exchanger activity occurred at a pH_i of ~6.7. By contrast, in P_i-depleted cells, half-maximal activity of the exchanger occurred at an approximate pH_i of 6.5, indicating a reduction in the sensitivity of the exchanger to increasing concentrations of intracellular protons (i.e., shift in the set point of the transporter towards a more acidic pH_i). This effect on intracellular proton sensitivity was accompanied by a marked reduction in the maximal activity of the exchanger. Under conditions that caused maximal stimulation of the exchanger (pH_i ~6.2), the activity of the exchanger in P_i-depleted cells was reduced by ~75% compared with control cells (Fig. 3).

Time course analysis for the inhibitory effect of P_i depletion on J_{H^+} mediated by the Na^+/H^+ exchanger is described in Fig. 4. Already after 2 h of incubation in

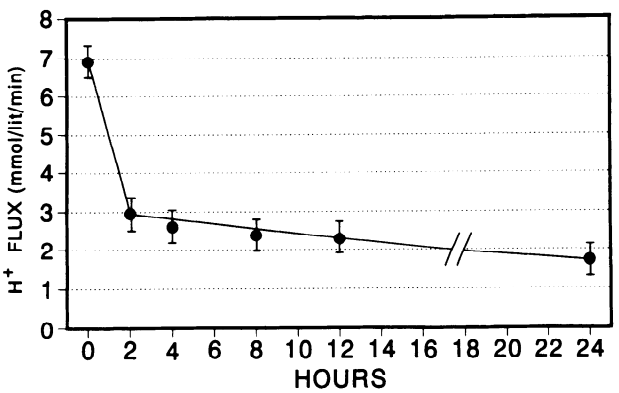

Fig. 4. Time course studies on inhibitory effect of P_i depletion on Na^+/H^+ exchange. OK cells were preincubated for indicated time periods in serum-free medium containing 1 mM P_i or 0 mM P_i. The cells were then loaded with BCECF and acidified by NH_3 prepulse following the same procedure as described in Fig. 1. On addition of NaCl, pH_i recovery in P_i-depleted cells was compared with control cells. J_{H^+} at indicated time points was calculated by multiplying initial rate of pH_i change by buffering power. Results are means ± SD (n = 3) from 8 independent experiments. * $P < 0.01$.

phosphate-free medium (which was associated with significant reduction in $[P_i]_i$ as shown in Table 2), P_i-depleted cells manifested ~60% reduction in J_{H^+} compared with control cells ($P < 0.01$). There was a continuous decrease in Na^+/H^+ activity thereafter, and at the end of 24-h incubation in phosphate-free media, J_{H^+} was 20% of the control value.

In Fig. 5 we studied the recovery of pH_i in Na^+-free conditions. As stated above, there is only minimal recovery in the absence of Na^+. The dpH_i/dt under these conditions was 0.022 ± 0.003 ΔpH/min in control cells (incubated for 24 h in 1 mM P_i) (Fig. 5*A*) and 0.029 ± 0.004 ΔpH/min in P_i-depleted cells (Fig. 5*B*). When one considers the difference in buffering power between control and P_i-depleted cells, the J_{H^+} ($\beta \times dpH_i/dt$) by the Na^+-independent pathway is not different between the two conditions (J_{H^+} = 0.58 ± 0.03 and 0.54 ± 0.04 $mmol \cdot l^{-1} \cdot min^{-1}$ in control and P_i-depleted cells, respectively; P = NS, $n = 4$).

The Na^+-independent pH_i recovery was augmented when cells were exposed to a high-K^+-containing solution (Fig. 5, *A* and *B*). This is in accord with the observations made by other investigators studying pH_i regulation in the OK cells (11, 12). The "Na^+-independent component of pH_i recovery" could thus represent either voltage-dependent H^+-conductive pathway or a K^+/H^+ exchange mechanism. At any rate, this component of pH_i recovery was not different between control and P_i-depleted cells.

The data presented in Figs. 1, 4, and 5 suggest that there are two components of the pH_i recovery from acid load in OK cells. The Na^+-dependent recovery (representing Na^+/H^+ exchange) is markedly inhibited in P_i-depleted cells in a reversible fashion, whereas the Na^+-independent pH_i recovery is not different between control and P_i-depleted cells.

$^{22}Na^+$ flux studies. We studied the activity of Na^+/H^+ exchange in control and P_i-depleted cells by using another measure of the exchanger, namely, amiloride-sensitive ^{22}Na uptake. pH_i was clamped at 6.4 and 6.9 by using the high-K^+-nigericin technique. As demonstrated in Fig. 6, at each given pH_i, amiloride-sensitive ^{22}Na uptake was substantially low in P_i-depleted cells compared with control cells. As expected, at pH_i 6.9, ^{22}Na uptake in each condition (control and P_i depletion) was significantly less than the corresponding condition at pH_i 6.4. However, there was still a marked difference between control and P_i-depleted cells at pH_i 6.9.

Na^+-K^+-ATPase activity. The luminal Na^+/H^+ exchanger in proximal tubular cells is a secondary active transporter that uses the energy from the transmembrane sodium gradient generated by the basolateral Na^+-K^+-ATPase activity. Thus any disturbance of this ion gradient can influence the transport rate of the exchanger. It was, therefore, necessary to study the activity of the Na^+-K^+-ATPase under control and P_i depletion. In doing so, we will be able to determine whether the reduced activity of the Na^+/H^+ exchange in P_i depletion is due to altered transmembrane sodium gradient or whether it is independent of modified function of the Na^+ pump.

The Na^+-K^+-ATPase function was first assayed by its hydrolytic activity. Figure 7 shows that the cellular ATPase activity was significantly decreased following 24-h incubation in phosphate-free medium. This finding is not unexpected considering the marked reduction in cellular ATP content under these conditions (Table 2). The first significant effect of P_i depletion on the ATPase activity was observed after 18 h of incubation in P_i-free medium. The enzymatic assay of Na^+-K^+-ATPase assesses the maximal activity or the total number of active pumps in the cells. To get a better insight into the influence of P_i depletion on the Na^+ pump, it was necessary to study how P_i depletion alters the function of existing pump units, an effect that could manifest itself faster than any influence on pump number. To this end we measured in control and P_i-depleted cells the ouabain-inhibitable ^{86}Rb uptake, a measure of the K-transporting rate of the pump. Figure 8 shows time-course studies of ^{86}Rb uptake in P_i-depleted cells. After 24 h of incubation in phosphate-free medium, ^{86}Rb uptake was 35,558 ± 2,500 compared with 68,129 ± 3,400 $counts \cdot min^{-1} \cdot well^{-1}$ observed in control cells. ($P < 0.01$, $n = 6$). At 2 and 6 h of incubation in phosphate-free

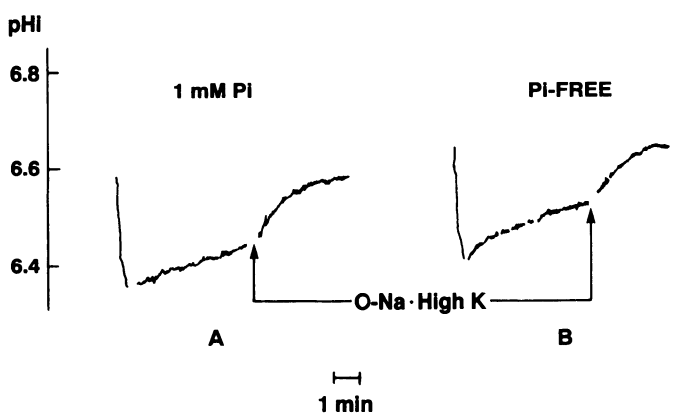

Fig. 5. Na^+-independent pH_i recovery in OK cells is unaffected by P_i depletion. OK cells were preincubated for 24 h in serum-free medium containing 1 mM P_i (*A*) or 0 mM P_i (*B*). On the day of experiment, cells were loaded with BCECF and then acidified by exposure to 0-Na NH_4Cl solution followed by removal of this medium and addition of 0-Na HEPES solution. Where indicated, 0-Na, high-K solution containing 1 μM valinomycin was added. Change in fluorescence was measured and calibrated to estimate pH_i. This experiment represents 1 of 4 similar experiments.

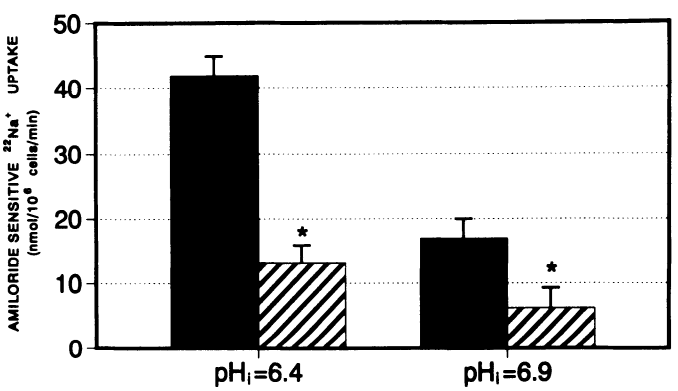

Fig. 6. OK cells were preincubated for 24 h in serum-free medium containing 1 mM P_i (solid bars) or zero phosphate (hatched bars). On the day of experiment, pH_i of cells was clamped at values of 6.4 and 6.9 by using high-K^+ nigericin technique. ^{22}Na uptake was then measured as described in METHODS. Results are means ± SD ($n = 4$) from 4 independent experiments. * $P < 0.05$.

conditions, there was only slight but nonsignificant reduction in ouabain-sensitive ^{86}Rb uptake compared with control.

$[Na^+]_i$ values. Table 3 describes the changes in $[Na^+]_i$ taking place in P_i-depleted cells. As expected, the decline in ATP and the resultant diminution in Na^+-K^+-ATPase activity during P_i depletion resulted in altered Na^+/K^+ ratio in the cells. A significant elevation in $[Na^+]_i$ was observed after 4 h of incubation in phosphate-free medium.

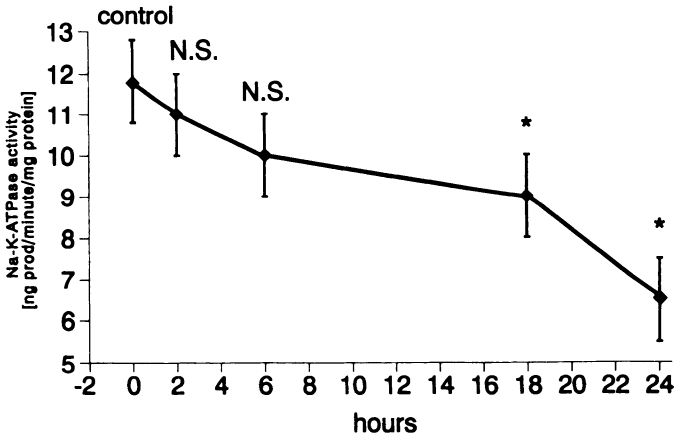

Fig. 7. Time course analysis of reduced Na^+-K^+-ATPase activity by P_i depletion. OK cells were preincubated for indicated time periods in serum-free medium containing 1 mM P_i or 0 mM P_i. At each time point, enzymatic assay of the pump was performed as described in METHODS. Results are means ± SD ($n = 4$) from 6 independent experiments. * $P <$ 0.01. NS, not significant. Na^+-K^+-ATPase activity in control cells did not change significantly over 24 h.

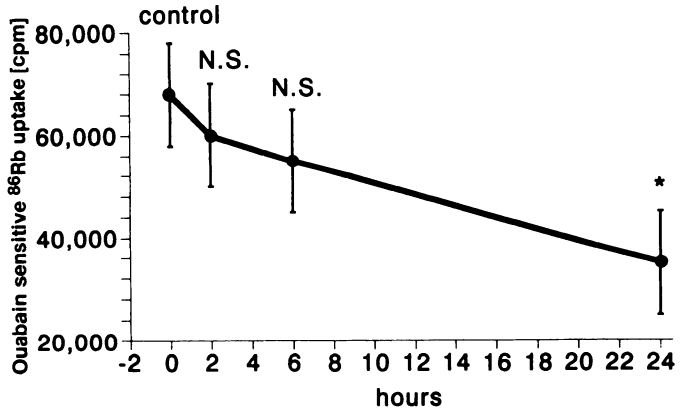

Fig. 8. OK cells were preincubated for indicated time periods in serum-free medium containing 1 mM P_i or 0 mM P_i. At each time point, ouabain-sensitive ^{86}Rb uptake was measured as described in METHODS. Results are means ± SD ($n = 4$) from 6 separate experiments. * $P <$ 0.01. ^{86}Rb uptake in control cells did not change significantly over 24 h.

Table 3. *Effect of P_i depletion on intracellular $[Na^+]$ and $[K^+]$*

	$[Na^+]_i$, mM	$[K^+]_i$, mM
Control	28±4.8	126±6.5
2 h	35±3.9	115±4.7
4 h	46±4.2*	102±3.2*
24 h	52±3.2*	90±5.5*

Values are means ± SD ($n = 4$) from 3 independent experiments. OK cells were preincubated in P_i-free medium for 2, 4, and 24 h. At each of these time points, cells were released from tissue culture flasks, and intracellular concentrations of Na^+ ($[Na^+]_i$) and K^+ ($[K^+]_i$) were determined as described in METHODS. Values obtained are compared with control cells that were incubated in 1 mM P_i for 24 h. * $P < 0.05$ vs. control.

DISCUSSION

The results described in this paper suggest that, when proximal tubular cells in culture are acutely deprived of phosphate (either by hours of incubation in P_i-free media or by minutes of exposure to fructose), the Na^+/H^+ exchanger is substantially inhibited. The inhibitory effect of P_i depletion on the exchanger was reversible. Thus when the cells were deprived of phosphate for 24 h and then acutely switched to a medium containing 1 mM P_i, there was almost a complete recovery of the Na^+/H^+ exchanger. This reversibility phenomenon was already apparent 1 h after replenishing the medium with P_i, which corresponds to restoration of $[P_i]_i$ under these circumstances. This observation is consistent with the adaptation of the phosphate transport process in phosphate-deprived proximal tubular cells (8). The adaptation results from an upregulation (i.e., increased protein synthesis) of the Na-P_i cotransport system after a 24-h period of phosphate depletion. The upregulated transport system will very rapidly replenish cellular stores of $[P_i]_i$ following the period of P_i deprivation once the cells are reexposed to phosphate in the cultured media. The repletion of $[P_i]_i$ probably normalizes the activity of the Na^+/H^+ exchanger.

The rapid recovery of the exchanger activity on P_i repletion, as well as other indexes for cell viability used in our study, indicate that the OK cells can survive for at least 24 h incubation in phosphate-free medium. In contrast to the OK cells, other proximal tubular cells show high degree of sensitivity to P_i depletion states. Thus freshly isolated rat proximal tubular cells show signs of cell injury (assessed by enhanced Ca^{2+} uptake and lactate dehydrogenase release) already following 40 min of exposure to P_i-free buffer (1). Aside from species variability, a possible explanation for this disparity may relate to the fact that freshly prepared tubular cells require high levels of energy because these cells are able to transport Na^+ vectorially. Cultured cells, on the other hand (e.g., OK cells), may be less active in transporting Na^+ vectorially and therefore will depend less on ATP supply than the freshly isolated tubules. Hence, a sharp drop in ATP during P_i depletion will damage the freshly isolated proximal tubules to a higher degree than cultured cells. A support to this notion is provided by the observation that another cultured cell system, LLC-PK_1, is also kept viable during several hours of ATP depletion (6). An alternative explanation to the differences in cell susceptibilities to P_i depletion lies in the fact that the medium used to incubate our cells under P_i-free conditions contained all the necessary nutrients including 0.6 mM glycine. The latter has been shown to protect proximal tubular cells from the damage induced by P_i depletion even though it does not prevent the fall in ATP under these circumstances (1).

The attenuated activity of the Na^+/H^+ exchanger activity in P_i depletion could theoretically result from diminished activity of the Na^+-K^+-ATPase. The Na^+/H^+

exchanger is a secondary active Na^+ transporter that harnesses energy from the transmembrane gradient generated by the active extrusion of Na^+ by the Na^+-K^+-ATPase. Since acute P_i depletion results in major depletion of cellular ATP content and impaired activity of the Na^+ pump, one could argue that accumulation of Na^+ inside the cell is responsible for slowing down the rate of the Na^+/H^+ exchanger. Our data, however, indicate that reduced transmembrane Na^+ gradient could not account for the inhibitory effect of P_i depletion on Na^+/H^+ exchanger activity. First, at a time when there was already 60% reduction in the exchanger activity (2-h incubation in phosphate-free medium), there was only a slight but nonsignificant elevation in $[Na^+]_i$. Second, for the experiments used to study the activation of the exchanger by intracellular acidosis, cells were incubated and washed several times in Na^+-free medium. Such has been the case both in the pH_i recovery assays (Figs. 1-4) and in the ^{22}Na flux studies (Fig. 6). The Na^+-free conditions would be expected to lower $[Na^+]_i$ to a significant degree. Thus our findings that P_i depletion attenuates the Na^+/H^+ exchanger activity under these conditions are consistent with an effect being independent of altered Na^+ gradients. Most importantly, time-course analysis revealed that the inhibition of Na^+-K^+ pump lagged several hours behind the reduced activity of the exchanger. Taken together, these data indicate that P_i depletion affects Na^+/H^+ exchanger independent of its inhibitory influence on the Na^+-K^+-ATPase.

It is conceivable that the intrinsic effect of P_i depletion on the Na^+/H^+ exchanger activity involves the phosphorylation state of this transporter. Stimulation of the Na^+/H^+ exchanger by intracellular acidification or osmotic stress has been observed to be attenuated in cells depleted of ATP, suggesting a role for kinase-mediated phosphorylation (6, 7, 14, 20). Also, the Na^+/H^+ exchanger residing in the apical membrane of the proximal tubule (including OK cells) has been shown to be regulated via protein phosphorylation carried out by specific protein kinases (PKC, PKA, calmodulin-dependent kinase) (16, 31). Recently, Sardet et al. (28) demonstrated phosphorylation of the exchanger in intact cells after incubation with a variety of growth factors. Since in our cells, P_i depletion leads to a reduction in exchanger activity in the absence of agonists, we speculate that under physiological conditions there is a tonic state of phosphorylation of the exchanger. In P_i depletion states, dephosphorylation of the exchanger (or a regulatory protein) may, therefore, take place and inhibit its activity both under basal conditions and during intracellular acidosis.

Our studies show that P_i depletion attenuates the Na^+/H^+ exchanger activity by shifting its dependence on the intracellular H^+ concentration to a more acidic pH level ("acid shift" of the transporter set point). Since the exchanger is allosterically activated by intracellular protons, this finding suggests that phosphorylation processes influence the degree to which allosteric mechanisms affect exchanger activity. In addition to the change in pH_i dependence, P_i depletion also reduces the maximal activity of the exchanger. This is also reflected by the fact that the inhibitory effect of P_i depletion was evident at all pH_i values examined. A combined effect of P_i depletion on both pH_i dependence and maximal activity of the exchanger has been also demonstrated in cultured rat aortic smooth muscle (20) and in LLC-PK_1 cells (6). It is possible that there are multiple sites of protein phosphorylation that would then explain a combined defect [i.e., pH_i dependence and maximal transport rate (V_{max})] in P_i depletion states. Alternatively, reduced V_{max} by P_i depletion could result from alteration in the rate of insertion of exchanger units from internal membrane sources into the cell membrane. Interestingly, despite the fact that P_i depletion inhibited the exchanger activity not only at acid pH_i but also at pH_i near resting levels (Fig. 3), there was not a difference in steady-state pH_i between control and P_i-depleted cells. It is thus possible that even though the exchanger activity is impaired, metabolic acid production by P_i-depleted cells is reduced at pH_i values near neutrality. This, in turn, will maintain resting pH_i in P_i-depleted cells at a value that is close to pH_i in control cells. Alternatively, it is possible that P_i depletion has in fact an initial acidifying effect on resting pH_i. However, this is not observed at steady-state conditions because other pH_i regulatory mechanisms will come into play to restore pH_i towards its original value. In the OK cells this function could be theoretically fulfilled by passive H^+ leak pathways.

The Na^+/H^+ exchanger serves as a major mechanism for proton secretion and reclamation of bicarbonate in the proximal tubule (5, 26). Therefore, impaired activity of the exchanger by acute P_i depletion may provide the cellular basis for the in vivo finding of impaired proximal reabsorption of bicarbonate and bicarbonaturia observed in experimental dietary deprivation of phosphorus (9, 10). P_i depletion accompanying hyperparathyroidism could also play a partial role in the inhibited function of the Na^+/H^+ exchanger and bicarbonaturia seen occasionally in parathyroid hormone excess states (3, 16, 25).

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Address for reprint requests: J. Green, Div. of Nephrology, Cedars-Sinai Medical Center, BB-220, 8700 Beverly Blvd., Los Angeles, CA 90048.

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