Mechanism and regulation of vitamin B₆ uptake by renal tubular epithelia: studies with cultured OK cells

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Mechanism and regulation of vitamin B₆ uptake by renal tubular epithelia: studies with cultured OK cells. Am J Physiol Renal Physiol 282: F465–F471, 2002; 10.1152/ajprenal.00267.2001.—The kidneys play an important role in regulating vitamin B₆ body homeostasis, but limited information exists regarding the mechanism of pyridoxine uptake by renal epithelial cells, and no study exists on its regulation. To address these issues, we used the renal opossum-derived tubular epithelial (opossum kidney; OK) cells and found pyridoxine uptake to be (1) be temperature and energy dependent, (2) be pH dependent, with a higher uptake at alkaline or neutral buffer pH compared with acidic pH, (3) be Na⁺ independent, (4) involve a saturable component (apparent Michaelis-Menten constant of 2.40 ± 0.23 μM), (5) be inhibited by structural analogs, and (6) be amiloride sensitive. Maintaining OK cells in a vitamin B₆-deficient growth medium (for 48 h) led to a significant upregulation of pyridoxine uptake. This upregulation was found to be specific for pyridoxine, inhibited by cyclohexamide and actinomycin D, reversible, and mediated via an increase in maximal velocity. Pretreating OK cells with modulates of a Ca²⁺/calmodulin-mediated pathway led to a significant downregulation in pyridoxine uptake via inhibition of maximal velocity. These results demonstrate that pyridoxine uptake by renal tubular epithelial OK cells is via a specialized pH-sensitive carrier-mediated mechanism. This mechanism appears to be regulated by extracellular vitamin B₆ levels and an intracellular Ca²⁺/calmodulin-mediated pathway.

MATERIALS AND METHODS

Custom-made [³H]pyridoxine (sp act 20 Ci/mmol, radiochemical purity > 97%) was obtained from American Radio-labeled Chemical (St. Louis, MO). All other chemicals and reagents used in this study were of analytical quality and were obtained from commercial sources.

Cell Culture

OK cells (American Type Culture Collection, passages 36–46) were grown in MEM in 75-cm² plastic flasks (Costar) at 37°C in a 5% CO₂-95% air atmosphere with media changes every 2–3 days. Cells were subcultured by trypsinization with 0.05% trypsin and 0.9 mM EDTA in Ca²⁺- and Mg²⁺-free phosphate buffered solution and plated onto 12-well plates at a concentration of 5 × 10⁵ cells/well. Uptake studies were performed on monolayers 2–3 days after confluence. Cell growth was observed by periodic monitoring with an inverted microscope. Viability of OK cells grown in control and vitamin B₆-deficient media was determined using the trypan blue dye exclusion method and was found to be >99%.

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VITAMIN B₆ IS AN ESSENTIAL micronutrient that exists in the diet as a mixture of three vitaminic forms in different proportions: pyridoxine, pyridoxal, and pyridoxamine and the corresponding phosphate derivatives. The vitamin is involved in a variety of critical metabolic reactions including carbohydrate metabolism, sphingolipid biosynthesis and degradation, amino acid metabolism (including that of homocysteines), and neurotransmitter metabolism. Therefore, deficiency of this essential micronutrient in humans leads to a variety of adverse conditions and to disturbances in normal cellular metabolism (for review, see Ref. 9).

Humans and other mammals lack the ability to synthesize vitamin B₆ and thus must obtain it from exog-
Uptake studies were performed at 37°C in Krebs-Ringer buffer containing (in mM) 123 NaCl, 4.95 KCl, 1.25 MgSO₄, 0.85 CaCl₂, 5 glucose, 5 glutamine, 10 HEPES, and 10 MES at pH 7.4 (unless otherwise stated). [³H]pyridoxine was added to the incubation buffer at the onset of incubation, and uptake was terminated at the desired time by the addition of 1 ml of ice-cold buffer and subsequent immediate removal by aspiration. Monolayers were then rinsed twice with ice-cold buffer, digested with 1 ml of 1 N NaOH, neutralized by HCl, and counted for radioactivity in a liquid scintillation counter. Protein contents of cell digests were estimated on parallel wells using a Bio-Rad protein assay kit.

In the study to determine the effects of maintaining OK cells in vitamin B₆-deficient growth medium on pyridoxine uptake, cells were grown for 48 h before uptake investigations in a vitamin B₆-deficient growth medium (with no added pyridoxine). Control cells were maintained in parallel wells in control growth media (i.e., medium that contained 5 µM pyridoxine). Both media were supplemented with 10% dialyzed fetal bovine serum. When the effects of actinomycin D and cyclohexamide on the observed induction of pyridoxine by the vitamin B₆-deficient condition was to be tested, these transcription/protein synthesis inhibitors were added at the onset of cell incubation with the vitamin B₆-deficient medium.

The metabolic form of the transported substrate after incubation with 30 nM [³H]pyridoxine was determined using the thin-layer chromatography procedure (with silica-precoated thin-layer plates) and a mobile phase containing a 8:7:4:1 vol/vol ratio of t-butanol, acetone, water, and diethylamine (10).

**Statistical Analysis**

Data are means ± SE of multiple separate uptake determinations performed on different occasions and are expressed in femtomoles or picomoles per milligram of protein per unit of time. Some variations in the absolute amount of pyridoxine uptake were seen with different cell batches; for per unit of time. Some variations in the absolute amount of pressurized in femtomoles or picomoles per milligram of protein minutions performed on different occasions and are ex-

\[ 1\text{mM} \]

**Mechanism for Pyridoxine Uptake and Its Specificity**

In the first study, we examined the initial rate of pyridoxine uptake as a function of substrate concentration in the incubation medium (0.015–10 µM). The results showed that pyridoxine uptake includes a saturable component (Fig. 4). Kinetic parameters of this component were determined as described in MATERIALS AND METHODS and found to be 2.44 ± 0.23 µM and 5.99 ±
0.23 pmol·mg protein\(^{-1}\)·3 min\(^{-1}\) for apparent \(K_m\) and \(V_{\text{max}}\), respectively.

In another study, we examined the specificity of the pyridoxine uptake process. This was performed by examining the effects of pyridoxine structural analogs and unrelated compounds on the initial rate of \(^3\)Hpyridoxine (15 nM) uptake. The results showed that pyridoxal, 4-deoxypyridoxamine, and pyridoxamine cause significant inhibition in pyridoxine uptake. On the other hand, pyridoxic acid (the principle urinary metabolite of vitamin B\(_6\), see Ref. 3) and pyridoxal-5-phosphate as well as the unrelated compounds tetraethylammonium chloride (TEA) and \(N\)-methylnicotinamide (NMN) were without effect (Table 1).

### Effects of Membrane Transport Inhibitors and Metabolic Poisons

In these studies, we examined the effects of the membrane transport inhibitors DIDS, probenecid, fur-

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### Table 1. Effect of pyridoxine structural analogs and unrelated compounds on the initial rate of uptake of \(^3\)Hpyridoxine

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration, (\mu)M</th>
<th>Uptake, fmol·mg protein(^{-1})·3 min(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>119.36 ± 2.57*</td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>3</td>
<td>82.40 ± 1.84*</td>
</tr>
<tr>
<td>4-Deoxypyridoxamine</td>
<td>25</td>
<td>70.67 ± 0.93*</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>25</td>
<td>82.03 ± 0.62*</td>
</tr>
<tr>
<td>Pyridoxic acid</td>
<td>25</td>
<td>66.01 ± 2.60*</td>
</tr>
<tr>
<td>Pyridoxal-5-phosphate</td>
<td>25</td>
<td>105.00 ± 4.01*</td>
</tr>
<tr>
<td>TEA</td>
<td>50</td>
<td>126.60 ± 2.40†</td>
</tr>
<tr>
<td>NMN</td>
<td>50</td>
<td>120.40 ± 3.64†</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>116.29 ± 3.85†</td>
</tr>
</tbody>
</table>

Values are means ± SE. Confuent monolayers of opossum kidney (OK) cells were incubated at 37\(^\circ\)C in Krebs-Ringer buffer at pH 7.4. Uptake of \(^3\)Hpyridoxine (15 nM) was measured during the initial rate (3 min). TEA, tetraethylammonium chloride; NMN, \(N\)-methylnicotinamide. \(P\) values were calculated using Student’s \(t\)-test. Comparisons were made relative to simultaneously performed controls. *\(P < 0.01\), †\(P = \) not significant.
Effect of extracellular substrate level. In this study, we examined the effect of growing OK cells in a vitamin B6-deficient growth medium on the ability of the cells to take up [3H]pyridoxine (see MATERIALS AND METHODS). Significant (P < 0.01) induction in pyridoxine (15 nM) uptake was observed in cells grown under vitamin B6-deficient conditions compared with those grown in control growth medium, with the induction being significantly (P < 0.01) inhibited when actinomycin D (0.23 μM) and cyclohexamide (40 μM) were present in the deficient growth medium (Fig. 5). In addition, this upregulation in [3H]pyridoxine (15 nM) uptake was found to be reversible after reintroduction of pyridoxine (5 μM) to the vitamin B6-deficient growth medium (uptake of [3H]pyridoxine of 119.99 ± 3.66, 223.52 ± 2.05, and 123.23 ± 2.05 fmol·mg protein⁻¹·3 min⁻¹ for cells grown in control growth medium, vitamin B6-deficient growth medium, and vitamin B6-deficient medium followed by incubation for 24 h in the presence of pyridoxine, respectively). In contrast to the effects of vitamin B6-deficient conditions on pyridoxine uptake, uptake of the unrelated vitamin, thiamin (30 nM), was found to be similar in cells grown in control and vitamin B6-deficient growth medium (98.1 ± 0.7 and 93.8 ± 4.0 fmol·mg protein⁻¹·3 min⁻¹, respectively).

In other studies, we determined whether the effects of the vitamin B6-deficient condition are mediated through effects on the V_max and/or the apparent K_m of the pyridoxine-uptake process. This was performed by examining pyridoxine uptake as a function of concentration in the two cell subtypes. The results (Fig. 6) showed a marked induction (P < 0.01) in the V_max of the pyridoxine uptake process in cells grown under vitamin B6-deficient conditions compared with those grown in control growth medium (V_max values of 7.25 ± 0.64 and 22.45 ± 1.03 pmol·mg protein⁻¹·3 min⁻¹ for cells grown in vitamin B6-deficient and control media, respectively) with a slight decrease in the apparent K_m (4.05 ± 1.07 and 2.22 ± 0.34 μM, respectively).

Role of intracellular regulatory pathways. In these studies, we examined the possible regulation of pyridoxine uptake by OK cells via specific intracellular regulatory mechanisms. We focused on pathways that have been shown to be involved in the regulation of other substrates (including other water-soluble vitamins) in renal and other epithelial cell membrane...
transport events including Ca\(^{2+}/\)calmodulin, nitric oxide (NO), and protein kinase A (PKA)-mediated pathways (6, 8, 12–15). The results showed that pretreating OK cells (for 1 h) with calmidazolium (25 \(\mu\)M; an inhibitor of the Ca\(^{2+}/\)calmodulin-mediated pathway) led to significant inhibition in pyridoxine uptake (122.6 ± 5.2 and 66.4 ± 4.9 fmol·mg protein\(^{-1}\)·min\(^{-1}\) for control and the presence of calmidazolium, respectively). Similarly, pretreatment with two other inhibitors of the Ca\(^{2+}/\)calmodulin-mediated pathway inhibitors, trifluoperazine (TFP) and KN62, also caused significant (\(P < 0.01\) for all) inhibition in pyridoxine (15 nM) uptake (132.3 ± 4.7, 94.0 ± 7.3, 76.4 ± 4.9, and 107.0 ± 2.6 fmol·mg protein\(^{-1}\)·min\(^{-1}\) for control and in the presence of 50 and 100 \(\mu\)M TFP and 25 \(\mu\)M KN62, respectively). We also examined the effects of pretreatment with calmidazolium (50 \(\mu\)M) on kinetic parameters (\(V_{\text{max}}\) and apparent \(K_m\)) of the pyridoxine-uptake process. The results (Fig. 7) showed that calmidazolium causes a significant (\(P < 0.01\)) inhibition in \(V_{\text{max}}\) but not in apparent \(K_m\) of the pyridoxine-uptake process (\(V_{\text{max}}\) of 8.06 ± 0.49 and 2.44 ± 0.09 pmol·mg protein\(^{-1}\)·min\(^{-1}\); apparent \(K_m\) of 2.74 ± 0.58 and 2.96 ± 0.38 \(\mu\)M for control and calmidazolium-pretreated cells, respectively).

The possible role of a NO-mediated pathway in the regulation of pyridoxine uptake by OK cells was also investigated. This was performed by examining the effect of the NO donor S-nitroso-N-acetylpenicillamine (SNAP) on pyridoxine (15 nM) uptake. The results showed that up to 1 mM SNAP had no effect on pyridoxine uptake (110.58 ± 4.98, 126.30 ± 1.80, 106.90 ± 0.30, and 116.16 ± 1.40 fmol·mg protein\(^{-1}\)·min\(^{-1}\) for control and in the presence of 0.1, 0.5, and 1 mM SNAP, respectively). Similarly, 8-bromo-cGMP (0.5 mM) was found to have no significant effect on pyridoxine uptake (130.4 ± 3.5 and 125.3 ± 2.5 for control and in the presence of 8-bromo-cGMP, respectively).

We also examined the potential role of a PKA-mediated pathway in the regulation of pyridoxine uptake. This was performed by examining the effects of modulators of this pathway on pyridoxine (15 nM) uptake. The results showed that pretreatment of OK cells for 1 h with dibutyryl cAMP (1 mM), 8-bromo-cAMP (1 mM), forskolin (0.1 mM), or H89 (1 \(\mu\)M) caused no significant effect on pyridoxine uptake (Table 3).

### DISCUSSION

Renal tubules play a critical role in salvaging filtered water-soluble nutrients such as vitamin B\(_6\). Any defect or interference with the normal vitamin B\(_6\) renal uptake process may lead to disturbance in normal vitamin B\(_6\) body homeostasis. Thus a detailed understanding of the renal uptake process of vitamin B\(_6\) is of significant importance. The present study examined the mechanism and regulation of uptake of pyridoxine, a member of the vitamin B\(_6\) family, by renal epithelial cells using the renal tubular epithelial OK cells as a model system. This well-established renal epithelial system has been widely used in similar investigations on cell physiology of renal transport (1, 7, 16, 17). The results showed that uptake of pyridoxine during the initial 3 min of incubation proceeded with no metabolic alterations in the transported substrate. The uptake process of pyridoxine was found to be both temperature and pH sensitive in nature. Lowering incubation temperature from 37 to 21 and 4°C was associated with a marked inhibition in pyridoxine uptake. With regard to pH sensitivity, although decreasing the incubation buffer pH from 8.0 to 7.0 did not affect the initial rate of pyridoxine uptake, further reduction in the buffer pH to 5.0 led to a gradual decrease in substrate uptake. Further investigations are required to establish the exact mechanism mediating the pH effect; i.e., whether it is mediated via an effect on the involved carrier system itself and/or through the pH gradient imposed across the membrane. In contrast to the effect of buffer pH, pyridoxine uptake by OK cells was found to be Na\(^+\) independent, because total replacement of Na\(^+\) in the incubation medium with equimolar concentrations of

![Fig. 7. Effect of calmidazolium on kinetic parameters of pyridoxine uptake by OK cells. Confluent monolayers of OK cells were pretreated (for 1 h) with 0 (control) or 25 \(\mu\)M calmidazolium. Uptake was then performed (for 3 min) in Krebs-Ringer buffer at pH 7.4 in the presence of different concentrations of pyridoxine. Uptake by the carrier-mediated system was calculated. Data are means ± SE of at least 3 separate uptake determinations. ○, Control cells; ●, calmidazolium-treated cells.](image-url)
other monovalent cations did not affect pyridoxine uptake. This is in contrast to the severe inhibition that was observed in the uptake of the unrelated vitamin biotin by the same cells after Na\(^+\) replacement in the incubation medium (biotin uptake by renal epithelial cells is known to be Na\(^+\)-dependent in nature; see Refs. 2 and 11). Our observation of a lack of a role for Na\(^+\) in pyridoxine uptake is similar to the previous observations by Bowman and McCormick (3) with isolated rat renal epithelial cells and by Middleton (10) with rat intestine. It is, however, different from a subsequent study by Bowman and colleagues (4) with renal brush-border membrane vesicles, which reported the process to be Na\(^+\)-driven. The cause of this discrepancy in the previous reports (3, 4) is not clear but cannot be attributed to species variations because rats were used in both of the referenced studies.

Uptake of pyridoxine by OK cells was found to be carrier mediated in nature. This conclusion is based on the observation that uptake of the substrate as a function of concentration includes a saturable component and by the observation of significant inhibition in [\(^3\)H]pyridoxine uptake by close structural analogs (pyridoxal and 4-deoxypyridoxamine). This pyridoxine carrier system displayed clear specificity, as indicated by the lack of effect of certain other analogs of the substrate (pyridoxic acid and pyridoxal-5-phosphate) and unrelated compounds (TEA and NMN) on its uptake. Uptake of pyridoxine by OK cells was found to be insensitive to the presence of membrane transport inhibitors DIDS, furosemide, and probenecid. However, a slight but significant inhibition in pyridoxine uptake was observed with the diuretic agent amiloride. The latter finding is similar to that reported previously for the vitamin uptake in isolated rat renal epithelial cells (3) and by certain strains of yeast (19).

After the characterization of the uptake mechanism of pyridoxine in OK cells, we used this cellular system to study regulation of the pyridoxine uptake by extracellular substrate-level and by intracellular regulatory mechanisms. Our results showed that growing OK cells in a vitamin B\(_6\)-deficient growth medium leads to a significant upregulation in pyridoxine uptake. This upregulation was significantly inhibited when the transcription or translation inhibitors actinomycin D and cyclohexamide, respectively, were present in the growth medium. The latter observations suggest possible involvement of transcription/translation mechanisms in the upregulation process of pyridoxine uptake under vitamin B\(_6\)-deficient conditions. Further studies at the molecular level are required to confirm this suggestion. The upregulation in pyridoxine uptake by vitamin B\(_6\)-deficient conditions was also found to be specific for pyridoxine, because uptake of the unrelated vitamin thiamine was not affected by such a condition. Furthermore, the induction was reversible after reintroduction of pyridoxine into the vitamin B\(_6\)-deficient growth medium. The induction in pyridoxine uptake by vitamin B\(_6\)-deficient conditions was found to be mainly mediated via a significant increase in the V\(_{\text{max}}\) of pyridoxine uptake with a slight change in the apparent K\(_{\text{m}}\). These findings suggest that the induction in pyridoxine is most likely the result of an increase in the number (and/or activity) of the pyridoxine-uptake carriers with slight changes in the corresponding affinities.

The possible role of intracellular regulatory pathways in the regulation of vitamin B\(_6\) uptake by renal epithelial cells was also investigated using this renal epithelial cell line as a model. In these investigations, we focused our effort on pathways that have been shown to play a role in regulating transport of other substrates, including other water-soluble vitamins (6, 8, 12–15). Our results showed that inhibition of the Ca\(^{2+}\)/calmodulin-mediated pathway with the use of different inhibitors leads to a significant inhibition (downregulation) of pyridoxine uptake. This inhibition was found to be mediated via an inhibition in the V\(_{\text{max}}\) of the pyridoxine-uptake process with no significant effect on the apparent K\(_{\text{m}}\). These findings suggest that the inhibition in pyridoxine uptake by the Ca\(^{2+}\)/calmodulin inhibitor calmidazolium is mediated via a decrease in the activity (and/or number) of the pyridoxine-uptake carriers with no effect on carrier affinity. Further studies are required to determine the precise mechanisms through which this intracellular regulatory pathway exerts its effect on pyridoxine uptake in renal epithelium. In contrast to the apparent role of the Ca\(^{2+}\)/calmodulin-mediated pathway in regulating pyridoxine uptake in OK cells, no role for NO- or PKA-mediated pathways was found. This conclusion is based on the observation that modulators of these pathways were without effect on pyridoxine uptake.

In summary, our study demonstrates that pyridoxine uptake by cultured renal tubular epithelial OK cells is via a pH-sensitive, Na\(^+\)-independent, specialized carrier-mediated mechanism. This uptake mechanism appears to be regulated by extracellular substrate levels and by an intracellular Ca\(^{2+}\)/calmodulin-mediated pathway. These findings should serve as a base for future investigations into the molecular mechanisms involved in the transport of vitamin B\(_6\) in renal epithelial cells and its regulation.

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