Transport Mechanism of Ceftibuten, a Dianionic Cephem, in Rat Renal Brush-Border Membrane

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The uptake mechanism of ceftibuten by rat renal brush-border membrane vesicles was investigated. Uptake was found to be independent of a Na^+ gradient and partially dependent on an inwardly directed H^+-gradient. Competition experiments between ceftibuten and several compounds demonstrated that the peptide-like structural features of inhibitors are more essential than their charge properties for inhibiting uptake. Anionic compound, such as *p*-aminohippuric acid, also inhibited ceftibuten uptake by renal brush-border membrane vesicles in the presence of an H^+-gradient. We conclude that ceftibuten, in spite of its anionic structure, is transported via the dipeptide transport systems, rather than the organic anion transport system.

KEY WORDS: ceftibuten; brush-border membrane vesicles; membrane transport; oligopeptide; renal proximal tubule; cephem antibiotic.

INTRODUCTION

Transport of the α-amino cephems in the renal brush-border membrane (BBM) is mediated by three transport systems, the H^+/organic cation antiport system [1], the organic anion transport system [2] and the dipeptide transport system [3,4]. The two former systems contribute to the renal tubular secretion of amino cephems, while H^+/dipeptide cotransport systems participate in the reabsorption of these antibiotics from the lumenal side. On the other hand, Tamai *et al.* [5] reported that cefixime, a dianionic cephem, is not recognized by the H^+/dipeptide transporter in the renal brush-border membrane although cefixime shared a common transport system with dipeptides and aminocephem in the intestinal BBM.

In the present study, the transport characteristics of ceftibuten, a new dianionic cephem, were investigated using isolated brush-border membrane vesicles from renal proximal tubule. This compound was previously reported to be transported via an H^+ coupled carrier-mediated transport system different from that for the amino cephems such as cephalexin and cefaclor, in small intestinal BBM [6–9].

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MATERIALS AND METHODS

Materials. Ceftibuten, compound V, and cephalexin were kindly donated by Shionogi Co. (Osaka, Japan), cephradine by Sankyo Co. (Tokyo, Japan), and cefixime by Fujisawa Pharmaceutical Co. (Osaka, Japan). All other chemicals were of the highest grade available and were used without further purification.

Preparation of the Brush-Border Membrane Vesicles. Renal brush-border membrane vesicles (BBMV) were prepared from the renal cortex of the male Wistar rat (200–300 g) by the calcium precipitation method according to Evers *et al.* [10]. Unless otherwise specified, the suspending buffer was 20 m*M* Hepes/Tris, PH 7.5, containing 100 m*M* D-mannitol and 100 m*M* HCl. Enrichment of the BBM fraction, was routinely more than 10-fold compared to the homogenate as revealed from the assessment of the specific activity of the membrane enzyme marker, alkaline phosphatase. Uptake experiments of tetraethylammonium (TEA^+), (a typical substrate for the cations transport system), revealed a functional integrity of the membrane.

Uptake Experiments. The uptake of ceftibuten by the freshly isolated membrane vesicles was performed at 25°C according to the method of Sugawara *et al.* [7] with minor modification. The reaction was initiated by mixing 40 μL of membrane vesicle suspension (10~15 mg protein/mL) with 200 μL of the transport buffer (unless otherwise, the transport buffer was composed of 100 m*M* D-mannitol, 100 m*M* KCl, 20 m*M* Mes/Tris, pH 5.5) containing substrates. Then, after a determined time, the reaction was terminated by diluting the reaction mixture with 4 mL of the ice-cold stop buffer (150 m*M* NaCl, 20 m*M* Hepes/Tris, pH 7.5) followed by filtration through a Millipore filter (HAWP, 0.45 μm, 2.5 cm diam.). The filter was then washed once with 8 mL of the ice-cold stop buffer. Ceftibuten trapped on the filter was extracted with 300 μL of the stop buffer.

Analytical Procedures. The detection of ceftibuten in BBMV was carried out with the use of high performance liquid chromatography as described previously [7,8,11,12]. Separation of ceftibuten was achieved on a reversed phase column (ODS Hitachi #3053, 5 μm, 4 mm i.d. × 250 mm) using a mobile phase consisting of acetonitrile/0.05 *M* citric acid-0.1 *M* KCl buffer, pH 2.5 (1:9). Samples were eluted at a flow rate of 0.7 mL/min. and the detection was set at 262 nm. Protein was measured by the method of Lowry *et al.* [13] with bovine serum albumin as standard.

RESULTS

Effect of Medium pH on Ceftibuten Uptake. The uptake of ceftibuten by renal BBMV during the first 1 min was greater at acidic pH medium, and the uptake over 30 min. was also dependent upon the pH of the medium (Fig. 1). The pKa_1 and pKa_2 of the carboxyl groups of ceftibuten are 2.3 and 3.2 [14]; therefore, ceftibuten exists predominantly as a dianion at the pH range 5.5–7.5, and the greater uptake observed at acidic pH medium is not simply due to an increase in the unionized form of ceftibuten according to the pH-partition theory. The greater uptake at acidic pH could be ascribed to changes in membrane surface potential accom-

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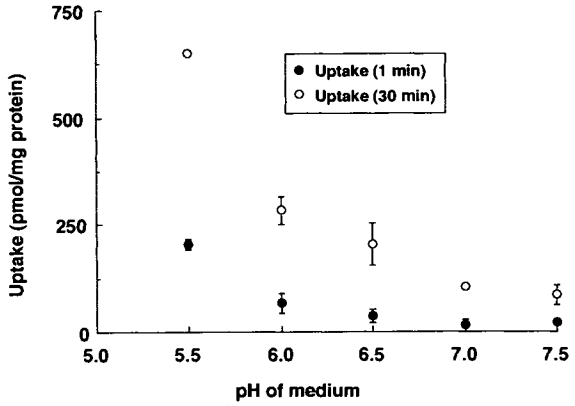

Fig. 1. Effect of pH of medium (pH_{inside} = $pH_{outside}$) on the uptake of ceftibuten (200 μ*M*) by rat renal BBMV. Mes/Tris buffers were used in the pH range 5.5–6.5 and Hepes/Tris buffers were used in the pH range 6.5–7.5. Each value is the mean ± S.D. (n = 3–5).

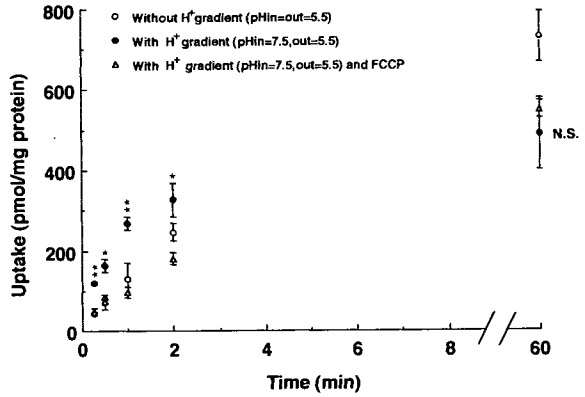

Fig. 2. Effect of H^+-gradient on the uptake of ceftibuten (200 μ*M*) by rat renal BBMV. The membrane vesicles were suspended in either Hepes/Tris buffer (pH 7.5) (△,●) or Mes/Tris buffer (pH 5.5) (○). The uptake buffer was a Mes/Tris buffer (pH 5.5). FCCP dissolved in absolute ethanol was added to the uptake buffer, with the addition of ethanol alone to the controls (●,○) (Final concentrations; FCCP, 50 μ*M*: ethanol, 0.5%, respectively. Each value is the mean ± S.D. (n = 3). $^*p < 0.05$, $^{**}p < 0.01$; significantly different from the uptake without H^+-gradient or from that with H^+-gradient and FCCP. N.S.; not significant compared with the uptake with H^+-gradient and FCCP, but significant when compared with the uptake without H^+ gradient ($p < 0.05$). The statistical analysis was assessed by ANOVA.

panying the changes in the pH of medium, as mentioned in our previous study in the case of intestinal BBM [15].

Inwardly Directed H^+ Gradient as Driving Force for Ceftibuten Uptake. The effect of an inwardly directed H^+ gradient on the uptake of ceftibuten by renal BBMV was examined (Fig. 2). Stimulation by an H^+-gradient was indeed observed, however, without an overshoot phenomenon, in contrast to results with intestinal BBMV [5,7,8]. In order to clarify whether the H^+-gradient dependency of ceftibuten uptake is due solely to the electrical gradient originated from the influx of H^+ into the intravesicular space, or it is also attributed to the chemical gradient of H^+, the time course of ceftibuten uptake under an inwardly directed H^+ gradient was determined in the presence and the absence of FCCP. The addition of the proton ionophore, FCCP, completely abolished the stimulating effect of the inwardly directed H^+ gradient.

Inhibitory Effect and Substrate Specificity of Ceftibuten Uptake. To further characterize substrate specificity of the transporter(s) of ceftibuten in the renal BBM, the inhibitory effects of dipeptides, cephem antibiotics, and several anionic compounds were examined (Table 1). In the presence of H^+-gradient, the uptake was markedly inhibited by all the compounds tested regardless of whether those compounds are cation(L-carnosine), zwitterion(L-Val-L-Pro, L-Ala-L-Pro, Gly-L-Sar, cephalexin, and cephradine), or anion (compound V[11], cefixime, *p*-aminohippuric acid(PAH) and hippurylphenyl-lactic acid), although the PAH interaction was weaker than the others.

Dixon Plot Analysis of Ceftibuten Uptake in the Pres-

Table I. Inhibitory Effects of Dipeptides, Cephem Antibiotics and Organic Acids on the Initial Uptake of Ceftibuten (0.5 mM) by Rat Renal BBMV

	In the presence of H^+ gradient (pH_{in} = 7.5, pH_{out} = 5.5)		In the absence of H^+ gradient (pH_{in} = pH_{out} = 5.5)	
Type of inhibitor	15-s uptake (% of control)	60-s uptake (% of control)	15-s uptake (% of control)	60-s uptake (% of control)
Control (without inhibitor)	100.0 ± 10.6	100 ± 2.9	100 ± 12.8	100 ± 7.2
L-Val-L-Pro	45.0 ± 9.9***	23.2 ± 6.5***	36.1 ± 14.7**	15.4 ± 1.1***
L-Ala-L-Pro	39.3 ± 14.5**	32.1 ± 4.1***	34.7 ± 10.7**	31.7 ± 10.6***
Gly-Sar	56.7 ± 15.5*	46.9 ± 5.9***	57.4 ± 5.2**	37.8 ± 7.8***
Cephalexin	66.9 ± 4.9*	58.1 ± 6.8**	82.5 ± 20.3	68.3 ± 3.3**
Cephradine	57.9 ± 3.7**	49.6 ± 14.8**	69.4 ± 12.6*	52.0 ± 6.4**
Carnosine	87.1 ± 5.0	78.7 ± 4.3**	59.2 ± 2.8*	59.7 ± 2.9**
Cefixime	54.2 ± 4.5***	43.9 ± 7.6***	58.8 ± 13.0*	45.1 ± 5.9***
Compound V	24.0 ± 3.7***	17.5 ± 2.9***	26.1 ± 6.0***	16.0 ± 1.7***
PAH	62.6 ± 5.2**	62.6 ± 8.4***	51.0 ± 7.2**	83.8 ± 3.6*
Succinic acid	121.2 ± 18.2	88.3 ± 9.6	96.5 ± 21.3	93.4 ± 15.9
Lactic acid	—	—	95.9 ± 7.4	88.4 ± 11.7
Hippurylphenyllactic acid	—	—	6.7 ± 6.6***	20.2 ± 0.5***

Inhibitors were added at a concentration of 10 mM. Each value is the mean ± S.D. (n = 3). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different from control.

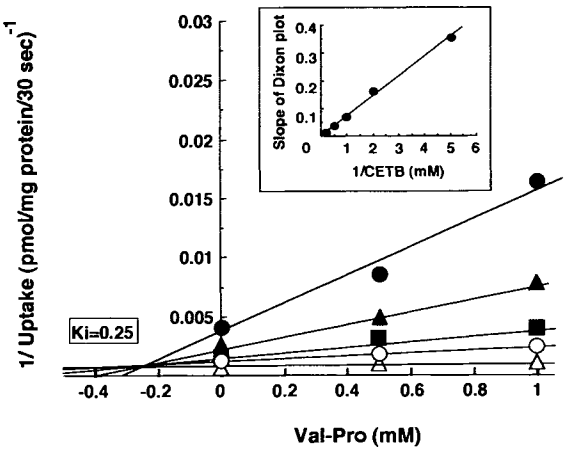

Fig. 3. Dixon plot analysis of ceftibuten initial (30 sec) uptake in the presence of L-Val-L-Pro and an inwardly directed H^+-gradient (pH_{in} = 7.5, pH_{out} = 5.5). The concentrations of ceftibuten were 0.2 (●), 0.5 (▲), 1 (■), 2 (○), or 5 m*M* (△). The inset shows a replot of the slopes of Dixon plot. Values are the mean of 3–6 measurements.

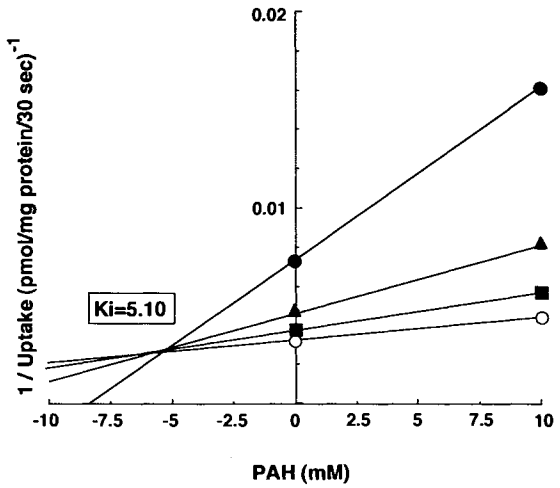

Fig. 5. Dixon plot analysis of ceftibuten initial (30 sec) uptake in the presence of PAH and an inwardly directed H^+-gradient (pH_{in} = 7.5, pH_{out} = 5.5). The concentrations of ceftibuten were 0.1 (●), 0.2 (▲), 0.3 (■), or 0.4 m*M* (○). Values are the mean of 4 measurements.

ence of Inhibitors. Figure 3 shows a Dixon plot of ceftibuten uptake with Val-Pro in the presence of an inwardly directed H^+-gradient. Val-Pro inhibited the uptake of ceftibuten competitively or partially competitively. The regression line obtained from the re-plot of the slopes of Dixon plot almost coincided with the origin (Fig. 3, inset). These results suggest that ceftibuten transport is mediated by a common transport system with oligopeptides such as Val-Pro. Similarly, as shown in Figures 4 and 5, both of the anionic compounds (compound V and PAH) demonstrated also a competitive or partially competitive effect on the uptake of ceftibuten. The apparent Ki values calculated from Dixon plots for Val-Pro, compound V and PAH were 0.25, 0.53 and 5.10 m*M*, respectively.

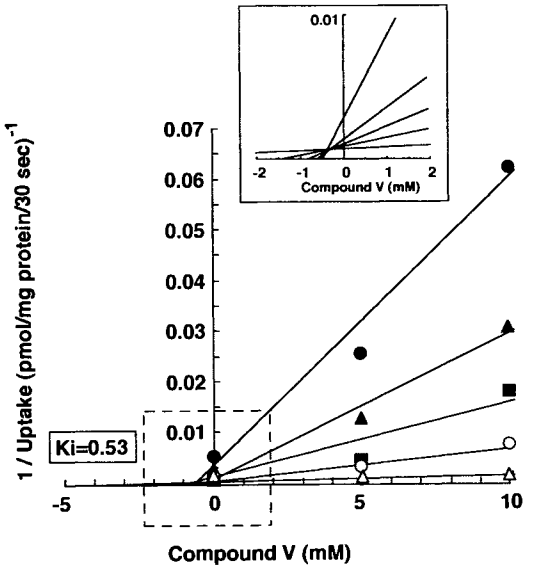

Fig. 4. Dixon plot analysis of ceftibuten initial (30 sec) uptake in the presence of compound V and an inwardly directed H^+-gradient (pH_{in} = 7.5, pH_{out} = 5.5). The concentrations of ceftibuten were as those in the caption for Figure 3. The inset shows an enlargement of the portion included in the dashed square in order to clarify the intersection of the lines of Dixon plot. Values are expressed as the mean of 6 measurements.

Inhibitory Effects of Anionic Compounds on Ceftibuten Uptake in the Presence of H^+-Gradient. Table 1 shows also the effects of dipeptides, aminocephem, and various anionic compounds on ceftibuten uptake in the absence of H^+-gradient ($pH_{in=out}$ 5.5). All the compounds having the amide-bonding (−CONH−) within the molecule exhibited significant inhibitions on the ceftibuten uptake, whereas lactic acid (a monocarboxylate) and succinic acid (a dicarboxylate) did not show any significant changes.

DISCUSSION

In the present study, ceftibuten uptake by the renal BBMV was found to be driven by an inward H^+-gradient, indicating that the direction of influx is from lumenal to intracellular side of the renal tubule, although there was no distinct overshoot phenomenon (Fig. 2). The inhibitory effect induced by the zwitter ionic and cationic dipeptides and by the peptide-like anionic compounds reveals that ceftibuten shares a common transport mechanism with dipeptides in the renal brush-border membrane. Oligopeptide transport in rat renal brush-border membrane is mediated by two transport systems, an H^+ dependent and an H^+ independent pathways [16]. This finding could account for the inhibition by dipeptides of ceftibuten uptake both in the presence and absence of a proton gradient. The dipeptide transport carrier(s) on the renal brush-border membrane may be operational even in the absence of H^+-movement. On the other hand, neither lactic acid nor succinic acid inhibited ceftibuten uptake. Furthermore, PAH inhibited H^+-driven transport of ceftibuten across the renal brush-border membrane from the lumen into the cell (reabsorption), although PAH is a typical substrate for the anion transporter in the renal brush-border membrane (tubular active secretion). These results provide further evidence that the inhibitory

effect is attributable to the peptide-like structure rather than the carboxyl group.

In contrast, cefixime, which has a similar structure to ceftibuten, was reported to be excreted through the renal BBM into the lumenal side predominantly via the organic anion transport systems [5], although this anionic antibiotic was transported via the dipeptide transport system in the intestinal BBM. In the present study, however, ceftibuten uptake from the lumenal side was mediated by dipeptide transporter(s), and was also inhibited by cefixime. These results document differences between renal and intestinal dipeptide transporters.

In conclusion, The present study demonstrated that ceftibuten is reabsorbed through the renal brush-border membrane via H^+-driven transport systems, and its transport was inhibited by dipeptides and peptide-like anionic compound.

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