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CARRIER-MEDIATED TRANSPORT OF AMINO-CEPHALOSPORINS BY BRUSH BORDER MEMBRANE VESICLES ISOLATED FROM RAT KIDNEY CORTEX

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Abstract—The uptake of cephalosporin antibiotics by brush border membrane vesicles isolated from rat renal cortex has been studied by a rapid filtration technique, demonstrating a carrier-mediated transport system for amino-cephalosporins such as cephalexin and cephradine. The antibiotics were taken up into an osmotically reactive intravesicular space. The uptake of cephalexin was saturable (apparent K_m 2.2 mM), was inhibited by structural analogues and sulfhydryl reagents, and was stimulated by the countertransport effect, although the Na^+ gradient did not affect the uptake. This transport system was essentially different from the transport system for *p*-aminohippurate in brush border membranes. The uptake properties for cephradine in brush border membrane vesicles appeared to be similar to those for cephalexin. The present results suggest the existence of a carrier-mediated transport system for amino-cephalosporins in brush border membranes. This system may be a part of the mechanism of tubular reabsorption of these antibiotics.

The renal handlings of cephalosporin antibiotics have been investigated by *in vivo* clearance or *in vitro* renal uptake studies, indicating that the processes responsible for the excretion of cephalosporins include glomerular filtration, tubular secretion and tubular reabsorption [1, 2]. Most cephalosporin antibiotics are actively secreted into the proximal tubular cell probably by the transport system for organic anions. The epithelium of the proximal tubule is characterized by cells with polarity. This asymmetry is evident ultrastructurally by the differentiation of the plasma membrane into two distinct components, the luminal brush border and the contraluminal basolateral membranes, and functionally by differences in the enzyme composition of the two membranes and in the mechanisms by which solutes cross the cell [3]. Therefore, it has been difficult to characterize the contribution of the brush border and the basolateral membranes in the transepithelial transport of cephalosporin antibiotics. In order to obtain further information about the mechanisms of cephalosporin excretion in the kidney, we have recently carried out a comparison of *in vitro* renal cortical uptake and the interaction with subcellular fractions isolated from the renal cortex, and indicated a relationship between active accumulation by cortical slices and specific interaction with basolateral membranes [4].

In recent years a methodology has been developed to use vesicles of the isolated brush border and basolateral plasma membranes for tubular transport studies [3, 5–7]. In particular, many studies have been presented on the mechanisms for D-glucose and neutral amino acid transport across both plasma membranes and the coupling of Na^+ with D-glucose and amino acid transport in brush border membranes [8–10]. These backgrounds prompted the present investigation, which was designed to access the uptake of cephalosporin antibiotics by brush border membrane vesicles. The uptake of cephalexin (amino-cephalosporin) was saturable, stimulated by the countertransport of the accumulated substrate, and inhibited by $HgCl_2$, *p*-chloromercuribenzoate, *p*-chloromercuribenzene sulfonate or *N*-ethylmaleimide. Thus, the present data suggest that a carrier-mediated transport system can play a role in the transport of amino-cephalosporins in brush border membranes, and it is essentially different from the transport system for *p*-aminohippurate (PAH).†

MATERIALS AND METHODS

Materials. Cephalexin and cephaloridine (Shionogi & Co., Osaka, Japan), cephradine (Sankyo Co., Tokyo, Japan), cefazolin (Fujisawa Pharmaceutical Co., Osaka, Japan) and cefotiam (Takeda Chemical Industries, Osaka, Japan) were kindly supplied. [^{3}H]PAH (374 mCi/mmole) was purchased from Amersham (U.K.). Tris, HEPES, *N*-ethylmaleimide, *p*-chloromercuribenzoic acid (sodium salt) and *p*-chloromercuribenzene sulfonic acid (monosodium salt) were obtained from Nakarai Chemicals (Kyoto, Japan). All other chemicals used for the experiments 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 (190–230 g) according to the calcium precipitation method, as previously described [4, 11]. The membranes were suspended in a buffer containing 100 mM mannitol and 20 mM HEPES–Tris (pH 7.5) (buffer A). The quality of the membrane preparations was evaluated

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† Abbreviations: HEPES, *n*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PAH, *p*-aminohippurate.

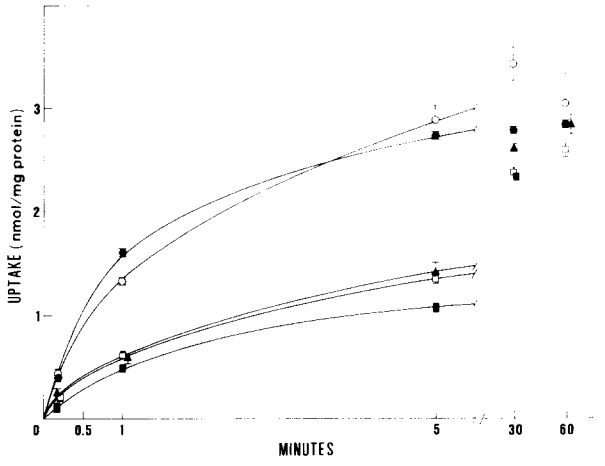

Fig. 1. Uptake of cephalosporin antibiotics by brush border membrane vesicles. Membrane vesicles were preincubated at 25° in 100 mM mannitol and 20 mM HEPES–Tris (pH 7.5) for 10 min. The vesicles (20 μl) were incubated at 25° with the substrate mixture (20 μl) containing 100 mM mannitol, 20 mM HEPES–Tris (pH 7.5), 200 mM NaCl and either 5 mM cephalexin (●), cephradine (○), cefazolin (□), cephaloridine (■) or cefotiam (▲). Final concentration: 100 mM NaCl, 2.5 mM antibiotics. Each point represents the mean ± S.E. of one or two experiments performed in duplicate determinations.

by marker enzymes; sp. acts of alkaline phosphatase (EC 3.1.3.1) and aminopeptidase (EC 3.4.11.2) were enriched approximately 10-fold compared to those found in the homogenate.

Transport assay. The uptake of substrates was measured by a rapid filtration technique. Membrane vesicles, dispersed in buffer A, were preincubated at 25° for 10 min (5–8 mg protein/ml). In most experiments the reaction was initiated rapidly by adding 20 μl of buffer A, containing substrate plus 200 mM NaCl or KCl, to 20 μl of membrane vesicle suspension at 25°. At the stated time points, the incubation was stopped by diluting a reaction sample with 1 ml of ice-cold buffer A. The tube contents were immediately poured onto Millipore filters (HAWP, 0.45 μm, 2.5 cm dia.) and washed with 5 ml of ice-cold buffer A. In separate experiments, non-specific adsorption was determined by the addition of substrate medium (20 μl) to 1 ml of ice-cold buffer A containing 20 μl of membrane vesicles. This value was subtracted from uptake data.

Analytical methods. Cephalosporins were analyzed by a high-pressure liquid chromatograph LC-3A (Shimadzu Co., Kyoto, Japan) as previously described [4]. The conditions used were as follows: column, Chemcosorb ODS 15 cm × 4.6 mm (Chemco Scientific Co., Ltd, Osaka, Japan); mobile phase, 0.03 M phosphate buffer (pH 7.0)/methanol = 82/18 for cephalexin, 80/20 for cephradine, 85/15 for cefazolin, 90/10 for cephaloridine and 75/25 for cefotiam; flow rate 1.3 ml/min; wavelength, 262, 262, 272, 240 and 260 nm for cephalexin, cephradine, cefazolin, cephaloridine and cefotiam, respectively; injection vol. 50 μl; temp. 40°. The cephalosporin trapped on the Millipore filter was extracted with 300 μl of distilled water, and was used for the determination. The recovery of cephalosporin antibiotics from the filter was more than 90%. Protein was determined, after precipitation with ice-cold 10% (w/v) trichloroacetic acid, by the method of Lowry *et al.* [12] with bovine serum albumin as a standard. The marker enzymes were assayed as previously described [11].

RESULTS

In order to estimate the general transport characteristics of cephalosporin antibiotics in brush border membranes, cephalexin and cephradine (aminocephalosporins), cefazolin, cephaloridine and cefotiam were used for the transport studies. Fig. 1 shows the uptake of cephalosporin antibiotics (2.5 mM) by brush border membrane vesicles in the presence of a 100 mM NaCl gradient (outside to inside). The rates of uptake for cephalexin and cephradine were faster than those for cefazolin, cephaloridine and cefotiam, and reached equilibrium at approximately 30 min. The uptake rates of these antibiotics were almost similar in the presence of an NaCl gradient or a KCl gradient (data not shown).

To ascertain that the uptake of cephalosporins by brush border membranes represented transport into vesicles rather than membrane binding, the uptake at 30 min was measured when the intravesicular space was decreased by increasing the medium osmolarity with mannitol. As shown in Fig. 2, there is a linear relationship between cephalexin or cefazolin uptake and the reciprocal of the medium osmolarity, suggesting that the antibiotics entered an interior space. Extrapolation of the line to infinite osmolarity, i.e. to zero intravesicular space, indicated that the binding was 10% for cephalexin and 5% for cefazolin under the incubation conditions normally used. Because of the small percentage of binding, the uptake data were not corrected for binding.

Fig. 3 shows the curves for the concn dependence of cephalexin and cefazolin uptake by brush border membrane vesicles. The relationship between concn and rate of uptake was nonlinear for cephalexin, providing evidence for saturability. In contrast, no evidence was observed for saturation of cefazolin uptake in the concn range used. The uptake of cephradine by brush boder membrane vesicles revealed a similar saturation phenomenon to that of cephalexin (data not shown).

In order to analyze the saturation of cephalexin

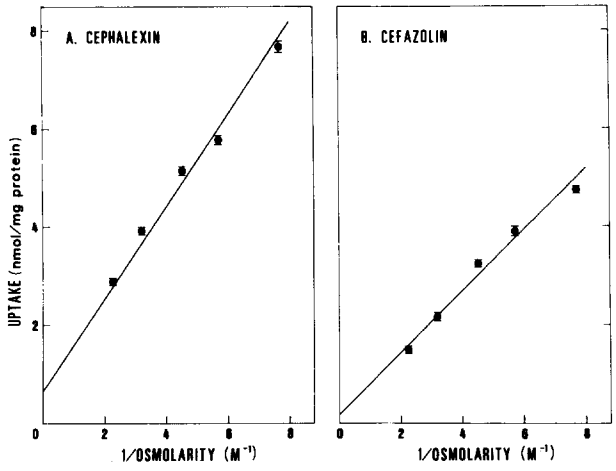

Fig. 2. Effect of osmolarity on the uptake of cephalexin (A) and cefazolin (B) by brush border membrane vesicles. The vesicles (20 μl) were incubated at 25° with 20 μl of 10 mM cephalexin or cefazolin for 30 min. The osmolarity was varied by addition of mannitol and is shown as the inverse of the osmolarity. Each point represents the mean ± S.E. of four determinations from a typical experiment.

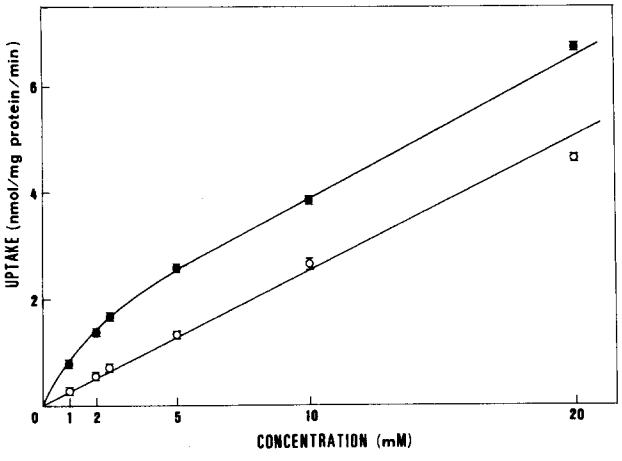

Fig. 3. Concn dependence of cephalexin and cefazolin uptake by brush border membrane vesicles. Cephalexin (●) and cefazolin (○) uptake for 1 min at concns between 1 and 20 mM was determined as described for Fig. 1. Each point represents the mean ± S.E. of four determinations from a typical experiment.

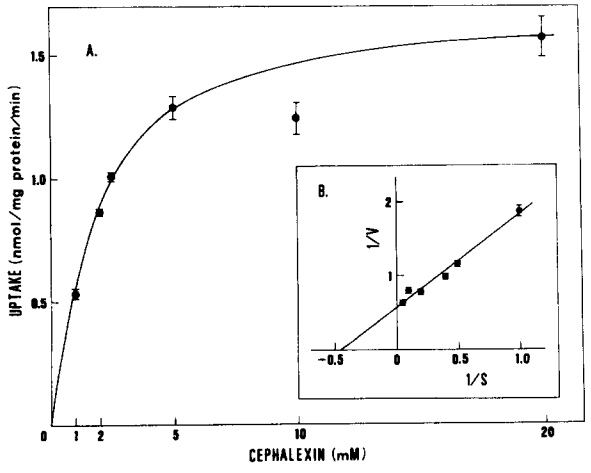

Fig. 4. Concn dependence of cephalexin uptake by brush border membrane vesicles. (A) Uptake rate was obtained from Fig. 3 by correcting for the nonsaturable component as indicated in the text. (B) Lineweaver–Burk plot.

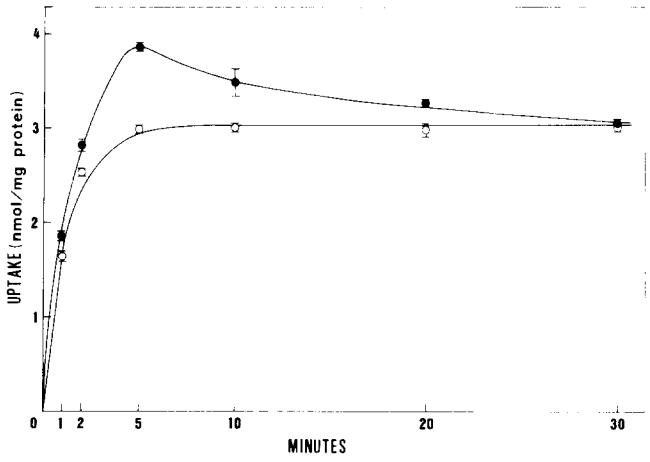

Fig. 5. Countertransport effect on cephalexin uptake by brush border membrane vesicles. The vesicles were preincubated in 100 mM mannitol and 20 mM HEPES–Tris (pH 7.5), with (●) or without (○) 25 mM cephradine for 30 min, and then aliquots (20 μl) were incubated with the substrate mixture (200 μl) containing 100 mM mannitol, 20 mM HEPES–Tris (pH 7.5), 100 mM NaCl and 2.5 mM cephalexin during the indicated periods. Each point represents the mean ± S.E. of two experiments performed in duplicate determinations.

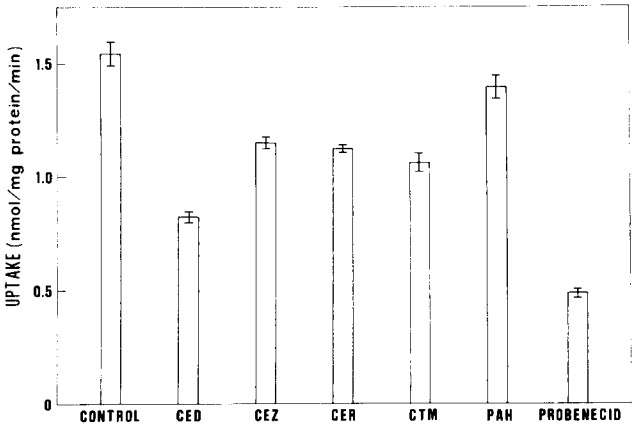

Fig. 6. Effect of various drugs on cephalexin uptake by brush border membrane vesicles. Cephalexin uptake for 1 min was determined as described for Fig. 1. Incubation medium contained 100 mM mannitol, 20 mM HEPES–Tris (pH 7.5), 100 mM NaCl and 2.5 mM cephalexin in the presence of various drugs (10 mM). CED, cephradine; CEZ, cefazolin; CER, cephaloridine; CTM, cefotiam; PAH, *p*-aminohippurate. Each column represents the mean ± S.E. of four to eight determinations from a typical experiment.

uptake, it was necessary to correct for the nonsaturable component. The contribution of the nonsaturable uptake could be estimated by employing the equation of the straight line generated at higher cephalexin concns, and subtracting this amount from the total uptake. After the correction for the nonsaturable component, the saturation phenomenon of cephalexin uptake became more apparent as shown in Fig. 4. A Lineweaver–Burk plot (Fig. 4B) of the uptake rates at concns between 1 and 20 mM revealed that the values of K_m and V_{max} were 2.2 mM and 1.7 nmoles/mg protein/min, respectively.

In order to confirm the existence of a carrier-mediated transport of cephalexin in brush border membranes, we studied the effect of countertransport. As shown in Fig. 5, the vesicles preloaded with a high concn of cephradine showed transient enhancement of cephalexin uptake, while no change of cephalexin uptake was observed in the vesicles preloaded with cefazolin (data not shown). These results suggest that cephalexin and cephradine can share a common carrier-mediated transport system in brush border membranes.

Fig. 6 shows the effect of various drugs (10 mM) on the uptake of cephalexin (2.5 mM) by brush border membrane vesicles. Among the cephalosporin antibiotics, cephradine had the strongest inhibitory effect on the uptake of cephalexin. Probenecid, a potent inhibitor of the organic anion transport system in the renal tubules, also strongly inhibited the uptake of cephalexin, although the inhibition by PAH was small. These results suggest that cephalexin could be transported across brush border membranes at least by two systems; a specific carrier-mediated transport system for amino-cephalosporins and an organic anion transport system.

Furthermore, the inhibitory effect of sulfhydryl reagents on the uptake of cephalexin and cefazolin

Table 1. Effect of sulfhydryl reagents on cephalexin, cefazolin and PAH uptake by brush border membrane vesicles

	Uptake (nmoles/mg protein/min)	% of control
Cephalexin		
Control	1.58 ± 0.03	
0.05 mM $HgCl_2$	0.93 ± 0.05	59
0.1 mM $HgCl_2$	0.75 ± 0.04	47
0.1 mM PCMB	0.65 ± 0.01	41
0.1 mM PCMBS	0.65 ± 0.02	41
0.5 mM *N*-ethylmaleimide	1.20 ± 0.06	76
Cefazolin		
Control	0.63 ± 0.02	
0.1 mM $HgCl_2$	0.56 ± 0.02	89
PAH		
Control	1.56 ± 0.04	
0.05 mM $HgCl_2$	1.50 ± 0.02	96
0.1 mM $HgCl_2$	1.51 ± 0.03	97
0.1 mM PCMB	1.50 ± 0.03	96

The vesicles were pretreated with $HgCl_2$, *p*-chloromercuribenzoate (PCMB), *p*-chloromercuribenzene sulfonate (PCMBS) or *N*-ethylmaleimide for 10 min at 25° prior to beginning the uptake. The uptake for 1 min was determined in the presence of sulhydryl reagents as described for Fig. 1. Incubation medium contained 100 mM mannitol, 20 mM HEPES–Tris (pH 7.5), 100 mM NaCl and sulfhydryl reagents with either 2.5 mM cephalexin, cefazolin or [^{3}H]PAH. Each value represents the mean ± S.E. of four to eight determinations from a typical experiment.

by brush border membrane vesicles were compared in Table 1, including the comparative study with PAH. The uptake of cephalexin was greatly inhibited in the presence of $HgCl_2$, *p*-chloromercuribenzoate, *p*-chloromercuribenzene sulfonate or *N*-ethylmaleimide. In contrast, these sulfhydryl reagents had a small or no inhibitory effect on cefazolin and PAH uptake by brush border membrane vesicles.

DISCUSSION

The present results demonstrated the existence of a carrier-mediated transport system for amino-cephalosporins such as cephalexin and cephradine in brush border membranes isolated from rat renal cortex. The uptake of cephalexin is saturable (apparent $K_m = 2.2$ mM), inhibited by structural analogues and sulfhydryl reagents, and undergoes a countertransport effect.

Among the transport properties of cephalexin and PAH in brush border membranes, there are a few differences concerning the saturability, counter-transport effect and specific inhibition. Kippen *et al.* [13] reported that PAH uptake by brush border membrane vesicles from rabbit kidney was not saturable. In our preliminary study [14], brush border membrane vesicles failed to display the capacity to accelerate the exchange of PAH and saturability of PAH uptake, although probenecid reduced PAH transport. In this study, furthermore, sulfhydryl reagents strongly inhibited the uptake of cephalexin, but did not inhibit PAH uptake. These reagents could be affecting the cephalexin uptake by a direct interaction with the translocating protein. Thus, in brush border membranes, caphalexin can be transported in part by a carrier-mediated system, which is different from the organic anion transport system.

It is well known that amino-penicillins and amino-cephalosporins are efficiently absorbed from the gastrointestinal tract even though they are ionized at physiological pH and have very low lipid solubilities. Recently several reports have demonstrated that some amino-penicillins and amino-cephalosporins could be absorbed by a carrier-mediated transport system in the intestinal mucosa [15–18]. Renal excretion is the principal route of elimination of these antibiotics, although they are handled differently by the renal tubule. Welles *et al.* [19] reported that cephalexin was secreted and reabsorbed by the renal tubule of dogs and rabbits. In this laboratory [20], renal handlings of cephalexin have been investigated by the clearance technique *in vivo*, indicating that the mechanisms responsible for the elimination are glomerular filtration, tubular secretion and tubular reabsorption. In particular, the degree of cephalexin reabsorption was larger than the estimate based on its lipid solubility. Therefore, the present results suggest that tubular reabsorption of cephalexin could be partially contributed to a carrier-mediated transport system in brush border membranes.

In conclusion, renal brush border membranes contain a carrier-mediated transport system for cephalexin that is different from the transport system for PAH. This system could be a part of the mechanism of tubular reabsorption for amino-cephalosporins.

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