Endogenous expression of the renal high-affinity H+-peptide cotransporter in LLC-PK₁ 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-PK₁ cells. Am. J. Physiol. 275 (Cell Physiol. 44): C1573–C1579, 1998.—The reabsorption of filtered di- and tripeptides as well as certain peptidomimetics 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-PK₁. Transport was assessed 1) by uptake studies using the radiolabeled dipeptide D-[³H]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 Km value for influx of 25.8 ± 3.6 µM, and affinities of differently charged dipeptides or β-lactam antibiotic cefadroxil to the binding site in the range of 20–80 µM. pH, measurements established the peptide transporter to induce pronounced intracellular acidification in LLC-PK₁ cells and confirm its postulated role as a cellular acid loader.

PEPT2; proximal tubule cell line LLC-PK₁; intracellular acidification; kinetic characterization

Although the characterization of the renal high-affinity transporter has been performed after heterologous expression (1, 3), a detailed analysis of its function in renal epithelial cells with regard to kinetics and acid-loading mechanisms has not been performed. This lack of information results mainly from the unavailability of suitable cell lines. Until now, the only cell line described to express the kidney-specific high-affinity H+-peptide cotransporter endogenously is SKPT-0193 CI.2 obtained by SV40 transformation of rat proximal tubular cells (6). In the present study we describe for the first time the endogenous expression of a high-affinity peptide transporter in the porcine kidney cell line LLC-PK₂. Because LLC-PK₁ cells have been shown to differentiate into epithelial cells that have been proven to be useful in the study of selected proximal cell processes (21, 31), they might provide a valuable model for studies on the characteristics and regulation of the renal high-affinity peptide transporter.

METHODS

Materials. Custom-synthesized D-[³H]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 β-lactams were purchased from Sigma Chemical. The pH-sensitive fluorescent dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-AM (BCECF-AM) was obtained from Bioprobe (Leiden, Netherlands). Fluorescent N-hydroxysuccinimidyl 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 Rener (Dannstadt, Germany). Rat tail collagen R was purchased from Serva. All reagents for RNA preparation and RT-PCR were from MBI Fermentas (Heidelberg, Germany), and the primers were custom synthesized by Eurogentec (Seraing, Belgium). 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-PK₁ 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 µg/ml streptomycin in a humidified incubator at 37°C under an atmosphere of 5% CO₂. Cells between passages 200 and 215 were seeded at a density of 5 × 10⁶ cells/well on Renner 6-well plastic cell culture plates or 2.2 × 10⁶ cells/well on 12-well plates subsequent to collagen coating of the wells with rat tail collagen.

Transport studies. Flux studies in LLC-PK₁ cells were performed in a buffer containing (in mM) 145 NaCl, 5.4 Cl, 1.8 CaCl₂, 1.8 MgSO₄, 20 glucose, and 25 HEPES/Tris (pH 7.4) or MES/Tris (pH ≤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-
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 at pH 7.4 (Fig. 3A).

Fig. 1. Uptake of 5 µM D-[3H]Phe-L-Ala into LLC-PK1 cells as a function of time after confluence (----) or presence of (○) 1 mM Gly-Gln.

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 confluence. Although LLC-PK1 cells have been described not to possess substantial peptide transport activity on the day of reaching confluence (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 postconfluence, since cell adherence to the culture wells 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).
Menten kinetics with an apparent Michaelis-Menten constant ($K_m$) of 25.8 ± 3.6 µM and a maximum velocity ($V_{max}$) of 33.4 ± 1.7 pmol·cm$^{-2}$·15 min$^{-1}$ (Fig. 3). The kinetic characteristics of D-Phe-Ala influx into LLC-PK$_1$ 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-PK$_1$ 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-L-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 $\beta$-lactam antibiotic cefadroxil. In contrast, benzylpenicillin, another $\beta$-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-PK$_1$ 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 ± 1.1 µM (Fig. 4). Cefadroxil uptake in oocytes mediated by the cloned PEPT2 (3) occurs with a $K_m$ of 25.8 ± 6.3 µM 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-PK$_1$.
cells. Moreover, affinities of the differently charged dipeptides clearly revealed PEPT2-like characteristics with $K_i$ values of $8.0 \pm 1.3 \mu M$ for Gly-Asp, $30.3 \pm 1.2 \mu M$ for Gly-Gln, and $151.8 \pm 1.4 \mu M$ for Gly-Lys, respectively.

Intracellular acidification of LLC-PK$_1$ cells is a consequence of $H^+$-peptide cotransport. $H^+$-coupled peptide transport with a concomitant decline in $pHi$ has so far been demonstrated for the renal transporter only for the acidic substrate Ala-Asp (17).

By using the $pHi$ 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 $pHi$ 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 $pHi$ returned to its initial values.

**Expression of a PEPT2 isoform in LLC-PK$_1$ cells.** Although the functional data obtained suggested that the transporter expressed in LLC-PK$_1$ cells is PEPT2-like, the porcine transporter has not been cloned, and therefore it is not known whether the transporter is expressed in these cells. We therefore performed RT-PCR analysis with specific primers derived from highly conserved regions of cloned PEPT2. It becomes evident from Fig. 6 that a product of 732 bp (specific for PEPT2 but not PEPT1) was amplified from LLC-PK$_1$ RNA samples. In addition, the amplified product revealed the same EcoV restriction site as rabbit PEPT2, suggesting that at least a very similar gene product is expressed in LLC-PK$_1$ cells.

**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 kidney 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 immunodetection, have not been reported.

**Table 1. Inhibition of $d$-[H]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±4.9*</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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</table>

Values are means ± SE. Uptake of 5 µM $d$-[H]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 pH, markedly, irrespective of the net charge of the substrates. By using the pH indicator BCECF, we demonstrate that intracellular acidification rates following perfusion with Gly-Asp, Gly-Gln, and Gly-Lys are very similar, whereas those generated by the $\beta$-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 pH, 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 having 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-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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