Interaction of β-Lactam Antibiotics with Histidine Residue of Rat H^+/Peptide Cotransporters, PEPT1 and PEPT2*

(Received for publication, October 14, 1997, and in revised form, December 19, 1997)

Tomohiro Terada, Hideyuki Saito, and Ken-ichi Inui‡

From the Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Kyoto 606-01, Japan

Peptide transporters mediate the H^+-coupled uphill transport of oligopeptides and peptide-like drugs such as β-lactam antibiotics in the intestinal and renal brush-border membranes. Two H^+/peptide cotransporters, PEPT1 and PEPT2, have been cloned and functionally characterized. In this study, we examined the interaction of the dipeptides and β-lactam antibiotics with the histidine residue of rat PEPT1 and PEPT2 transfected into the renal epithelial cell line LLC-PK1, Diethylpyrocarbonate (DEPC), which is a histidine residue modifier, abolished the glycylsarcosine uptake by both transfec-
tants. The DEPC-induced inhibition of glycylsarcosine uptake via PEPT1 or PEPT2 was attenuated by an excess of dipeptide or aminopeptidase. In contrast, anionic cephalosporins without an α-amino group and bestatin, which is an antineoplastic drug with a β-amino group, did not attenuate the DEPC-induced inactivation of PEPT1 and PEPT2. The DEPC inactivation of PEPT1 was almost prevented by various charged dipeptides, which suggests that the inability of the drugs without an α-amino group to prevent the DEPC inactivation was not due to their ionic charge. These findings suggest that the α-amino group of β-lactam antibiotics interacts with the histidine residue of PEPT1 and PEPT2 and may be involved in the mechanism of substrate recognition by the peptide transporters.

In the small intestine and kidney, epithelial assimilation of oligopeptides is mediated by H^+-coupled peptide transport systems (1–3). Because there are 20 amino acids that comprise oligopeptides, there can be 400 dipeptides and 8000 tripeptides with various charges and molecular sizes. In addition to the native small peptides, the peptide transporter recognizes a wide variety of peptide-like drugs such as orally active β-lactam antibiotics (4–6), bestatin (an antineoplastic drug) (7, 8), and angiotensin converting enzyme inhibitors (9). The peptide transporter shows such a broad range of substrate specificity; however, the mechanism that recognizes substrates by the peptide transporter has been incompletely understood. Previously, we have cloned rat H^+/peptide transporters, PEPT1 (10) and PEPT2 (11), and constructed PEPT1- and PEPT2-expressing transfectants (12–14). Using these transfectants, we demonstrated that both PEPT1 and PEPT2 recognized various orally active β-lactam antibiotics (14). When PEPT1-expressing cells were treated with diethylpyrocarbonate (DEPC), 1 which is a histidine residue modifier, cefibuten (anionic cephalosporin without an α-amino group) uptake was completely abolished (12). Furthermore, using the site-directed mutagenesis technique, the histidine residues at positions 57 and 121 of rat PEPT1 were suggested to be involved in substrate recognition and/or responsible for the intrinsic activity of the transporter (12).

To elucidate the diversity of the substrate recognition by the peptide transporters, it is needed to clarify the mechanisms involved in the interaction of the substrates with the essential residues of PEPT1 and PEPT2. Because histidine residues of the peptide transporters have been indicated as the most important key amino acid residues (12, 15, 16), we investigated the functional role of histidine residues to examine the preventive effect of various substrates on the DEPC-induced inactivation of PEPT1 and PEPT2. We report here that the DEPC-sensitive histidine residue of rat PEPT1 and PEPT2 can serve as the binding site of the α-amino group of the substrates.

EXPERIMENTAL PROCEDURES

Cell Culture—The parental LLC-PK1 cells obtained from the American Type Culture Collection (ATCC CRL-1392) were cultured in complete medium, which consisted of Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), supplemented with 10% fetal bovine serum (Whittaker Bioproducts Inc., Walkersville, MD) without antibiotics in an atmosphere of 5% CO2, 95% air at 37 °C. The LLC-PK1 cells, transfected with rat PEPT1 cDNA (LLC-rPEPT1) and with rat PEPT2 cDNA (LLC-rPEPT2) were used as described, previously (14). In the uptake experiments, the cells were cultured for 6–7 days in complete medium.

Uptake Studies by Cell Monolayers—Uptake of [14C]glycylsarcosine was measured in cells grown in 60-mm plastic dishes as described previously (14). The protein content of the cell monolayers solubilized in 1 N NaOH was determined by the method of Bradford (17) using a Bio-Rad protein assay kit with bovine γ-globulin as the standard.

Materials—Cefadroxil (Bristol Meyers Co., Tokyo, Japan), cefixime (Fujisawa Pharmaceutical Co., Osaka, Japan), cefibuten (Shionogi and Co., Osaka, Japan), cephradine (Sankyo Co., Tokyo, Japan), cephalexin (Takeda Chemical Industries, Osaka, Japan), and bestatin (2R,3S)-3-amino-2-hydroxy-4-phenylbutanoyl- L-leucine) (Nippon Kayaku Co., Tokyo, Japan) were gifts from the respective suppliers. [14C]Glycylsarcosine (1.78 GBq/mmol) was obtained from Daiichi Pure Chemicals Co., Ltd. (Ibaraki, Japan). Glycylsarcosine and lysyl-t-leucine were obtained from Sigma. Glutamyl-t-glutamic acid was purchased from the Peptide Institute Inc. (Osaka, Japan). Captopril and DEPC were obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals used were of the highest purity available.

RESULTS

At first, we examined the concentration dependence for the inhibitory effect of DEPC on [14C]glycylsarcosine uptake. When the LLC-rPEPT1 and LLC-rPEPT2 cells were treated with

---

* This work was supported in part by a Grant-in-Aid for Scientific Research (B) and a Grant-in-Aid for Scientific Research on Priority Areas of “Channel-Transporter Correlation” from the Ministry of Education, Science, and Culture of Japan and by a grant from the Japan Research Foundation for Clinical Pharmacology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Pharmacy, Kyoto University Hospital, Sakyo-ku, Kyoto 606-01, Japan. Tel.: 81-75-751-3577; Fax: 81-75-751-4207.

1 The abbreviation used is: DEPC, diethylpyrocarbonate.
various concentrations of DEPC, the half-maximal inhibition for \([^{14}\text{C}]\text{glycylsarcosine}\) uptake in both transfectants was observed at about 0.4 mM, and the maximal inhibition was at 1 mM (Table I). Therefore, the concentration of DEPC for subsequent studies was 1 mM. Fig. 1 shows the effect of DEPC treatment on \([^{14}\text{C}]\text{glycylsarcosine}\) uptake by LLC-rPEPT1 or LLC-rPEPT2 cells and the preventive effect of glycylsarcosine on the DEPC-induced inhibition. The uptake of \([^{14}\text{C}]\text{glycylsarcosine}\) by the transfectants was inhibited markedly by the pretreatment with 1 mM DEPC. This inhibition was abolished mostly by unlabeled 10 mM glycylsarcosine.

Next, we examined whether the DEPC-induced inactivation of PEPT1 and PEPT2 was affected by the pH of the incubation medium. As shown in Fig. 2, glycylsarcosine uptake at both pH 6.0 (with a H\(^+\) gradient) and pH 7.4 (without a H\(^+\) gradient) was blocked by pretreatment with DEPC in the LLC-rPEPT1 and LLC-rPEPT2 cells.

The histidine residues might have been located with the PEPT1 substrate binding site, and therefore, we examined the effect of two cephalosporins on the DEPC inactivation of PEPT1. Fig. 3A shows the effect of cephradine (aminocephalosporin) and cefditoren (anionic cephalosporin without an \(-\text{amino group}\)) concentration on the DEPC-induced inhibition of the glycylsarcosine uptake by the LLC-rPEPT1 cells. Rat PEPT1 has a much higher affinity to cefditoren than to cephradine (13, 14). Cephradine prevented the DEPC-induced inhibition of glycylsarcosine uptake at 5–10 mM, but cefditoren had no preventive effect even at 10 mM. Similar results were observed for the LLC-rPEPT2 cells (Fig. 3B).

To elucidate the effect of cefditoren, we examined the effect of the charge of the substrates on the DEPC-induced inactivation of PEPT1. As shown in Fig. 4A, the DEPC-induced inhibition of \([^{14}\text{C}]\text{glycylsarcosine}\) uptake by LLC-rPEPT1 cells was

---

**TABLE I**

*Effect of DEPC concentration on \([^{14}\text{C}]\text{glycylsarcosine}\) uptake by LLC-rPEPT1 and LLC-rPEPT2 cells*

The cells were preincubated at 25 °C for 10 min with various concentrations of DEPC (pH 6.0). After preincubation, the cells were rinsed once with the incubation medium and then incubated with \([^{14}\text{C}]\text{glycylsarcosine}\) (20 \(\mu\text{M}\), pH 6.0) for 15 min at 37 °C. The radioactivity of the solubilized cells was determined. Each value represents the mean ± S.E. of three independent monolayers. The values in parentheses represent the percentage of control.

| DEPC concentration | \([^{14}\text{C}]\text{Glycylsarcosine uptake}\) |
|--------------------|---------------------------------|
| \(\text{mM}\)      | LLC-rPEPT1 cells | LLC-rPEPT2 cells |
| 0                  | 920 ± 6 (100) | 298 ± 11 (100) |
| 0.01               | 909 ± 19 (99) | 306 ± 19 (103) |
| 0.1                | 909 ± 28 (99) | 311 ± 19 (104) |
| 0.5                | 240 ± 12 (26) | 110 ± 4 (37)   |
| 1                  | 56 ± 5 (6)    | 58 ± 4 (20)    |
| 5                  | 56 ± 9 (6)    | 70 ± 5 (24)    |

---

**Fig. 1.** Effect of DEPC pretreatment on \([^{14}\text{C}]\text{glycylsarcosine}\) uptake by LLC-rPEPT1 (A) and LLC-rPEPT2 cells (B). The cells were preincubated at 25 °C for 10 min with 1 mM DEPC (pH 6.0) in the absence or the presence of glycylsarcosine at 10 mM. After preincubation, the cells were rinsed once with the incubation medium and then incubated with \([^{14}\text{C}]\text{glycylsarcosine}\) (20 \(\mu\text{M},\) pH 6.0) for 15 min at 37 °C. The radioactivity of the solubilized cells was determined. Each column represents the mean ± S.E. of three independent monolayers. GLY-SAR, glycylsarcosine.

**Fig. 2.** Effect of DEPC pretreatment on \([^{14}\text{C}]\text{glycylsarcosine}\) uptake either at pH 6.0 or 7.4 by LLC-rPEPT1 (A) and LLC-rPEPT2 cells (B). The cells were preincubated at 25 °C for 10 min in the absence (open columns) or the presence (shaded columns) of 1 mM DEPC (pH 6.0). After preincubation, the cells were rinsed once with the incubation medium and then incubated with \([^{14}\text{C}]\text{glycylsarcosine}\) (20 \(\mu\text{M}\)) at pH 6.0 or pH 7.4 for 15 min at 37 °C. The radioactivity of the solubilized cells was determined. Each column represents the mean ± S.E. of three independent monolayers.
prevented by various charged substrates but not by ceftibuten. When the inhibitory effect of these compounds was examined, all the dipeptides, cephradine, and ceftibuten inhibited [14C]glycylsarcosine uptake (Fig. 4B). These results suggested that the preventive effect of substrates was independent of either their ionic charges or affinity to the transporters. For the LLC-rPEPT2 cells, these substrates prevented the DEPC-induced inhibition of glycylsarcosine uptake in a manner similar to that used for inhibiting LLC-rPEPT1 cells (data not shown).

We investigated whether the preventive effects depended on the \(\alpha\)-amino group of the substrates. As shown in Fig. 5A, the substrates with an \(\alpha\)-amino group such as glycylsarcosine, cephradine, and cefadroxil (aminopenicillamin) prevented the DEPC-induced inhibition of [14C]glycylsarcosine uptake by LLC-rPEPT1 cells. However, all peptide-like drugs without an \(\alpha\)-amino group such as cephibutin, cefixime, bestatin, and captopril (angiotensin converting enzyme inhibitor) had no effect at a concentration of 10 mM in the LLC-rPEPT1 cells. Cyclacillin did not have the preventive effect despite its having an \(\alpha\)-amino group. Similar results were obtained in LLC-rPEPT2 cells except for cefadroxil (Fig. 5B). In the absence of DEPC, the pretreatment of cefadroxil at 10 mM had an inhibitory effect on glycylsarcosine uptake (data not shown). Therefore, cefadroxil at 10 mM might have a cis-inhibitory effect on the [14C]glycylsarcosine uptake, considering that cefadroxil had a higher affinity for PEPT2 with an apparent inhibition constant of 3 \(\mu\)M (14).

**DISCUSSION**

In the present study, glycylsarcosine uptake by the PEPT1- and PEPT2-expressing transfecants was inhibited by pretreatment with DEPC in the absence and the presence of \(\text{H}^+\) gradient, which suggests that the histidine residue modified by DEPC at least served as the substrate binding site. This could be supported by the fact that the various dipeptides and aminopenicillaminin prevented the DEPC-induced inactivation of PEPT1 and PEPT2. On the other hand, the peptide-like drugs without an \(\alpha\)-amino group had no preventive effect, although they can be transported by the peptide transporter (5–7, 9). These findings suggest that the histidine residue located in the recognition site is involved in the binding site of the \(\alpha\)-amino group of the dipeptide and aminopenicillaminins. Because only the unprotonated imidazole ring reacts with DEPC (18), it is
possible that the imidazole group of the histidine residue located at the recognition site is protonated by the α-amino group of the dipeptides and aminopenicillins but not by the peptide-like drugs that do not have an α-amino group. The α-amino group of the substrates might interact with the imidazole ring of the histidine residue of peptide transporters by proton binding. It is noted that these results were observed for PEPT1 and PEPT2 in a similar manner, which suggests that the DEPC-sensitive histidine residue plays the same role in both transporters.

Among the substrates examined that had an α-amino group, only cyclacillin did not show the preventive effect against the DEPC inactivation. This may be due to the structure of cyclacillin. Cyclacillin has an α-carbon group as part of its cyclohexane ring; therefore, the cyclohexane ring may interfere with the α-amino group-histidine interaction. Nevertheless, cyclacillin was recognized by PEPT1 at a relatively high affinity (14, 19).

A possible explanation is that the hydrophobic NH₂-terminal side chain of cyclacillin interacts with the peptide transporters instead of the α-amino group-histidine interaction. As reported by Daniel et al. (20), the marked hydrophobicity of the NH₂-terminal side chain of aminopenicillins increased the affinity to the renal H⁺/peptide cotransporter. For the peptide-like drugs without an α-amino group such as ceftibuten and ceftixime, which are very hydrophilic, there might be interactions between these drugs and the binding site of the peptide transporter other than the α-amino group-histidine interaction.

We have previously reported that histidines 57 and 121, which are located at the predicted transmembrane domains 2 and 4 of rat PEPT1, are involved in substrate binding and/or are responsible for the intrinsic activity of the transporter (12). In contrast to our results, Fei et al. (21) demonstrated that histidine 57 of human PEPT1 was absolutely essential for the catalytic activity but histidine 121 of human PEPT1 did not appear to play an essential role for the catalytic activity. The reason for this discrepancy regarding the role of histidine 121 in the rat and human PEPT1 remains unknown. However, it is possible that at least two histidine residues are involved in the transport activity of PEPT1. Indeed, Steel et al. (22) proposed that one histidine residue as a cation site was responsible for the proton coupling and that a second histidine residue was adjacent to the peptide binding site in the studies to determine differently charged dipeptide-H⁺ flux coupling ratios. Mackenzie et al. (23) demonstrated that human PEPT1 bound H⁺ first and then the substrate as demonstrated by the biophysical and kinetic analysis of the human PEPT1. These findings suggest that two histidine residues are necessary, because the histidine residue with a protonated imidazole ring cannot bind the α-amino group of the substrates as shown by the present study. Although we cannot identify the histidine residue of the binding site from this study, either histidine 57 or 121 of PEPT1 might be the candidate residue for the binding site of the α-amino group of the substrates.

In conclusion, this is the first demonstration that the α-amino group of the dipeptides and aminopenicillins interacts with the DEPC-sensitive histidine residue of rat PEPT1 and PEPT2. The present findings represent the first step for understanding the substrate recognition mechanisms by peptide transporters.

REFERENCES

1. Leibach, F. H., and Ganapathy, V. (1996) Annu. Rev. Nutr. 16, 99–119
2. Daniel, H., and Herget, M. (1997) Am. J. Physiol. 273, F1–F8
3. Adibi, S. A. (1997) Gastroenterology 113, 322–340
4. Okano, T., Inui, K., Maegawa, H., Takano, M., and Hori, R. (1986) J. Biol. Chem. 261, 14130–14134
5. Tsuji, A., Terasaki, T., Tamai, I., and Hirooka, H. (1987) J. Pharmacol. Exp. Ther. 241, 594–601
6. Muranushi, N., Yoshikawa, T., Yoshida, M., Oguma, T., Hirano, K., and Yamada, H. (1989) Pharm. Res. 6, 308–312
7. Inui, K., Tomita, Y., Katsura, T., Okano, T., Takano, M., and Hori, R. (1992) J. Pharmacol. Exp. Ther. 260, 482–486
8. Saito, H., and Inui, K. (1993) Am. J. Physiol. 265, G289–G294
9. Swaan, P. W., Stehouwer, M. C., and Tukker, J. J. (1995) Biochim. Biophys. Acta 1256, 31–38
10. Saito, H., Okuda, M., Terada, S., Sasaki, S., and Inui, K. (1995) J. Pharmacol. Exp. Ther. 275, 1631–1637
11. Saito, H., Terada, T., Okuda, M., Sasaki, S., and Inui, K. (1996) Biochim. Biophys. Acta 1280, 173–177
12. Terada, T., Saito, H., Mukai, M., and Inui, K. (1996) FEBS Lett. 394, 196–200
13. Terada, T., Saito, H., Mukai, M., and Inui, K. (1997) J. Pharmacol. Exp. Ther. 281, 1415–1421
14. Terada, T., Saito, H., Mukai, M., and Inui, K. (1997) Am. J. Physiol. 273, F706–F711
15. Miyamoto, Y., Ganapathy, V., and Leibach, F. H. (1996) J. Biol. Chem. 261, 16133–16140
16. Kato, H., Maegawa, H., Okano, T., Inui, K., and Hori, R. (1989) J. Pharmacol. Exp. Ther. 251, 745–754
17. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
18. Miles, E. W. (1977) Methods Enzymol. 47, 431–442
19. Ganapathy, M. E., Brandsch, M., Prasad, P. D., Ganapathy, V., and Leibach, F. H. (1995) J. Biol. Chem. 270, 25672–25677
20. Daniel, H., and Adibi, S. A. (1993) J. Clin. Invest. 92, 2215–2223
21. Fei, Y.-J., Liu, W., Prasad, P. D., Kekuda, R., Oblak, T. G., Ganapathy, V., and Leibach, F. H. (1997) Biochemistry 36, 452–460
22. Steel, A., Nussberger, S., Romero, M. F., Boyer, W. F., Boyd, C. A. R., and Hediger, M. A. (1997) J. Biol. Chem. 272, 5863–5869
23. Mackenzie, B., Loo, D. D. F., Fei, Y.-J., Liu, W., Ganapathy, V., Leibach, F. H., and Wright, E. M. (1996) J. Biol. Chem. 271, 5430–5437