Biochemical Pharmacology, Vol. 45, No. 9, pp. 1763–1768, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

TRANSPORT OF BESTATIN IN RAT RENAL BRUSH-BORDER MEMBRANE VESICLES

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(*Received* 14 *December* 1992; *accepted* 18 *February* 1993)

Abstract—Bestatin [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine] is a dipeptide, comprising L-leucine and an unusual β-amino acid. We studied its transport mechanism in rat renal brush-border membrane vesicles. Uptake of cephradine, an aminocephalosporin, by isolated brush-border membrane vesicles was trans-stimulated and cis-inhibited by bestatin, indicating that these drugs are transported via the same transport system(s). The uptake of bestatin was trans-stimulated by preloading the vesicles with glycylsarcosine, and was cis-inhibited by substrates for the H^+/dipeptide cotransport system. Bestatin inhibited tetraethylammonium (an organic cation) uptake, and bestatin uptake was cis-inhibited by substrates for the H^+/organic cation antiport system. In addition, bestatin uptake was stimulated by an outward H^+ gradient (the driving force for the H^+/organic cation antiport system). These findings suggest that bestatin, in spite of being a dipeptide, is transported via not only the H^+/dipeptide cotransport system but also the H^+/organic cation antiport system in rat renal brush-border membrane.

Bestatin (Fig. 1), a dipeptide containing an unusual β-amino acid [1, 2], has been used clinically as an anticancer agent [3]. It is well absorbed following oral administration [4, 5] and excreted rapidly from the kidney largely in an intact form. We reported previously that bestatin is actively absorbed from the intestine via a H^+/dipeptide cotransport system, which is one of the reasons why it can be given orally [6, 7].

In intestinal brush-border membrane, bestatin as well as dipeptides and cephalosporins is transported via the H^+/dipeptide cotransport system with an inward H^+ gradient (luminal pH < intracellular pH) serving as the driving force [6–11]. Similarly, dipeptides and cephalosporins are reabsorbed via the H^+/dipeptide cotransport system across the renal brush-border membrane [9, 12, 13]. In renal brush-border membrane, however, cephalosporins are also secreted via a H^+/organic cation antiport system and an organic anion transport system [14, 15]. So, an inward H^+ gradient, the driving force for the reabsorption of cephalosporins via the H^+/dipeptide cotransport system, is at the same time the driving force for secretion via the H^+/organic cation antiport system.

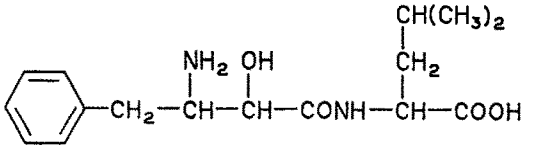

Fig. 1. Structure of bestatin.

As bestatin is excreted from the kidney, the transport mechanism of the drug in renal brush-border membrane is of great interest. However, such a study has not been reported yet. Therefore in the present study, we investigated the transport mechanism of bestatin using isolated renal brush-border membrane vesicles.

MATERIALS AND METHODS

Materials. Bestatin and [^{3}H]bestatin (308 mCi/mmol, 11.4 GBq/mmol) (Nippon Kayaku Co., Tokyo, Japan), cephradine (Sankyo Co., Tokyo, Japan) and cephalexin (Shionogi and Co., Osaka, Japan) were gifts. [1-^{14}C]Tetraethylammonium bromide (4.8 mCi/mmol, 178 MBq/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA, U.S.A.). Tris, 2-(*N*-morpholino)ethanesulfonic acid (Mes‡) and HEPES were purchased from Nacalai Tesque (Kyoto, Japan). Glycylsarcosine was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and other dipeptides were from the Peptide Institute (Osaka, Japan). All other chemicals were of the highest purity available.

Preparation of brush-border membrane vesicles. Brush-border membrane vesicles were isolated from the renal cortex of male Wistar albino rats (200–230 g) by the Mg/EGTA precipitation method of Biber *et al.* [16] and our previous report [17] with several modifications. All steps were performed on ice or at 4°. The cortex was homogenized with a Universal Homogenizer (Nihonseiki Co., Tokyo,

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‡ Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; EGTA, ethylene glycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Japan) at full speed for 2 min in an appropriate volume of 300 mM mannitol, 12 mM Tris–HCl (pH 7.1) and 5 mM EGTA, and then the same buffer was added to make a 10% homogenate. After diluting with distilled water (1:1), $MgCl_2$ was added to a final concentration of 10 mM, and the homogenate was allowed to stand for 15 min. The homogenate was centrifuged at 1900 *g* for 15 min in a Hitachi High Speed Refrigerated Centrifuge SCR 20B (rotor RPR20-2). The supernatant was centrifuged at 24,000 *g* for 30 min. The pellet was resuspended in an appropriate volume of the buffer comprised of 150 mM mannitol, 6 mM Tris–HCl (pH 7.1) and 2.5 mM EGTA, and homogenized in a glass/Teflon Potter homogenizer with 10 strokes at 1000 rpm. $MgCl_2$ was added to a final concentration of 10 mM, and the suspension was centrifuged at 1900 *g* for 15 min. The supernatant was centrifuged at 24,000 *g* for 30 min. The final pellet (purified brush-border membrane) was resuspended in a buffer comprised of 100 mM mannitol and 10 mM HEPES (pH 7.5) by 10 passages through a fine needle (0.4 × 19 mm) attached to a plastic syringe. For the transport experiments, brush-border membrane was diluted in the experimental buffer and centrifuged at 24,000 *g* for 30 min. The pellet was resuspended in the experimental buffer to give the final protein concentration of 5–9 mg/mL by 10 passages through a fine needle as described above. In general, the experimental buffer contains either 100 mM mannitol, 100 mM KCl and 10 mM HEPES (pH 7.5 or 7.0), or 100 mM mannitol, 100 mM KCl and 10 mM Mes (pH 6.0), and the pH was adjusted with KOH.

Transport studies. The uptake of each substrate by brush-border membrane vesicles was measured by a rapid filtration technique. The reaction was initiated by the addition of 80 or 200 μL of buffer containing the substrate to 20 μL of membrane suspension (5–9 mg protein/mL) at 25° or 37°. At the stated times, the incubation was stopped by diluting the reaction mixture with 1 mL of ice-cold stop solution containing 150 mM KCl, 20 mM HEPES/Tris (pH 7.5) and 0.1 mM $HgCl_2$. The mixture was poured immediately onto Millipore filters (HAWP, 0.45 μm, 2.5-cm diameter) which were washed once with 5 mL of ice-cold stop solution. In separate experiments, non-specific adsorption was estimated by the addition of substrate mixture to 1 mL of ice-cold stop solution containing 20 μL of membrane vesicles. This value was subtracted from uptake data for background correction.

Analytical methods. The radioactivity of [^{3}H]-bestatin was determined by liquid scintillation counting, using an external standard to correct for quench. Cephradine was analysed by HPLC LC-3A (Shimadzu Co., Kyoto, Japan) with Chemcosorb 5-ODS-H column, 15 cm × 4.6 mm (Chemco Scientific Co., Osaka, Japan) as described previously [14]. Protein was determined, after precipitation with 10% (w/v) trichloroacetic acid, by the method of Lowry *et al.* [18], with bovine serum albumin as a standard.

Statistical analysis. Data were analysed statistically using one-way analysis of variance followed by Dunnett's *t*-test.

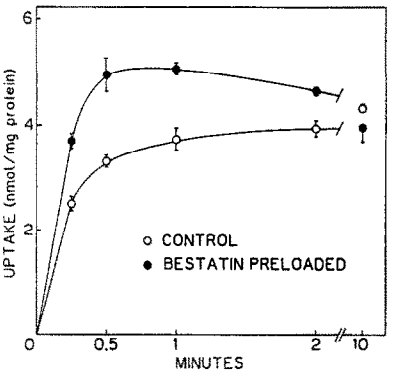

Fig. 2. Trans-stimulation effect of bestatin on cephradine uptake by renal brush-border membrane vesicles. Membrane vesicles were preincubated at room temperature for 1 hr in 100 mM mannitol, 100 mM KCl and 10 mM HEPES (pH 7.0) with or without 10 mM bestatin, and then aliquots (20 μL, 0.120 mg protein) were incubated at 37° with the substrate mixture (200 μL) comprising 100 mM mannitol, 100 mM KCl, 10 mM HEPES (pH 7.0) and 1.1 mM (final 1 mM) cephradine. Control (○), bestatin preloaded (●). Each point represents the mean ± SE of three determinations.

RESULTS

Effect of bestatin on cephradine uptake

In intestinal brush-border membrane vesicles, bestatin is transported via the same transport system as cephradine, an aminocephalosporin antibiotic [6, 7]. Therefore, we first studied the effect of bestatin on cephradine uptake by renal brush-border membrane vesicles. Figure 2 shows cephradine uptake by brush-border membrane vesicles preloaded with bestatin. Cephradine uptake by the preloaded vesicles was markedly stimulated compared to that by control vesicles (trans-stimulation effect).

As reported previously, aminocephalosporins are transported via the H^+/dipeptide cotransport system, the H^+/organic cation antiport system and the organic anion transport system in renal brush-border membrane vesicles [12, 14, 15]. In our preliminary experiments, bestatin did not affect the uptake of *p*-aminohippurate, a typical organic anion (data not shown). Therefore, the effect of bestatin on the cephradine uptake was examined in the presence of either an inward H^+ gradient (the driving force for the H^+/dipeptide cotransport system) or an outward H^+ gradient (the driving force for the H^+/organic cation antiport system). As shown in Fig. 3, bestatin inhibited cephradine uptake in the presence of an inward H^+ gradient (Fig. 3B) as effectively as in the presence of an outward H^+ gradient (Fig. 3A).

Effect of H^+ gradient on bestatin uptake

The effect of an inward and an outward H^+ gradient on bestatin uptake was examined (Fig. 4). In the presence of an inward H^+ gradient (pH_{in} =

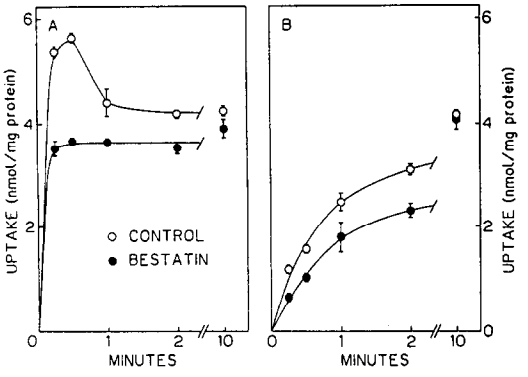

Fig. 3. Effect of bestatin on cephradine uptake by renal brush-border membrane vesicles. Membrane vesicles [20 μL; 0.128 mg protein (A), 0.170 mg protein (B)], suspended in 100 mM mannitol, 100 mM KCl and either 10 mM Mes [pH 6.0 (A)] or 10 mM HEPES [pH 7.5 (B)], were incubated at 37° with the substrate mixture (200 μL), comprising 100 mM mannitol, 100 mM KCl, 1.1 mM (final 1 mM) cephradine and either 10 mM HEPES [pH 7.5 (A)] or 10 mM Mes [pH 6.0 (B)] in the presence (●) or absence (○) of 10 mM (final) bestatin. Each point represents the mean ± SE of three determinations.

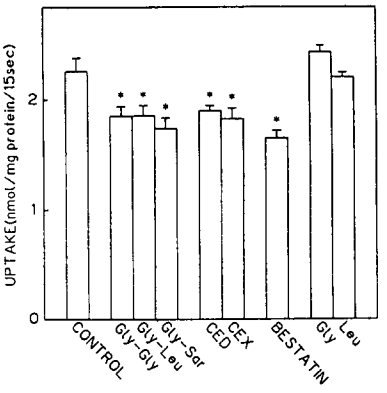

Fig. 5. Effect of various compounds on bestatin uptake by renal brush-border membrane vesicles. Membrane vesicles (20 μL, 0.179 mg protein), suspended in 100 mM mannitol, 100 mM KCl and 10 mM HEPES (pH 7.5), were incubated at 37° for 15 sec with the substrate mixture (200 μL) comprising 100 mM mannitol, 100 mM KCl, 10 mM Mes (pH 6.0), 1.1 mM (final 1 mM) labeled bestatin and 11 mM (final 10 mM) added compounds: Gly-Gly, glycylglycine; Gly-Leu, glycylleucine; Gly-Sar, glycylsarcosine; CED, cephradine; CEX, cephalexin; bestatin; Gly, glycine; Leu, leucine. Each value represents the mean ± SE of three determinations. *$P < 0.05$, significant difference from control.

7.5, $pH_{out} = 6.0$), bestatin uptake was slightly stimulated rather than inhibited, compared to that in the absence of a H^+ gradient ($pH_{in} = pH_{out} = 6.0$) (Fig. 4A). In the presence of an outward H^+ gradient ($pH_{in} = 6.0$, $pH_{out} = 7.5$), bestatin uptake was markedly stimulated, compared to that in the absence of a H^+ gradient ($pH_{in} = pH_{out} = 7.5$) (Fig. 4B). As the equilibrium values of bestatin uptake were similar under these conditions, intravesicular volume seems not to be affected by these pHs.

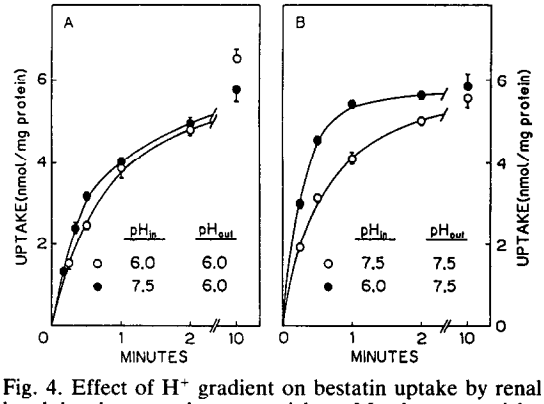

Fig. 4. Effect of H^+ gradient on bestatin uptake by renal brush-border membrane vesicles. Membrane vesicles [20 μL; 0.096 mg protein (pH 6.0), 0.097 mg protein (pH 7.5)], suspended in 100 mM mannitol, 100 mM KCl and either 10 mM HEPES [pH 7.5 (A) (●), (B) (○)] or 10 mM Mes (pH 6.0 (A) (○), (B) (●)], were incubated at 37° with the substrate mixture (200 μL), comprising 100 mM mannitol, 100 mM KCl, 1.1 mM (final 1 mM) labeled bestatin and either 10 mM Mes [pH 6.0 (A)] or 10 mM HEPES [pH 7.5 (B)]. Each point represents the mean ± SE of three determinations.

Bestatin uptake via the dipeptide transport system

The effect of various compounds on bestatin uptake was studied in the presence of an inward H^+ gradient (Fig. 5). The substrates for the H^+/dipeptide cotransport system (glycylglycine, glycylleucine, glycylsarcosine, cephradine and cephalexin) and unlabeled bestatin significantly inhibited [^{3}H]bestatin uptake, while amino acids did not.

Figure 6 shows bestatin uptake by brush-border membrane vesicles preloaded with glycylsarcosine. Bestatin uptake was stimulated by preloading the vesicles with glycylsarcosine.

Bestatin uptake via the organic cation transport system

To study the interaction of bestatin with the H^+/organic cation antiport system, we examined the effect of bestatin on tetraethylammonium uptake. As reported previously [15], tetraethylammonium uptake by renal brush-border membrane vesicles was markedly stimulated by an outward H^+ gradient against its concentration gradient (overshoot phenomenon, Fig. 7A). On the other hand, such an overshoot uptake was not observed in the absence of a H^+ gradient (Fig. 7B), though tetraethylammonium is taken up by a carrier-mediated process. Bestatin inhibited tetraethylammonium uptake both in the presence (Fig. 7A) and in the absence (Fig. 7B) of an outward H^+ gradient. As shown in Fig. 8, bestatin uptake was inhibited by tetraethylammonium in both the presence (Fig. 8A) and the absence (Fig. 8B) of an outward H^+ gradient.

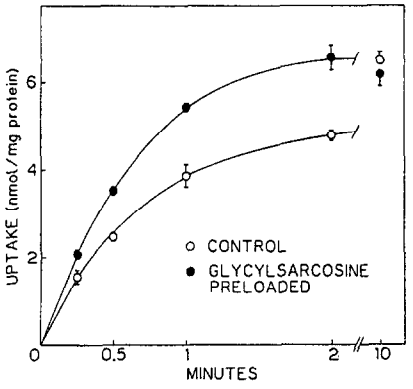

Fig. 6. Trans-stimulation effect of glycylsarcosine on bestatin uptake by renal brush-border membrane vesicles. Membrane vesicles were preincubated at room temperature for 1 hr in 100 mM mannitol, 100 mM KCl and 10 mM Mes (pH 6.0) with or without 10 mM glycylsarcosine, and then aliquots (20 μL, 0.096 mg protein) were incubated at 37° with the substrate mixture (200 μL) comprising 100 mM mannitol, 100 mM KCl, 10 mM Mes (pH 6.0) and 1.1 mM (final 1 mM) labeled bestatin. Control (○), glycylsarcosine preloaded (●). Each point represents the mean ± SE of three determinations.

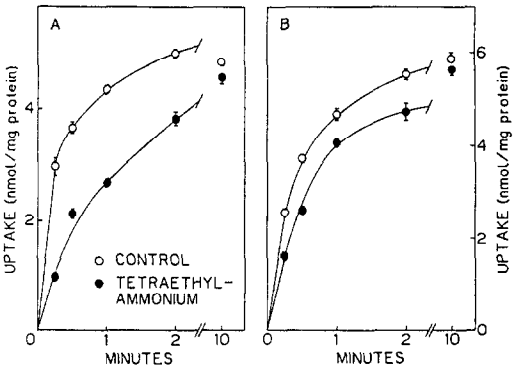

Fig. 8. Effect of tetraethylammonium on bestatin uptake by renal brush-border membrane vesicles. Membrane vesicles [20 μl; 0.120 mg protein (A), 0.124 mg protein (B)], suspended in 100 mM mannitol, 100 mM KCl and either 10 mM Mes [pH 6.0 (A)] or 10 mM HEPES [pH 7.0 (B)] were incubated at 37° with the substrate mixture (200 μL), comprising 100 mM mannitol, 100 mM KCl, 10 mM HEPES [pH 7.5 (A) or pH 7.0 (B)] and 1.1 mM (final 1 mM) labeled bestatin in the presence (●) or absence (○) of 10 mM (final) tetracthylammonium. Each point represents the mean ± SE of three determinations.

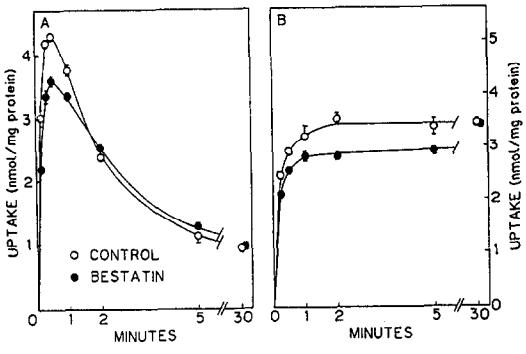

Fig. 7. Effect of bestatin on tetraethylammonium uptake by renal brush-border membrane vesicles. Membrane vesicles (20 μL, 0.124 mg protein), suspended in 100 mM mannitol, 100 mM KCl and either 10 mM Mes [pH 6.0 (A)] or 10 mM HEPES [pH 7.0 (B)] were incubated at 25° with the substrate mixture (80 μL), comprising 100 mM mannitol, 100 mM KCl, 10 mM HEPES [pH 7.5 (A) or pH 7.0 (B)] and 0.3125 mM (final 0.25 mM) (A) or 1.25 mM (final 1 mM) (B) labeled tetraethylammonium in the presence (●) or absence (○) of 10 mM (final) bestatin. Each point represents the mean ± SE of three determinations.

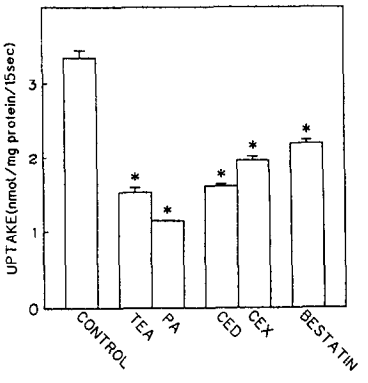

Fig. 9. Effect of various compounds on bestatin uptake by renal brush-border membrane vesicles. Membrane vesicles (20 μL, 0.181 mg protein), suspended in 100 mM mannitol, 100 mM KCl and 10 mM Mes (pH 6.0), were incubated at 37° for 15 sec with the substrate mixture (200 μL) comprising 100 mM mannitol, 100 mM KCl, 10 mM HEPES (pH 7.5), 1.1 mM (final 1 mM) labeled bestatin and 11 mM (final 10 mM) added compounds. TEA, tetraethylammonium; PA, procainamide; CED, cephradine; CEX, cephalexin; bestatin. Each value represents the mean ± SE of three determinations. *$P < 0.05$, significant difference from control.

To clarify further the relationship between bestatin transport and the H^+/organic cation antiport system, the effect of various compounds on bestatin uptake was studied in the presence of an outward H^+ gradient (Fig. 9). The substrates for the H^+/organic cation antiport system (tetraethylammonium, procainamide, cephradine and cephalexin) and unlabeled bestatin inhibited [^{3}H]bestatin uptake.

DISCUSSION

In this report, the characteristics of bestatin transport were studied in renal brush-border membrane vesicles. The trans-stimulation and cis-inhibition effect of bestatin on cephradine uptake indicate that bestatin is transported via the same

transport system(s) as cephradine. Thus, the transport system by which bestatin was transported may be the H^+/dipeptide cotransport system, the H^+/organic cation antiport system and/or the organic anion transport system. First, the role of the H^+/dipeptide cotransport system was investigated. The uptake of bestatin was inhibited by the substrates for the H^+/dipeptide cotransport system and was trans-stimulated by glycylsarcosine. Therefore, the H^+/dipeptide cotransport system should be involved in bestatin transport in renal brush-border membrane. Secondly, the role of the H^+/organic cation antiport system was studied: bestatin inhibited tetraethylammonium uptake and *vice versa*. The mutual inhibition was observed in both the presence and the absence of an outward H^+ gradient. Therefore, the inhibition should not be due to the facilitated dissipation of H^+ gradient but to the competition between tetraethylammonium and bestatin during the transport process. In addition, bestatin uptake was stimulated by an outward H^+ gradient, the driving force for the H^+/organic cation antiport system. These findings suggest that bestatin should also be transported via the H^+/organic cation antiport system. On the other hand, the organic anion transport system seems to contribute little, if any, to bestatin transport, because there was no effect of bestatin on *p*-aminohippurate transport.

In intestinal brush-border membrane, bestatin and cephalosporins are transported via the same transport system as dipeptides (the H^+/dipeptide cotransport system) [6, 7]. Their uptake by intestinal brush-border membrane vesicles is stimulated in the presence of an inward H^+ gradient (the driving force for the H^+/dipeptide cotransport system) and reduced in the presence of an outward H^+ gradient, compared to in the absence of a H^+ gradient [7, 10]. The H^+/dipeptide cotransport system also exists in renal brush-border membrane and reabsorbs dipeptides from the luminal fluid. It is not obvious whether this dipeptide transport system is the same as that in intestinal brush-border membrane, but many of the same dipeptides as well as cephalosporins are transported via these H^+/dipeptide cotransport systems [9]. In the present study bestatin was also found to be transported via the dipeptide transport system in renal brush-border membrane vesicles.

In renal brush-border membrane vesicles, cephalosporins are transported not only via the H^+/dipeptide cotransport system but also via the H^+/organic cation antiport system [12, 15]. The H^+/organic cation antiport system transports organic cations from the cells to the lumen. Uptake of tetraethylammonium, a typical substrate for the H^+/organic cation antiport system, by renal brush-border membrane vesicles is stimulated in the presence of an outward H^+ gradient (the driving force for the H^+/organic cation antiport system) and reduced in the presence of an inward H^+ gradient, compared to in the absence of a H^+ gradient [19]. On the other hand, uptake of bestatin and cephalosporins by renal brush-border membrane vesicles exhibited a different pattern. Our previous report showed that cephalexin uptake by renal brush-border membrane vesicles was stimulated by an outward H^+ gradient but not reduced by an inward H^+ gradient, compared to in the absence of H^+ gradient [15]. In the present study, similar findings were obtained for bestatin uptake by renal brush-border membrane vesicles. This seems to be due to the contribution of the H^+/dipeptide cotransport system to the transport of bestatin and cephalosporins, because an inward H^+ gradient is the driving force for the H^+/dipeptide cotransport system.

In conclusion, bestatin is transported not only via the H^+/dipeptide cotransport system but also via the H^+/organic cation antiport system in the renal brush-border membrane. The H^+/organic cation antiport system in the renal brush-border membrane may have a broad substrate specificity and transport not only organic cations but also zwitterions, such as bestatin and cephalosporins as substrates.

Acknowledgement—This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture in Japan.

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