Endogenous expression of the renal high-affinity H\(^{+}\)-peptide cotransporter in LLC-PK\(_1\) cells

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Wenzel, Uwe, Daniela Diehl, Martina Herget, and Hannelore Daniel. Endogenous expression of the renal high-affinity H\(^{+}\)-peptide cotransporter in LLC-PK\(_1\) cells. Am. J. Physiol. 275 (Cell Physiol. 44): C1573–C1579, 1998.—The reabsorption of filtered di- and tripeptides as well as certain peptide mimetics from the tubular lumen into renal epithelial cells is mediated by an H\(^{+}\)-coupled high-affinity transport process. Here we demonstrate for the first time H\(^{+}\)-coupled uptake of dipeptides into the renal proximal tubule cell line LLC-PK\(_1\). Transport was assessed 1) by uptake studies using the radiolabeled dipeptide d-[\(^{3}\)H]Phe-L-Ala, 2) by cellular accumulation of the fluorescent dipeptide d-Ala-Lys-AMCA, and 3) by measurement of intracellular pH (pHi) changes as a consequence of H\(^{+}\)-coupled dipeptide transport. Uptake of d-Phe-L-Ala increased linearly over 11 days postconfluency and showed all the characteristics of the kidney cortex high-affinity peptide transporter, e.g., a pH optimum for transport of d-Phe-L-Ala of 6.0, an apparent high-affinity peptide transporter, e.g., a pH optimum for and showed all the characteristics of the kidney cortex high-affinity peptide transporter, e.g., a pH optimum for transport of d-Phe-L-Ala of 6.0, an apparent high-affinity transporter PEPT1 mRNA is also found to operate in an electrogenic mode by coupling of

Recent studies on the characteristics and regulation of the renal high-affinity peptide transporter.

METHODS

Materials. Custom-synthesized d-[\(^{3}\)H]Phe-L-Ala (9 Ci/mmol) and unlabeled d-Phe-L-Ala were obtained from Zeneca (Cheshire, UK) and Bachem (Heidelberg, Germany), respectively. All other peptides and \(\beta\)-lactams were purchased from Sigma Chemical. The pH-sensitive fluorescent dyes 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-AM (BCECF-AM) was obtained from BioProbes (Leiden, Netherlands). Fluorescent N-hydroxy-succinimidyl 7-aminomethylcoumarin-3-acetic acid (AMCA-NHS) was obtained from Pierce (Rockford, IL). All the materials needed for cell culture were either from Gibco (Eggenstein, Germany) or Serva. All reagents for RNA preparation and RT-PCR were from MBI Fermentas (Heidelberg, Germany). Teriary butyl-oxy-carbonyl-d-Ala-L-Lys-tertiary butylester (Boc-d-Ala-L-Lys-OtBu) was a generous gift from Prof. H. Brückner (Giessen, Germany).

Cell culture. LLC-PK\(_1\) cells (American Type Culture Collection, CRL 1392, passage 195) were cultured and passaged in Dulbecco's modified Eagle's medium (GIBCO 41965) supplemented with 10% fetal calf serum, 2 mM glutamine, 1% MEM nonessential amino acids (GIBCO 01140), 10 mM HEPES, 1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 \(\mu\)g/ml streptomycin in a humidified incubator at 37°C under an atmosphere of 5% CO\(_2\). Cells between passages 200 and 215 were seeded at a density of 5 \(\times\) \(10^5\) cells/well on Renner 6-well plates subsequent to collagen coating of the wells with rat tail collagen.

Transport studies. Flux studies in LLC-PK\(_1\) cells were performed in a buffer containing (in mM) 145 NaCl, 5.4 Cl\(_{-}\), 1.8 CaCl\(_2\), 1.8 MgSO\(_4\), 20 glucose, and 25 HEPES/Tris (pH 7.4) or MES/Tris (pH \(\leq\) 6.5), respectively. For uptake, cell monolayers grown in six-well plates were washed free of serum-containing medium and incubated with substrates or inhibi-
tors for 15 min at 37°C. After the incubation period the cells were washed three times with ice-cold incubation buffer, scraped off with a rubber policeman after addition of 600 µL TEN buffer/well (in mM: 150 NaCl, 40 Tris, 1 EDTA), and digested with 20 µL of tissue solubilizer. Cellular accumulation of d-[3H]Phe-L-Ala was measured subsequent to the addition of scintillation cocktail by liquid scintillation spectrophotometry. Binding of tracer to the cells was determined as the residual radioactivity associated with the cells in the presence of excess nonlabeled (20 mM) Gly-Gln. Uptake of d-[3H]Phe-L-Ala over 15 min was linear for all pH values and substrate concentrations tested.

Synthesis of d-Ala-L-Lys-AMCA and fluorescence microscopy. Conjugation of AMCA with the ε-amino group of lysine has been carried out using Boc-d-Ala-L-Lys-OkBu and AMCA-NHS as starter molecules (2). After removal of protective groups, d-Ala-L-Lys-AMCA was purified by two-dimensional preparative thin-layer chromatography. Determination of the compound’s concentration was based on its molar extinction coefficient (absorption maximum at 340 nm) and fluorescent properties (emission maximum at 450 nm when excited at 340 nm).

For transport studies with the fluorescent dipeptide analog d-Ala-L-Lys-AMCA, LLC-PK1 cells were grown on collagen-coated coverslips inserted into the six-well plates. Incubation with 5 µM fluorophore-linked dipeptide was performed as described above for the radiolabeled peptide. After the cells were washed with ice-cold buffer, they were fixed with 3% p-formaldehyde and 1% glutaraldehyde for 15 min at room temperature. The coverslips were taken from the wells and washed three times with PBS, pH 7.4, and one drop of embedding medium was applied to the cells before adhesion to polylysine-coated coverslips. Specific uptake of d-Ala-L-Lys-AMCA was assessed by fluorescence microscopy using a Leitz Aristoplan microscope, and cells were observed in either fluorescent light using filter block A (band-pass filter 340–380 nm for excitation; long-pass filter 425 nm for emission) or by using Nomarski optics as described previously (12).

Intracellular pH measurements. For intracellular pH (pHi) measurements, LLC-PK1 cells grown in 12-well plates were loaded with BCECF by preincubation with 5 µM lipophilic acne (BCECF-AM) at 37°C for 30 min. Subsequently, the monolayers were washed with buffer at pH 7.4, and the buffers with or without substrates were changed by superfusion at the time points indicated in the graphs. Intracellular apparent H⁺ activity was determined by measuring the intensity of emission at 538 nm after excitation of the fluorophore at 444 nm (isosbestic point) and 490 nm (pH-sensitive wavelength), respectively, using a microtiter plate reader (Fluoroskan Ascent, Labsystems, Meriken Diagnostika, Bornheim-Hersel, Germany). The 444/490 fluorescence ratio was converted to pHi values based on a calibration curve generated by estimation of the fluorescence ratio in buffers of various pH (5).

RT-PCR from RNA of LLC-PK1 cells. RNA from LLC-PK1 cells was isolated by using the Tristar RNA-clean kit from MBI Fermentas (Heidelberg, Germany). RT-PCR was performed with 5 µg of isolated RNA. First-strand cDNA synthesis was accomplished with a primer representing nucleotides 1889–1879 (back primer: 5’-CTTGTCAGCAAGACATGACC-3’ of the protein-coding region of rabbit PEPT2. PCR amplification of a 732-bp product was achieved with a forward primer, representing nucleotides 1167–1188 (5’-CTGAGCATGAGCGCATGCAG-3’) of the rabbit PEPT2 protein-coding region, and the back primer. Amplification was performed with 35 cycles (95°C denaturation for 1 min, 55°C hybridization for 2 min, 72°C extensions for 2 min; Personal.

RESULTS

Uptake of d-[3H]Phe-L-Ala and d-Ala-L-Lys-AMCA into LLC-PK1 cells in the postconfluent state. Peptide transport into LLC-PK1 cells was measured by uptake of radiolabeled d-Phe-L-Ala at various time points after the cells had reached confluency. Although LLC-PK1 cells have been described not to possess substantial peptide transport activity on the day of reaching confluency (7), uptake of d-[3H]Phe-L-Ala at pH 6.0 increased almost linearly for up to 11 days in the postconfluent state (Fig. 1). Moreover, transport rates were suppressed to rates similar to that of confluent cells (0 days) by the addition of 1 mM Gly-Gln (Fig. 1). All further uptake experiments were performed at day 9 of postconfluenccy, since cell adherence to the culture well decreased subsequent to this time point. The uptake of dipeptides or dipeptide mimetics into LLC-PK1 cells in the postconfluent state was also demonstrated by transport studies, using the fluorescent dipeptide d-Ala-L-Lys-AMCA as a substrate. Although cells displayed a bright blue fluorescence when incubated with the fluorophore-conjugated dipeptide, simultaneous application of 5 mM Gly-Gln reduced staining of the cells to background levels (Fig. 2).

Characteristics of d-[3H]Phe-L-Ala influx into LLC-PK1 cells. Transport of D-Phe-L-Ala into LLC-PK1 cells as a function of apical pH increased fourfold when buffer pH values were reduced from 7.4 to 6.0 (Fig. 3, inset). At pH values <5.5, transport rates were moderately reduced when compared with the transport optimum at pH 6.0–5.5. Uptake of d-Phe-L-Ala as a function of substrate concentration followed Michaelis-Menten kinetics with a Vmax of 2.5 nmol/mg protein/5 min and a Km of 40 µM.

Fig. 1. Uptake of 5 µM d-[3H]Phe-L-Ala into LLC-PK1 cells as a function of time after confluence; 5 x 10⁶ cells/well were seeded in collagen-coated plastic 6-well culture plates. Cells reached confluency on the day postseeded. Fresh medium was given every second day until day 4 postconfluently, after which medium was renewed daily. Uptake of d-[3H]Phe-L-Ala is shown at indicated time intervals postconfluently in absence (●) or presence (○) of 1 mM Gly-Gln.
Menten kinetics with an apparent Michaelis-Menten constant ($K_m$) of $25.8 \pm 3.6 \mu M$ and a maximum velocity ($V_{max}$) of $33.4 \pm 1.7 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{min}^{-1}$ (Fig. 3). The kinetic characteristics of D-Phe-Ala influx into LLC-PK₁ cells therefore closely resemble those found for the same substrate when assessed in Xenopus oocytes expressing the cloned renal PEPT2 transporter (11).

Substrate specificity. The substrate specificity of the expressed activity in LLC-PK₁ was assessed by determining the ability of a variety of dipeptides and peptidomimetics to inhibit influx of D-[3H]Phe-L-Ala (Table 1). Gly-Gln, Gly-Asp, and Gly-Lys were all strong inhibitors of D-Phe-Ala uptake, irrespective of the different net charges they bear at pH 6.0. Although a dipeptide and a tripeptide consisting of alanine also inhibited dipeptide transport, the free amino acid failed to reduce D-Phe-Ala uptake significantly. The ability of the transporter to interact also with peptide mimetics is shown by the inhibition of transport by the amino β-lactam antibiotic cefadroxil. In contrast, benzylpenicillin, another β-lactam, failed to reduce transport. Moreover, captopril, an angiotensin-converting enzyme inhibitor that has been demonstrated to interact with PEPT1 (4) but not with PEPT2 (3), was also unable to inhibit D-Phe-l-Ala influx into LLC-PK₁ cells. The high-affinity phenotype interaction of cefadroxil with the transporter was confirmed by its dose-dependent inhibition with an apparent $K_i$ value of $15.2 \pm 1.1 \mu M$ (Fig. 4). Cefadroxil uptake in oocytes mediated by the cloned PEPT2 (3) occurs with a $K_m$ of $25.8 \pm 6.3 \mu M$ but showed an ~30-fold lower affinity when studied with the cloned intestinal isoform PEPT1 (4). In addition, the apparent $K_i$ values of three differently charged dipeptides (Gly-Gln, Gly-Asp, Gly-Lys) were determined. This was of interest since we recently demonstrated for PEPT2, when expressed in oocytes, that affinities for the peptides decreased in the order Gly-Asp, Gly-Gln, Gly-Lys (1). From Fig. 4 it becomes evident that all substrates displayed a high affinity to the binding site of the peptide transporter in LLC-PK₁.

Fig. 2. Uptake of 5 µM D-Ala-Lys-AMCA into LLC-PK₁ cells. Uptake of the fluorescent dipeptide was determined 9 days postconfluently at pH 6.0 and is shown in absence (A, C) or presence (B, D) of 5 mM Gly-Gln. Shown are photomicrographs using Nomarski optics (A, B) or conventional fluorescence microscopy (C, D).
![Graph](image1)

**Fig. 3.** Uptake of $\text{d-}[\text{3H}]\text{Phe-L-Ala}$ into LLC-PK$_1$ cells as a function of substrate concentration and incubation pH. Transport was measured at pH 6.0 at day 9 postconfluently in the presence of increasing concentrations of $\text{d-Phe-L-Ala}$. Radioactivity associated with the cells in the presence of 20 mM Gly-Gln was assumed to represent substrate diffused or bound, and the amount was subtracted from total counts. Curves were fitted to a Michaelis-Menten equation by nonlinear regression analysis, and the apparent $K_m$ value was derived by the least-squares method. Insert: transport of 5 µM $\text{d-}[\text{3H}]\text{Phe-L-Ala}$ when measured in a range between pH 5.0 and 8.0.

![Graph](image2)

**Fig. 4.** $K_i$ values for differently charged dipeptides and the $\beta$-lactam, cefadroxil, with regard to $\text{d-}[\text{3H}]\text{Phe-L-Ala}$ uptake into LLC-PK$_1$ cells. Uptake of 5 µM $\text{d-Phe-L-Ala}$ at pH 6.0 was measured in the presence of increasing concentrations of cefadroxil (●), Gly-Asp (○), Gly-Gln (▲), or Gly-Lys (△), respectively. Data represent means ± SE from 3–6 wells per concentration tested.

Cellular acidification of LLC-PK$_1$ cells is a consequence of H$^+$-peptide cotransport. H$^+$-coupled peptide transport with a concomitant decline in pH$_i$ has so far been demonstrated for the renal transporter only for the acidic substrate Ala-Asp (17). By using the pH indicator BCECF, here we show that superfusion of LLC-PK$_1$ cells with acidic (Gly-Asp), neutral (Gly-Gln), or basic (Gly-Lys) dipeptides at extracellular pH 6.0 leads to strong intracellular acidification that reached steady-state levels at a pH$_i$ of 6.25 and did not differ significantly between those peptides chosen (Fig. 5). In contrast, the acidification induced by cefadroxil was markedly smaller (Fig. 5). After the substrates were washed out by perfusion with buffer pH 7.4, cells totally recovered from the peptide or peptide mimetic-induced acid load, and pH$_i$ returned to its initial values.

### DISCUSSION

Reabsorption of short-chain peptides in the mammalian renal tubule has been described as mediated by two different H$^+$-coupled transport systems that differ considerably in substrate affinities (9, 10, 23). Although the high-affinity-type transport system PEPT2 is prominent on the mRNA level and the functional level, Northern blot analysis suggested that the mRNA of the low-affinity-type transporter PEPT1 is also expressed in kidney but at low levels (19, 20, 22). Brandsch et al. (6) suggested that PEPT1 and PEPT2 may be located in different sections of the nephron. According to their hypothesis, the concentration of small peptides increases from proximal to distal parts of the nephron, due to progressive hydrolysis of filtered oligopeptides by brush-border membrane peptidases. Therefore, the presence of the high-affinity-type PEPT2 in more proximal and of the low-affinity-type PEPT1 in more distal parts of the tubule would be advantageous with regard to the most efficient conservation of amino acid nitrogen. However, so far the proposed different localizations of both transporter isoforms along the tubule, e.g., by in situ hybridization techniques or immunolocalization, have not been reported.

### Table 1. Inhibition of $\text{d-}[\text{3H}]\text{Phe-L-Ala}$ uptake into LLC-PK$_1$ cells by selected peptides and peptidomimetics

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Uptake, % of control</th>
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<tr>
<td>Gly-Gln</td>
<td>1.1 ± 0.5*</td>
</tr>
<tr>
<td>Gly-Lys</td>
<td>9.4 ± 4.5*</td>
</tr>
<tr>
<td>Gly-Asp</td>
<td>2.1 ± 0.7*</td>
</tr>
<tr>
<td>Carnosine</td>
<td>5.7 ± 2.3*</td>
</tr>
<tr>
<td>L-Ala</td>
<td>93.4 ± 1.8t</td>
</tr>
<tr>
<td>L-Ala-L-Ala</td>
<td>2.6 ± 0.9*</td>
</tr>
<tr>
<td>L-Ala-L-Ala-L-Ala</td>
<td>1.0 ± 0.3*</td>
</tr>
<tr>
<td>Cefadroxil</td>
<td>6.7 ± 2.5*</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>105.2 ± 6.8t</td>
</tr>
<tr>
<td>Captopril</td>
<td>90.3 ± 8.5t</td>
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</tbody>
</table>

Values are means ± SE. Uptake of 5 µM $\text{d-}[\text{3H}]\text{Phe-L-Ala}$ into LLC-PK$_1$ cells was measured 9 days postconfluently at pH 6.0 in the absence (control) or presence of 1 mM inhibitors. *P < 0.001. †Not significantly different from control.
Madin-Darby canine kidney cells, which display characteristics of cells of the distal tubule (18) or collecting duct (24), have been demonstrated to express a low-affinity-type peptide (PEPT1-like) transport activity, suggesting that distal parts of the tubule express solely functional PEPT1 carriers. Moreover, it was shown that this transporter activity is regulated by calmodulin-dependent processes (7).

Although, recently, expression of the high-affinity PEPT2 transporter in rat proximal tubular cells immortalized by SV40 transformation (6) has been demonstrated, these cells are not readily available. The well-established renal proximal cell line LLC-PK₁ (16, 21, 28), on the other hand, was reported not to possess endogenous peptide transport activity (7, 29, 30), a feature that was exploited to use LLC-PK₁ as a host for heterologous expression of PEPT1 and PEPT2 (29–31). Confirming the results of these studies, it was shown here that, before reaching the confluent state, LLC-PK₁ cells possess indeed only very low peptide transport activity. However, we also clearly demonstrate that endogenous peptide transport activity resembling the PEPT2 type is expressed in LLC-PK₁ after cells have reached confluency. Phenotypical characteristics of PEPT2, such as the pH optimum for D-[3H]Phe-L-Ala influx, the kinetics of dipeptide influx, and the existence of a PEPT2-specific mRNA present in LLC-PK₁ cells, justify this conclusion. In addition, the apparent affinities for dipeptides and cefadroxil determined in LLC-PK₁ are almost identical to those reported in Xenopus laevis oocytes (3, 11) or Pichia pastoris (12) expressing the cloned rabbit renal PEPT2 or in Hela cells expressing the human PEPT2 (14). Affinities of the same substrates for interaction with the intestinal transporter are ~20-fold lower when determined in oocytes expressing PEPT1 (4, 11). In the postconfluent state LLC-PK₁ cells not only transport D-[3H]Phe-L-Ala but also the dipeptide mimetic D-Ala-L-Lys-AMCA. This fluorescent dipeptide has recently been demonstrated to display influx characteristics in PEPT2-expressing P. pastoris that were almost identical to those obtained by use of radiolabeled D-Phe-L-Ala (12). The coumarin-conjugated dipeptide therefore might allow investigators to study peptide transport in LLC-PK₁ cells independently of expensive custom synthesis of radiolabeled substrates.

Although the pH dependency of dipeptide transport and the rheogenic character of PEPT2-mediated influx...
of neutral dipeptides already suggested that H+ is the cotransported ion species, this has been verified experimentally in renal cells only for Ala-Asp (17). Here we show that translocation of peptide substrates is associated with H+ influx that reduces pHi markedly, irrespec-
tive of the net charge of the substrates. By using the pHi indicator BCECF, we demonstrate that intracellular hydrolysis of the dipeptide Gly-Asp, Gly-Gln, and Gly-Lys were very similar, whereas those generated by the β-lactam cefadroxil are significantly lower. When currents associated with transport of Gly-Asp, Gly-Gln, and Gly-Lys were determined in voltage-clamped oocytes expressing the rabbit PEPT2, we observed that the same three substrates generated the same maximal current responses, independently of the net charge of the substrates at extracellular pH 6.5 (1). Although pHi could not be measured in oocytes expressing PEPT2, we suggested that the different dipeptides were transported by the same peptide-H+ flux-coupling ratio and that this is the consequence of transport of only the zwitterionic form of the substrates. Our present findings in LLC-PK1 cells confirm this hypothesis by almost identical intracellular acidification rates in the presence of the three differently charged peptides, which may also result from similar if not identical flux-coupling ratios and maximal transport rates.

That pHi is more reduced by dipeptides than by cefadroxil suggests a higher maximal transport capacity for dipeptides. This may be a consequence of the configuration, e.g., when peptides consisting of L-amino acids are compared with substrates with a D-configuration in the amino-terminal position, such as in cefadroxil or D-Phe-L-Ala. This hypothesis is supported by the fact that pHi changes induced by D-Phe-L-Ala are comparable with those of cefadroxil but smaller than those induced by dipeptides consisting of L-amino acids only (data not shown). However, it needs to be emphasized that a rapid intracellular hydrolysis of the dipeptides consisting of L-α-amino acids could also contribute to the more pronounced decrease in pHi observed for the natural dipeptides.

The demonstration that pHi is markedly reduced when dipeptides are taken up by the renal peptide transporter addresses the physiological importance of these transport-mediated pHi changes. Because di- and tripeptides are present in plasma and are continuously filtered in the glomerulus, the renal peptide transporter operates as a constant acid loader in tubular cells. This is important for both the pHi recovery systems and their regulation, as well as for other metabolic events such as increased renal ammoniagenesis in response to a low pHi. Because a number of protein kinase recognition sites have been identified in the coding sequence of PEPT2, regulation of transport activity, in particular in relation to changes in pHi, needs to be investigated. For this purpose LLC-PK1 might provide a very useful cellular model.

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