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Phosphate deprivation inhibits NH_4^+ transport in OK cells

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Deprivation of dietary phosphate leads to a decrease in urinary excretion of ammonium in rats which may be mediated through an alteration in ammonium transport in the proximal tubule. In the present study the OK renal epithelial cell line, a model for the proximal tubule, was used to determine if NH_4^+ transport was changed during acute phosphate deprivation. The intracellular pH after perfusion with NH_4Cl solution was used for calculation of intracellular NH_4^+ concentration. Intracellular [NH_4^+] increased linearly during the first 2 min of acidification with NH_4Cl. NH_4^+ transport, defined by the initial rate of the increase in intracellular [NH_4^+], was decreased by 32% ($P < 0.005$) in phosphate-deprived cells. This transport process was inhibited by barium chloride, but not by DIDS, amiloride or ouabain, suggesting that the NH_4^+ transport pathway may utilize K^+ channels. Acute phosphate deprivation may inhibit NH_4^+ transport in OK cells by decreasing membrane K^+ permeability.

Introduction

Acute and chronic deprivation of dietary phosphate is associated with alterations in several organs including the kidney. The changes in renal function are accompanied by a fall in plasma phosphate, elevated plasma calcium, decreased urinary excretion of phosphate and increased urinary calcium excretion [1]. Chronic phosphate deprivation also leads to increased bicarbonate excretion [2] and decreased excretion of ammonium [1].

The proximal tubule is the major site of renal ammonia production and this production is regulated by acid-base status [3]. One mechanism by which NH_3 excretion is decreased during phosphate deprivation may be a decrease in renal production [4]. It has been proposed that the decrease in NH_3 production is due to a rise in intracellular pH. While increased intracellular pH occurs in liver and muscle in response to phosphate deprivation, it is not clear if a similar change occurs in the kidney [5]. Initial NMR studies, for example, indicate that intracellular pH of proximal tubules decreases during acute phosphate deprivation [6]. Another mechanism which could contribute to decreased excretion of ammonia is a change in membrane transport which would lead to a decrease in the rate of ammonia exit from the proximal tubule cell.

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Diffusion trapping may not account for all ammonia secretion. There may be a transport process in the proximal tubule which preferentially secretes NH_4^+ across the apical plasma membrane into the tubular lumen [7]. Although Na^+/NH_4^+ exchange may occur it has been suggested that this is not the predominant mechanism, at least in the rat with chronic metabolic acidosis [7]. Competition between NH_4^+ and K^+ for transport on the Na^+/K^+-ATPase pump in the basolateral membrane of rabbit proximal tubules also has been suggested, based on measurement of ouabain-sensitive oxygen consumption [8]. If K^+ channels are present in the apical membrane of the mammalian proximal tubule, ammonia could also pass through these channels (as suggested for the thick limb of Henle [9]) due to the similar ionic radius of NH_4^+ and K^+ [10]. However, the exact mechanism has not been identified. In the present study we used OK cells, an established cell line with certain proximal tubule characteristics [11,12], to explore the possible mechanism of NH_4^+ transport and the influence of acute phosphate deprivation on this process.

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Methods

Cell culture and intracellular pH measurement

OK cells were used between passages 15 to 30. The cells were cultured routinely in monolayers in a 1:1 mixture of Dulbecco's modified Eagle's medium

(DMEM) and Ham's F12, containing 25 mM $NaHCO_3$, 10% fetal calf serum, 10 mM Hepes and 100 IU/ml penicillin G. The cultures were kept at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells were resuspended by incubation for 6 min at 37°C in solution containing 0.05% trypsin, 138 mM NaCl, 5 mM KCl, 7 mM $NaHCO_3$, 5.5 mM glucose, and 0.6 mM EDTA. The cell suspension was seeded on plastic coverslips in 60 mm plastic dishes at a density of $2 \cdot 10^5$ cells/coverslip and cultured for 2–3 days. On the day of the experiment the confluent coverslips from the same passage were divided into two groups. The culture medium was removed from both groups and replaced, in one group, with a serum-free phosphate-deficient DMEM medium containing 0.1% bovine serum albumin. The other group (controls) was given the same medium containing Na_2HPO_4. Phosphate content of control and phosphate-deficient media was determined to be 1.028 mM and 0.002 mM, respectively. Both groups of cells were washed carefully in the appropriate serum-free medium prior to addition and incubation. The control and phosphate-deprived groups were incubated for 3 h at 37°C and treated identically from this point.

Intracellular pH measurement

Intracellular pH was measured with the fluorescent pH indicator 2',7'-bis(2-carboxyethyl)-5(and-6)-carboxyfluorescein (BCECF). At the end of the 3 h incubation, the acetoxymethyl ester of BCECF (dissolved in dimethyl sulfoxide) was added to the medium at a final concentration of 8.3 μM and the cells were loaded with the dye for 30 min at 37°C. The coverslip was then washed three times with NaCl solution (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM glucose, 7 mM Hepes, pH 7.4 with Tris) and inserted into a perfusion cuvette for measurement of fluorescence. The exact method for BCECF fluorescence measurement was described previously in detail [13]. Briefly, using a Perkin-Elmer fluorimeter (Model LS5), fluorescence was measured by alternating the excitation wavelengths between 500 nm and 440 nm (1 cycle/30 s) and using a constant emission wavelength of 535 nm. The cuvette, maintained at room temperature, was perfused first with the NaCl solution for 5 min and then with NMDG solution containing NH_4Cl (125 mM *N*-methyl-D-glucamine chloride, 20 mM NH_4Cl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM glucose, 7 mM Hepes, pH 7.4 with Tris) for 4 min. Extracellular NH_4^+ was then washed away by additional perfusion with NMDG solution which contained no NH_4Cl. After finishing this procedure, the excitation ratio (500:440) for BCECF fluorescence was calibrated with intracellular pH for each coverslip. The calibration method [13] used 10 μM nigericin, a K^+/H^+ ionophore, and solutions with high K^+ concentration (110 mM KCl, 20 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 18 mM glucose, 7 mM Hepes, and 4 mM Mes) with pH values ranging from 6.2 to 7.7 (adjusted with KOH). A representative calibration experiment is shown in Fig. 1. There was a linear relationship between pH and the excitation ratio (500:440) for BCECF fluorescence. Construction of a calibration curve for every coverslip and use of fluorescence ratios will eliminate changes due to differences in dye loading or cell number.

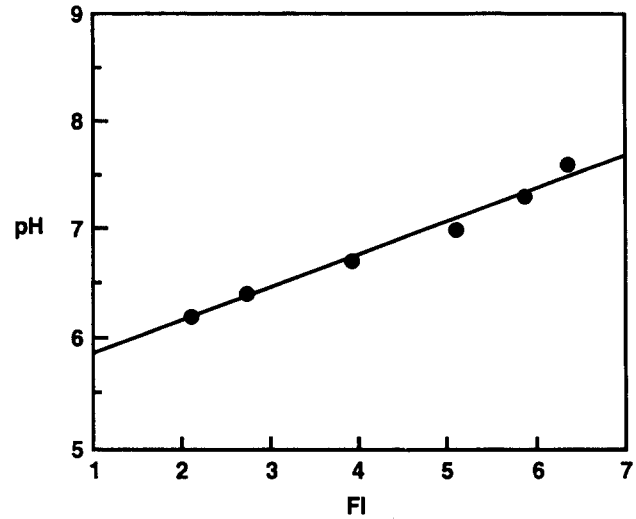

Fig. 1. Calibration curve showing linear relationship between intracellular pH in OK cell monolayers and fluorescence intensity (FI) of BCECF expressed as the ratio at excitation wavelengths of 500:440 nm.

Calculation of NH_4^+ transport

As shown in Fig. 2, perfusion with 20 mM NH_4Cl led to rapid alkalinization followed by acidification. We

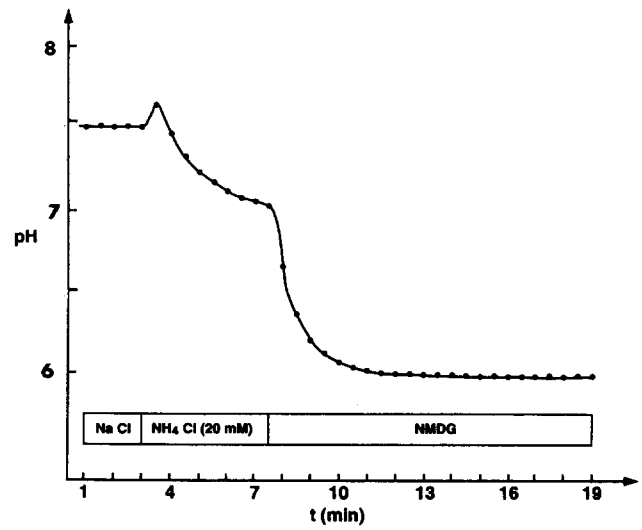

Fig. 2. Standard protocol showing time-course of changes in intracellular pH in OK cell monolayers induced by successive perfusion with NaCl medium, NH_4Cl medium, and NH_4^+-free *N*-methyl-D-glucamine (NMDG) medium. See Methods for complete composition of all solutions. Data presented are from a single experiment.

assume the early part of the acidification process represents NH_4^+ transport into the cell. The calculation of intracellular NH_4^+ concentration from intracellular pH has been described previously [14,15]. Rearranging the equation:

$$pH_{in} = pK_a + \log[NH_3]_{in}/[NH_4^+]_{in}$$

gives:

$$\text{intracellular } [NH_4^+] = [NH_3]_{in} \times 10^{pK_a}/10^{pH_{in}}$$

where $pK_a = 9.3$ [16] and pH_{in} is the measured intracellular pH. NH_3 is assumed to rapidly equilibrate across the cell membrane such that $[NH_3]_{in} = [NH_3]_{out}$ [14]. The latter is calculated from the Henderson-Hasselbalch equation for the equilibrium distribution between NH_3 and NH_4^+ at pH 7.4 in the extracellular solution. As shown below (Results), the initial change in intracellular $[NH_4^+]$ was a linear process. The rate of change in intracellular $[NH_4^+]$ can be taken as an index of NH_4^+ transport into the cell based on the following analysis:

Rate of change of total $[\text{ammonia}]_{in}$

= increase in $[NH_4^+]_{in}$ + change in equilibrium $[NH_3]_{in}$

The change in equilibrium $[NH_3]_{in}$ is due to the changes in pH_{in} and $[NH_4^+]_{in}$:

$$[NH_3]_{in} = 10^{pH_{in}-9.3} \cdot [NH_4^+]_{in}$$

During the initial 2 min of the acidification process the intracellular pH will be in the range 7.2–7.9 (Fig. 2) and the equilibrium $[NH_3]_{in}$ is estimated to be 1–4% of $[NH_4^+]_{in}$. This is very small and can be neglected compared to $[NH_4^+]_{in}$. Furthermore the rate of change of intracellular NH_4^+ concentration ($\text{mol cm}^{-3}\,\text{min}^{-1}$) can be converted to transport rate ($\text{mol cm}^{-2}\,\text{min}^{-1}$) by multiplying by the ratio of cell volume/cell surface area. Therefore in the following discussion we will use the rate of change of intracellular $[NH_4^+]$ as the index of the membrane transport of NH_4^+.

Other assays

Intracellular Na^+ and K^+ in OK cell monolayers were determined by atomic absorption spectroscopy, as described previously in detail [17,18]. Monolayers of OK cells in 35 mm dishes were incubated in normal or phosphate-deficient medium for 3.5 h and then washed six times with ice-cold 300 mM mannitol, 10 mM Hepes-Tris (pH 7.4). The washed cells were ruptured by addition of 1 ml water followed by freezing at −20°C. After thawing and a second freeze-thaw cycle, the water was removed and used directly for measure-

Protein was determined by a modification [11] of the Lowry procedure after solubilizing cells in 0.5% Triton X-100. Phosphate content of cell culture medium was determined by a colorimetric assay [19] after extraction with 5% perchloric acid.

Control and phosphate-deprived cells were always compared in parallel in the same experiment. Statistically significant differences between control and phosphate-deprived cells were analyzed with the Student's *t*-test for group comparisons.

Results

Perfusion with 20 mM NH_4Cl produced a rapid and significant ($P < 0.005$) initial alkalinization of intracellular pH in both control and phosphate-deprived OK cells (Fig. 3). However, the intracellular pH was not significantly different between the two groups at the peak of alkalinization (Fig. 3). This indicates that there are no significant differences in intracellular $[NH_4^+]$ between the two groups at the alkalinization point. Since extracellular NH_4^+ remains constant, the concentration gradient driving NH_4^+ entry into the cells will be similar in both groups.

The increase in intracellular $[NH_4^+]$ was linear with time for the first 2 min of the acidification step (Fig. 4). This linear relationship was expected for NH_4^+ transport because the initial intracellular $[NH_4^+]$ was far less than the Nernst equilibrium $[NH_4^+]$ inside the cell. The latter was estimated to be 281 mM based on a membrane potential of −70 mV in OK cells [12]. Within the linear phase the rate of change can be calculated

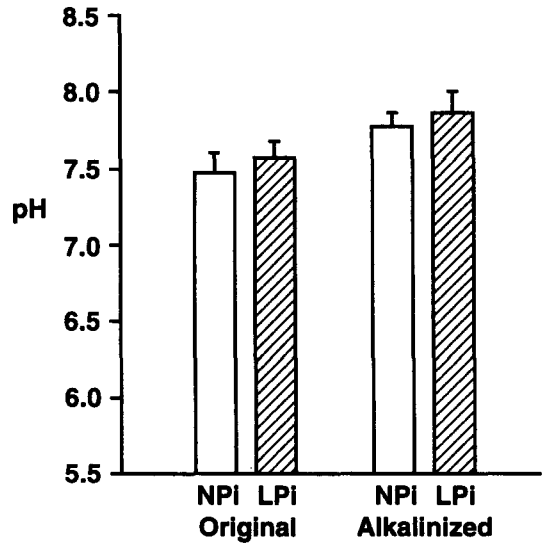

Fig. 3. Intracellular pH in control (NPi) and phosphate-deprived (LPi) OK cell monolayers. Total period of phosphate deprivation was 3.5 h. Measurements were made before perfusion with NH_4Cl solution (Original) and at peak of initial alkalinization (Fig. 2) induced by NH_4Cl perfusion (Alkalinized). Data are means + S.D. from six ex-

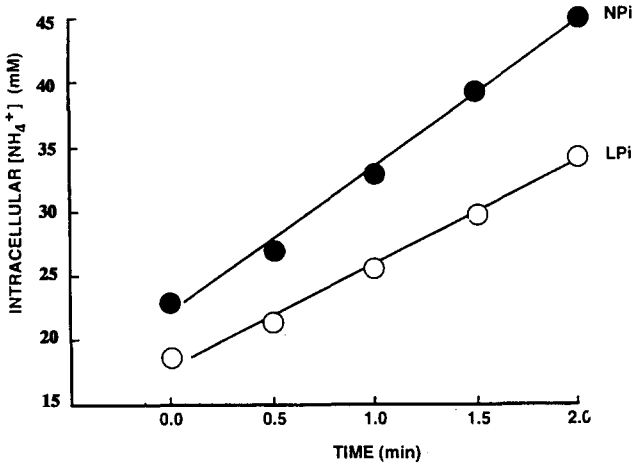

Fig. 4. Change in intracellular concentration of NH_4^+ during acidification of control (NPi) and phosphate-deprived (LPi) groups. Phosphate deprivation was for 3.5 h. The regression coefficient was 0.996 for both lines. Each data point is the mean value from six experiments.

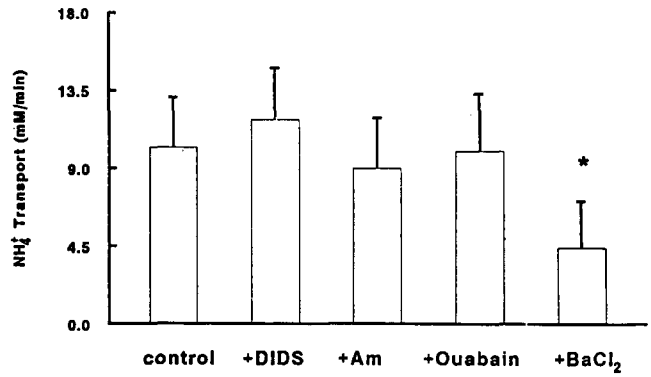

Fig. 6. Ammonium transport by OK cell monolayers (normal phosphate medium) in the presence and absence (control) of 0.4 mM 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), 0.5 mM amiloride (Am), 0.1 mM ouabain, and 10 mM $BaCl_2$. Inhibitors were added to the NH_4Cl perfusion solution at the final concentrations indicated. Data are means ± S.D. from five experiments. * Significantly different ($P < 0.02$) compared to control group.

from any two time points. The data in the next figure (Fig. 5) were calculated from the first 1.5 min of this time-course.

In phosphate-deprived cells, the rate of entry of NH_4^+ was significantly decreased by 32% compared to controls (Fig. 5). Since the NH_4^+ concentration gradient was not different, this change probably reflects a decrease in membrane permeability to NH_4^+ in the phosphate-deprived cells. Due to the exponential relationship between $[NH_4^+]_{in}$ and intracellular pH, we compared the rate of pH change in the two groups. The rate of pH_{in} change also was significantly decreased ($P < 0.05$) from 0.16 ± 0.03 in controls to 0.12 ± 0.03 pH units/min (mean ± S.D., $n = 6$) in phosphate-deprived cells. Thus there was an altered response to the NH_4Cl solution after phosphate deprivation.

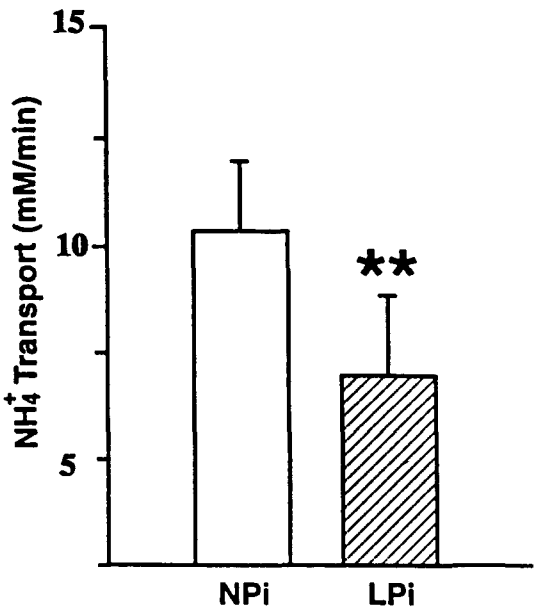

Fig. 5. Rate of NH_4^+ transport in control (NPi) and phosphate-deprived (LPi) OK cell monolayers. Phosphate deprivation was for 3.5 h. Data are means ± S.D. from six experiments. ** Significantly different ($P < 0.005$) compared to control group.

In control cells maintained in medium with a normal phosphate level, the rate of NH_4^+ transport was not blocked by DIDS (Fig. 6), ruling out a role for Cl^-/HCO_3^- exchange in decreasing pH_i by allowing HCO_3^- exit. $BaCl_2$ but neither amiloride nor ouabain inhibited the acidification (Fig. 6), suggesting NH_4^+ entry may be via K^+ channels rather than by Na^+/NH_4^+ exchange [7] or by Na^+/K^+-ATPase [8]. This would be consistent with the report that a K^+ permeable channel of about 22 pS has been observed in the OK cell apical plasma membrane [20], and with the report that barium can eliminate the electrogenic NH_4^+ induced depolarization in mouse proximal tubule in the absence of bicarbonate [21].

Perfusion with NMDG solution for up to 12 min after NH_4Cl removal led to no recovery of pH (Fig. 2), suggesting that NH_4^+ exit is difficult to detect with this experimental system. One reason for this may be that NH_3 exits first, making the cell acidic and closing K^+ channels so that NH_4^+ cannot leave the cell. It was suggested previously that K^+ permeability in OK cells is regulated by intracellular pH [12].

Intracellular K^+ was 1029 ± 39 (mean ± S.E., $n = 3$) in control cells and 953 ± 13 nmol/mg protein in phosphate-deprived cells. Intracellular Na^+ was 58 ± 3 in controls and 57 ± 4 nmol/mg protein in phosphate-deprived cells. These values were not significantly different between the two groups.

Discussion

The 3.5 h period of phosphate deprivation used in these studies was shown previously [11] to induce an adaptive increase by about 50% in Na^+/phosphate cotransport. This is qualitatively similar to the response of the phosphate transport system of the renal proximal tubule in phosphate-deprived rats, suggesting that OK renal epithelial cells represent a useful and versatile system for studying the cellular mechanism of renal adaptation to phosphate deprivation. It should be noted that phosphate deprivation of OK cells is accomplished by incubation in medium containing almost no phosphate, a situation which the proximal tubule cells rarely experience. However, in both cases the signal for the adaptive response may be the change in intracellular free phosphate which may be comparable between the cultured cell model and proximal tubules of phosphate-deprived rats.

The baseline intracellular pH of OK cells in Hepes-buffered Na medium was slightly higher than the buffer (pH 7.4), which is consistent with the previous studies [22]. However, there was no significant difference between the control and phosphate-deprived OK cells both in the baseline pH and in the pH at the alkalinization point. This suggests that at normal pH range the intracellular buffer capacity, estimated by the NH_3 entry induced alkalinization [14], was not altered by phosphate deprivation. So the initial linear increase in intracellular [NH_4^+] (Fig. 4), calculated by the intracellular pH change, can be used as an index of NH_4^+ transport into the cell. This measurement though indirect may be useful for estimating NH_4^+ transport in other cultured cells.

A change in NH_4^+ transport, in the absence of changes in the gradient, could be due not only to a change in membrane permeability to NH_4^+ but also to a change in membrane potential. However, phosphate deprivation does not change significantly the electrogenic Na^+/proline cotransport in OK cells [11], suggesting that any change in membrane potential after phosphate deprivation is small. In addition, phosphate deprivation did not change intracellular [K^+] and [Na^+], the major cations determining the membrane potential. This suggests that any change in membrane potential must be a secondary result of a change in membrane K^+ permeability. Since NH_4^+ transport into the cells may occur via K^+ channels (Fig. 6), we suggest that phosphate deprivation could alter primarily the membrane permeability for K^+ and that this may be the major reason why NH_4^+ transport is decreased.

NH_4^+ excretion is decreased in phosphate-deprived rats [1]. This may occur because intracellular alkalinization decreases NH_4^+ formation in the kidney during phosphate deprivation [4]. However, this mechanism is not supported by our results (Fig. 3) which showed no significant effect of acute phosphate deprivation on intracellular pH. On the basis of the present data we cannot determine if acute phosphate deprivation of OK cells is accompanied by a change in NH_4^+ formation. Our other findings (Figs. 4 and 5) suggest, indirectly, that acute phosphate deprivation may lead to a change in membrane permeability to NH_4^+. Caution is necessary when using a cultured cell model to understand changes in proximal tubule cell function in vivo. The role of other nephron segments [7] in determining urinary NH_4^+ excretion also has to be considered. However, decreased formation and decreased permeability to NH_4^+ in proximal tubule cells would be consistent with the finding of decreased NH_4^+ excretion in phosphate deprived rats [1].

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