Recognition of L-Amino Acid Ester Compounds by Rat Peptide Transporters PEPT1 and PEPT2

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ABSTRACT

Peptide transporters (PEPT1 and PEPT2) in epithelia play an important role in the absorption of small peptides and peptide-like drugs. Recently, it was demonstrated that various nonpeptidic compounds can be transported by these transporters. In the present study, we focused on the L-amino acid ester compounds and examined the mechanisms of their interaction with rat PEPTs (rPEPTs) using stable transfectants. Valacyclovir, the L-valyl ester prodrug of the antiviral agent acyclovir, competitively inhibited [14C]glycylsarcosine uptake in the rPEPT1- or rPEPT2-expressing cells. Dixon plot analyses showed that the inhibition constant (K_i) values of valacyclovir were 2.7 and 0.22 mM for rPEPT1 and rPEPT2, respectively, indicating that Val-OMe is also a high-affinity substrate for rPEPT2. Val-OMe had a trans-stimulation effect on [14C]glycylsarcosine efflux from both transfectants, suggesting the translocation of L-valine methyl ester via rPEPTs. Val-OMe showed the most potent inhibitory effect among the several L-amino acid methyl esters examined. We conclude that Val-OMe, as well as valacyclovir, could be recognized and transported by rPEPT1 and rPEPT2 and that these L-valyl esters showed higher affinity for rPEPT2 as do most substrates of these transporters. Our results suggest that L-valine is a desirable L-amino acid for the esterification of poorly permeable drugs to enhance their oral bioavailability targeting intestinal PEPT1.

Peptide transporters (PEPT1 and PEPT2) play an important role in the maintenance of protein nutrition by mediating the transport of dipeptides and tripeptides across the brush-border membranes in the small intestine and kidney (Leibach and Ganapathy, 1996; Daniel and Herget, 1997). Due to their broad substrate specificity, PEPT1 and PEPT2 are able to transport various peptide-like drugs structurally related to small peptides such as β-lactam antibiotics (Ganapathy et al., 1995; Saito et al., 1995; Terada et al., 1997b) and the anticancer agent bestatin (Saito et al., 1996). In addition, intestinal PEPT was used to improve the intestinal absorption of poorly absorbed pharmacologically active amino acid analogs through the production of dipeptidyl derivatives (Hu et al., 1989; Tsuji et al., 1990). Therefore, the determination of the structural requirements for substrate recognition of PEPTs is important for the molecular design of pharmacologically active peptide-like drugs and prodrugs.

Recently, valacyclovir, the L-valyl ester of the antiviral agent acyclovir, was demonstrated to be transported by intestinal PEPT1 (Balimane et al., 1998; Han et al., 1998a,b). The bioavailability of acyclovir after oral valacyclovir administration was considerably greater than that after oral administration of acyclovir (Perry and Faulds, 1996). Therefore, L-valyl esterification seems to be a very useful strategy for improving the intestinal absorption of drugs with low oral bioavailability. However, it has not been fully understood whether other L-amino acid ester compounds, as well as valacyclovir, are recognized by PEPTs. Such information will provide the molecular basis for a rational design of oral prodrugs targeting the intestinal PEPT1. In addition, there is little information about the interaction of L-amino acid ester compounds with PEPT2. A comparison of this interaction between PEPT1 and PEPT2 will be important to characterize the biological aspects of the PEPTs.

The aim of this study was to gain more information regarding the interaction of L-amino acid ester compounds with PEPTs. We examined the effects of valacyclovir and a series of L-amino acid alkyl esters on [14C]glycylsarcosine uptake by rat PEPT1- or PEPT2-expressing transfectant. Furthermore, we focused L-valine methyl ester (Val-OMe) as a typical of L-amino acid alkyl ester and investigated its inhibition kinet-
ics and trans-stimulation effect on [14C]glycylsarcosine uptake by both sets of transfectants.

Experimental Procedures

Materials. Valacyclovir was supplied by Glaxo Wellcome Research and Development (Hertfordshire, UK). [14C]Glycylsarcosine (1.78 GBq/nmol) was obtained from Daiichi Pure Chemicals Co., Ltd. (Ibaraki, Japan). All l-valine alkyl esters were purchased from Sigma Chemical Co. (St. Louis, MO). L-Alanine- and l-phenylalanine methyl esters were obtained from Nacalai Tesque (Kyoto, Japan). Other l-amino acid methyl esters were obtained from Sigma Chemical Co. All other chemicals used were of the highest purity available.

Cell Culture. The parental LLC-PK1 cells were obtained from American Type Culture Collection (ATCC CRL-1392; Rockville, MD). The LLC-PK1 cells transfected with rPEPT1 cDNA (LLC-rPEPT1) or with rPEPT2 cDNA (LLC-rPEPT2) were constructed as described previously (Terada et al., 1997a,b). Those transfectants were cultured in complete medium consisting of Dulbecco’s modified Eagle’s medium (Gibco Life Technologies, Grand Island, NY) supplemented with 10% FBS (Whittaker Bioproducts Inc., Walkersville, MD) with G418 (Gibco) in an atmosphere of 5% CO2/95% air at 37°C. In the uptake experiments, the cells were cultured for 6 days in complete medium without G418.

Uptake Studies by Cell Monolayers. The uptake of [14C]glycylsarcosine was measured in cells grown on 35-mm plastic dishes as described previously (Terada et al., 1997b). The protein contents of cell monolayers solubilized in 1 N NaOH were determined according to the method of Bradford (1976) with a Bio-Rad (Hercules, CA) protein assay kit with bovine γ-globulin as the standard.

Statistical Analysis. Data were analyzed statistically with non-paired t test or one-way ANOVA, followed by Scheffe’s test when multiple comparisons were needed.

Results

Interaction of Valacyclovir with PEPTs. Previously, we established and characterized rPEPT1- or rPEPT2-expressing transfectant (LLC-rPEPT1 and LLC-rPEPT2 cells, respectively; Terada et al., 1997a,b). With the use of these transfectants, the mechanism involved in the interaction of valacyclovir with rPEPT1 and rPEPT2 was examined. As shown in Fig. 1, A and B, valine and acyclovir, which are constituents of valacyclovir, had no inhibitory effect, whereas valacyclovir had a potent inhibitory effect on [14C]glycylsarcosine uptake by both transfectants. The inhibiton kinetics of valacyclovir on [14C]glycylsarcosine uptake revealed that valacyclovir was found to decrease the affinity of glycylsarcosine for the transporters (Km values for control versus those in the presence of valacyclovir = 1.9 ± 0.1 versus 3.2 ± 0.3 mM for rPEPT1 and 0.09 ± 0.01 versus 0.15 ± 0.03 mM for rPEPT2). In contrast, the maximal velocities showed no significant changes (Vmax values for control versus those in the presence of valacyclovir: 66 ± 2 versus 62 ± 5 nmol/mg protein/15 min for rPEPT1 and 1.1 ± 0.1 versus 1.0 ± 0.2 nmol/mg protein/15 min for rPEPT2). These results showed that valacyclovir competitively inhibited [14C]glycylsarcosine uptake by both rPEPT1 and rPEPT2.

To determine the inhibition constant (Ki) values of valacyclovir, Dixon plot analyses were performed. As illustrated in Fig. 1, C and D, plots were linear in both transfectants. The Ki values calculated from the points of intersection were 2.7 mM for rPEPT1 and 0.22 mM for rPEPT2. These observations indicated that rPEPT2 had higher affinity for valacyclovir than PEPT1.

Interaction of l-Valine Alkyl Esters with PEPTs. To clarify whether other ester compounds are recognized by rPEPT1 and rPEPT2, we next investigated the interactions of a series of l-amino acid alkyl esters with both transporters. We first examined the effects of l-valine alkyl esters on [14C]glycylsarcosine uptake by LLC-rPEPT1 and LLC-rPEPT2 cells. As shown in Fig. 2, [14C]glycylsarcosine uptake
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by both transfectants was inhibited by various L-valine alkyl esters, as well as by valacyclovir, but not by valine.

Inhibition Kinetics and trans-Stimulation Effect of Val-OMe. Because Val-OMe was the smallest molecule of the L-valine alkyl esters examined, we were interested in this compound to study the minimal structural requirements for the recognition by PEPTs and examined its inhibition kinetics on [14C]glycylsarcosine uptake by both transfectants. As shown in the insets in Fig. 3, the presence of Val-OMe decreased the affinity of glycylsarcosine to the transporters (Km values for control versus in the presence of Val-OMe: 1.7 ± 0.02 versus 3.1 ± 0.09 mM for rPEPT1 and 0.97 ± 0.003 versus 0.16 ± 0.03 mM for rPEPT2). The maximal velocities were not significantly changed (Vmax values for control versus in the presence of Val-OMe: 57 ± 3 versus 53 ± 3 nmol/mg protein/15 min for rPEPT1 and 1.0 ± 0.1 versus 0.9 ± 0.2 nmol/mg protein/15 min for rPEPT2). These results showed that Val-OMe competitively inhibited [14C]glycylsarcosine uptake by both rPEPT1 and rPEPT2, as well as by valacyclovir.

Figure 4 shows the results of Dixon plot analyses of Val-OMe. The plots also suggested that Val-OMe inhibited [14C]glycylsarcosine uptake in a competitive manner with K values of 3.6 and 0.83 mM for rPEPT1 and rPEPT2, respectively.

trans-Stimulation studies have been done to show whether inhibitors can be substrates for the PEPT (i.e., translocation by the PEPT; Okano et al., 1986; Inui et al., 1992). Because preloading of the transfectants with Val-OMe was difficult due to the conversion of this compound to L-valine and methanol, we examined the trans-stimulation effects of the Val-OMe on the efflux of [14C]glycylsarcosine from transfectants preloaded with [14C]glycylsarcosine. As shown in Fig. 5, A and B, efflux of [14C]glycylsarcosine was significantly enhanced by unlabeled glycylsarcosine and Val-OMe but not by valine. In another experiment, we further tested whether the L-valine benzyl ester (the largest molecule of L-valine alkyl esters examined) exhibited the trans-stimulation effect. L-Valine benzyl ester also showed the trans-stimulation effect on the efflux of [14C]glycylsarcosine by transfectants expressing rPEPT1 (control, 290 ± 12 pmol/mg protein/5 min; 5 mM L-valine benzyl ester, 510 ± 9 pmol/mg protein/5 min, mean ± S.E. of three monolayers, P < .01) or rPEPT2 (control, 30 ± 0.3 pmol/mg protein/5 min; 1 mM L-valine benzyl ester, 42 ± 0.5 pmol/mg protein/5 min, mean ± S.E. of three monolayers, P < .01).

Interaction of L-Amino Acid Methyl Esters with PEPTs. Finally, we examined the effects of various L-amino acid derivatives of methyl ester on [14C]glycylsarcosine uptake by LLC-rPEPT1 and LLC-rPEPT2 cells. As shown in Fig. 6, [14C]glycylsarcosine uptake by LLC-rPEPT1 cells was inhibited markedly by the presence of Val-OMe or L-isoleucine methyl ester. On the other hand, in LLC-rPEPT2 cells, L-valine, L-leucine, and L-isoleucine methyl ester potently inhibited [14C]glycylsarcosine uptake.

Discussion

It has been believed that peptide bonds are required for recognition by PEPTs as substrates. However, recent investigations have demonstrated that various compounds with no peptide bonds, such as 4-aminophenylacetic acid (Temple et al., 1998), δ-aminolevulinic acid (which has a ketomethylene
group instead of peptide bond; Döring et al., 1998a), and ω-amino fatty acids with more than 4CH₂ units in the backbone (Döring et al., 1998b), are recognized and transported by PEPTs. Valacyclovir, the L-valyl ester prodrug of the antitherpetic agent acyclovir, has also shown to interact with PEPTs (Balimane et al., 1998; Ganapathy et al., 1998; Han et al., 1998a,b). Oral administration of valacyclovir produced a higher bioavailability, followed by the L-isoleucyl, L-alanyl, glycyl, and L-leucyl esters. This order was comparable with the inhibitory potencies of L-amino acid methyl esters on [14C]glycylsarcosine uptake by rPEPT1-expressing cells in the present study. Therefore, the findings of Beauchamp et al. (1992) may reflect the affinities of these compounds to intestinal PEPT1. In support of these suggestions, glycyl ester acyclovir showed less transport by human PEPT1 than the L-valyl ester acyclovir valacyclovir (Han et al., 1998a,b).

Taken together, interaction potencies of L-amino acid ester compounds with PEPTs were dependent on L-amino acids, and L-valine was suggested to be a preferable L-amino acid for this purpose.

A comparison of Kᵢ values showed that both valacyclovir and Val-OMe had higher affinity for rPEPT2 than for rPEPT1. These findings were comparable with those of previous reports that PEPT2 showed higher affinity for chemically diverse dipeptides (Ramamoorthy et al., 1995) and amino β-lactam antibiotics (Ganapathy et al., 1995; Terada et al., 1997b) compared with PEPT1. Therefore, the peptide bonds appear to not be important for determination of the affinity of both transporters. We previously demonstrated that the ω-amino group of substrates was one of the determinants for the high affinity interaction with rPEPT2 (Terada et al., 1997b). Because both valacyclovir and Val-OMe have a free ω-amino group originating from L-valine, our assumption was shown to be true in this case.

In conclusion, we demonstrated that valacyclovir and Val-OMe are recognized by rPEPT1 and rPEPT2 and that valacyclovir and Val-OMe showed higher affinity for rPEPT2 than for rPEPT1. These findings suggest that L-valyl ester uptake via PEPT1 (Balimane et al., 1998; Han et al., 1998a,b) and the present results concerning the trans-stimulation effect of L-valine benzyl ester. Val-OMe was a probable substrate for the PEPTs, whereas L-valine was not. The only difference in the chemical structures of these compounds is COO⁻ versus COOCH₃. These findings raised the question of how the transporters discriminate between these two compounds. The size or configuration of the ester methyl group of Val-OMe may be an important determinant for recognition by PEPTs. Alternatively, the carboxylic anion charge of L-valine might interfere with the interaction with PEPTs. The precise mechanisms of the different interactions of L-valine and Val-OMe with PEPTs were unclear based on the results of the present study, and further studies are needed to clarify these issues. Recently, using a series of ω-amino fatty acids as model compounds, Döring et al. (1998b) demonstrated that 5-amino pentanoic acid satisfied the minimal structural requirements for substrates of rabbit PEPT1. Considering the differences of L-valine and Val-OMe, it should be reasonable that Val-OMe is also one of the substrates to satisfy the minimal structural requirements.

Fig. 5. Efflux of [14C]glycylsarcosine from LLC-rPEPT1 (A) and LLC-rPEPT2 (B) cells. The cell monolayers were preloaded by incubation with 20 μM [14C]glycylsarcosine for 15 min at 37°C. The efflux of [14C]glycylsarcosine was measured after incubation with either incubation medium (control) or each test compound for 5 min at 37°C. Each column represents the mean ± S.E. of six monolayers from two separate experiments. *P < .05, **P < .01, significantly different from control.

Fig. 6. Effects of L-amino acid methyl esters on [14C]glycylsarcosine uptake by LLC-rPEPT1 (A) and LLC-rPEPT2 (B) cells. The cell monolayers were incubated with incubation medium containing [14C]glycylsarcosine (20 μM, pH 6.0) for 15 min at 37°C. The efflux of [14C]glycylsarcosine was measured after incubation with either incubation medium (control) or each test compound for 5 min at 37°C. Each group represents the mean ± S.E. of three monolayers. *P < .05, **P < .01, significantly different from control.
compounds can be generally accepted as substrates of PEPT1 and PEPT2, similar to other substrates of PEPTs. From the viewpoint of drug delivery system, L-valyl esterification of poorly absorbed drugs may be a useful and developmental strategy for improving their intestinal absorption.

References


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