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Uptake of dipeptide and β-lactam antibiotics by the basolateral membrane vesicles prepared from rat kidney

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Abstract

The transport of dipeptides and β-lactam antibiotics across the rat renal basolateral membrane was examined. The initial uptake of glycylsarcosine and cefadroxil by rat renal basolateral membrane vesicles was inhibited by the presence of all the di- and tripeptides and β-lactam antibiotics that were tested in this study. However, the uptake of both substrates was not inhibited by glycine, an amino acid. The initial uptake of zwitterionic β-lactam antibiotics, cefadroxil, cephradine, and cephalexin, was stimulated by preloaded glycylsarcosine (countertransport effect). On the other hand, the uptake of dianionic β-lactam antibiotics, ceftibuten and cefixime, was not affected. A concentration-dependent initial uptake of glycylsarcosine and cefadroxil suggested the existence of a carrier-mediated mechanism, whereas the transport of ceftibuten did not show any saturated uptake. The transporter that participates in the permeation of dipeptides and β-lactam antibiotics across basolateral membranes showed lower affinity than did PEPT1 and PEPT2. This is the first study that showed an evidence for a peptide transporter, expressed in the rat renal basolateral membrane, that recognizes zwitterionic β-lactam antibiotics using basolateral membrane vesicles isolated from normal rat kidney.

Keywords: Kidney; Basolateral membrane; Transport; Dipeptide; β-lactam antibiotic

1. Introduction

Peptide transporters expressed in brush-border membranes of intestinal and renal epithelial cells contribute to the efficient absorption and reabsorption of oligopeptides. It is known that the dipeptide transport system in renal tubular epithelial cells contributes to reabsorption of several β-lactam antibiotics in the kidney [1,2]. In the brush-border membrane, it has been demonstrated that peptides are transported by H^+-coupled co-transport systems [3]. The results of experiments using purified brush-border membrane vesicles from the rat kidney cortex suggest that there are at least two distinct peptide transporters in the brush-border membranes of renal proximal tubules and that these dipeptide transporters also recognize β-lactam antibiotics [4–6]. Furthermore, molecular cloning studies have revealed the existence of the two distinct peptide transporters, PEPT1 [7] and PEPT2 [8].

In contrast to the brush-border membrane, there is little functional and molecular information available on the peptide transport system localized at the basolateral membranes in the kidney. Terada et al. [9] suggested the existence of a novel peptide transporter in renal basolateral membranes based on the results of experiments using Mardin–Darby canine kidney (MDCK) cells. However, there are few reports about the transport mechanism of dipeptides and β-lactam antibiotics across basolateral membranes of epithelial cells using normal tissue.

In the present study, characteristics of the transport of peptide and β-lactam antibiotics as well as a dipeptide, glycylsarcosine, across the rat renal basolateral membrane were investigated using isolated basolateral membrane vesicles prepared from the renal proximal tubule.

2. Materials and methods

2.1. Materials

Ceftibuten (Shionogi, Osaka, Japan) and cefixime (Fujisawa Pharmaceutical, Osaka, Japan) were kindly donated. [^{3}H]Glycylsarcosine (92.8 GBq/mmol) was purchased from

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Moravec Biochemicals (Brea, CA). All the other chemicals were of the highest grade available and were used without further purification.

2.2. Preparation of basolateral membrane vesicles

Basolateral membrane vesicles were isolated according to the self-orienting Percoll-gradient centrifugation method of Kobayashi et al. [10] with minor modifications for the renal cortex. Kidneys were excised from adult male Wistar rats, 180–250 g, under anesthesia (50 mg/kg sodium pentobarbital, i.p.) and rinsed with ice-cold saline. All the following procedures were performed on ice or at 4 °C. The renal cortex was sliced and homogenized for 4 min at 16500 rpm with a blender (Ace Homogenizer, Nihonseiki Seisakusho, Tokyo, Japan) in 80 ml of an ice-cold buffer containing 250 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM triethanolamine/HCl, pH 7.5 (solution A). The homogenate was centrifuged for 15 min at $1500 \times g$, and the supernatant was recentrifuged for 20 min at $20500 \times g$. The fluffy upper layer was collected, resuspended in 28.2 ml of solution A containing 12% Percoll, and homogenized with a glass/Teflon Dounce-type homogenizer with 10 strokes. The crude membrane suspension was centrifuged for 60 min at $50{,}000 \times g$. The third fraction from the bottom (approximately 10 ml) was withdrawn and diluted to 32 ml of a buffer consisting of 100 mM D-mannitol, 100 mM KCl, 5 mM EGTA, and 20 mM HEPES/Tris pH 7.5 (solution B). After centrifugation of this fraction for 30 min at $48{,}000 \times g$, the pellet was collected and resuspended in 20-ml solution B, and $MgCl_2$ was added to a final concentration of 10 mM. The basolateral membrane was precipitated by centrifugation for 15 min at $3400 \times g$. The pellet was suspended in 20 ml of a buffer (experimental buffer; 100 mM D-mannitol, 100 mM KCl, 20 mM HEPES/Tris pH 7.5) and homogenized in a glass/Teflon Dounce-type homogenizer with 10 strokes. After a final centrifugation at $27{,}000 \times g$ for 30 min, basolateral membrane vesicles were suspended in the experimental buffer by passing through a fine needle (0.4×19 mm) with a glass syringe 10 times. Basolateral membrane vesicles were used for transport studies within 4 h after preparation.

2.3. Uptake experiments

Uptake experiments were performed at 25 °C according to the method of Sugawara et al. [11]. The reaction was initiated by mixing 40 μl of membrane vesicle suspension with 200 μl of the transport buffer (unless otherwise stated, the transport buffer was composed of 100 mM D-mannitol, 100 mM KCl, and 20 mM HEPES/Tris, pH 7.5) containing substrates. Then, after a determined time, the reaction was terminated by diluting the reaction mixture with 5 ml of ice-cold stop buffer (150 mM KCl, 20 mM HEPES/Tris, pH 7.5) followed by filtration through a Millipore filter (HAWP, 0.45 μm, 2.5-cm diameter). The filter was then washed once with 5 ml of the ice-cold stop buffer. β-Lactam antibiotics trapped on the filter were extracted with 300 μl of distilled water.

The countertransport effect was examined by the method of Muranushi et al. [12] with minor modifications. Membrane vesicles were suspended in the experimental buffer solution to a final concentration of about 20 mg protein/ml. A portion of the vesicle suspension (20 μl) was preloaded with 20 μl of the experimental buffer solution containing 20 mM of glycylsarcosine for 30 min at 25 °C, and then the uptake experiment was carried out as described above.

2.4. Analytical procedures

The detection of β-lactam antibiotics trapped on the filter was carried out using high-performance liquid chromatography as previously reported [13]. Separation of β-lactam antibiotics was carried out on a reversed phase column (ODS Hitachi #3053) using a mobile phase of 12% acetonitrile containing 0.05 M citric acid and 0.01 M KCl with pH adjusted to 2.5 with NaOH. For the separation of cephradine and cephalexin, 20% acetonitrile containing 0.04 M citric acid and 0.08 M KCl (pH 2.5) was used. The flow rate and the detection wavelength were 0.7 ml/min and 262 nm, respectively. [^{3}H]Glycylsarcosine was measured by liquid scintillation counting. The uptake value was normalized with the amount of membrane protein. The concentration of membrane protein was measured by the method of Lowry et al. [14] with bovine serum albumin as a standard. The activity of Na^+, K^+-ATPase was measured by the method of Proverbio and Del Castillo [15]. Alkaline phosphatase activity was assayed using an Alkaline Phospha K-test kit (Wako Pure Chemical Industries, Osaka, Japan). The results are expressed as mean ± S.E. Student's t test was used to determine the statistical significance, and P values less than 0.05 were considered significant.

3. Results and discussion

3.1. Enzyme activity and transport activity of membrane vesicles

To confirm the purity and transport function of the membrane vesicles, we examined the activity of marker enzyme and transport of typical substrates that are transported through basolateral membrane via active transporters. The enrichment factors for the respective marker enzyme of renal basolateral and brush-border membranes, Na^+, K^+-ATPase, and alkaline phosphatase were calculated. The activity of Na^+, K^+-ATPase in the basolateral membrane was 7.4 times higher than that in the renal cortex homogenate, whereas the activity of alkaline phosphatase showed only 1.3 times of enrichment. To confirm that the basolateral membrane forms vesicles, the equilibrium uptake (60 min) of

cefadroxil was assessed at various transmembrane osmotic gradients. As shown in Fig. 1, the uptake of cefadroxil decreased with an increase in medium osmolarity, indicating formation of vesicles. Extrapolation of the fitted line to the vertical axis showed slight adsorption of cefadroxil to the basolateral membrane vesicles.

We also examined the activity of known transporters in the rat renal basolateral membrane according to the method of Pritchard [16]. The uptake of *p*-aminohippuric acid (PAH; a typical substrate for an organic anion transporter) by renal basolateral membrane vesicles in the presence of an inward Na^+ gradient and glutaric acid was examined. Stimulation of the uptake of PAH by an inward gradient of Na^+ and glutaric acid was observed (Fig. 2). Glutaric acid would be concentrated by Na^+-dependent dicarboxylate cotransporter-1 (NaDC-1) into the vesicle, and this would be followed by exchange of PAH for glutarate via organic anion transporter-1 (OAT-1) [16]. Therefore, it was shown that the basolateral membrane vesicles prepared in this study were adequate to examine the function of transporters in the basolateral membrane.

3.2. Effects of peptides and β-lactam antibiotics on the uptake of glycylsarcosine and cefadroxil by basolateral membrane vesicles

To characterize the transport of glycylsarcosine in the renal basolateral membrane, the inhibitory effects of glycylsarcosine, alanylalanine, and β-lactam antibiotics were examined. The uptake of glysylsarcosine was inhibited by all of the tested compounds, and the degrees of the inhibition were almost the same (Table 1). We also examined the effects of di- and tripeptides, β-lactam antibiotics, and glycine, an amino acid, on the uptake of cefadroxil by the renal basolateral membrane. As shown in Table 2, the uptake of cefadroxil was inhibited by all of the peptides and β-lactam antibiotics. On the other hand, glycine did not show any effect. These results suggest that a transporter that recognizes both peptides and β-lactam antibiotics is expressed in the renal basolateral membrane. In this study, there was no driving force in any of the experiments. There is no H^+ gradient on the basal side of epithelial cells which is a driving force for the transporters PEPT1 and PEPT2 expressed in the brush-border membrane. Moreover, the uptake of cefadroxil by basolateral membrane vesicles was not affected by an inward Na^+ gradient (not shown). Therefore, although a role of H^+ gradient should be examined further, it is possible that glycylsarcosine and cefadroxil are transported via a facilitative transport system.

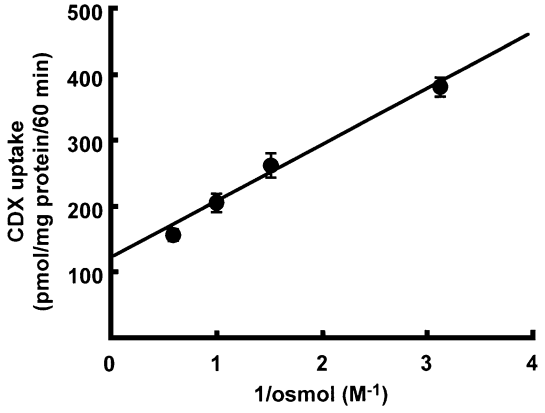

Fig. 1. Uptake of cefadroxil as a function of osmolarity of the extravesicular medium. Membrane vesicles (40 μl) were suspended in 100 mM KCl, 100 mM D-mannitol, and 20 mM HEPES/Tris, pH 7.5. Uptake study was performed by adding an incubation medium (200 μl) consisting of 100 mM KCl, 100 mM D-mannitol, and 20 mM HEPES/Tris, pH 7.5 containing 0–270 mM D-cellobiose. The uptake was performed at 25 °C, and the concentration of cefadroxil was 1 mM. Each point represents the mean ± S.E. ($n=9$) from three different preparations.

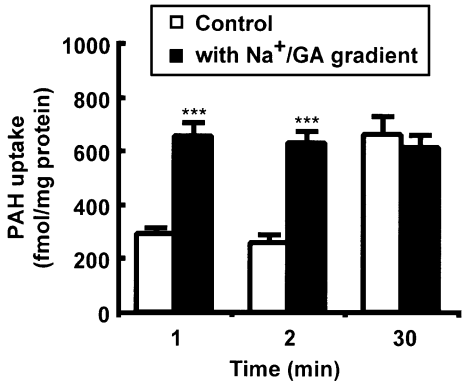

Fig. 2. Effects of inward gradient of Na^+ and glutaric acid (GA) on the uptake of *p*-aminohippuric acid (PAH; 2 μM) by rat renal basolateral membrane vesicles. Membrane vesicles (40 μl) were suspended in 100 mM KCl, 100 mM D-mannitol, and 20 mM HEPES/Tris (pH 7.5). Uptake study was performed by adding a buffer (200 μl) containing 100 mM NaCl, 100 mM D-mannitol, 12 μM gllutaric acid, and 20 mM HEPES/Tris (pH 7.5) with 2.4 μM *p*-aminohippuric acid. Medium temperature was 25 °C. Each column represents the mean ± S.E. ($n=10$) from three different preparations of vesicles. The symbol (***) indicates significant difference from the control ($P<0.001$).

Table 1
Effects of dipeptides and β-lactam antibiotics on the uptake of [^{3}H]glycylsarcosine by rat renal basolateral membrane vesicles

Inhibitor	Uptake (percent of control)
None (control)	100.0 ± 5.6
Gly–Sar	45.0 ± 4.8***
Ala–Ala	39.2 ± 4.5***
Cefadroxil	58.8 ± 2.4***
Ceftibuten	53.0 ± 5.9***
Cephradine	46.5 ± 5.9***
Cephalexin	47.5 ± 4.6***

Membrane vesicles (40 μl) were suspended in 100 mM KCl, 100 mM D-mannitol and 20 mM HEPES/Tris buffer, pH 7.5. Uptake was performed by adding the buffer (200 μl) containing 24 μM [^{3}H]glycylsarcosine in the absence (control) or presence of 24 mM of an inhibitor at 25 °C for 2 min. Each value represents the mean ± S.E. ($n=9$) from three different preparations of vesicles. The symbol (***) indicates significant difference from the control ($P<0.001$).

Table 2
Effects of peptides, glycine and β-lactam antibiotics on the uptake of cefadroxil by rat renal basolateral membrane vesicles

Inhibitor	Uptake (percent of control)
Peptides or glycine†	
None (control)	100.0 ± 9.2
Gly–Sar	74.1 ± 5.4**
Gly–Pro	65.1 ± 7.1***
Phe–Ala	78.3 ± 3.6*
Ala–Ala	58.5 ± 7.6***
Phe–Gly–Gly	73.9 ± 6.6**
Gly	111.7 ± 2.5
β-lactam antibiotics††	
None (control)	100.0 ± 4.6
Cephalexin	65.2 ± 5.4***
Cefixime	78.3 ± 5.8**

Membrane vesicles (40 μl) were suspended in 100 mM KCl, 100 mM D-mannitol and 20 mM HEPES/Tris buffer, pH 7.5. Uptake was performed by adding the buffer (200 μl) containing 1.2 mM cefadroxil in the absence (control) or presence of 24 mM of an inhibitor either at 37 °C for 5 min† or at 25 °C for 2 min††. Each value represents the mean ± S.E. ($n=9$) from three different preparations of vesicles. The symbols (*), (**), and (***) indicate significant differences of $P<0.05$, $P<0.01$, and $P<0.001$ from the control, respectively.

3.3. Countertransport effects of glycylsarcosine on the uptake of β-lactam antibiotics by basolateral membrane vesicles

Countertransport (trans-stimulation) effects of glycylsarcosine on the uptake of various β-lactam antibiotics were examined (Fig. 3). The uptake of cefadroxil, cephradine, and cephalexin was stimulated in vesicles preloaded with glycylsarcosine. On the other hand, the uptake of ceftibuten and cefixime was not affected. These results suggest that β-lactam antibiotics that have β-amino groups in their molecules shared a common transporter, whereas ceftibuten and cefixime, di-anionic compounds, are not transported. Although ceftibuten and cefixime, as well as cefadroxil, are well-known substrates for PEPT1 and PEPT2, they have different structures [17]. These results suggest that it is important that an α-amino moiety be transported via a transporter common to dipeptides such as glycylsarcosine.

3.4. Kinetics of the transport of glycylsarcosine, cefadroxil, and ceftibuten by renal basolateral membrane vesicles

The concentration dependence of the initial uptake (2 min) of glycylsarcosine, cefadroxil, and ceftibuten by rat renal basolateral membrane vesicles was studied (Fig. 4). The uptake of glycylsarcosine and cefadroxil was saturated with an increase in concentration (Fig. 4A and B), whereas that of ceftibuten was not (Fig. 4C). The kinetic parameters of glycylsarcosine and cefadroxil were estimated by nonlinear least-squares regression analysis using the following equation:

$$v = V_{max} \times [S]/(K_m + [S]) + K_d \times [S]$$

where v is the uptake amount (pmol/mg protein/2 min), $[S]$ is the concentration of the substrate (mM), K_m is the Mechaelis–Menten constant (mM), V_{max} is the maximum transport rate of the saturated part, and K_d is the rate constant of nonsaturated permeation (nl/mg protein/2 min). For glycylsarcosine, K_m, V_{max}, and K_d were estimated to be 8.0 ± 4.1 (mM), 1620 ± 760 (pmol/mg protein/2 min), and 136.7 ± 19.6 (nl/mg protein/2 min), respectively. For cefadroxil, they were estimated to be 10.4 ± 5.3 (mM), 2126 ± 1083 (pmol/mg protein/2 min), and 27.4 ± 24.2 (nl/mg protein/2 min), respectively. The kinetic parameters for the saturated process were similar between glycylsarcosine and cefadroxil, and nonsaturated permeation of glycylsarcosine was greater than that of cefadroxil.

Terada et al. [17] estimated the K_i value of the inhibitory effect of cefadroxil on the uptake of glycylsarcosine using LLC-PK1 cells that stably express PEPT1 or PEPT2, which are expressed on the brush-border membranes of renal epithelial cells. The estimated K_i values for PEPT1 and PEPT2 were 2.1 mM and 3 μM, respectively. The K_m values of glycylsarcosine for PEPT1 and PEPT2 were 1.1 and 0.11 mM, respectively. As shown in this study, the affinities of glycylsarcosine and cefadroxil for the system that participates in the transport across the basolateral membrane were much lower than those for PEPT1 and PEPT2. Moreover, the uptake of ceftibuten by basolateral membrane vesicles did not saturate, although it is transported via both PEPT1 and PEPT2. The results of this study clearly showed a difference between substrate specificities of transporters expressed on the brush-border and basolateral membranes.

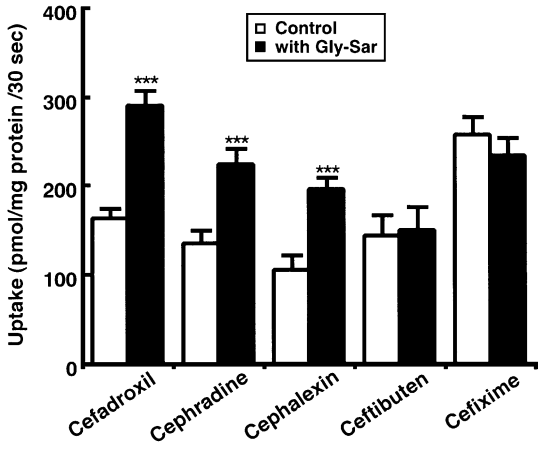

Fig. 3. Countertransport effects of Gly–Sar on the uptake of various β-lactam antibiotics by rat renal basolateral membrane vesicles. Membrane vesicles (40 μl) were preincubated at 25 °C for 30 min in 100 mM KCl, 100 mM D-mannitol, and 20 mM HEPES/Tris buffer, pH 7.5, in the absence (control) or presence (with Gly–Sar) of 10 mM Gly–Sar. Uptake was performed by adding the buffer (200 μl) containing 1.2 mM β-lactam antibiotic in the presence (control) or absence (with Gly–Sar) of 2 mM glycylsarcosine (final concentration of Gly–Sar outside the vesicles being 1.7 mM). Medium temperature was 25 °C. Each column represents the mean ± S.E. ($n=9$) from three different preparations of vesicles. The symbol (***) indicates significant difference from the control ($P<0.001$).

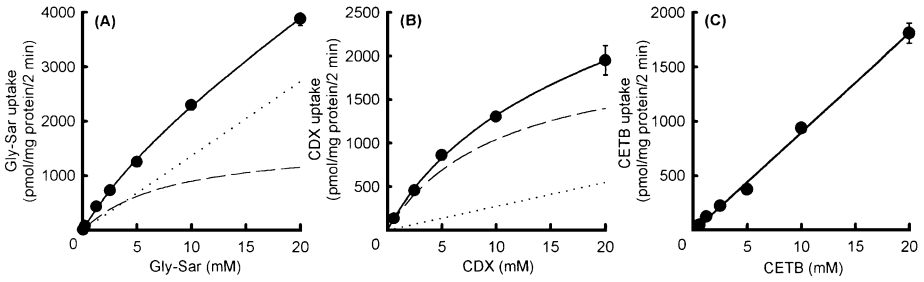

Fig. 4. Concentration dependence of the uptake of glycylsarcosine (A), cefadroxil (B), and ceftibuten (C) by rat renal basolateral membrane vesicles. Membrane vesicles (40 μl) were suspended in 100 mM KCl, 100 mM D-mannitol, and 20 mM HEPES/Tris buffer, pH 7.5. Uptake was performed by adding the buffer (200 μl) containing a substrate at 25 °C for 2 min. Each point represents the mean ± S.E. ($n = 3–9$) of different preparations of vesicles. Dotted and dashed lines indicate passive (nonsaturated) and saturated processes, respectively.

Terada et al. [18] and Sawada et al. [19] estimated the K_m value of the transport (uptake) of glycylsarcosine through the basolateral membrane using MDCK cells cultured on a porous filter. They also estimated K_i values of inhibitory effects of ceftibuten and cefadroxil on the uptake of glycylsarcosine. The K_m value of the uptake of glycylsarcosine was estimated to be 74 μM, and the K_i values of ceftibuten and cefadroxil were estimated to be 2.1 mM and 1 μM, respectively. In the present study, however, K_m values of glycylsarcosine and cefadroxil were estimated to be 8.0 and 10.4 mM, respectively. These values are very different from the values in MDCK cells. We did not examine the K_i value of the inhibitory effect of ceftibuten on the uptake of glycylsarcosine. However, the data on uptake of ceftibuten by basolateral membrane vesicles suggest that ceftibuten is not transported by the common system to glycylsarcosine and cefadroxil because the uptake of ceftibuten does not saturate up to 20 mM. Although there is great difference in kinetic parameters, there are two similar points between the transporter that was suggested by the results of experiments using MDCK cells and that in this study. One point is that both transporters were inhibited by dipeptides and β-lactam antibiotics but not by amino acid, and the other is that cefadroxil had higher affinity than did ceftibuten. At this stage, we do not know the reason for the differences in K_m. Difference in experimental conditions or in species might be a reason.

In this study, K_m values of the transport of glycylsarcosine and cefadroxil were 8.0 and 10.4 mM, respectively. Although we did not examine the transport of these compounds via organic anion transporters, these values are larger than reported K_i values of cloned rat organic anion transporters for β-lactam antibiotics [20,21]. Moreover, kinetic analysis showed that the uptake of these substrates fitted with one phase Michaelis–Menten equation and there was no higher affinity phase. Therefore, we concluded that the contribution of organic anion transporters is very small in this experimental condition.

In the kidney, the active glucose transporters SGLT1 and SGLT2 (sodium-dependent glucose transporters [22,23]), which have high affinity and low capacity, are expressed in the brush-border membrane of the proximal tubule. In the basolateral membrane, on the other hand, the facilitative glucose transporter GLUT2 [24,25], which has low affinity and high capacity, is expressed. The basolateral transporter that was characterized in the present study also has low affinity and high capacity compared to the transporter in the brush-border membrane. The peptide transporter expressed in basolateral membrane might have similar function to GLUT2 in reabsorption of peptides. However, if the transport of peptides is coupled with proton like PEPT1 and PEPT2 is still unclear. In the intestine it is reported that the transport of glycylproline across basolateral membrane is driven by proton gradient [26]. Therefore, it is possible that proton works as a driving force although it may not work concentrative in physiological condition. Further study should be done to clarify this point.

In summary, this is the first report on transport characteristics of dipeptides and β-lactam antibiotics, determined using basolateral membrane vesicles prepared from the rat kidney. The results suggest that there is a transporter that transports dipeptides and zwitterionic β-lactam antibiotics across the rat renal basolateral membrane. This transporter, however, does not transport dianionic β-lactam antibiotics such as ceftibuten. They are likely blockers of the transporter rather than substrates. So far, no peptide transporter other than PEPT1 and PEPT2 that is expressed on the brush-border membrane of epithelial cells has been cloned. Further molecular biological studies are needed to elucidate the transport characteristics in the basolateral membrane.

Acknowledgements

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