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Characterization of the transport system for β-lactam antibiotics and dipeptides in rat renal brush-border membrane vesicles by photoaffinity labeling

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The uptake of the α-aminocephalosporin cephalexin into brush-border membrane vesicles from rat renal cortex was independent on an inward H^+-gradient in contrast to the intestinal transport system. The transport system could be irreversibly inhibited by photoaffinity labeling. Two binding polypeptides for β-lactam antibiotics and dipeptides with apparent molecular weights 130 000 and 95 000 were identified by photoaffinity labeling with [^{3}H]benzylpenicillin and *N*-(4-azido[3,5-^{3}H]benzoyl) derivatives of cephalexin and glycyl-L-proline. The uptake of cephalexin and the labeling of the respective binding proteins was inhibited by β-lactam antibiotics and dipeptides as with intestinal brush-border membranes. These data indicate that the transport systems for β-lactam antibiotics and dipeptides in the brush-border membrane from rat kidney and small intestine are similar but not identical.

β-Lactam antibiotics are excreted predominantly by the kidney [1–3]. A carrier-mediated transport of the polar α-aminocephalosporins across the renal brush-border membrane was shown [4–6]. The uptake of α-aminocephalosporins was competitively inhibited by dipeptides [6] and its has been suggested that α-aminocephalosporins are transported across the renal brush-border membrane by the reabsorption system for dipeptides [7–11] as in the small intestine [12–15]. A specific binding of cephalosporins to plasma membrane fractions from rat renal cortex has been reported [16]. However, the protein components of the transport system for β-lactam antibiotics and dipeptides in the renal brush-border membrane have not yet been identified. Therefore, in the present study the renal transport system for β-lactam antibiotics and dipeptides was characterized and the respective binding polypeptides were identified by photoaffinity labeling. The results are compared with recent data on the identification of the transport system for β-lactam antibiotics and dipeptides in the small intestine [20–22].

Brush-border membrane vesicles from rat kidney cortex were prepared by the Mg^{2+}-precipitation method [23]. The enrichment factors of the specific activities of the brush border enzymes γ-glutamyltransferase (EC 2.3.2.2) and leucine aminopeptidase (EC 3.4.11.2) were 23 ± 6 and 51 ± 7, respectively. The quality of the vesicles was tested by the Na^+-dependent uptake of D-glucose. Uptake of cephalexin into brush-border membrane vesicles was measured by the membrane filtration technique [24,25] and the cephalexin taken up by the vesicles was determined by high pressure liquid chromatography as described [21].

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For photoaffinity labeling with [^{3}H]benzylpenicillin (specific radioactivity 18 Ci/mmol, Amersham Buchler GmbH, Braunschweig, F.R.G.), *N*-(4-azido[3,5-^{3}H]benzoyl)cephalexin (specific radioactivity 50 Ci/mmol [21]) and *N*-(4-azido[3,5-^{3}H]benzoyl)glycyl-L-proline (specific radioactivity 48.9 Ci/mmol [21]), 200 μg of rat renal brush-border membrane vesicles were incubated with 1–5 μCi of the respective photolabile ligands in 10 mM sodium phosphate buffer (pH 7.4)/150 mM NaCl for 5 min in the dark and subsequently illuminated in a Rayonet photochemical reactor RPR 100 equipped with 16 RPR 2530 Å lamps. With [^{3}H]benzylpenicillin the irradiation time was 2 min, whereas the *N*-(4-azidobenzoyl) derivatives were irradiated 20 s for photoaffinity labeling. Subsequently the vesicles were washed and protein was precipitated [26]. After SDS-gel electrophoresis on 0.7 × 200 × 150 mm gels [27], the Serva Blue R-250 stained gels were scanned with a Densitometer Desaga CD 50 (Heidelberg, F.R.G.) and radioactivity was determined by liquid scintillation counting after slicing the gels into 2 mm pieces and digestion of proteins by Biolute [21].

In order to characterize the transport systems for β-lactam antibiotics in the brush-border membrane of the rat renal proximal tubule, the uptake of the α-aminocephalosporin cephalexin into brush-border membrane vesicles from kidney cortex was measured. In the presence and in the absence of an inward H^+-gradient ($pH_{out} = 6.0$, $pH_{in} = 7.4$) similar uptake rates were found (Fig. 1), whereas the uptake of dipeptides is stimulated by an inward H^+-gradient [10,11]. It has been shown that dipeptides inhibit the uptake of cephalexin by rat renal brush-border membrane vesicles [6]. Consequently, common transport systems for α-aminocephalosporins and dipeptides in the renal brush-border membrane have been suggested [6] as in the small intestine where both, the uptake of α-aminocephalosporins and dipeptides is stimulated by an inward H^+-gradient [14,15,17–19]. In order to investigate the the specificity of the renal transport system for β-lactam antibiotics, the effect of various compounds on the uptake of cephalexin was determined. Table I shows that compounds with peptide structure, dipeptides and β-lactam antibiotics, penicillins as well as cephalosporins, inhibited the uptake of cephalexin by renal brush-border membrane vesicles, whereas amino acids had no effect. These results indicate that the specificities of the renal and the intestinal [13,15] uptake systems for α-aminocephalosporins are very similar.

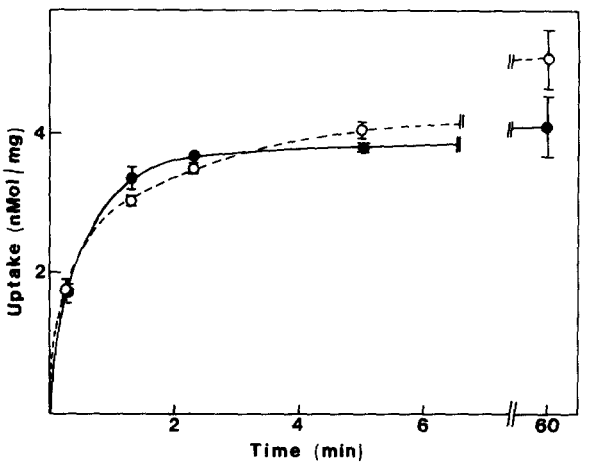

Fig. 1. Uptake of cephalexin by rat renal brush-border membrane vesicles. Rat renal brush-border membrane vesicles (100 μg, 20 μl) loaded with 10 mM Tris-Hepes buffer (pH 7.4), 300 mM mannitol were incubated at 30 °C with 180 μl of 2 mM cephalexin either in 10 mM citrate-Tris buffer (pH 6.0)/140 mM KCl (○) or 10 mM Tris-Hepes buffer (pH 7.4)/140 mM KCl (●).

TABLE I

INHIBITION OF CEPHALEXIN UPTAKE INTO RAT RENAL BRUSH-BORDER MEMBRANE VESICLES

Rat renal brush-border membrane vesicles (100 μg, 20 μl) loaded with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol, were incubated at 30 °C for 1 min with 180 μl 10 mM citrate-Tris buffer (pH 6.0)/140 mM KCl containing 2 mM cephalexin in the absence or in the presence of the indicated substances.

Additive	Uptake (nmol·mg^{-1}·min^{-1})	% of control
None	2.15 ± 0.14	100
25 mM glycine	2.18 ± 0.12	101.4 ± 5.5
25 mM glycyl-L-proline	1.07 ± 0.06	49.76 ± 2.8
25 mM L-carnosine	1.67 ± 0.09	77.67 ± 4.1
25 mM cefadroxil	0.90 ± 0.03	41.86 ± 1.4
25 mM benzylpenicillin	1.02 ± 0.07	47.44 ± 3.2
12 mM amoxicillin	1.03 ± 0.11	47.90 ± 5.1

The protein components of the transport system for β-lactam antibiotics and dipeptides in the small intestine have recently been identified by photoaffinity labeling. With photolabile derivatives of cephalexin and glycyl-L-proline [21,22] as well as by direct photoaffinity labeling with [^{3}H]benzylpenicillin [20,22] membrane polypeptides of molecular weights 127000, 100000, 94000 and 86000 were labeled in rat intestinal brush-border membranes with predominant labeling of the polypeptide of molecular weight 127000. In order to identify the respective binding proteins for β-lactam antibiotics and dipeptides in the rat renal brush-border membrane, membrane vesicles from the kidney cortex were submitted to photoaffinity labeling. To investigate whether the photoprobes interact with the renal transport system for β-lactam antibiotics and dipeptides, the uptake of cephalexin into rat renal brush-border membrane vesicles was measured in the presence of *N*-(4-azidobenzoyl)cephalexin and benzylpenicillin. Both compounds led to a concentration-dependent inhibition of the uptake of cephalexin. 1.6 mM of *N*-(4-azidobenzoyl)cephalexin and 30 mM benzylpenicillin produced half-maximal inhibition of cephalexin uptake. This inhibition of cephalexin uptake by *N*-(4-azidobenzoyl)cephalexin and benzylpenicillin indicates a specific interaction of these photoprobes with the renal dipeptide transport system. To test whether the inhibition of the cephalexin uptake was reversible without photolysis, the membrane vesicles were preincubated with or without the photolabile ligands under red lighting. After washing vesicles free of *N*-(4-azidobenzoyl)cephalexin, no inhibition of cephalexin uptake was observed as compared to control vesicles (Fig. 2A). This experiment demonstrated that the inhibition by *N*-(4-azidobenzoyl)cephalexin under subdued light was completely reversible. Next, experiments were performed to determine whether photolysis of vesicles in the presence of the photoaffinity probes would result in an irreversible inhibition of cephalexin uptake. Irradiation of membrane vesicles at 254 nm for 30 s led to a slight reproducible stimulation of cephalexin uptake (20–25%, Fig. 2B, left bar). However, a significant irreversible inhibition was seen in vesicles pretreated with *N*-(4-azidobenzoyl)cephalexin after photoaffinity labeling (Fig. 2B, right bar). With benzylpenicillin also a significant irreversible inhibition of cephalexin uptake was obtained after irradiation at 254 nm for 2 min (Fig. 2C). When substrates of this transport system, dipeptides and β-lactam antibiotics, were simultaneously present during photoaffinity labeling, the transport system could be protected from irreversible inhibition. Table II shows that the irreversible inhibition of cephalexin uptake by photoaffinity labeling with *N*-(4-azidobenzoyl)cephalexin was reduced by the presence of β-lactam antibiotics and dipeptides compared to vesicles, where no protective substrate was present or which were incubated with amino acids. These experiments

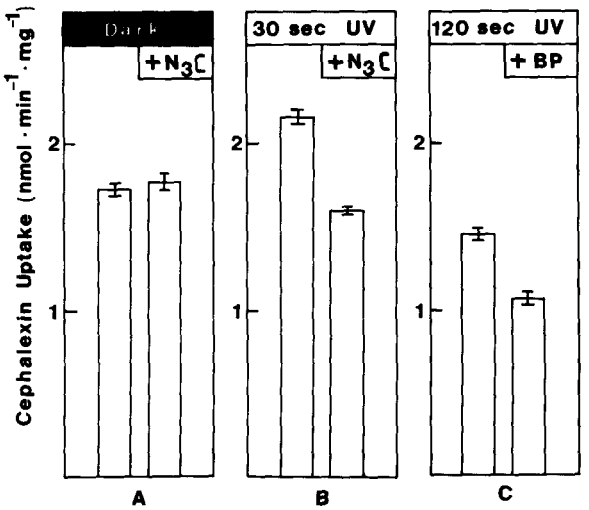

Fig. 2. Effect of irradiation and pretreatment with *N*-(4-azidobenzoyl)cephalexin and benzylpenicillin on the uptake of cephalexin into rat renal brush-border membrane vesicles. (A) Lacking inhibition in the dark. Vesicles (300 μg) were incubated for 10 min in the dark in the absence (left bar) and in the presence of 2 mM *N*-(4-azidobenzoyl)cephalexin (right bar). (B) Irreversible inhibition after photolabeling with *N*-(4-azidobenzoyl)cephalexin. Vesicles (300 μg) were irradiated for 30 s at 254 nm in the absence (left bar) and in the presence of 2 mM *N*-(4-azidobenzoyl)cephalexin (right bar). (C) Irreversible inhibition after photolabeling with benzylpenicillin. Vesicles (300 μg) were irradiated for 120 s at 254 nm in the absence (left bar) and in the presence of 50 mM benzylpenicillin (right bar). The incubation buffer contained 20 mM Tris-Hepes buffer (pH 7.4)/100 mM mannitol/100 mM KCl. After washing the vesicles in 20 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol, cephalexin uptake (2 mM) in 20 mM Tris-Hepes buffer (pH 7.4)/100 mM mannitol/100 mM NaCl was measured for 1 min at 30 °C. (N_3C, *N*-(4-azidobenzoyl)cephalexin; BP, benzylpenicillin).

TABLE II

SUBSTRATE PROTECTION OF THE RENAL TRANSPORT SYSTEM FOR β-LACTAM ANTIBIOTICS AND DIPEPTIDES FROM PHOTOINACTIVATION

Brush-border membrane vesicles from rat renal cortex (300 μg) were photolyzed for 30 s at 254 nm in the absence or in the presence of 2 mM *N*-(4-azidobenzoyl)cephalexin and the indicated concentrations of L-alanine, glycyl-L-proline and cefadroxil. After washing of the vesicles the uptake of 2 mM cephalexin in 20 mM Tris-Hepes buffer (pH 7.4)/100 mM mannitol/100 mM NaCl into 100 μg of vesicles was measured for 1 min at 30 °C. (N_3C, *N*-(4-azidobenzoyl)cephalexin).

Additive	[N_3C] (mM)	Uptake rate (nmol · min^{-1} · mg^{-1})	% inhibition
None	0	3.38 ± 0.08	0
None	2	2.87 ± 0.02	15.1 ± 0.10
50 mM L-alanine	2	2.26 ± 0.08	33.1 ± 1.17
50 mM glycyl-L-proline	2	2.98 ± 0.04	11.8 ± 0.15
40 mM cefadroxil	2	3.03 ± 0.02	10.3 ± 0.06

demonstrate that the photolabile derivatives previously used for the identification of the intestinal dipeptide transporter [20–22] are also well suited photolabile substrates for the identification of the renal transport system for β-lactam antibiotics and dipeptides.

Photoaffinity labeling of renal brush-border membrane vesicles with the respective radioactively labeled compounds, [^{3}H]benzylpenicillin, *N*-(4-azido[3,5-^{3}H]benzoyl)cephalexin or *N*-(4-azido[3,5-^{3}H]benzoyl)glycyl-L-proline resulted with all three derivatives in the covalent incorporation of radioactivity into two membrane polypeptides with apparent molecular weights of 130000 (Fig. 4, peak a) and 95000 (Fig. 3, peak b). Dependent upon the membrane preparation occasionally a polypeptide of molecular weight 165000 (Fig. 3, peak c) becomes labeled. The occurrence of this labeled polypeptide had no influence on the uptake of cephalexin suggesting that it is not involved in the transport of β-lactam antibiotics and dipeptides. No additional labeled polypeptides of higher or lower molecular weight could be detected by varying of the gel concentration from 6–15%. Coelectrophoresis of renal and intestinal brush-border membrane vesicles labeled with photoreactive derivatives of β-lactam antibiotics and dipeptides showed only one radioactively labeled band in the molecular weight range 127000–130000. Fig. 3 shows that the distribution of radioactivity with all three photoaffinity labels

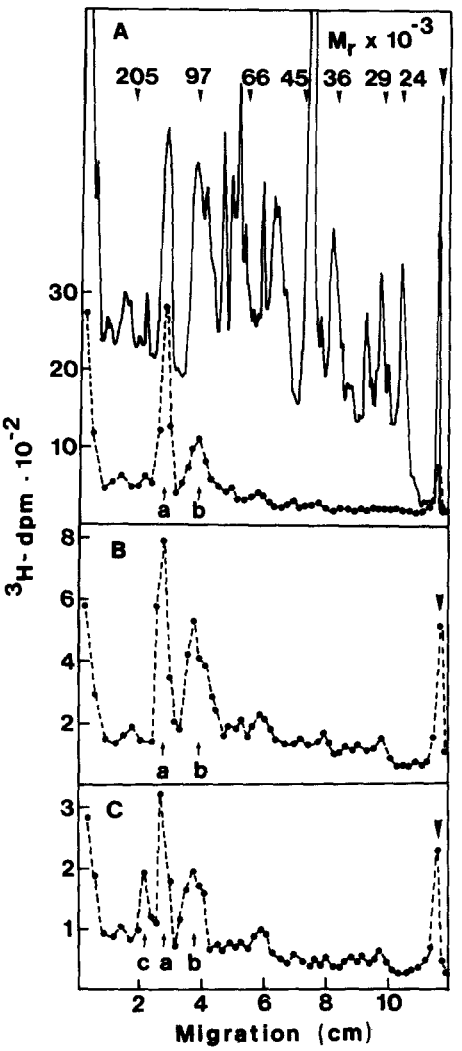

Fig. 3. Distribution of radioactivity after SDS gel electrophoresis of rat renal brush-border membranes after photoaffinity labeling. 200 μg of rat renal brush-border membrane vesicles were photolabeled with (A) 0.83 μM (2.5 μCi) [^{3}H]benzylpenicillin, (B) 0.14 μM (1.4 μCi) *N*-(4-azido[3,5-^{3}H]benzoyl)cephalexin and (C) 0.09 μM (1.35 μCi) *N*-(4-azido[3,5-^{3}H]benzoyl)glycyl-L-proline and subsequently submitted to SDS gel electrophoresis on 9% gels. The drawn line shows the densitometer scanning of the Serva-Blue stained polypeptides whereas the dotted line indicates the distribution of radioactivity. The positions of the marker proteins (M_r) and the tracking dye Bromophenol blue are indicated by arrows.

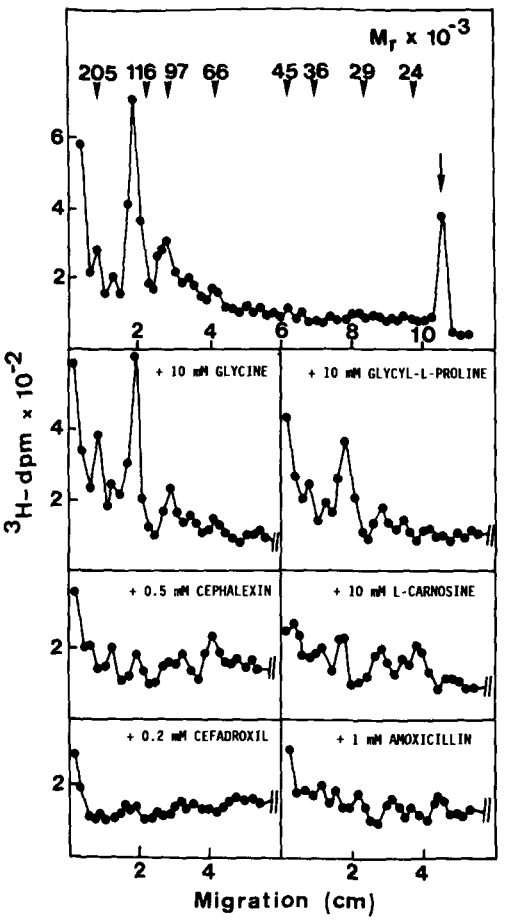

Fig. 4. Effect of various compounds on the labeling of binding proteins for β-lactam antibiotics and dipeptides in rat renal brush-border membrane vesicles. 200 μg of rat renal brush-border membrane vesicles were photolabeled with 1.1 μM (3.75 μCi) [^{3}H]benzylpenicillin in the absence (A) and in the presence of 10 mM glycine (B), 10 mM glycyl-L-proline (C), 0.5 mM cephalexin (D), 10 mM L-carnosine (E), 0.2 mM cefadroxil (F) and 1 mM amoxicillin (G). For all, other conditions see legend to Fig. 3.

is different from the distribution of Serva-Blue stained polypeptides indicating a specific labeling of the respective binding proteins for β-lactam antibiotics and dipeptides.

To determine the specificity of the renal binding proteins, competition labeling experiments were performed. Fig. 4 shows that amino acids such as glycine or proline had no effect on the labeling pattern. Dipeptides, however, and β-lactam antibiotics, penicillins as well as cephalosporins, clearly inhibited the labeling of the respective membrane polypeptides. The specificity of the binding proteins for β-lactam antibiotics and dipeptides in the renal brush-border membrane is very similar to that of the respective proteins from the intestinal brush-border membrane [20–22].

In conclusion, the experiments performed with renal and small intestinal brush-border membrane vesicles indicate that the transport systems shared by β-lactam antibiotics and dipeptides in the brush-border membrane of the renal cortex and the small intestine are similar. Both transport systems have similar specificity for transport and binding and their protein components have similar molecular weights as determined by SDS gel electrophoresis. They differ, however, in the dependence of the uptake of α-aminocephalosporins on an inward H^+-gradient. These results suggest that the transport systems for β-lactam antibiotics and dipeptides in the brush-border membrane from kidney cortex and small intestine are related, but not identical.

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