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Stereoselective uptake of β-lactam antibiotics by the intestinal peptide transporter

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1 The stereoselective transport of β-lactam antibiotics has been investigated in the human intestinal epithelial cell line, Caco-2, by use of D- and L-enantiomers of cephalexin and loracarbef as substrates.

2 The L-isomers of cephalexin, loracarbef and dipeptides displayed a higher affinity for the oligopeptide/H^+-symporter in Caco-2 cells than the D-isomers. This was demonstrated by inhibition of the influx of the β-lactam, [^{3}H]-cefadroxil.

3 By measurement of the substrate-induced intracellular acidification in Caco-2 cells loaded with the pH-sensitive fluorescent dye BCECF (2',7'-*bis*(2-carboxyethyl)-5-(6)-carboxy-fluorescein), it was demonstrated for the first time that L-isomers of β-lactams not only bind to the peptide transporter with high affinity but are indeed transported.

4 Efficient proton-coupled transport of L-β-lactam antibiotics was also shown to occur in *Xenopus laevis* oocytes expressing the cloned peptide transporter PepT1 from rabbit small intestine.

5 Both cell systems therefore express a stereoselective transport pathway for β-lactam antibiotics with very similar characteristics and may prove useful for screening rapidly the oral availability of peptide-derived drugs.

Keywords: Proton-coupled transport; dipeptide; cephalosporin; Caco-2 cells; enterocytes; intracellular pH; β-lactam antibiotics

Introduction

β-Lactam antibiotics are bactericidal agents that act by inhibition of bacterial cell wall synthesis. They exert their effects by interrupting the transpeptidation and hence the linkage of individual peptidoglycan chains of the bacterial cell wall (Boyd, 1982; Neu, 1985). The β-lactam group encompasses the penicillins, cephalosporins, carbapenems and monobactams (Donowitz & Mandell, 1988). The general structure of the cephalosporins resembles the backbone of a tripeptide with the second peptide bond incorporated into a lactam ring. The excellent oral availability in particular of aminocephalosporins is explained by the fact that they serve as substrates for the intestinal peptide transporter(s). In the apical membrane of intestinal epithelial cells and the human colon carcinoma cell line, Caco-2, di- and tripeptides (Ganapathy *et al.*, 1984; Thwaites *et al.*, 1994b), peptide-mimetics, including selected β-lactam antibiotics (Tsuji *et al.*, 1986; Dantzig *et al.*, 1994a), immunostimulants (Saito & Inui, 1993; Takano *et al.*, 1994) and angiotensin-converting-enzyme inhibitors (Boll *et al.*, 1994; Thwaites *et al.*, 1994a), are transported by a common electrogenic H^+/peptide symport system. Although an interaction of L-isomers of peptides and β-lactam antibiotics with the substrate binding site of the intestinal peptide transporter(s) has been demonstrated by competition experiments in tissue preparations (Matthews & Adibi, 1976; Matthews, 1987; Tamai *et al.*, 1988), brush border membrane vesicles (Rajendran *et al.*, 1985; Tsuji *et al.*, 1986) and Caco-2 cells (Dantzig & Bergin, 1990; Thwaites *et al.*, 1994a) direct evidence for a stereoselective transport of these compounds was absent. In addition, studies with a reconstituted putative peptide transport protein isolated from rabbit small intestine failed to detect any transport of the L-isomer of the aminocephalosporin, cephalexin (Kramer *et al.*, 1992). For these reasons we addressed the question of the stereoselectivity of peptide and β-lactam transport by investigating whether (a) the cloned rabbit intestinal peptide transporter (PepT1) expressed in *Xenopus laevis* oocytes transports aminocephalosporin antibiotics when presented as L-isomers and (b) whether in Caco-2 cells a stereoselective transport of aminocephalosporins is found when influx is assessed by measuring intracellular acidification as a consequence of proton-coupled β-lactam cotransport.

Since peptides and β-lactams in L-configuration are rapidly hydrolyzed by intracellular peptidases, the demonstration of transport of β-lactams in L-configuration followed by intracellular cleavage could be helpful in the development of a peptide prodrug strategy.

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Methods

Studies in Xenopus laevis *oocytes expressing PepT1*

Expression and flux studies Preparation and handling of oocytes have been described previously (Boll *et al.*, 1994). Oocytes were injected with 50 nl of water (controls) or 50 nl of RNA solution containing 5 ng of transporter complementary RNA (cRNA). All studies were performed after 3 days by incubating oocytes in a buffer composed of (mM): NaCl 100, KCl 2, $CaCl_2$ 1, $MgCl_2$ 1, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 5, 2-(N-morpholino)ethanesulphonic acid (MES)/Tris 5, pH 6.5.

Intracellular pH recordings Changes in intracellular pH in oocytes were measured by dual emission fluorometry based on the pH-dependent fluorescence changes of the dextran-coupled dye (mol.wt. 10.000) SNARF-1. Emission of SNARF-1 after excitation at 535 nm was determined at peaks at 590 nm and 635 nm, resulting from its acidic and basic forms, respectively. The emission ratio was converted to intracellular pH by a calibration curve in which intracellular pH was measured by changing extracellular pH in the presence of the ionophore FCCP (40 μM). Three days after injection of water (control) or 5 ng cRNA a volume of 50 nl of the dye, (10 mM) was injected into individual oocytes. Two hours after injection of the dye,

oocytes were placed in a perfusion chamber which allowed monitoring of fluorescence changes of the intracellular dye in the absence and the presence of substrates. All fluorometric measurements with oocytes were carried out in a Shimadzu-RF-500 spectrofluorophotometer at 37°C. The pH traces show typical experiments from three individual oocytes. All uptake experiments were repeated in 5–8 oocytes from at least two separate batches.

Studies in Caco-2 cells

Cell culture Caco-2 cells (ATCC, HTB 37, passage 31) were cultured and passaged in Dulbecco's Modified Eagle Medium (Gibco 02430, Germany) supplemented with 10% foetal calf serum, 2 mM glutamine, 1% MEM non-essential amino acids (Gibco 01140, Germany) and 70 $\mu g\ ml^{-1}$ gentamicin in a humidified incubator at 37°C under an atmosphere of 5% CO_2. To generate functional epithelial monolayers, Caco-2 cells were seeded onto Transwell polycarbonate membranes (Costar 3401, Germany) with a density of 0.6×10^5 cells cm^{-2}. All cells were used between passages 38 and 56. After three weeks Caco-2 cells were fully differentiated and used for flux experiments. Transepithelial electrical resistance was measured with an epithelial voltmeter (EVOM, WPI, U.S.A.) and resistances of $\geqslant 300\ \Omega\ cm^{-2}$ indicated the presence of an intact monolayer (Artursson, 1990; Riley *et al.*, 1991).

Transport studies Flux studies in Caco-2 cells were performed in a modified Krebs buffer containing (mM): NaCl 1.37, KCl 5.4, $CaCl_2$ 2.8, $MgSO_4$ 1.0, NaH_2PO_4 0.3, KH_2PO_4 0.3, glucose 10 and HEPES-Tris 10 for incubation buffer pH 7.4 or Mes-Tris 10 mM for incubation buffer pH 6.5 respectively. Cell monolayers were washed free of serum-containing medium and incubated at the apical side in the presence of substrates and inhibitors dissolved in incubation buffer pH 6.5 containing in addition 100 μM of the peptidase inhibitor, amastatin. The basolateral side of the monolayers was bathed in incubation buffer pH 7.4. After an incubation period of 30 min at 37°C the monolayers were washed with ice cold buffer without substrates, filter inserts were cut out and cells were subsequently digested with a solubilizer. Tissue accumulation of substrates was determined by liquid scintillation spectroscopy. A control experiment in triplicate was carried out for each batch of cells and the results of inhibition experiments are generally expressed as uptake in percentage of controls (without competing substrates).

Intracellular pH (pH_i) measurements For pH_i measurements the Caco-2 monolayers were loaded with BCECF by incubation of the cells with 5 μM of the lipophilic ester (BCECF-AM), in both apical and basal compartments (40 min at 37°C). Subsequently the monolayer inserts were placed in a 24 mm diameter perfusion chamber mounted on the stage of an inverted fluorescence microscope (Nikon Diaphot). Perfusion of the apical and basolateral chambers was accomplished by a compressed air-driven system (flow rate 5 $ml\ min^{-1}$) which allowed the exchange of the apical incubation media during perfusion. The apical and basolateral bath volumes of 0.5 and 1.0 ml could be completely changed in 6 or 12 s, respectively. All experiments were performed at 37°C. Intracellular pH was estimated by measuring the intensity of emission of BCECF at 520 nm after excitation of the fluorescent dye at the pH-insensitive wavelength at 440 nm and the pH-sensitive wavelength at 490 nm, respectively. The 440/490 nm ratio of 5–10 cells was monitored on-line with a photon counting system (Newcastle Photometric Systems). The ratio was converted to pH_i by calibration of the cells with nigericin (10 μM) and high $[K^+]$-solutions (Thomas *et al.*, 1979).

Materials

Custom-synthesized [^{3}H]-cefadroxil (39 Ci $mmol^{-1}$) was obtained from Amersham Buchler, Braunschweig, Germany. β-Lactams, peptides, amastatin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and the pH sensitive fluorescent dye 2',7'-*bis*-(2-carboxyethyl)-5-(6)-carboxyfluorescein-acetoxymethylester (BCECF-AM) were purchased from Sigma (Deisenhofen, Germany). The dextran-coupled pH-sensitive dye, SNARF-1 (mol.wt. 10000) was obtained from Bioprobes (Eugene, OR, U.S.A.). D- and L-isomers of cephalexin and loracarbef were kindly provided by Eli Lilly (Indianapolis, IN, U.S.A.).

Calculations

All calculations including kinetic analysis by non linear regression analysis were performed by using INPLOT (GraphPAD, Los Angeles, CA, U.S.A.). For each variable at least 3 independent experiments (3–6 monolayers, 3–10 oocytes) were carried out. Data are given as the mean + s.d.

Results

Affinities of L- and D-isomers of aminocephalosporins for the Caco-2 peptide transporter

The apparent affinities for inhibition of cefadroxil uptake into Caco-2 cells of enantiomers of loracarbef, cephalexin, the dipeptide alanyl-alanine (Ala-Ala) as well as two other dipeptides and three further β-lactams were determined. Inhibition studies allowed classification of the competing compounds into three groups depending on their inhibitory potency (Figure 1). A first class of peptides (e.g. D-alanyl-D-alanine and D-phenylalanine) and β-lactams (eg. benzylpenicillin) failed to inhibit [^{3}H]-cefadroxil uptake. The second group, including the orally acive β-lactams, D-cephalexin, D-amoxicillin and D-loracarbef as well as the alanyl peptides containing only one D-amino acid residue, showed significant inhibition of cefadroxil uptake into Caco-2 cells ranging from 27.2% to 47.8%. A third group, represented by the naturally occurring dipeptides consisting of L-amino acids (L-alanyl-L-alanine, glycyl-L-sarcosine and glycyl-L-glutamine) but also the L-enantiomers of loracarbef and cephalexin, displayed the highest inhibitory potency ($\geqslant$60% inhibition) of all substrates tested.

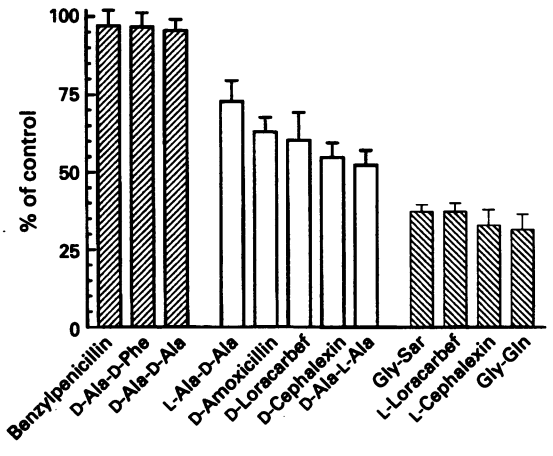

Figure 1 Inhibition of [^{3}H]-cefadroxil uptake into Caco-2 cells by stereoisomers of alanyl-peptides and selected β-lactam-antibiotics. In addition, influx inhibition by the dipeptides glycyl-sarcosine (Gly-Sar) and glycyl-glutamine (Gly-Gln) and the β-lactams, amoxicillin and benzylpenicillin, is presented. Uptake of 1 mM cefadroxil into Caco-2 cells was measured at apical buffer pH 6.5 for 30 min at 37°C in the absence (control) or presence of 10 mM of the inhibitors. The aminopeptidase inhibitor, amastatin, was present at a concentration of 100 μM in each experiment. Values are expressed as the mean ± s.d. of 3–6 monolayers. Hatched (/) columns: no significant inhibition; open columns: $\leqslant$50% inhibition; hatched columns (\): $\geqslant$60% inhibition.

Since uptake studies employing the L-isomers of peptides and β-lactams might be hampered by hydrolysis of the compounds by membrane-bound peptidases, all uptake studies were performed in the presence of 100 μM amastatin. Cefadroxil transport is not affected at this concentration. There was only a modest increase in the inhibitory potency (≤9.4%) of the L-enantiomers of peptides and β-lactams when transport of cefadroxil was studied in the presence of the aminopeptidase inhibitor, amastatin (data not shown). This indicates that the compounds are not subjected to large scale hydrolysis during uptake studies.

To demonstrate competitive interaction of loracarbef and cephalexin enantiomers with the substrate binding site of the peptide carrier, the uptake rates of 0.5, 2 and 5 mM cefadroxil were determined in the presence of increasing concentrations of L- or D-isomers of loracarbef and cephalexin. As shown in Figure 2 all enantiomers showed competitive inhibition of cefadroxil uptake. However the apparent K_i-values for D-loracerbef ($K_i = 1.8 \pm 0.25$ mM) and D-cephalexin ($K_i = 1.4 \pm 0.35$ mM) were almost threefold higher than the apparent affinity constants of L-loracarbef ($K_i = 0.7 \pm 0.14$ mM) and L-cephalexin ($K_i = 0.5 \pm 0.20$ mM). Although the high affinity and competitive interaction with the carriers substrate binding site already suggested that L-enantiomers may also serve as substrates, we have measured substrate-mediated influx of protons into Caco-2 cells for a direct demonstration of β-lactam transport.

Transport of loracarbef and cephalexin enantiomers in Caco-2 cells

It was shown previously that the transport of dipeptides across the apical membrane of Caco-2 cells is coupled to H^+-flow into the cells causing a significant decrease in pH_i reversed by perfusion with substrate-free medium (Thwaites *et al.*, 1993a, b). Caco-2 cells were superfused at the basal side with buffer pH 7.4 throughout the experiment and apical pH was changed from pH 7.4 to pH 6.5 (Figure 3). Superfusion of monolayers with an apical buffer pH 6.5 caused a slight decrease of intracellular pH representing a substrate-independent proton permeability of the brush border membrane. This decrease of pH_i to pH 7.30 showed saturation after 2 min, suggesting activation of pH_i regulatory mechanisms by the cells.

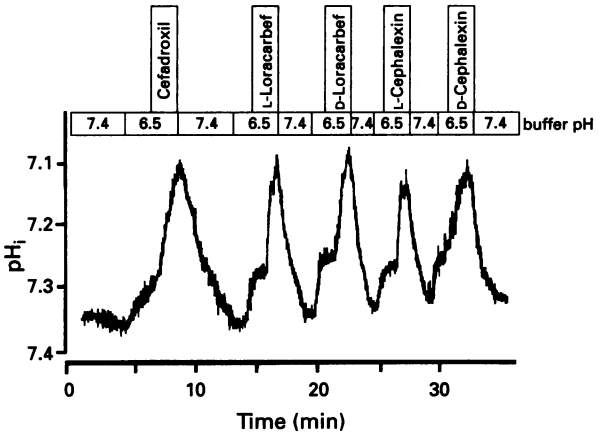

Figure 3 Intracellular pH changes in BCECF-loaded Caco-2 monolayers. The figure represents the effects of exposure of the monolayer to an apical pH of 6.5 containing none or either 5 mM of cefadroxil, L- and D-loracarbef and L- and D-cephalexin respectively. Basolateral pH was maintained at pH 7.4.

Introduction of an apical medium of pH 6.5 containing 5 mM cefadroxil, or 5 mM D- or L-loracarbef respectively initiated a rapid fall in pH_i within 2.5 min to approximately pH 7.1. Superfusion with a substrate-free buffer of pH 7.4 led to a complete intracellular pH recovery from the substrate-induced acid load. As shown in Table 1, the slope of the initial linear phase of pH_i-decrease was reduced with D-loracarbef when compared to the L-enantiomer, indicating that the L-form of this β-lactam is transported more efficiently. Similar results were obtained when perfusion was performed with a medium containing 5 mM D-cephalexin or L-cephalexin (Table

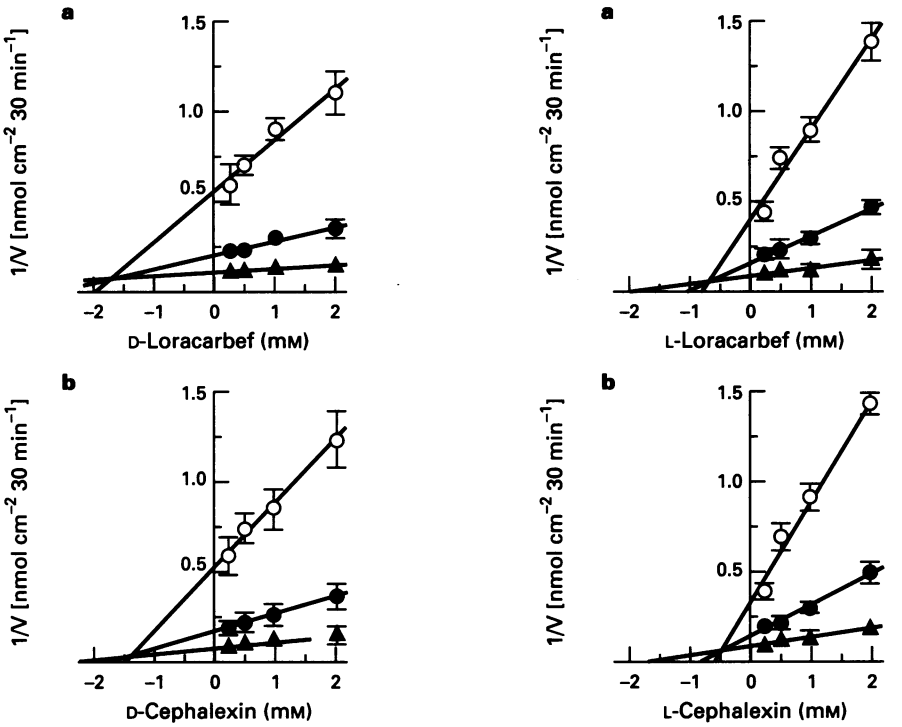

Figure 2 Dixon-Webb plots of [^{3}H]-cefadroxil uptake into Caco-2 cells in the presence of D- and L-enantiomers of loracarbef (a) and cephalexin (b). Uptake of 0.5 mM (○), 2 mM (●) and 5 mM (▲) cefadroxil was measured for 30 min at 37°C at apical buffer pH 6.5 in the presence of 0.25, 0.5, 1 and 2 mM of D- and L-β-lactam enantiomers. Values are given as mean ± s.d. of 3–6 monolayers. Apparent K_i-values were determined by linear regression analysis from the Dixon plots.

Table 1 Substrate-induced intracellular acidification rates in Caco-2 cells

Compound [5 mM]	ΔpH_i (min^{-1})
D-Cephalexin	0.082 ± 0.015
L-Cephalexin	0.181 ± 0.031
D-Loracarbef	0.130 ± 0.020
L-Loracarbef	0.201 ± 0.018
Cefadroxil	0.102 ± 0.022
Benzylpenicillin	ND
Benzylpenicillin + cefadroxil	0.112 ± 0.025

Caco-2 cells were perfused from the apical side with buffer pH 6.5 containing the β-lactam antibiotics (basolateral pH 7.4). Intracellular pH-changes due to the addition of the different β-lactams are shown in Figures 3 and 4 respectively. Initial acidification rates (ΔpH_i min^{-1}) induced by the compounds were calculated from the linear portion of the traces from three individual experiments by linear regression analysis.
ND, not detectable.

1). The initial acidification rate in Caco-2 cells caused by cefadroxil addition was very similar to that caused by the D-isomers of loracarbef and cephalexin (Table 1). In contrast, benzylpenicillin neither induced a significant decline of pH_i below the pH-drop by buffer alone (pH 6.5) (Table 1), nor inhibited the acidification during the apical perfusion with cefadroxil (Figure 4, Table 1). The lack of effect of benzylpenicillin on intracellular pH is in accordance with its failure to inhibit [^{3}H]-cefadroxil influx into Caco-2 cells (Figure 1).

Transport of loracarbef and cephalexin enantiomers in PepT1 cRNA injected oocytes

After we had established that L-isomers of peptides as well as aminocephalosporins are transported by the Caco-2 cell peptide transporter, we investigated whether they are also transported by the peptide transporter from rabbit small intestine. Previous studies had suggested that L-cephalexin is not transported by the purified brush border membrane peptide transporter from rabbit small intestine when reconstituted into liposomes (Kramer *et al.*, 1992) although L-cephalexin was found to inhibit the uptake of its D-enantiomer into proteoli-

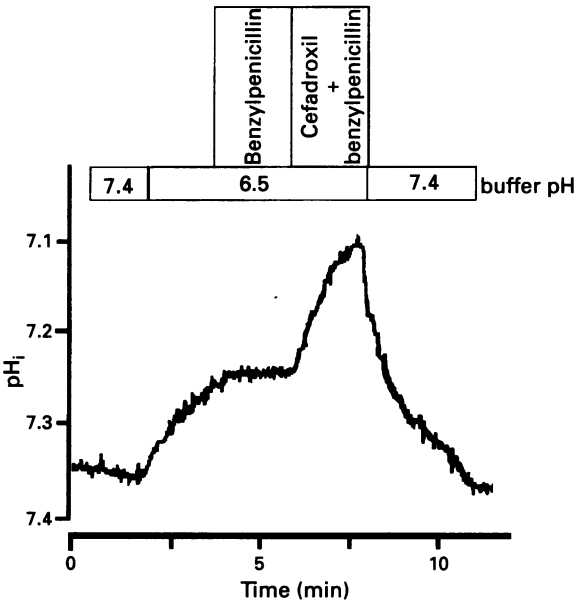

Figure 4 Intracellular pH-measurements determined during apical superfusion with benzylpenicillin (5 mM) alone, followed by a solution containing both cefadroxil (5 mM) and benzylpenicillin (5 mM). Basolateral pH was maintained at 7.4.

Table 2 Inhibition of uptake of [^{3}H]-cefadroxil into oocytes expressing PepT1 by D- and L-isomers of loracarbef and cephalexin

Compound [5 mM]	*% inhibition*
D-Cephalexin	45.1 ± 3.2
L-Cephalexin	67.4 ± 4.4
D-Loracarbef	40.2 ± 4.3
L-Loracarbef	60.5 ± 5.7

Oocytes were injected with 50 nl (5 ng per oocyte) of PepT1 cRNA. Three days post-injection, uptake of 1 mM cefadroxil was measured at pH 6.5 after 30 min of incubation. Cefadroxil influx was determined in the absence or the presence of 5 mM of D- and L-loracarbef or cephalexin respectively. Data are presented as the means ± s.d. for 5–10 oocytes per individual experiment.

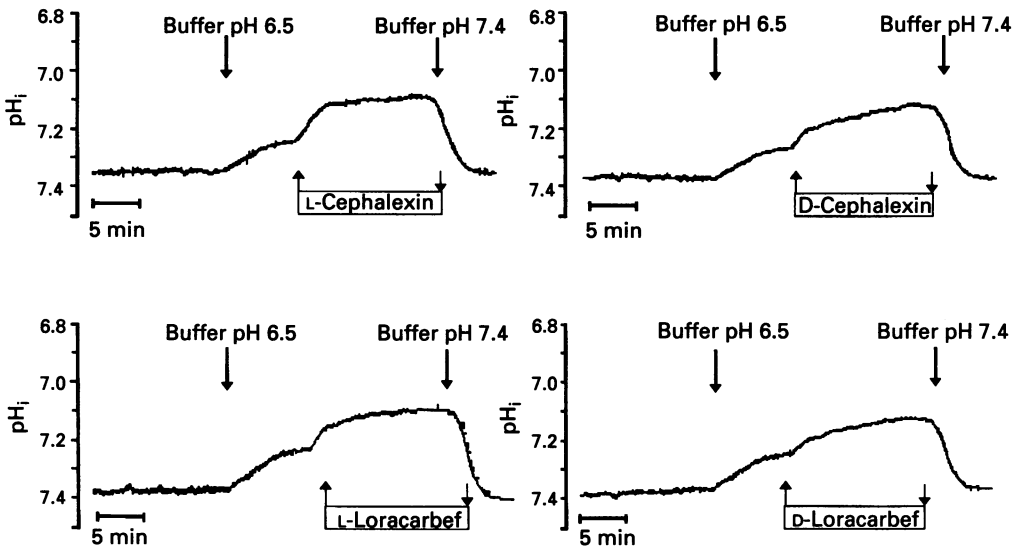

Figure 5 Intracellular pH measured in dextran-SNARF-1 loaded oocytes expressing the peptide transporter from rabbit small intestine (PepT1). Three days after injection of 5 ng of PepT1-cRNA per oocyte, each oocyte was microinjected with the pH-sensitive dye dextran-SNARF-1 as described in the Methods section. Subsequently, perfusion of ooyctes was carried out in a buffer pH 6.5 in the absence and presence of 5 mM D- and L-enantiomers of loracarbef and cephalexin. Shown are characteristic individual traces from three experiments employing the different β-lactams.

Table 3 Substrate-induced initial pH_i decline in oocytes expressing the rabbit small intestinal peptide transporter PepT1

Compound [5 mM]	ΔpH_i (min^{-1})
D-Cephalexin	0.041 ± 0.008
L-Cephalexin	0.058 ± 0.010
D-Loracarbef	0.030 ± 0.011
L-Loracarbef	0.056 ± 0.009

Three days after injection of 5 ng PepT1 cRNA, the oocytes were loaded with 50 nl of dextran-coupled SNARF-1 as described in the Methods section. β-Lactam induced initial acidification rates (ΔpH_i min^{-1}) were calculated from the linear portion of the pH_i traces of three individual oocytes (Figure 5). Oocytes were perfused with buffer pH 6.5 and 5 mM of each of the compounds in buffer pH 6.5. The initial decline of pH_i was determined by linear regression analysis.

posomes. Since differences in the functional features of the transporters expressed in human intestinal (Caco-2) cells and rabbit small intestine cannot be excluded, we used the cloned peptide transporter PepT1 expressed in *Xenopus laevis* oocytes to determine stereoselectivity of uptake of aminocephalosporins by pH_i measurements. Functional expression of peptide transport in oocytes was assessed by measuring pH dependent influx of [^{3}H]-cefadroxil and cefadroxil-mediated H^+ influx by dual emission fluorescence of the pH sensitive dye SNARF-1. As shown previously (Boll *et al.*, 1994) cefadroxil uptake (1 mM) into oocytes at pH 6.5 is increased about twofold when compared to buffer pH 7.4. Addition of 5 mM of D- and L-loracarbef significantly reduced cefadroxil influx at pH 6.5 by 40% and 60%, whereas 5 mM D- and L-cephalexin inhibited influx by 45% and 67% respectively (Table 2). When pH_i was measured in oocytes expressing PepT1, perfusion with a buffer pH 6.5 reduced intracellular pH by 0.1 units. As demonstrated earlier in PepT1-cRNA-injected oocytes (Boll *et al.*, 1994), perfusion with 5 mM cefadroxil at pH 6.5 caused a substrate specific decrease in pH_i by 0.2 pH units. In the present study, perfusion of oocytes expressing PepT1 with both D- and L-enantiomers of loracarbef and cephalexin caused a significant and reversible intracellular acidification (Figure 5). Similar to the studies in Caco-2 cells, initial acidification rates in oocytes were higher with the L-isomers compared to the D-isoforms (Table 3). Based on pH_i measurements in both Caco-2 cells and oocytes, we conclude that D- and L-enantiomers of the β-lactams cause proton influx as a consequence of carrier mediated substrate/H^+ cotransport and therefore, that both groups of compounds are transported by a common peptide transport system.

Discussion

Administration of drugs by the oral route is a major aim of drug delivery. However, many hydrophilic compounds have very low absorption rates. Even though amino-β-lactam antibiotics have tripetide-like structures and, therefore, are highly hydrophilic and ionized at physiological pH, they are generally considered to undergo acceptable oral absorption (Bergan, 1984). The high oral availability of most of the cephalosporins is explained by intestinal absorption via a saturable transport mechanism shared by di- and tripeptides (Iseki *et al.*, 1984; Okano *et al.*, 1986; Tsuji *et al.*, 1986). Transport of β-lactam antibiotics and di/tripeptides by a common transport system has also been demonstrated in the human intestinal cell line Caco-2 (Inui *et al.*, 1992; Matsumoto *et al.*, 1994). In addition, other peptide-derived drugs like angiotensin-converting-enzyme inhibitors (Thwaites *et al.*, 1994b) and the immunostimulant bestatin (Saito & Inui, 1993; Takano *et al.*, 1994) share the H^+-coupled peptide transporter in the apical membrane of epithelial cells. Although the natural substrates of the intestinal peptide transporters in the vertebrate intestine are short chain peptides consisting almost exclusively of L-amino acids, the peptide transporters also interact with peptides and peptide mimetics containing D-amino acids. As has been shown previously (Asatoor *et al.*, 1973; Thwaites *et al.*, 1994) the incorporation of a D-amino acid into the N-terminus of a di- or tripeptide reduces the rate of absorption significantly. This study demonstrates the stereoselective interaction of alanyl peptides with the transporter in Caco-2 cells. Incorporation of D-alanine into the N-terminus reduces affinity for interaction with the transporter, although incorporation of D-amino acids into the C-terminal position of dipeptides appears to restrict affinity even more. Furthermore, peptides consisting only of D-amino acids do not serve as substrates (Figure 1). Therefore, a D-configuration in the N-terminus of a peptide substrate does still allow efficient transport. All orally active β-lactams (Amidon & Sinko, 1988; 1989) carry an unsubstituted or modified D-phenylglycine side group in the N-terminal position and, therefore, should have affinities similar to di- and tripeptides with an N-terminal D-amino acid. Although the restricted conformational freedom in the lactam ring system may reduce overall substrate affinity, the aminocephalosporins generally have K_m and K_i values which are very similar to kinetic constants obtained for di- and tripeptides (Ganapathy *et al.*, 1984; Kudo *et al.*, 1989).

Since transport studies using peptides consisting of L-amino acids are hampered by hydrolysis (at both the brush border membrane and in the cytosol) resistant peptides (including glycyl-sarcosine) are used most frequently. However, glycyl peptides do not allow the direct determination of transport characteristics of different stereoisomers and radiolabelled β-lactams employed in transport studies are always D-isomers. Hence, studies on the stereoselectivity of transport for the two different groups of substrates have been limited to competition experiments and have yielded conflicting data about structural and conformational requirements for substrate transport. Therefore, in addition to flux studies and competition experiments, a method which allows direct assessment of substrate specific acidification of cells expressing the intestinal peptide transporter was employed (Thwaites *et al.*, 1993a, b).

This study demonstrates (in two cell systems and by applying different experimental approaches) that aminocephalosporins are transported into cells expressing the peptide transporter regardless of whether they are supplied as L- or D-enantiomers. In addition, the comparison of uptake of substrates into Caco 2 cells and oocytes expressing the rabbit intestinal peptide transporter PepT1 reveals no major functional differences, indicating that the transport systems from both sources have similar characteristics.

In a series of papers Kramer *et al.* described the isolation and reconstitution of a putative peptide transporter protein from rabbit small intestine (Kramer *et al.*, 1992). A 127 kDa membrane protein was identified on the basis of photoaffinity labelling with [^{3}H]-benzylpenicillin and was purified by wheatgerm-lectin chromatography and cation-exchange chromtography. Its apparent molecular weight suggests that it could be similar to a 120 ± 10 kDa protein from Caco-2 cells identified by Dantzig *et al.* (1994b) as a protein associated with peptide transport. This cloned putative transporter was found to belong to the cadherin superfamily of calcium dependent, cell-cell adhesion proteins. Although certain features of the 127 kDa protein strongly suggested that it was identical to the intestinal peptide transporter from the rabbit intestine, not all findings were consistent with features of the transporter characterized in membrane vesicles and tissue preparation. Finally, the reconstitution of the 127 kDa protein into liposomes demonstrated its capability for transporting D-cephalexin which was most convincing in its suggested role as the intestinal peptide transporter. However, the proteoliposomes did not transport D-cephalexin, questioning whether the transporter indeed discriminates L-isomers or may represent a protein with a function other than transport. With the cloning of PepT1 from the

same source and its functional expression in oocytes a re-evaluation of the question of stereoselectivity of β-lactam transport appeared important.

Since the rabbit small intestinal peptide transporter (PepT1) and the peptide transporter in Caco-2 cells both transport D- as well as L-isomers of aminocephalosporins, it is plausible to conclude that the lack of transport of L-cephalexin in the proteoliposomes containing the 127 kDa protein from rabbit intestine is not caused by species differences of the peptide transporters. As an alternative explanation we could assume that the 127 kDa represents a second transport pathway which, beside PepT1, is responsible for aminocephalosporin transport. However, as demonstrated by Fei *et al.* (1994) and our group (data not shown), hybrid depletion of rabbit small intestine mRNA with PepT1-antisense-mRNA indicate that PepT1 is the only transporter responsible for transport of dipeptides as well as aminocephalosporins in the rabbit intestine. In addition there is no kinetic evidence from either studies in Caco-2 cells (Dantzig *et al.*, 1992; Brandsch *et al.*, 1994) nor rabbit intestinal vesicles (Ganapathy *et al.*, 1984; Inui *et al.*, 1988) for a second system responsible for transport of neutral peptides or aminocephalosporins. Since the 127 kDa protein had initially been identified and purified by its ability to bind benzylpenicillin, it is surprising that benzylpenicillin in our studies failed to inhibit transport of cefadroxil in Caco-2 cells (Figure 1) and oocytes expressing PepT1 (Boll *et al.*, 1994) and moreover, failed to induce cytosolic acidification by a proton-coupled transport mechanism (Figure 4). Therefore, it remains to be determined which role the 127 kDa protein plays in β-lactam absorption. Although it is apparently not necessary for brush border transport of peptides and β-lactams, it might act as an additional membrane-binding protein.

In conclusion, our studies demonstrate that oocytes expressing the peptide transporter PepT1 from rabbit small intestine take up both D- as well as L-enantiomers of aminocephalosporins. Our studies also suggest that the L-isomers of the β-lactams are transported by a PepT1 like protein across the apical membrane of the human Caco-2 cells. The higher affinity of the L-isoforms for interaction with the substrate binding site of the transporters confirms the concept of stereoselective substrate recognition by the carrier protein. These data may be of special interest in rational drug design for peptide prodrugs.

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