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Uptake of cyclic dipeptide by PEPT1 in Caco-2 cells: phenolic hydroxyl group of substrate enhances affinity for PEPT1

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Abstract

Uptake of cyclic dipeptides by H^+/oligopeptide cotransporter (PEPT1) was studied in monolayers of the human intestinal cell line, Caco-2. The cyclic dipeptides studied were cyclic glycylphenylalanine (cyclo(Gly-Phe)), cyclic phenylalanylserine (cyclo(Phe-Ser)), cyclic seryltyrosine (cyclo(Ser-Tyr)) and cyclic glycyltyrosine (cyclo(Gly-Tyr)). These molecules have both peptide bonds and aromatic rings, and are similar in structure to cephalexin and cephadroxil, which are transported by PEPT1. Cellular uptake of these cyclic dipeptides was pH dependent, and was inhibited by the addition of PEPT1 substrates such as glycylsarcosine, indicating PEPT1-mediated transport. Michaelis constants (K_m) for these cyclic dipeptides were cyclo(Ser-Tyr) < cyclo(Phe-Ser), and cyclo(Gly-Tyr) < cyclo(Gly-Phe), indicating that tyrosine possessing phenol moiety has higher affinity for PEPT1 than phenylalanine possessing benzen moiety. The K_m for cephadroxil possessing phenol moiety was reportedly lower than that for cephalexin possessing benzen moiety. Therefore, it was concluded that the phenolic hydroxyl group of the substrate may enhance affinity for PEPT1.

Introduction

Intestinal H^+/oligopeptide cotransporter (PEPT1), which transports oligopeptides (Ganapathy & Leibach 1985; Fei et al 1994; Tamai et al 1994), also transports several drugs including cephadroxil and cephalexin. The function of PEPT1 causes efficient absorption of drugs from the intestine (Sinko & Amidon 1998). Thus, PEPT1 plays an important role in intestinal absorption of not only nutrients but also drugs, suggesting that intestinal transport via PEPT1 may be useful for oral drug delivery. However, the structural requirements for PEPT1-mediated transport have not yet been determined.

Some cyclic peptides have biological activity (Prasad et al 1977; Walter et al 1979; Morley & Levin 1980; Sakurada et al 1982; Lin et al 1994). In a previous study, we found that cyclic dipeptides are much more stable than linear dipeptides, and can be transported by PEPT1 in isolated rat small intestine (Mizuma et al 1997). This stability allows the exclusion of metabolic degradation when evaluating transportability. Furthermore, PEPT1-mediated transport of cyclic seryltyrosine (cyclo(Ser-Tyr)) was greater than that of cyclic glycylphenylalanine (cyclo(Gly-Phe)) in isolated rat small intestine (Mizuma et al 1998).

Most cephalosporins, such as cephalexin and cephadroxil, which are transported by PEPT1, possess a phenol or benzen moiety.

In the present study, to determine which structural factors are important in the transport of PEPT1 substrates, we examined cyclic phenylalanylserine (cyclo(Phe-Ser)), cyclic glycyltyrosine (cyclo(Gly-Tyr)), cyclo(Ser-Tyr) and cyclo(Gly-Phe). Cellular uptake of cyclic dipeptides into human intestinal Caco-2 cells was studied to characterize PEPT1-mediated transport.

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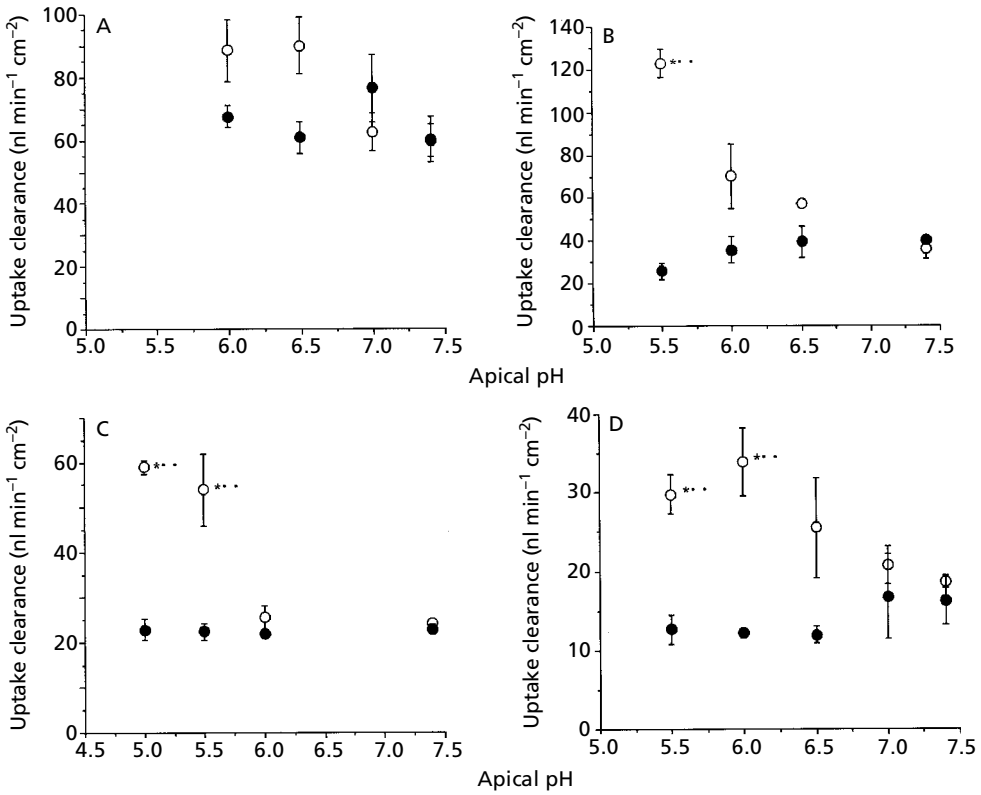

Figure 1 Effects of apical pH and PEPT1 substrate on the uptake of 0.1 mM cyclic glycylphenylalanine (A), cyclic phenylalanylserine (B), cyclic glycyltyrosine (C) and cyclic seryltyrosine (D) into Caco-2 cell monolayers. Incubation time: 10 min. Open and closed circles represent data in the absence and presence of PEPT1 substrate, respectively. Data represent mean±s.e.m., n = 3. *$P < 0.05$ vs at pH 7.4; ¨$P < 0.05$ vs in the presence of PEPT1 substrate, 20 mM glycylleucine (A and B) or 20 mM glycylsarcosine (C and D).

Materials and Methods

Chemicals

Cyclo(Gly-Phe), cyclo(Phe-Ser) and cyclo(Ser-Tyr) were purchased from Bachem Feinchemikalien AG (Switzerland). Cyclo(Gly-Tyr) was purchased from Iwaki Glass (Chiba, Japan). Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline, fetal bovine serum albumin (FBS), non-essential amino acids solution (MEM), and trypsin EDTA liquid were obtained from Invitrogen (CA, USA). All other chemicals were of reagent grade.

Uptake experiment

Caco-2 cells were purchased from the American Type Culture Collection (MD, USA). Dishes (9 cm^2; Corning) were inoculated with 1×10^6 cells (passage 49–81) in 2 mL of DMEM supplemented with 10% FBS, 1% MEM non-essential amino acids, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, pH 7.4. Cells were given fresh culture medium every day and were grown in an atmosphere of 5% CO_2/95% air at 37°C for 10–20 days. Cyclic dipeptide solution (100 μM, 2 mL) at pH 6.0 was placed onto the apical side at 37°C after the medium was aspirated. The following procedure was performed immediately after or periodically after addition of the cyclic dipeptide solution. Caco-2 cells were washed five times with cold incubation solution without cyclic dipeptide. Cells were then dissolved in 400 μL of a 0.1% Triton X, 0.5% perchloric acid solution containing internal standard. Lysate was centrifuged at 10 000 rev min^{-1} for 5 min, and the resulting supernatant was saved for high-performance liquid chromatography (HPLC).

HPLC assay

Cyclic peptides were determined by a previously reported method (Mizuma et al 1997, 1998). Briefly, the HPLC system (Hitachi, Japan) consisted of a pump (655A-11), a UV detector (655A-21) set at 194 nm, an integrator (D-2500) and an ODS column (TSKgel ODS-120T, 4.6 mm i.d, 25 cm length; Tosoh, Japan). For cyclo(Gly-Phe) and cyclo(Phe-Ser), the mobile phase consisted of 10% methanol and 0.05% phosphoric acid. For cyclo(Ser-Tyr), the mobile phase consisted of 3% methanol and 0.05% phosphoric acid. For cyclo(Gly-Tyr), the mobile phase consisted of 2% methanol, 0.05% phosphoric acid and 1-heptane-

sulfonic acid (100 mg L^{-1}). The flow rate of the mobile phase was 1.5 mL min^{-1} and the internal standard was 250 μM *p*-aminosalicylic acid.

Data analysis

Kinetic parameters were calculated by fitting pooled data to equation 1 with a non-linear least square fitting computer program (MULTI; Yamaoka et al 1981) and the Dumping Gauss-Newton method.

$$\text{Uptake rate} = V_{max} \times C/(K_m + C) + CL_{pas} \times C \quad (1)$$

where V_{max} and K_m are the maximum rate and Michaelis constant for PEPT1-mediated transport, respectively, and CL_{pas} is transport clearance for passive transport.

Statistical treatment was done by Dunnett's multiple comparison post-test following analysis of variance or by Student's *t*-test.

Results and Discussion

The time course experiment for cyclic dipeptide uptake showed a linear relationship between the uptake amount and the incubation time during the 10 min after starting incubation (data not shown), indicating that the initial rate of uptake can be calculated from the uptake amount during 10 min of incubation. The profiles of uptake rate versus apical pH for cyclo(Gly-Phe), cyclo(Phe-Ser), cyclo(Gly-Tyr) and cyclo(Ser-Tyr) in the absence and presence of PEPT1 substrate are shown in Figure 1. In the absence of glycylsarcosine or glycylleucine (PEPT1 substrate), the uptake rate was dependent on apical pH, and the uptake rates at pH < 7.4 were greater than that at pH = 7.4. On the other hand, uptake rates in the presence of the PEPT1 substrate were almost constant over the pH range tested. Glycylsarcosine and glycylleucine decreased the uptake rates of cyclic dipeptides at an apical pH less than 7.4, but did not significantly decrease the uptake rates at an apical pH of 7.4. These results indicate that cyclic dipeptides are taken up by H^+-dependent PEPT1 into the inside of cells.

Concentration dependency of cyclic dipeptides uptake was also studied. Eadie-Hofstee plots of the uptake of cyclo(Gly-Phe), cyclo(Phe-Ser), cyclo(Gly-Tyr) and cyclo(Ser-Tyr) by Caco-2 cell monolayers showed a curved data profile (data not shown), indicating that uptake of cyclic dipeptides consists of saturable and non-saturable (passive) components. Thus, the kinetic parameters were calculated by fitting data to equation 1. V_{max}, K_m and CL_{pas} were 21.9 μM, 2.57 pmol min^{-1} cm^{-2} and 30.6 nL $min^{-1}cm^{-2}$ for cyclo(Ser-Tyr), and 118 μM, 1.84 pmol min^{-1} cm^{-2} and 40.8 nL $min^{-1}cm^{-2}$ for cyclo(Phe-Ser), respectively. V_{max}, K_m and CL_{pas} of cyclo(Gly-Tyr) and cyclo(Gly-Phe) were 62.9 μM, 2.24 pmol min^{-1} cm^{-2} and 36.3 nL $min^{-1}cm^{-2}$, and 90.9 μM, 2.30 pmol min^{-1} cm^{-2} and 54.2 nL $min^{-1}cm^{-2}$, respectively. For glycine-possessing cyclic dipeptides, the K_m value for cyclo(Gly-Tyr) uptake was less than that for cyclo(Gly-Phe). For serine-containing cyclic dipeptides, the K_m value for cyclo(Ser-Tyr) uptake was considerably less than that for cyclo(Phe-Ser). This suggests that the tyrosine moiety enhances the affinity of cyclic dipeptide for PEPT1 compared with the phenylalanine moiety. Studies on the transport of cephalosporins in intestine (Sinko & Amidon 1998) and kidney (Daniel & Adibi 1993) reported that the K_m value for cephadroxil was less than that for cephalexin. These reports support our result that the phenol moiety of PEPT1 substrate leads to greater affinity for PEPT1 compared with the benzen moiety. Hence, the phenolic hydroxyl group may enhance affinity for PEPT1. On the other hand, for tyrosine-containing cyclic dipeptides, the K_m value of the uptake of cyclo(Ser-Tyr) was less than that of cyclo(Gly-Tyr), but the K_m value for the uptake of cyclo(Phe-Ser) was not greater than that of cyclo(Gly-Phe) for phenylalanine-containing cyclic dipeptides. Therefore, the contribution of the alcoholic hydroxyl group to the affinity for PEPT1 cannot be generalized. From these results, it is considered that the phenolic hydroxyl group has the potential to interact with neighbouring atom/groups through hydrogen bonding, and that this result provides information for exploring steric structures of the binding site of PEPT1 in plasma membrane.

In conclusion, cyclic dipeptides are transported by PEPT1 in Caco-2 cells, and the phenolic hydroxyl group of the substrate may enhance affinity for PEPT1. Constituents of the binding site of PEPT1 interact with the phenolic hydroxyl group to increase binding affinity. This may be an important factor in designing a rational drug aimed at oral delivery via PEPT1.

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