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Interaction of the orally active dianionic cephalosporin cefixime with the uptake system for oligopeptides and α-amino-β-lactam antibiotics in rabbit small intestine

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Abstract—The uptake of two orally active β-lactam antibiotics of different chemical structure, the zwitterionic α-aminocephalosporin cephalexin and the dianionic carboxymethoxyimino-cephalosporin cefixime, by brush border membrane vesicles obtained from rabbit small intestine and their molecular interaction with the H^+/oligopeptide transport system were investigated. The uptake of both compounds was stimulated by an inwardly directed H^+-gradient with a profound pH-maximum for cephalexin at pH $6_{outside}$ and pH 7.4_{inside} whereas cefixime uptake was maximal below pH $5_{outside}$. Modification of histidyl residues of membrane proteins led to a complete loss of pH dependence of transport of both cephalosporins. The uptake of cephalexin was competitively inhibited by cefixime and dipeptides and vice versa that of cefixime by cephalexin and dipeptides. The uptake of cefixime was trans-stimulated by cephalexin and glycyl-L-proline whereas cephalexin uptake could only be trans-stimulated by glycyl-L-proline, not by cefixime. Photoaffinity labeling with [^{3}H]benzylpenicillin as a direct photoaffinity probe of the H^+/oligopeptide transport system demonstrated a direct molecular interaction of both cephalexin and cefixime with this transporter in the pH range of 5–8. Thermal pretreatment of membrane vesicles inhibited the cephalexin transport system temperature-dependently, whereas cefixime uptake was not inhibited, but stimulated. Taken together we conclude that dianionic cephalosporins like cefixime bind to the transport system shared by oligopeptides and α-amino-β-lactam antibiotics. Their transport across the enterocyte brush border membrane, however, may occur to a significant extent by a different transport system.

α-Amino-β-lactam antibiotics are peptide-derived drugs carrying the structural elements of a tripeptide [1]. Their uptake into intestinal cells occurs by a saturable, H^+-activated active transport system [2–6] as the uptake of small peptides [7, 8]. Transport studies have suggested that orally active α-amino-β-lactam antibiotics share this intestinal transport system for oligopeptides [4–6, 9]. The identity of the transport systems for penicillins, cephalosporins and small peptides was demonstrated by photoaffinity labeling with photoreactive derivatives of penicillins, cephalosporins and dipeptides. An integral membrane protein of M_r 127,000 was identified as (a component of) the peptide transport system in the brush border membrane of enterocytes from the small intestine of rabbit, rat and pig [5, 10–14]. Recently, new orally active cephalosporins without an α-amino group have been found [15] which are well absorbed from the small intestine. Kinetic experiments suggested that these dianionic cephalosporins like cefixime are exclusively taken up by the H^+-dependent dipeptide transport system [16–18]. In one study however, a multiplicity of the intestinal peptide transport system has been suggested for α-aminocephalosporins and cefixime being recognized by only one of these systems [19]. In this study the molecular interaction of cefixime with the intestinal transport system for α-amino-β-lactam antibiotics and oligopeptides was investigated.

Materials and Methods

Materials. [Phenyl-4-(*n*)-^{3}H]benxylpenicillin (specific radioactivity 18–31 Ci/mmol) was obtained from Amersham (Braunschweig, F.R.G.), cefixime (FK 027) a gift from Dr Adam, Hoechst Aktiengesellschaft. Cephalexin and DEP were obtained from the Sigma Chemical Co. (München, F.R.G.) and cellulose nitrate filters (type HAWP 0.45 mm, 25 mm diameter) for the transport studies from Millipore (Eschborn, F.R.G.). Solvents for HPLC and all other substances were from commercial sources and of analytical grade.

Methods. Brush border membrane vesicles (BBMV*) from rabbit small intestine were prepared by the Mg^{2+}-precipitation method as described previously [5, 10, 20]. Protein was determined according to Bradford [21] using the BioRad kit (BioRad, München, F.R.G.). The uptake of cephalexin and cefixime was measured by the membrane filtration method [22] as described [5, 6, 10, 23, 24]. The composition of the incubation media is given in the legends to figures. Trans-stimulation was initiated by mixing of 20 μL membrane vesicle suspension (100 μg of membrane protein) with 180 μL medium containing the corresponding cephalosporin (2 mM) and uptake was measured for 1 min. Cephalexin and cefixime taken up by the vesicles were measured by HPLC [5, 6, 10, 14]. As mobile phase acetonitrile/30 mM sodium phosphate buffer (pH 7.0)/10 mM tetraethylammonium chloride with the ratios 22:78 for cephalexin and 14:86 for cefixime was used with detection by ultraviolet absorption at 262 nm. In all experiments the indicated values are the means ± SD of three to six individual determinations using a single membrane preparation. Each experiment was performed at least three times with different membrane preparations. Photoaffinity labeling [5, 10, 14], sodium dodecylsulfate (SDS) gel electrophoresis and detection of radioactivity [5, 10, 13] were performed as described previously.

Results and Discussion

The acidic microclimate pH of the luminal surface of the small intestine [25] serves as a driving force for the uptake system for small peptides [4, 7, 8]. The uptake of cefixime by rabbit BBMV was as the uptake of α-amino-β-lactam antibiotics stimulated by an inwardly directed H^+-gradient [17, 19, data not shown]. Figure 1, upper panel, shows that the uptake of cephalexin was maximal at a pH gradient $pH_{out} = 6$ and $pH_{in} = 7.4$; at higher or lower pH values of the medium the transport activity greatly decreased. In contrast, the uptake of cefixime increased by lowering of

* Abbreviations: BBMV, brush border membrane vesicles; DEP, diethylpyrocarbonate; SDS, sodium dodecylsulfate.

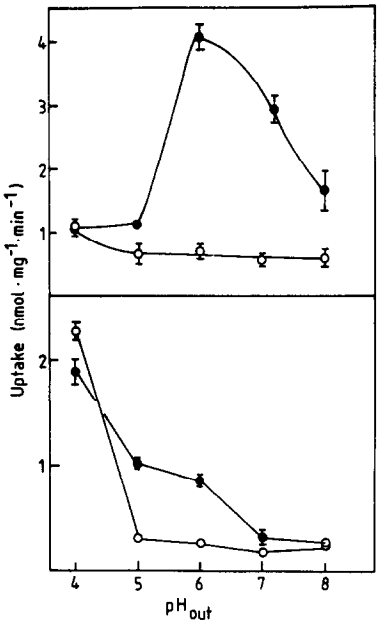


Fig. 1. Effect of extravesicular pH and treatment with DEP on the uptake of cephalexin (upper panel) and cefixime (lower panel) into BBMV from rabbit small intestine. BBMV preloaded with 10 mM Tris–Hepes-buffer (pH 7.4)/300 mM mannitol were incubated for 10 min at 20° either with 20 mM potassium phosphate buffer (pH 6.4/280 mM mannitol/0.9% ethanol) or 10 mM DEP in the above buffer. After washing with 10 mM Tris–Hepes buffer (pH 7.4)/300 mM mannitol and resuspending of the vesicles in this buffer, control (●) and DEP-treated vesicles (○) (100 μg of protein, 20 μL) were mixed with 180 μL of either 50 mM citrate–Tris buffer (pH 4.0, 5.0 or 6.0)/125 mM KCl or 50 mM Tris–Hepes buffer (pH 7.0 or 8.0)/125 mM KCl containing either 2 mM cephalexin or 2 mM cefixime and uptake was measured for 1 min.

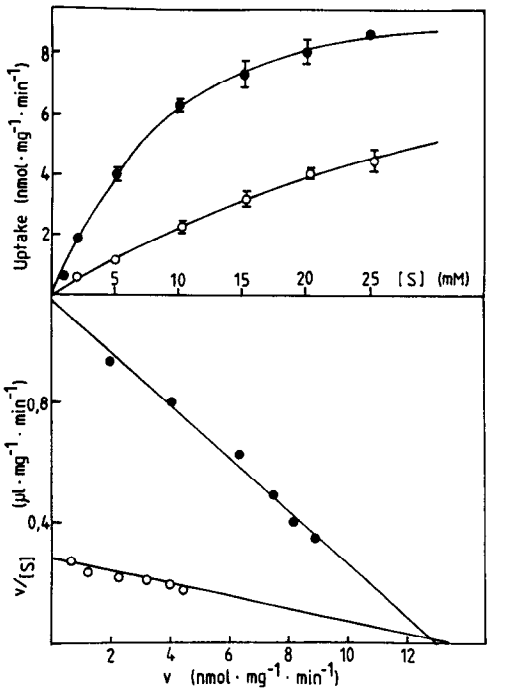


Fig. 2. Effect of cefixime on the uptake of cephalexin into BBMV from rabbit small intestine. The uptake of cephalexin at the indicated concentrations into BBMV preloaded with 10 mM Tris–Hepes buffer (pH 7.4)/300 mM mannitol (100 μg of protein, 20 μL) in 180 μL 10 mM citrate–Tris buffer (pH 6.0)/140 mM KCl was measured for 1 min either in the absence (●) or in the presence of 4 mM cefixime (○). The upper panel shows the concentration dependence, the lower panel the kinetic analysis in a $v/[s]$ vs v diagram.

the external pH and was maximal below pH 5 (Fig. 1, lower panel). Treatment of BBMV with diethylpyrocarbonate (DEP) led to a complete loss of the pH-dependence of the transport activity for cephalexin and cefixime (Fig. 1, ○).

Figure 2 (upper panel) shows the concentration-dependent uptake of cephalexin into BBMV in the absence and the presence of 4 mM cefixime. Kinetic analysis revealed a competitive inhibition (Fig. 2, lower panel) under these conditions ($pH_{out} = 6.0$, $pH_{in} = 7.4$). *Vice versa*, the uptake of cefixime was also competitively inhibited by cephalexin, benzylpenicillin and dipeptides in accordance with previously published results [17–19]. If cephalexin and cefixime share a common transport system, their uptake into BBMV should be stimulated by preloading of the vesicles with substrates for the intestinal peptide transport system. Figure 3 shows that a dipeptide such as glycyl-L-proline was able to trans-stimulate the uptake of both cephalexin and cefixime in the presence of an inwardly directed pH gradient ($pH_{out} = 5.0$, $pH_{in} = 7.4$), whereas no trans-stimulation occurred in the absence of such a gradient. Cefixime however was not able to trans-stimulate the uptake of cephalexin, neither in the presence nor in the absence of a pH gradient. In contrast, cephalexin preloading of the vesicles stimulated the uptake of cefixime into BBMV in the presence of an inwardly directed pH-gradient ($pH_{out} = 5.0$, $pH_{in} = 7.4$). These findings are partially different from those reported by Inui *et al.* [19] who have found that preloading of the vesicles with cefixime stimulated the uptake of the α-aminocephalosporin cephradine. In these studies, the equilibrium uptake values for cephradine in control and cefixime-preloaded vesicles were considerably different making an interpretation as counter-transport questionable. In our studies, cefixime preloading was not able to trans-stimulate cephalexin uptake in the pH range of 5–7.4 with measurement of cephalexin uptake either under iso-pH conditions ($pH_{out} = pH_{in}$) or gradient conditions ($pH_{out} < pH_{in}$).

To detect a direct molecular interaction of cefixime with the intestinal oligopeptide transporter, competition photoaffinity labeling experiments were performed. Figure 4A shows that the labeling of the 127 kDa binding protein for β-lactam antibiotics and dipeptides by photoactivated [^{3}H]benzylpenicillin was specifically decreased in a concentration-dependent manner by cefixime indicating a binding of cefixime to the 127 kDa protein. According to Inui *et al.* [19], cefixime does not interact with the transport system for α-amino-β-lactam antibiotics at neutral pH and is transported by a different peptide carrier in an acidic pH region. If so, cefixime should not decrease the extent of labeling of the 127 kDa polypeptide at pH 7 or 8. Figure 4B shows that in the pH range of 5–8, both orally active cephalosporins, cephalexin and cefixime, decreased the extent of labeling of the 127 kDa protein demonstrating that cefixime also interacts at neutral and lightly alkaline pH values with the intestinal oligopeptide transporter. This does not rule out the possibility that the photolabeled

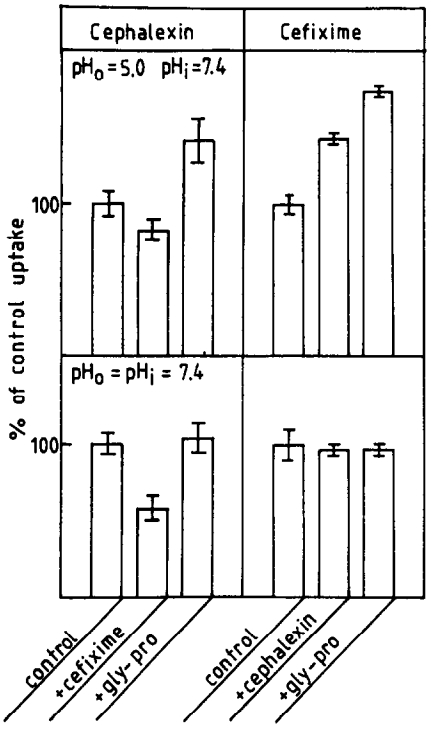
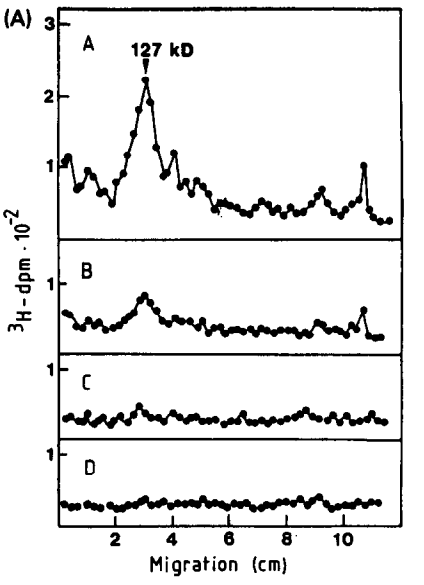


Fig. 3. Trans-stimulatory effects of cephalexin, cefixime and glycyl-L-proline on the uptake of cephalexin and cefixime into BBMV from rabbit small intestine. BBMV were incubated at 20° for 1 hr with 5 mM cephalexin, 5 mM cefixime or 20 mM glycyl-L-proline in 10 mM Tris–Hepes buffer (pH 7.4)/140 mM KCl. Trans-stimulation was started by a 20-fold dilution of the incubation mixtures either with 10 mM citrate–Tris buffer (pH 5.0)/140 mM KCl (upper panel) or 10 mM Tris–Hepes buffer (pH 7.4)/140 mM KCl (lower panel) containing 2 mM cephalexin or 2 mM cefixime. The uptake of cephalexin and cefixime was measured at 30° for 1 min and uptake is expressed as percentage of the respective controls.



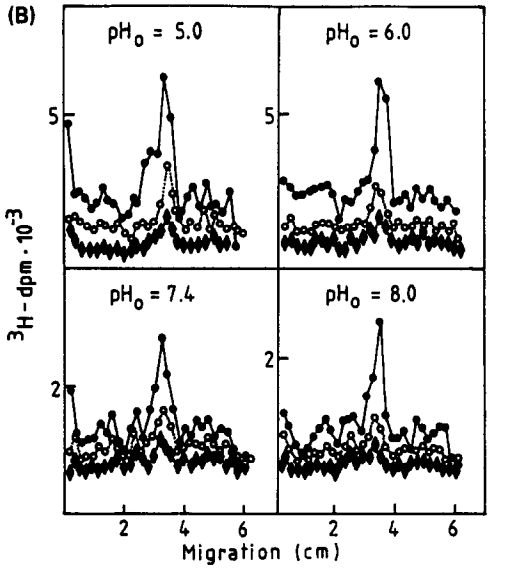


Fig. 4. Influence of cefixime on photoaffinity labeling of BBMV from rabbit small intestine with [^{3}H]benzylpenicillin. (A) BBMV (200 μg of protein) were photoaffinity-labeled with 0.83 μM (3 μCi) [^{3}H]benzylpenicillin either in the absence (A) or in the presence of 100 μM (B), 400 μM (C) or 1000 μM (D) cefixime. (B) BBMV (200 μg of protein, 20 μL) were mixed with 180 μL of buffers of different pH values (50 mM citrate–Tris buffer (pH 5 or 6)/125 mM KCl or 50 mM Tris–Hepes (pH 7–8)/125 mM KCl) containing either no inhibitor (●), 100 μM cefixime (○) or 100 mM cephalexin (◆). After 10 min of incubation in the dark, [^{3}H]benzylpenicillin (5 μCi) was added to achieve a final concentration of 2 μM. Subsequent to further 5 min of incubation in the dark, the vesicles were irradiated for 2 min at 254 nm. After washing the vesicles, the membrane proteins were separated by SDS–polyacrylamide gel electrophoresis on 7.5% gels and the distribution of radioactivity was determined by liquid scintillation counting after slicing the gels in 2 mm pieces and digestion of proteins with Biolute S.

127 kDa band contains a family of closely related transport proteins with different but overlapping specificities.

The trans-stimulation experiments described above may be interpreted that more than one transport system is involved in the uptake of zwitterionic and dianionic cephalosporins and it is probable that both transport systems show a different temperature sensitivity. Therefore, BBMV were heated to 70° for 30 min prior to transport studies and subsequently the uptake of cephalexin and cefixime was measured into control and heat-pretreated vesicles. Figure 5A shows that such a thermal pretreatment of BBMV greatly decreased the time-dependent uptake of cephalexin indicating an impairment of the oligopeptide transport system. The uptake of cefixime, however, was not inhibited by such a thermal treatment. This astonishing result was reproduced in several independent experiments with different membrane preparations. By varying the incubation temperature from 20 to 70°, the 30-sec uptake rates for cephalexin began to decrease above 50°. In contrast, the 30-sec uptake rates for cefixime were stimulated beginning above 40° and ending with an approximately 3-fold uptake rate after 60 and 70° pretreatment. Equilibrium uptake measurements of both compounds into control and heat-pretreated vesicles

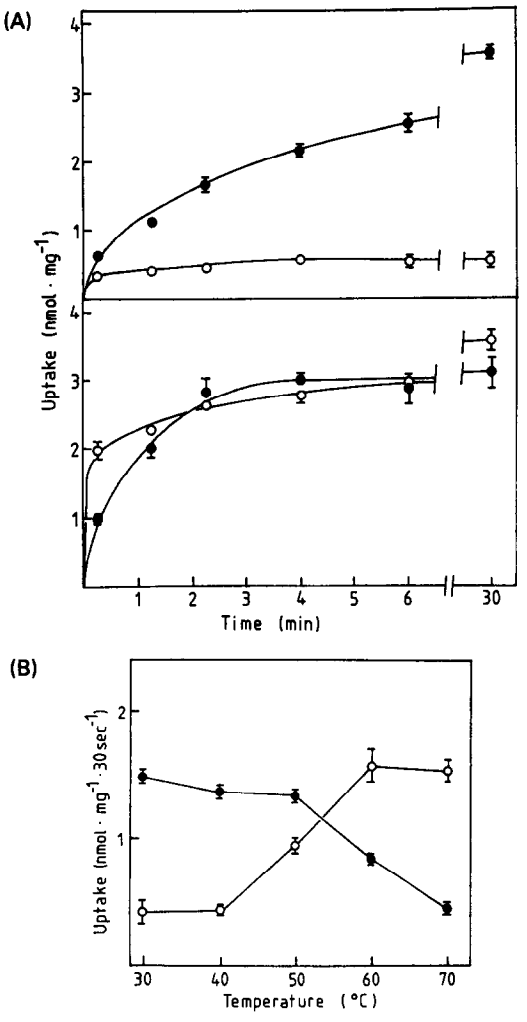


Fig. 5. Effect of thermal pretreatment of BBMV from rabbit small intestine on the H^+-dependent uptake of cephalexin and cefixime. (A) BBMV suspended in 10 mM Tris–Hepes buffer (pH 7.4)/300 mM mannitol were incubated for 30 min either at 30° (controls) or 70°. Subsequently, 20 μL (100 μg of protein) of these vesicle suspensions were mixed with 180 μL of 10 mM citrate–Tris buffer (pH 6.0)/140 mM KCl containing either 2 mM cephalexin (upper panel) or 2 mM cefixime (lower panel) and uptake was measured for 1 min. (●) Uptake into control vesicles. (○) Uptake into thermally pretreated vesicles. (B) BBMV suspended in 10 mM Tris–Hepes buffer (pH 7.4)/300 mM mannitol were held at the indicated temperatures for 30 min and subsequently the uptake either of cephalexin (●) or cefixime (○) was measured for 30 sec in the presence of a H^+-gradient (pH_{out} = 6.0, pH_{in} = 7.4).

dependent on the medium osmolarity did not give an indication for an increased binding of cefixime to thermally pretreated vesicles. Furthermore, in efflux studies with cephalexin and cefixime from control and heat-pretreated vesicles no indication for an increased leakage for cephalexin compared to cefixime was found. The reason for this different behavior of cephalexin and cefixime remains unclear; a similar dissociating effect of heat pretreatment was observed for the brush border enzymes sucrase and isomaltase [26].

Taken together these findings suggest that the uptake of cefixime into enterocytes across the brush border membrane occurs by more than only the oligopeptide transport system. From the data obtained we conclude that dianionic cephalosporins such as cefixime bind to the peptide transport system as was demonstrated by competitive inhibition of uptake of α-amino-β-lactam antibiotics and by photoaffinity labeling studies; their transport across the enterocyte brush border membrane however may occur to a significant extent by a different transport system.

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