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Research Paper

Quantitative Evaluation of PEPT1 Contribution to Oral Absorption of Cephalexin in Rats

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Purpose. PEPT1 mediates the intestinal absorption of many drugs, but its contribution to oral absorption of drugs is still controversial. The objective of this study is to quantitatively evaluate the contribution of PEPT1 to oral absorption of cephalexin, a typical substrate for PEPT1, in rats.
Materials and Methods. The absorbability of cephalexin *via* PEPT1 or passive diffusion was assessed in five intestinal segments by utilizing glycyl-proline as a competitive inhibitor by *in-situ* closed loop method. Absorption kinetics of cephalexin after oral administration was predicted by GI-Transit-Absorption model.
Results. Absorbability of cephalexin was segment-dependent, and concentration-dependent in all the segments except for the lower ileum. Intrinsic absorption rate constant *via* PEPT1 ranged from 0.64 to 4.07 h^{-1}. The absorption rate constants *via* passive diffusion ranged from 0.78 to 1.24 h^{-1}. Plasma concentration–time profile of cephalexin was successfully predicted and the substantial contribution of PEPT1 to the oral absorption was calculated to be from 46% to 60% of total absorption. Simulation study indicated that 83% bioavailability would be expected for cephalexin even though PEPT1 does not function.
Conclusions. PEPT1 substantially contributes to oral absorption of cephalexin, around a half of total absorption. However, the function of PEPT1 can be compensated by passive diffusion for cephalexin.

KEY WORDS: cephalexin; GITA model; oral absorption; passive diffusion; PEPT1.

INTRODUCTION

The intestinal absorption of many drugs are mainly *via* passive diffusion, but several membrane transporters are believed to be involved in the intestinal absorption of drugs at the same time. However, the substantial contribution of carrier-mediated transport mechanism or passive diffusion mechanism to the absorption of drugs orally administered is still ambiguous.

Proton-coupled oligopeptide transporter PEPT1 is well known as a transporter for peptide-mimetic drugs such as β-lactam antibiotics as well as oligopeptides (1–5). PEPT1 is driven by an inward transmembrane proton gradient by sequential actions of Na^+/K^+ ATPase and Na^+/H^+ antiporter in the epithelial cells of small intestine (6, 7). Since most drugs, of which absorption can be mediated by PEPT1, have hydrophilic property, which is usually limiting the membrane transport *via* passive diffusion, PEPT1 has been thought to be an important transporter for the absorption of such drugs. Actually the significant contribution of PEPT1 was recognized (2–6,8,9), but it was also suggested that passive diffusion was involved in the absorption of PEPT1 substrates (9–13).

PEPT1 activity is determined by the expression level, H^+ gradient as driving force and the pH-dependent conformation change (14–16). PEPT1 expression profile along through the small intestine is still controversial, because the expression and/or function of PEPT1 is influenced by various factors (17–26) including the circadian rhythm (21–23) and food condition (23–25). The value of pH in the luminal fluid is also variable, but it is generally accepted that around 6 in the proximal segment ascends to around 7 in the distal one throughout the small intestine (27,28). The conformation change in PEPT1 by environmental pH was suggested to influence the transport activity (14–16). These would be reasons for why the estimation of actual contribution of PEPT1 to drug absorption after oral administration is still ambiguous.

Cephalexin, a β-lactam antibiotics, is one of typical substrates for PEPT1 (1–5) and believed to be absorbed mainly *via* PEPT1, because cephalexin is well absorbed after oral administration in spite of its hydrophilic property (29,30). Actually, many researchers evidenced that cephalexin is transported *via* PEPT1 and indicated the relatively high affinity to PEPT1 (4,5,31). The expression levels of hPEPT1 were significantly correlated with the uptake of cephalexin into hPEPT1-overexpressed Caco-2 cells (9). On the other hand, it was also suggested that the passive diffusion substantially contributed to the absorption of cephalexin (9–

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13). The absorbability of cephalexin is much higher in the upper segment of small intestine than that in the lower segment (30,32), but the expression level of PEPT1 is even higher in the lower segment under the fed condition (33). Furthermore, the expression levels along through the small intestine did not correlate with the absorption function estimated for cephadroxil, a very similar with cephalexin in terms of chemical structure, K_m and transport characteristics (5), by *in-situ* closed loop method (33). These reports indicate that PEPT1 is involved in the absorption of cephalexin, but that the substantial contribution is still controversial at the same time.

Furthermore, drug absorption after oral administration is influenced by many physiological factors including gastrointestinal transit (34,35). Residence time of drugs in each segment is one of the most important factors regulating drug absorption after oral dosing (35). Utilizing GI-Transit-Absorption model (GITA model) mainly based on the drug absorbability in each segment and GI-transit kinetics, we have predicted and analyzed the absorption kinetics of several drugs including cephalexin (30,35–38). In our previous studies, the absorbability of cephalexin was higher in the upper segments from duodenum to lower jejunum, but the substantial contribution after oral dosing was higher in lower jejunum and upper ileum because of residence time (30) and the segmental contribution was variable dependent on the change in gastrointestinal transit (39). This is also a great factor that makes it difficult to evaluate the actual role of PEPT1 in drug absorption after oral administration.

In the present study, therefore, we assumed that carrier-mediated absorption of cephalexin would be ascribed to PEPT1 alone in rats, and tried to quantitatively evaluate the substantial contribution of PEPT1 to the absorption of cephalexin after oral administration by GITA model, considering the absorbability *via* PEPT1 and passive diffusion and the effect of gastrointestinal transit.

MATERIALS AND METHODS

Materials. Cephalexin anhydrate and sodium ampicillin, an internal standard, were purchased from Sigma Chemical Co. (St. Louis, MO). Glycyl-proline (Gly-Pro) was obtained from Peptide Institute Inc. (Minoh, Japan). All other chemicals and reagents were analytical grade commercial products.

Animals. Male Wistar rats (Japan SLC, Hamamatsu, Japan), maintained at 25°C and 55% humidity under 12-h lighting condition (8:00–20:00), were allowed free access to standard laboratory chow (Clea Japan, Tokyo, Japan) and water. They were fasted for 24 h prior to and during the experiment, but were allowed free access to water. Rats weighing 185–255 g were randomly assigned to each experimental group. Every animal experiment was started around 14:00 to avoid the effect of diurnal change in PEPT1 activity (21–23). Our investigations were performed after approval by our local ethical committee at Okayama University and in accordance with "Principal of Laboratory Animal Care (NIH publication # 85-23)".

In-situ Closed Loop Study. The absorption experiments were performed in five different intestinal segments from duodenum to lower ileum with a conventional *in-situ* closed loop method (38, 41). The length of each segment used for the absorption study was as follows: duodenum, ca. 6 cm; upper and lower jejunum, ca. 10 cm; upper and lower ileum, ca. 10 cm. Cephalexin with or without Gly-Pro was introduced into each segment at 0.5 mL of isotonic phosphate buffer solution (pH 6.5 for duodenum and jejunum, and pH 7.0 for ileum, which was determined based on physiological data reported by Kararali (28)). Gly-Pro was chosen as a competitive inhibitor for the absorption of cephalexin, because Gly-Pro has a very high affinity for PEPT1 and is not hydrolyzed by peptidase (42–44). The luminal fluid was completely collected from each segmental loop and the remaining amounts of cephalexin were determined at fixed time periods after starting the absorption study. Then, the apparent first-order absorption rate constant (ka^{app}) was obtained by linear regression.

In-vivo Oral and Intravenous Administration Studies. One day before drug administration, the jugular vein of a rat was cannulated with vinyl tubing (i.d., 0.5×0.8 mm; Dural Plastics & Engineering, Australia) under ether anesthesia. In the case of oral administration, cephalexin dissolved in saline was intragastrically administered at a dose of 5 mg/5 mL/kg (2.74 mM), corresponding to a standard therapeutic dose, under light ether anesthesia. For intravenous administration, cephalexin dissolved in saline was administered into the tail vein at the dose of 5 mg/1 mL/kg under light ether anesthesia. Blood samples were periodically taken from the cannulated jugular vein. Plasma obtained by centrifugation was deproteinized by methanol. The resulting supernatant was introduced into HPLC for the analysis of cephalexin.

Analytical Method. Cephalexin was determined by HPLC, which consists of a model LC-6A HPLC pump (Shimadzu, Kyoto, Japan), a model SIL-6A system controller (Shimadzu), and a model SPD-6A UV detector (Shimadzu) set at 260 nm. Synergi 4u Fusion-RP 80A (150×4.6 mm i.d., Phenomenex, Inc. California, USA) was used at room temperature. The mobile phase was 10 mM acetate buffer (pH 6.0):methanol (85:15, *v*/*v*) delivered at 1.0 mL/min. The standard curves (0.1–1 μg/mL or 1–30 μg/mL) gave the coefficients of variation (CV) ranged from 8.90% to 23.73% or 3.08% to 12.39%, respectively. The correlation coefficients were over 0.948.

Pharmacokinetic Analysis. Pharmacokinetic parameters describing the plasma concentration–time profile of cephalexin after intravenous administration were obtained based on a two-compartment model by the non-linear least-squares regression program MULTI (45). The plasma concentration-time profile is generally expressed by the following equation:

$$Cp = A \cdot \exp(-\alpha \cdot t) + B \cdot \exp(-\beta \cdot t) \quad (1)$$

where α and β are rate constants for the distribution phase and elimination phase, respectively. A and B are hybrid constants shown as $D\cdot(\alpha-k_{21})/Vc/(\alpha-\beta)$ and $D\cdot(k_{21}-\beta)/Vc/(\alpha-\beta)$, respectively. D, k_{21} and Vc mean dose, first-order rate constant from peripheral to central compartment and distribution volume in central compartment, respectively.

Concentration-dependency of inhibitory effect by Gly-Pro against cephalexin absorption was analyzed by MULTI (45) based on Hill equation described below:

$$v_{abs} = V_{min} + \frac{V_{max} - V_{min}}{1 + \left(\frac{C_{Gly-Pro}}{IC_{50}}\right)^{P}} \quad (2)$$

where v_{abs} means the initial absorption rate of cephalexin, which is calculated by multiplying ka^{app} obtained in the *in-situ* closed loop study with the initial dose of cephalexin. V_{max} and V_{min} mean the maximal and minimal values of the absorption rate, respectively. $C_{Gly\text{-}Pro}$ represents the concentration of Gly-Pro and IC_{50} means the concentration that inhibits the absorption of cephalexin by 50%. P is the shape factor that accommodates the shape of the curve.

Parameters describing the absorbability of cephalexin from each segment were obtained based on Michaelis–Menten type equation with passive diffusion process.

$$v^{tot} = \left(\frac{V_{max}/V_l}{K_m + C} + ka^{pass}\right) \cdot C \cdot V_l \quad (3)$$

where v^{tot} means the total initial absorption rate of cephalexin, which is calculated by multiplying ka^{app} obtained in the *in-situ* closed loop study with the initial dose of cephalexin. V_{max} indicates the maximal absorption rate of cephalexin. K_m, C and V_l represent the affinity of cephalexin for PEPT1, initial concentration of cephalexin, and the volume of drug solution introduced into each segment in the *in-situ* closed loop study, respectively. ka^{pass} is the rate constant for cephalexin absorption *via* passive diffusion. Each parameter was obtained by simultaneous fitting study for data obtained in the *in-situ* closed loop studies without and with Gly-Pro by utilizing Eqs. 3 and 4, respectively. Fitting studies were performed by utilizing MULTI program (45). Equation 4 expresses the initial absorption rate of cephalexin only *via* passive diffusion (v^{pass}), which should be observed in the *in-situ* closed loop studies with Gly-Pro.

$$v^{pass} = ka^{pass} \cdot C \cdot V_l \quad (4)$$

Prediction of plasma concentration–time profile of cephalexin after oral administration. The prediction was performed by the convolution method using the GI-transit rate constants (k_i) for solution and the absorption rate constant (ka_i^{app}) in each segment based on GITA model (Fig. 1). ka_i^{app} means the sum of ka_C^{PEPT} and ka^{pass} in segment i. ka_C^{PEPT} was defined as an apparent absorption rate constant at a given concentration of cephalexin (C) as follows:

$$ka_C^{PEPT} = \frac{V_{max}/V_l}{K_m + C} \quad (5)$$

Furthermore, we defined an intrinsic absorption rate constant *via* PEPT1 as follows:

$$ka_{int}^{PEPT} = \frac{V_{max}/V_l}{K_m} \quad (6)$$

The absorption of cephalexin obeys a non-linear kinetics as shown in Eq. 3, and the concentration of cephalexin in luminal fluid after oral dosing would be changeable because of the absorption and transit. This phenomenon is too complicated mathematically and experimentally to reflect or follow it. In the present study, therefore, the apparent first-order kinetics was assumed in the case of oral absorption, and prediction and several evaluations were performed under the two extreme conditions for cephalexin concentration: the concentration of dosing solution (2.74 mM) and the extremely low concentration that can be neglected comparing with K_m value. In the latter case, Eq. 6 is available for describing the absorption of cephalexin *via* PEPT1.

The outline of the prediction method based on GITA model is as follows:

First of all, the amount of drug *versus* time profile (X-time profile) in each segment is calculated by means of the convolution method. Laplace transform of drug amount in segment $i+1\left(\tilde{X}_{i+1}(s)\right)$ is described by Eq. 7.

$$\tilde{X}_{i+1}(s) = \frac{k_i \cdot \tilde{X}_i(s)}{s + k_{i+1} + ka_{i+1}^{app}} \quad (7)$$

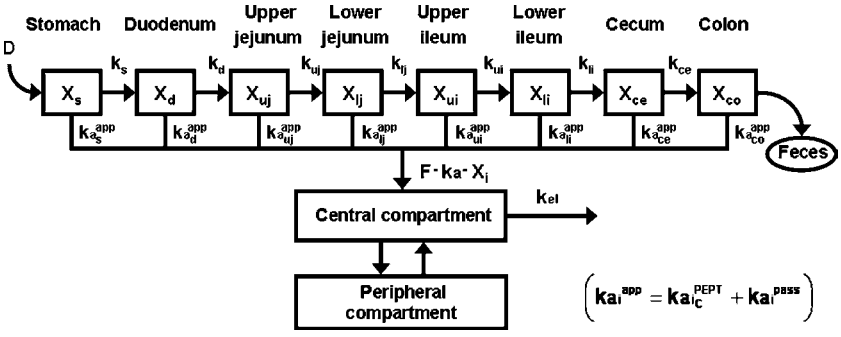

Fig. 1. Scheme for GI-Transit-Absorption model (GITA model). D intragastrically administered dose of cephalexin, X_i amount of drug in the segment i, k_i first-order transit rate constant of drug from the segment i, ka_i^{app} apparent first-order absorption rate constant for the segment i ($ka_i^{app} = ka_i^{PEPT} + ka_i^{pass}$), *kel* first-order elimination rate constant from central compartment, F availability.

The fraction of dose available for the absorption in segment i+1 (F_{i+1}) can be given by Eq. 8 using its Laplace transform$\left(\tilde{f}_{i+1}(s)\right)$.

$$\tilde{f}_{i+1}(s) = \frac{k_i \cdot \tilde{f}_i(s)}{s + k_{i+1} + ka_{i+1}^{\text{app}}} \tag{8}$$

Then, the Laplace transform of the absorption rate in the segment i, $\tilde{fa}_i(s)$, is expressed as follows:

$$\tilde{fa}_i(s) = ka_i^{\text{app}} \cdot \tilde{f}_i(s) \tag{9}$$

Plasma concentration of cephalexin orally administered can be described by the following Eq. 10 as Laplace transform.

$$\tilde{Cp}^{\text{po}}(s) = \frac{D_{\text{po}}}{D_{\text{iv}}} \cdot \sum_{i=\text{d}}^{\text{co}} \tilde{fa}_i(s) \cdot \tilde{Cp}^{\text{iv}}(s) \tag{10}$$

where $\tilde{Cp}^{\text{po}}(s)$ and $\tilde{Cp}^{\text{iv}}(s)$ express Laplace transforms of plasma concentration of cephalexin after oral and intravenous administrations, respectively. D_{po} and D_{iv} are doses of cephalexin for oral and intravenous administration, respectively. The inverse Laplace transformation of Eq. 10 by a convolution program (46) gives the plasma concentration–time profile of cephalexin after oral administration.

Western Blot Analysis. Western blot analysis was performed utilizing brush border membrane fraction of rat intestinal epithelial cells. Brush border membrane vesicles (BBMVs) were prepared using the method reported by Kessler *et al.* (47) with minor modification (48). Final BBMVs were purified at least around 17 fold compared with the corresponding mucosal homogenate, which was judged based on the activity of alkaline phosphatase determined with the method of Murer *et al.* (49).

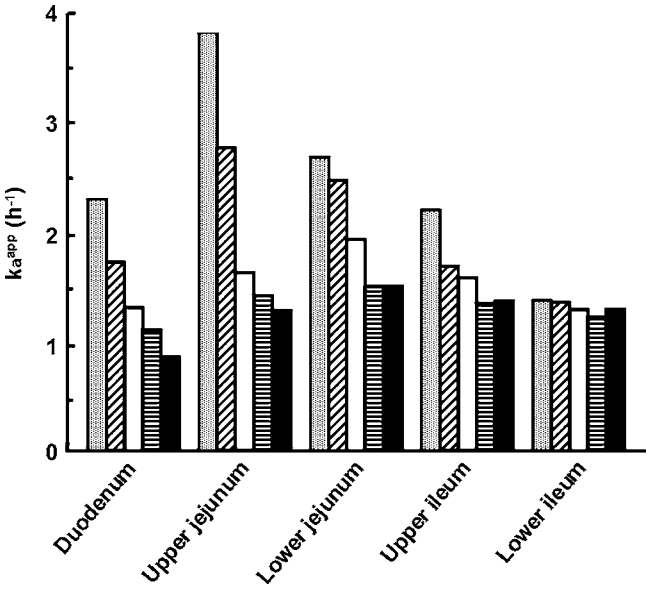

Fig. 2. Segment- and concentration-dependency of cephalexin absorption from rat small intestine. The apparent first-order absorption rate constant, ka^{app}, was obtained by *in-situ* closed loop study from at least three experiments for each time point. Initial concentrations of cephalexin were as follows: ▒, 1.37 mM; ▨, 4.10 mM; □, 9.58 mM; ▤, 19.16 mM; ■, 37.48 mM.

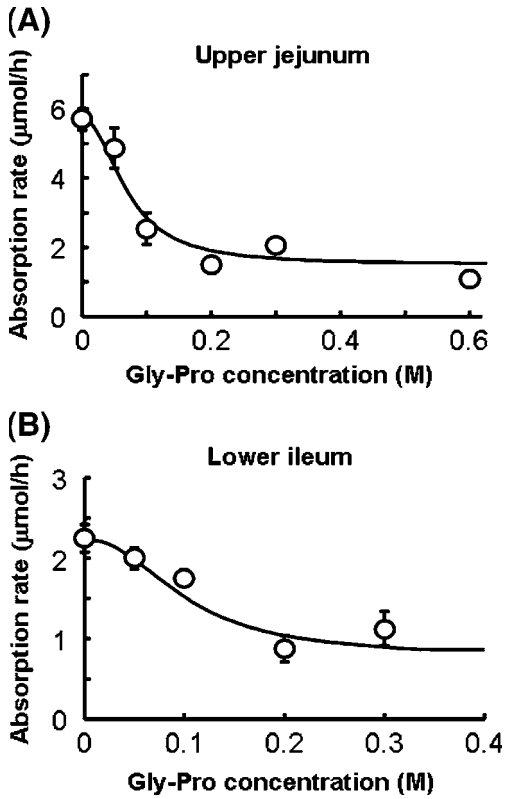

Fig. 3. Concentration-dependency of inhibitory effect by glycylproline on cephalexin absorption. Initial absorption rate of cephalexin (v_{abs}), of which the initial concentration was 2.85 mM, was determined by *in-situ* closed loop study. Results are expressed as the mean with S.E. of at least three experiments. *Solid lines* reveal the fitting lines obtained by utilizing Hill's equation (Eq. 2).

All the equipments and chemicals used in the Western blot analysis were obtained from Bio-Rad (Hercules, CA) unless otherwise specified. BBMVs resuspended in the sample buffer were separated by SDS-PAGE using 12.5% polyacrylamide gel (Ready Gel J, Bio-Rad) according to the method of Laemmli (50), and transferred to nitrocellulose membranes. The blots were blocked with phosphate-buffered saline containing 5% nonfat milk by 1.5-h incubation at room temperature, and incubated with the PEPT1 polyclonal antibody, H-235 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or the villin polyclonal antibody, C-19 (Santa Cruz Biotechnology, Inc.), for 1 h. The blots were then incubated for 1 h with anti-rabbit or anti-goat HRP antibody (Kirkegaard & Perry Laboratories, Guildford, UK). The blots were developed with an ECL kit (Amersham Pharmacia, Buckinghamshire, UK) and exposed to ECL hyperfilm (Amersham Pharmacia). The quantification of bands was performed by densitometric analysis using the Scion image (Scion Co., Frederick, MD).

Statistical Analysis. Results are expressed as the mean ± S.E. of at least three experiments. Statistical significance in the differences of the means was determined by Tukey's multiple comparison test. Statistical significance of the

correlation between observed and calculated values of plasma concentrations was determined by Pearson's method.

RESULTS AND DISCUSSION

To evaluate the absorbability of cephalexin *via* PEPT1 and passive diffusion in each intestinal segment, first of all, initial total absorption rates of cephalexin were determined in five segments at five different concentrations of cephalexin by *in-situ* closed loop study and apparent first-order absorption rate constants (ka^{app}) calculated are shown in Fig. 2. The decrease in ka^{app} with increase in cephalexin concentration was found in all the segments except for lower ileum and particularly greater decrement was observed in the upper segments such as duodenum and jejunum. In the lower concentration ranges, such upper segments also provided

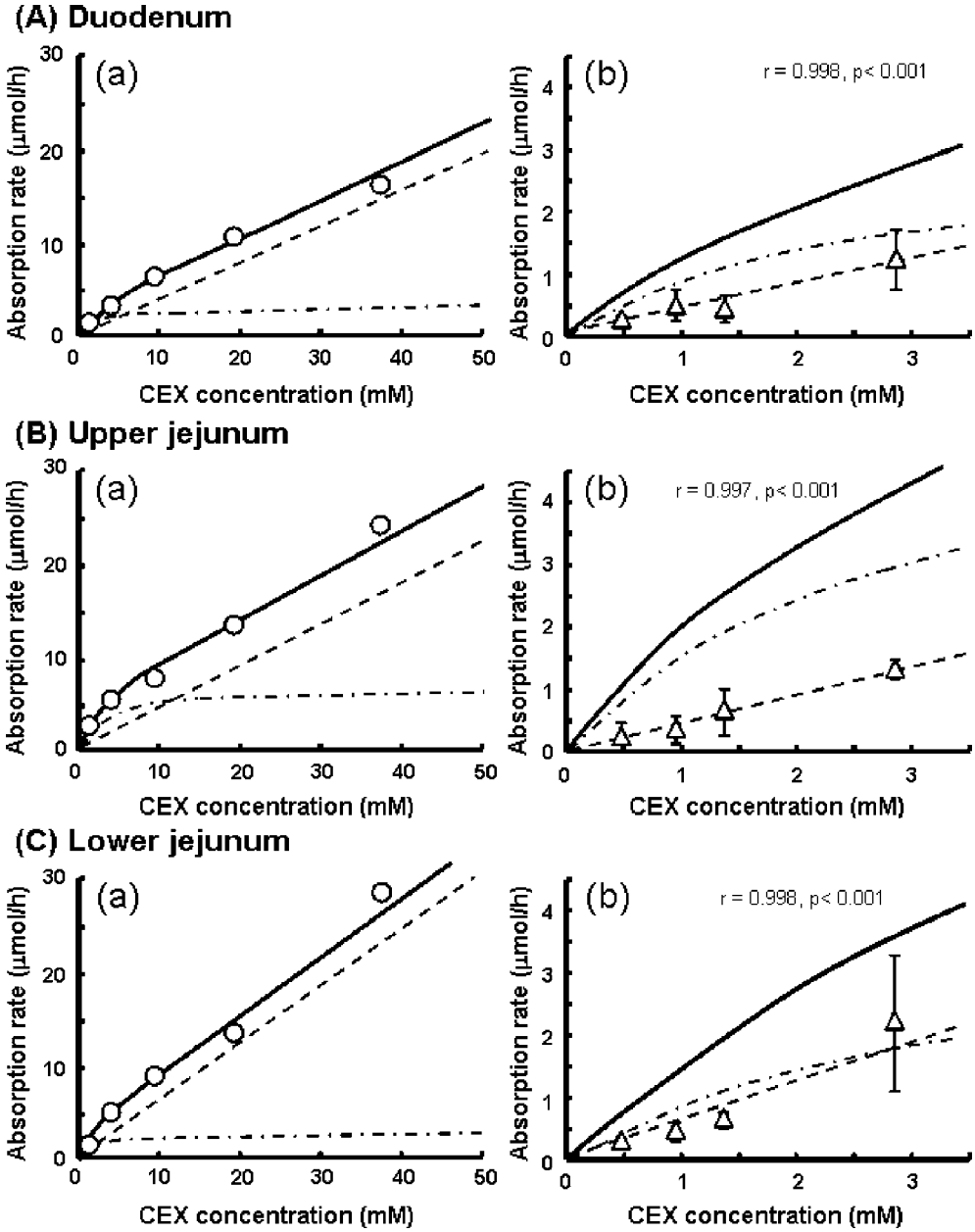

Fig. 4. Kinetic analysis of cephalexin absorption from each intestinal segment. Initial absorption rates of cephalexin were determined by *in-situ* closed loop study. (**A**) Whole profile from 0 to 50 mM, (**B**) lower concentration range from 0 to 3.5 mM. Total absorption rates calculated by using ka^{app} shown in Fig. 1 are expressed as *open circles*. Absorption rates *via* passive diffusion determined by the study with 0.3 M Gly-Pro are expressed as *open triangles* (mean) with S.E. bar of at least three experiments. Simultaneous fitting was performed utilizing Eq. 3 and obtained lines are expressed as follows: ——, total absorption rate; — · —, absorption rate *via* PEPT1; – – –, absorption rate *via* passive diffusion. Statistical significance of the correlation between observed and calculated values were examined by Pearson's method.

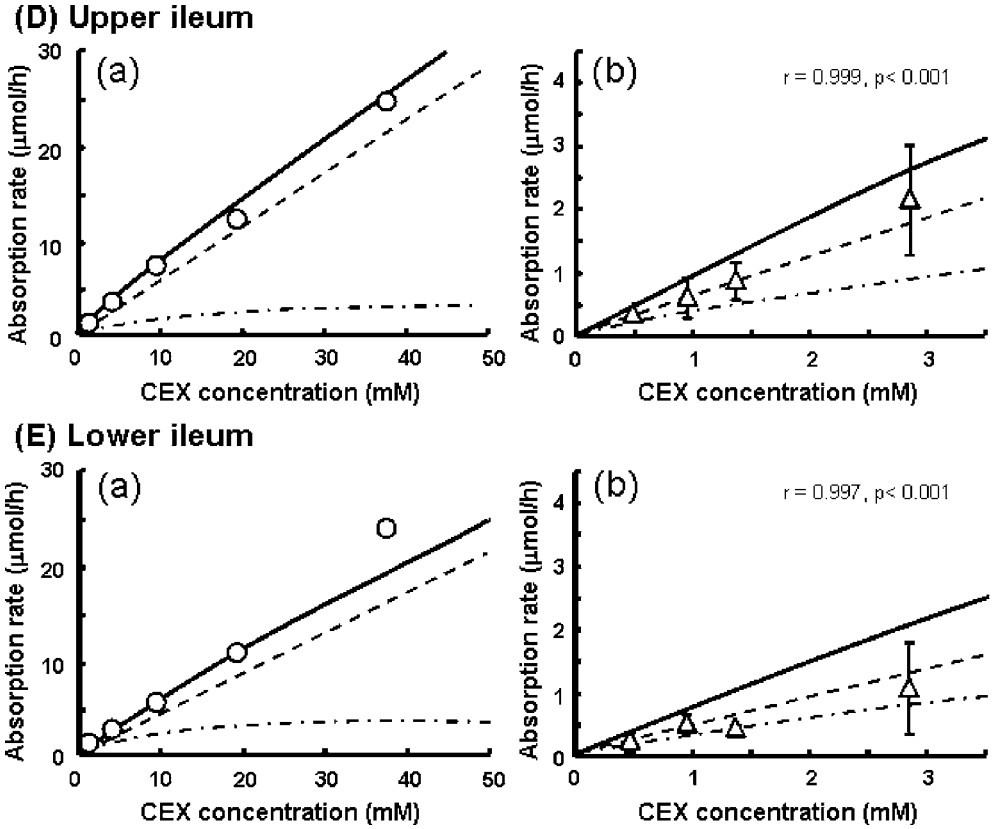

Fig. 4. (continued)

larger values of ka^{app} than lower segments, which coincides with previous reports (30,32). On the other hand, no concentration-dependency was revealed in lower ileum, suggesting that carrier-mediated transport would not be involved so much in cephalexin absorption from the segment. Furthermore, over 19.16 mM, ka^{app} values seem to be constant and very similar in each segment, suggesting that the contribution of passive diffusion could be very large in such higher concentration range.

To select the adequate concentration of Gly-Pro for inhibiting cephalexin absorption *via* PEPT1, the concentration-dependency was examined in two segments (Fig. 3). The absorption of cephalexin, of which the initial concentration was 2.85 mM, was strongly inhibited by Gly-Pro and reached the lowest plateau level by over 0.2 or 0.3 M Gly-Pro. Considering the much higher affinity of Gly-Pro for PEPT1 (42,44) than that of cephalexin (1,4,31,51–54), the stability against peptidases (42), the difference in concentration used from cephalexin and the saturation of the inhibitory effect by over 0.2 M, the absorption of cephalexin *via* PEPT1 is almost completely inhibited by over 0.2 or 0.3 M Gly-Pro. Therefore, 0.3 M was selected as a concentration for the inhibition study to evaluate ka^{pass}. Parameters obtained by fitting analyses with Eq. 2 are as follows: for upper jejunum; V_{max}=5.71 μmol/

Table I. Pharmacokinetic Parameters Describing Absorption of Cephalexin from Each Intestinal Segment

	K_m (mM)	V_{max} (μmol/h)	ka_{int}^{PEPT} (h^{-1})	ka^{pass} (h^{-1})	$ka_{2.74}^{PEPT}$ (h^{-1})	AIC
Duodenum	2.67	3.11	2.33	0.78	1.15	−18.75
Upper jejunum	2.91	5.92	4.07	0.89	2.10	−15.05
Lower jejunum	2.91	3.55	2.44	1.24	1.26	−15.05
Upper ileum	13.59	4.82	0.71	1.19	0.59	−10.11
Lower ileum	13.59	4.38	0.64	0.89	0.54	−10.11

Pharmacokinetic parameters were obtained by the simultaneous fitting study. K_m value was assumed to be the same within jejunum or ileum segment. ka^{pass} means the absorption rate constant of cephalexin *via* passive diffusion. ka_{int}^{PEPT} and $ka_{2.74}^{PEPT}$ represent the intrinsic value and the value at 2.74 mM of absorption rate constant *via* PEPT1 for cephalexin, respectively. AIC means Akaike's information criteria.

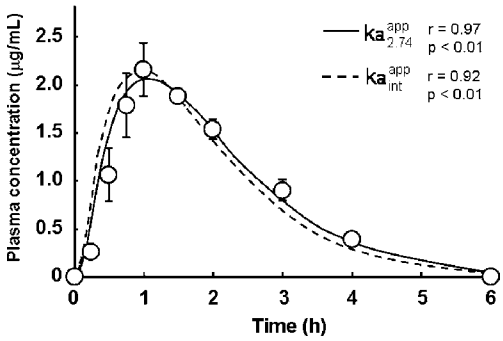

Fig. 5. Prediction of plasma concentration–time profile of cephalexin after oral administration. Observed values (*open circle*) are expressed as the mean with S.E. bar of five experiments. ——, calculated by utilizing $ka_{2.74}^{app} (= ka_{2.74}^{PEPT} + ka^{pass})$; – – –, calculated by utilizing $ka_{int}^{app} (= ka_{int}^{PEPT} + ka^{pass})$. Statistical significance of the correlation between observed and calculated values were examined by Pearson's method.

h (observed data), V_{min}=1.42 μmol/h, IC_{50}=0.07 M: for lower ileum; V_{max}=2.25 μmol/h (observed data), V_{min}=0.80 μmol/h, IC_{50}=0.10 M: P=1.50 for both segments.

In Fig. 4, initial total absorption rates of cephalexin (v^{tot}) and absorption rates *via* passive diffusion determined in the *in-situ* closed loop study utilizing 0.3 M Gly-Pro as a competitive inhibitor were plotted against the initial concentration of cephalexin for each intestinal segment. To obtain the intrinsic parameters describing the absorption characteristics *via* PEPT1 such as V_{max} and K_m, and ka^{pass}, simultaneous fitting was performed for the data by utilizing Eq. 3. Obtained fitting lines for total absorption rates and absorption rates *via* passive diffusion were significantly correlated with the observed data for every segment examined as evidenced by Pearson's method. The values of K_m obtained here (Table I) were in the range of reported values from 1.39 to 17.9 mM (1,4,31,51–54), but a larger value was obtained in ileum than upper segments (Table I), which might be explained by the effect of environmental pH on the conformational change (14–16). There was not a large difference in V_{max}, but upper jejunum provided the largest value. The intrinsic absorption rate constants *via* PEPT1, ka_{int}^{PEPT}, reveal that the functional activity was higher in the upper segments than the lower segments. On the other hand, the values of ka^{pass} did not show a large difference among the segments (Table I). The apparent absorption rate constant *via* PEPT1 at 2.74 mM of cephalexin, a concentration in dosing solution, ka_{int}^{PEPT} was also calculated (Table I). The parameter shows that the increase in cephalexin concentration apparently decreases the apparent rate constant *via* PEPT1.

Fig. 5 shows the plasma concentration–time profile of cephalexin after oral administration at 5 mg/5 mL/kg (2.74 mM) and prediction curves calculated by utilizing $ka_{2.74}^{app} (= ka_{2.74}^{PEPT} + ka^{pass})$ and $ka_{int}^{app} (= ka_{int}^{PEPT} + ka^{pass})$. Plasma concentration profile after intravenous administration was described by Cp= 12.92 (± 0.68)·EXP(−5.77 (± 0.38)·t)+3.72 (± 0.44)·EXP(−1.14 (± 0.11)·t) μg/mL (n=6, Dose=5 mg/mL/kg, unit of time=h), which was used as a weight function. Gastrointestinal transit rate constants used are as follows: Stomach, 1.12 h^{-1}; Duodenum, 28.75 h^{-1}, Upper jejunum, 18.07 h^{-1}; Lower jejunum, 4.21 h^{-1}; Upper ileum, 1.16 h^{-1}; Lower ileum, 0.46 h^{-1} (30), where only gastric emptying rate constant was experimentally determined under light ether anesthesia, because the delay of gastric emptying at early time periods was expected. As mentioned in "MATERIALS AND METHODS", the apparent first-order absorption was assumed and prediction was performed under the two extreme conditions. Both calculated profiles were in good agreement with the observed profile (Fig. 5). Pharmacokinetic parameters calculated based on the calculated curves also clearly indicate the excellent prediction by GITA model (Table II).

Based on the predicted results, the segmental contribution to cephalexin absorption after oral administration was evaluated for both calculation conditions (Fig. 6(A)). In the case of $ka_{2.74}^{PEPT}$ use for calculation, the segmental contribution ranged from 6.6% for duodenum to 32.1% for upper ileum. Utilization of ka_{int}^{PEPT} made a small change in the contribution profile, ranging from 10.1% for duodenum to 34.2% for lower jejunum, where the absorption site for cephalexin slightly moves to the proximal region. However, it would be mentioned that the major segments for cephalexin absorption are lower jejunum and upper ileum regardless of ka^{PEPT} values. Although ka^{app} and ka^{PEPT} values in Table I show that upper jejunum has the highest activity for the absorption of cephalexin, the substantial contribution after oral dosing would be the third among the segments of small intestine. On the other hand, upper ileum, of which ka^{app} and ka^{PEPT} values are the forth largest, provides the second greatest contribution to cephalexin absorption. This discrepancy between the absorbability estimated by *in-situ* closed loop study and the substantial contribution evaluated by *in-vivo* oral absorption study is explained by the residence time of drug in each segment after oral administration (30).

Table II. Pharmacokinetics Parameters Describing Absorption of Cephalexin after Oral Administration into Rats

Calculation		T_{max} (h)	C_{max} (μg/mL)	AUC (μg/mL·h)	MRT (h)	Bioavailability (%)
From observed values		1.10 (0.10)	2.32 (0.22)	5.18 (0.11)	1.83 (0.11)	90.5 (0.02)
From predicted values	$ka_{2.74}^{app}$	1.07	2.07	5.19	1.91	90.8
	ka_{int}^{app}	0.92	2.20	5.29	1.81	92.5

Pharmacokinetic parameters calculated "from observed values" were expressed as the mean with S.E. in parentheses of five experiments. The parameters "from predicted values" were calculated based on the predicted plasma profiles calculated by utilizing $ka_{2.74}^{app}$ or ka_{int}^{app} with GITA model.

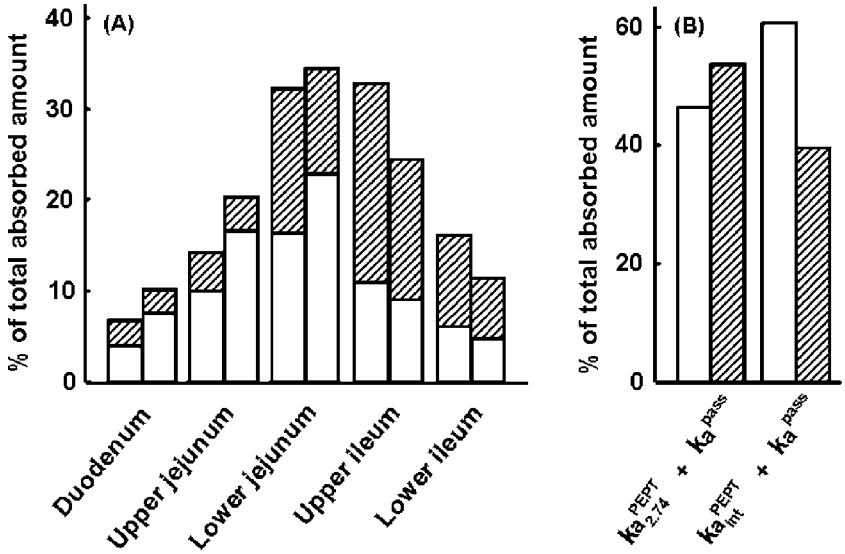

Fig. 6. Contribution of PEPT1 and passive diffusion to cephalexin absorption in each intestinal segment after oral administration. Results were obtained by the integration of absorption rate–time profile with GITA model. (**A**) *Left or right bar* in each segment represents the contribution calculated with $ka_{2.74}^{PEPT}$ and ka^{pass} or ka_{int}^{PEPT} and ka^{pass}, respectively. (**B**) Contribution of PEPT1 and passive diffusion in whole small intestine. □, PEPT1; ▨, passive diffusion.

Duodenum and upper jejunum are superior to upper and lower ileum in absorption activity for cephalexin, but from those segments drugs are moved to the next segment extremely quickly as shown by gastrointestinal transit rate constants.

Fig. 6(A) also shows the contribution of PEPT1 and passive diffusion to cephalexin absorption in each segment after oral dosing. When $ka_{2.74}^{PEPT}$ was used for the calculation, the absorption *via* PEPT1 was superior to that *via* passive diffusion in duodenum and upper jejunum. The highest contribution of PEPT1 (16%), which is almost equal to that of passive diffusion, was found in lower jejunum. In the both ileum segments, passive diffusion was predominant and the

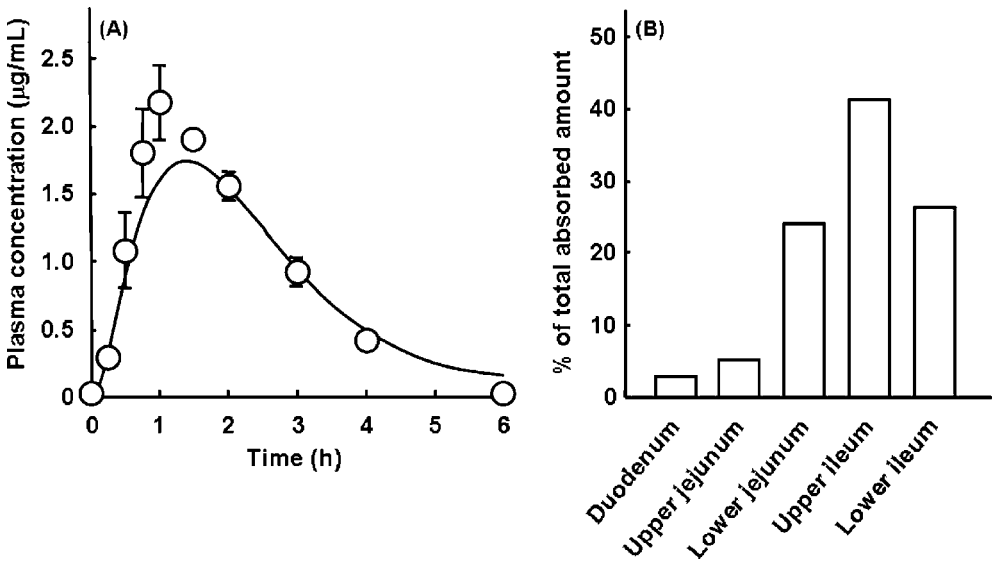

Fig. 7. Simulation of cephalexin absorption only *via* passive diffusion after oral administration. (**A**) Plasma concentration–time profile: observed values (*open circle*) are revealed again for comparison. *Solid line* expresses the line calculated by utilizing only ka^{pass}. (**B**) Segmental contribution to cephalexin absorption. Results were obtained by the integration of absorption rate–time profile calculated with GITA model by utilizing only ka^{pass}.

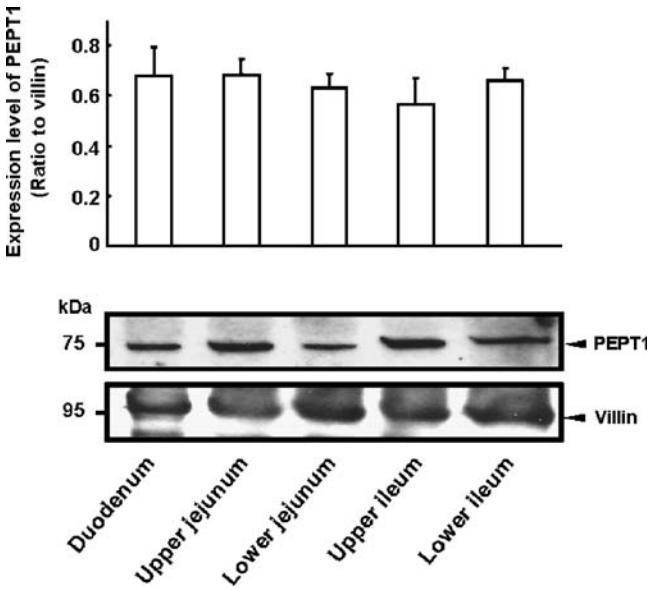

Fig. 8. Expression level of PEPT1 in each intestinal segment. PEPT1 in brush border membrane was detected by Western blot analysis. *Upper panel*: Results are expressed as the mean with S.E. bar of four experiments. *Lower panel*: a typical image of Western blot for PEPT1 and villin.

highest contribution (21.5%) was shown in the upper ileum. Total contribution of PEPT1 was around 46% throughout the small intestine (Fig. 6(B)). The utilization of $ka_{\text{int}}^{\text{PEPT}}$ for calculation resulted in the increase in the contribution of PEPT1, especially in the proximal region, but still passive diffusion was predominant in the distal region (Fig. 6(A)). Total contribution of PEPT1 was calculated to be around 60% (Fig. 6(B)). Considering the assumption employed in this approach, the utilization of $ka_{2.74}^{\text{app}}$ and $ka_{\text{int}}^{\text{app}}$ leads to the under- and over-estimation of the contribution of PEPT1 to the absorption of cephalexin, respectively. Taken all together, therefore, the substantial contribution of PEPT1 to cephalexin absorption could be almost even with that of passive diffusion after oral administration of a standard therapeutic dose.

Our approach allowed to simulate the absorption kinetics of cephalexin only *via* passive diffusion. Fig. 7(A) shows the plasma concentration–time profile of cephalexin absorbed only *via* passive diffusion. Although the calculated line was not able to catch up with $C_{\max}$ of observed concentration profile, the line was not estranged from the observed profile so much. Bioavailability was estimated to be 83.2% in this case, which means that the loss of PEPT1 can be almost compensated by the absorption *via* passive diffusion. The segmental contribution profile was shifted to the distal segment, comparing with the results shown in Fig. 6 (Fig. 7 (B)). This result is quite reasonable because the decrease in *ka* leads to the delay of drug absorption and the increase in the delivery of drugs to the more distal segment. Although gene knockout animals are often used to estimate the role of a given protein, terrible physiological condition caused by the lack of a given gene or functional compensation by other proteins (55) might result in misunderstanding. To the contrary, the present approach clearly informed that both PEPT1 and passive diffusion were almost equivalently involved in the absorption of cephalexin from rat small intestine, and that the lack of PEPT1 could be compensated by the absorption *via* passive diffusion after oral administration of a standard therapeutic dose, based on the information obtained under the physiological condition.

Cephalexin is a well-known substrate for PEPT1 (1–5), and the predominant or significant contribution of PEPT1 was suggested for the absorption from rat small intestine (8, 9). However, the substantial contribution of passive diffusion was also indicated by several researchers (9–13). Furthermore, it was reported that nutrition including oligopeptides or standard food did not affect the absorption of cephalexin in rats, humans (56) or dogs (57). These previous reports support our present findings.

Finally, the expression level of PEPT1 was confirmed throughout the small intestine. Fig. 8 shows quantitative evaluation (upper panel) and typical image of Western blot (lower panel). Under the normal feeding condition, the expression level of PEPT1 was reported to be higher in the distal region than that in the proximal region at mRNA level (33). However, the starvation induced the expression of PEPT1 (25). Particularly the induction was remarkable at the segments where the expression level was lower and 48-h starvation made the expression level almost equal throughout the small intestine (33). Twenty four-hour starvation also increased the expression level of PEPT1 three times in jejunum (58). The result shown in Fig. 8 confirmed that the expression of PEPT1 on brush border membrane was longitudinally almost constant in rat small intestine under 24-h fasted condition. Obviously, this expression level profile is not correlated with the functional profile throughout the small intestine as shown in Fig. 1 and Table I. But this discrepancy between the expression level and the functional activity was reported in terms of mRNA level (33), which could be partly explained by the difference in pH value influencing the transport activity as a driving force and the conformational change (14–16).

Although the involvement of other putative peptide transporters such as HPT1 (cadherin transporter) and PHT1 (peptide/histidine transporter) cannot be excluded completely (5), their contribution might be small considering their expression level lower than that of PEPT1 in rats (59,60). In the case of humans, PEPT1 are expressed mainly in upper segments such as duodenum and jejunum (59,61) and the expression level is much lower than that in rats (60). Considering the very short residence time in such upper segments as well (30,35,62), the contribution of PEPT1 to cephalexin absorption might not be so large in humans. It was also suggested that passive diffusion might contribute to cephalexin absorption in humans (9). HPT1 might be paid attention to because the expression level is much higher than that of PEPT1 in humans (60).

In the present study, we focused on cephalexin as a typical substrate for PEPT1, but different compounds have different values of K_m for PEPT1, lipophilicity and diffusivity. Therefore, the relative contribution of PEPT1 and passive diffusion to the oral absorption is dependent on the compound of interest and its therapeutic dose as well.

In conclusion, it was confirmed that PEPT1 was significantly involved in the absorption of cephalexin after oral

administration to rats. However, the contribution would be almost the same with that of passive diffusion, which could almost compensate the loss of PEPT1, at a typical therapeutic dose.

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