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Uptake of the cephalosporin, cephalexin, by a dipeptide transport carrier in the human intestinal cell line, Caco-2

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The transport of the orally absorbed cephalosporin, cephalexin, was examined in the human epithelial cell line, Caco-2 that possesses intestinal enterocyte-like properties when cultured. In sodium-free buffer, the cells accumulated 1 mM D-[9-^{14}C]cephalexin against a concentration gradient and obtained a distribution ratio of 3.5 within 180 min. Drug uptake was maximal when the extracellular pH was 6.0. Uptake was reduced by metabolic inhibitors and by protonophores indicating that uptake was energy- and proton-dependent. Kinetic analysis of the concentration dependence of the rate of cephalexin uptake showed that a non-saturable component (K_d of 0.18 ± 0.01 nmol/min per mg protein per mM) and a transport system with a K_m of 7.5 ± 2.8 mM and a V_{max} of 6.5 ± 0.9 nmol/min per mg protein were responsible for drug uptake. Uptake was competitively inhibited by dipeptides. The transport carrier exhibited stereospecificity for the L-isomer of cephelaxin. Drug uptake was not affected by the presence of amino acids, organic anions, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid or 4,4′-diisothiocyano-2,2′-disulfonic stilbene. Therefore, Caco-2 cells take up cephalexin by a proton-dependent dipeptide transport carrier that closely resembles the transporter present in the intestine. Caco-2 cells represent a cellular model for future studies of the dipeptide transporter.

Introduction

Intestinal enterocytes are polarized cells with a microvillous luminal surface and a physically distinct basolateral membrane on the serosal side. These epithelial cells function in the degradation and absorption of nutrients and solutes from the lumen of the intestine [1]. To facilitate absorption, the cells have many transport carriers. Several of the intestinal transporters, such as the sodium-dependent glucose transporter, are not commonly found in other tissues. The elucidation of transport mechanisms in human tissue has been difficult due to a lack of available tissue, and the rapid loss of viability when excised [2].

The human intestinal cell line Caco-2 provides a cellular model for the study of the differentiated function of intestinal enterocytes [3]. Caco-2 cells spontaneously differentiate in culture to polar cells possessing microvilli and enterocytic properties [4]. Confluent monolayers form tight junctions between cells [3,4], and exhibit dome formation [5] and electrical properties characteristic of an epithelium [6]. Like the small intestinal enterocyte, Caco-2 cells possess several biochemical markers asymmetrically distributed between the two cellular surfaces. For example, alkaline phosphatase [4], isomaltase-sucrase [4], and aminopeptidases [4] reside in the apical membrane, and the receptors for transferrin [7] and epidermal growth factor [8] are located predominately on the basolateral membrane. Several transport carriers normally found in the small intestine are present in Caco-2 cells such as: the folic acid transport carrier [9], the sodium-dependent phosphate transporter [10] and a sodium-dependent glucose transporter [11].

Abbreviations: cephalexin, 7-(D-α-amino-α-phenylacetamidocarbonyl)-3-methyl-3-cephem-4-carboxylic acid, monohydrate; cefaclor, 7-(D-α-amino-α-phenylacetamidocarbonyl)-3-chloro-3-cephem-4-carboxylic acid, monohydrate; EBSS, Earle's balanced salt solution; Mes, 2-(*N*-morpholino)ethanesulfonic acid; HPLC, high performance liquid chromatography; DR, distribution ratio; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; L-Phe-Gly, L-phenylalanylglycine; Gly-L-Pro, glycyl-L-proline; L-Pro-Gly, L-prolylglycine; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic stilbene.

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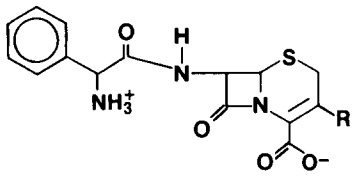

Drug	—R
Cephalexin	$-CH_3$
Cefaclor	—Cl

Fig. 1. Structures of two orally absorbed cephalosporin antibiotics, cephalexin and cefaclor. At acidic pH values these two antibiotics exist predominantly as zwitterions with the free amino group being protonated and the carboxylic acid group being deprotonated. (The pK_a of the carboxylic acid group of cephalexin is 2.57 and of cefaclor is approximately 1.5, and the pK_a of the amino group of both antibiotics is 7.17.)

The present study was undertaken to evaluate the potential usefulness of the Caco-2 cell line as an in vitro model for the study of the properties of the human dipeptide transport carrier. This transporter is known to be located in the intestine and the kidney and mediates the uptake of dipeptides, tripeptides and the orally absorbed cephalosporin antibiotics [12–27]. Because dipeptides and cephalosporins share certain structural features such as a peptide bond with an α-amino group and a terminal carboxylic acid group (as illustrated in Fig. 1 for cephalexin and cefaclor), it is not surprising that these compounds share a common transport mechanism. Unlike dipeptides, these orally absorbed antibiotics are not hydrolyzed by intestinal peptidases; consequently, the cephalosporins are ideal substrates to characterize the dipeptide carrier in intact tissue. In the present study, the uptake of the orally absorbed cephalosporin, cephalexin was examined. A proton-dependent dipeptide transport carrier mediated drug uptake into Caco-2 cells.

Materials and Methods

Materials. New England Nuclear was the supplier for radioactive [^{3}H]inulin. D-[9-^{14}C]Cephalexin (12.25 Ci/mol) was prepared as previously described [28]. Cephalexin and cefaclor were obtained from Eli Lilly and Co., Indianapolis, IN. Bumetanide was a gift of Leo Pharmaceuticals, Sweden. A collagen dispersion was kindly supplied by Ethicon, Inc., Somerville, NJ. Growth media and EBSS was purchased from Gibco, Grand Island, NY. The other reagents were purchased from Sigma Chemical Company, St. Louis, MO.

Cell culture. The human adenocarcinoma cell line Caco-2 was obtained from Dr. J. Fogh at the Research Unit of Memorial Sloan-Kettering Cancer Center in Rye, NY [29]. The cells were passaged in Dulbecco's Modified Eagle medium containing 10% fetal calf serum and 1% Minimal Essential Media non-essential amino acid solution without addition of sodium pyruvate or antibiotics [4]. For flux measurements, $(0.5–1.0) \cdot 10^5$ cells were grown in collagen-coated [30] multiwell dishes (24 well) for 13–15 days and the medium was replaced every two to three days. The cells were mycoplasma-free and were used between passage numbers 28 and 40.

Transport measurements. Drug uptake was measured at 37 °C using D-[9-^{14}C]cephalexin and a cluster-tray technique [31]. The protocol for the flux measurement has been reported previously [32]. The flux buffer was bicarbonate-free EBSS containing 25 mM Mes titrated to pH 6.0 with KOH, and choline chloride replaced the sodium chloride. The osmolality of the flux buffer was adjusted to 300 ± 5 mosmol/kg with choline chloride [31,32]. To assess the effect of extracellular pH on uptake, the flux buffers contained either 25 mM citric acid (pH 5.0 and 5.2), 25 mM Mes (pH 5.5–6.5), or 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 6.8 and 7.0) and were titrated to the proper pH with KOH. [^{3}H]Inulin was used as a marker for the extracellular fluid that adhered to the cells during the washing procedure; this value was used to estimate the zero time for the determination of the rate of uptake. Fresh solutions of the cephalosporins, dipeptides, SITS, and DIDS were prepared for each experiment. Protein was measured by the method of Lowry et al. [33].

Calculations. Initial uptake rates were calculated by linear regression from points determined at 0, 1, 2, 3, and 4 min. Initial rates of 1 mM cephalexin uptake ranged from 0.9 to 2.2 nmol/min per mg protein depending on the lot of serum and collagen. Percent inhibition was calculated based on the control uptake rate measured in each experiment. Kinetic parameters were determined by a computer fit of the data using the Marquardt algorithm, a general nonlinear curve-fitting procedure (IBM Share No. 3094; Ref. 34).

Intracellular concentration of cephalexin. To determine whether cephalexin was metabolized, Caco-2 cells were incubated with D-[9-^{14}C]cephalexin in flux buffer for 180 min; subsequently, the cells were washed and lysed. The constituents of a 50 μl sample of the lysate were separated by HPLC using an isocratic solvent system described previously [28] and the eluant was monitored at 262 nm. Both the cephalexin standard and the D-[9-^{14}C]cephalexin in the lysates eluted as a single peak at 4.8 min. The eluant was collected with an Isco fraction collector and aliquots were counted to determine radioactivity. The cellular volume of Caco-2 cells has been reported to be 3.49 and 3.66 μl/mg protein [35,11]. The value of 3.66 μl/mg protein was used to calculate the distribution ratio, DR, which equals the intracellular drug concentration/extracellular drug concentration.

Results

Time course for cephalexin uptake

As a first step in the characterization of drug uptake, the accumulation of 1 mM cephalexin by Caco-2 cells was examined at pH 6.0 which is within the estimated pH range of 5.5 to 6.3 present in the intestine [36,37]. Caco-2 cells were incubated with 1 mM cephalexin over a 180 min time course in the presence or absence of sodium in the flux buffer. As shown in Fig. 2, the cells took up the drug linearly for the first five min (inset). Within 180 min, a steady state was achieved with a DR of 3.5 ± 0.1 ($n = 4$). By contrast, when the cells were incubated at 4°C (bottom curve, Fig. 2) the drug reached a plateau within 30 min with a DR of 0.9 ± 0.1 ($n = 4$), indicating that binding was not responsible for the concentrative uptake observed at 37°C. Moreover, cephalexin was not metabolized by 180 min. When cell lysates were analyzed by HPLC, 99.9% of the D-[9-^{14}C]cephalexin eluted as a single peak that corresponded to the cephalexin standard. Therefore, the rate of drug uptake into Caco-2 cells is a true measure of transport and is not affected by metabolism or binding. These data indicate that cephalexin uptake is mediated by a sodium-independent mechanism and that the drug is concentrated intracellularly. In the studies described below, initial uptake rates were determined over a four min time course using sodium-free flux buffer.

pH dependence of uptake

The effect of varying the extracellular pH from 5.0 to 7.0 on the rate of 1 mM cephalexin uptake was measured. As shown in Fig. 3, drug uptake was influenced by the pH of the flux buffer and was maximal at pH 6.0. To examine the role of an inwardly directed proton gradient across the plasma membrane on uptake, the proton gradient was dissipated prior to measuring drug uptake. The cells were pH-clamped by incubation in flux buffer containing 135 mM potassium and the ionophore, nigericin, which exchanges protons with potassium ions across the membrane. As shown in Fig. 3 (bottom curve), cephalexin uptake was substantially reduced over the pH range.

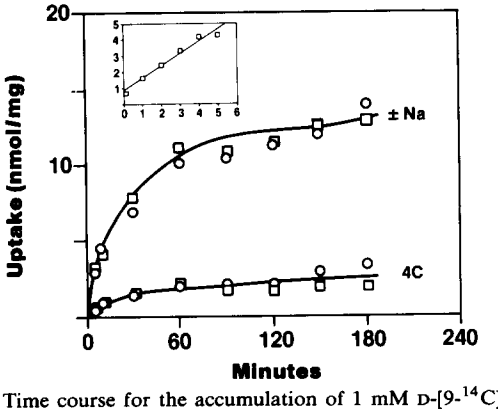

Fig. 2. Time course for the accumulation of 1 mM D-[9-^{14}C]cephalexin. Cells were incubated at 37°C or 4°C in flux buffer (pH 6.0) containing either sodium chloride (○) or choline chloride (□). Inset shows drug uptake measured in sodium-free flux buffer over a five min time course, where the value for the zero time was calculated from the adhered extracellular fluid as indicated in Materials and Methods. The curves shown are representative of four independent experiments.

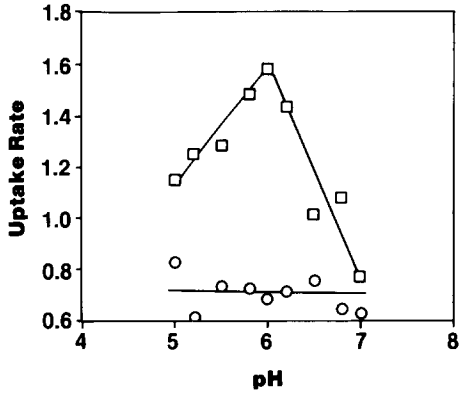

Fig. 3. Effect of extracellular pH on the rate of 1 mM D-[9-^{14}C]cephalexin uptake. Cells were incubated 5–10 min in flux buffer solutions of different pH values and washed once before measuring the initial uptake rate in the same flux buffer as indicated in Materials and Methods. The flux buffer contained 4 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 5.5 mM glucose, 0.1 μg/ml phenol red and either 135 mM choline chloride (□) or 8 μM nigericin, 135 mM KCl (○). The uptake rate is expressed as nmol/min per mg protein. The curves shown are representative of two to four independent experiments.

To further evaluate the role of the pH gradient on drug uptake, cells were incubated at 37°C in flux buffer at pH 7.3 (close to the intracellular pH of 7.27, M.H. Montrose, personal communication) and the accumulation of 1 mM cephalexin was measured over a 180 min time period. A steady-state drug level was achieved at pH 7.3 within 60 min with a DR of 1.0 ± 0.1 ($n = 4$; data not shown). Therefore, the ability of the cells to concentrate cephalexin intracellularly when incubated at pH 6.0 (Fig. 2) is dependent on the presence of an inwardly directed pH gradient.

Energy poisons

If cephalexin uptake is proton-dependent then drug uptake would be energy-dependent. Consequently, the effect of metabolic inhibitors was examined on the initial rate of drug uptake measured at pH 6.0 in the absence of sodium. As shown in Table I, incubation of the cells for 15 min with sodium azide, 2,4-dinitrophenol, or oligomycin reduced uptake by 30–64% and the addition of the protonophores, nigericin and FCCP, reduced drug uptake by 60%. When measured in

TABLE I

Effect of metabolic inhibitors and protonophores on the rate of cephalexin uptake

Cells were incubated 15 min with the indicated compound and washed prior to measuring the rate of 1 mM D-[9-^{14}C]cephalexin uptake in sodium-free buffer. The uptake rate for the untreated cells was 2.19 nmol/min per mg protein. Each value is the mean ± S.D. of four determinations measured in two independent experiments.

Compound	% Inhibition
Oligomycin (25 μg/ml)	30 ± 13
2,4-Dinitrophenol (0.5 mM)	57 ± 8 [a]
Sodium azide (10 mM)	64 ± 6 [a]
Nigericin (10 μg/ml)	59 ± 4 [a]
FCCP (10 μg/ml)	62 ± 5 [a]

[a] Data were analyzed by Student's *t*-test ($P < 0.005$).

sodium-containing buffer, the addition of 1mM ouabain (an inhibitor of the Na^+/K^+-ATPase responsible for maintaining the sodium gradient) or 1 mM amiloride (an inhibitor of the Na^+/H^+ antiporter) had little effect on uptake (by 19% and 5%, respectively), which is consistent with drug uptake being sodium-independent (Fig. 2). These data indicate that transport is energy- and proton-dependent.

Concentration dependence of cephalexin uptake

To examine the kinetics of cephalexin transport by Caco-2 cells, initial rates of drug uptake were measured at pH 6.0 over the concentration range of 0.5 to 35 mM

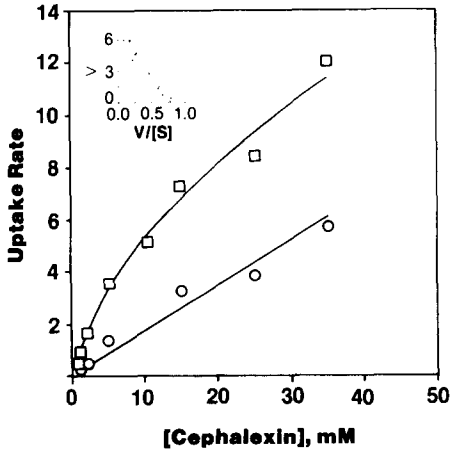

Fig. 4. Concentration dependence of the rate of D-[9-^{14}C]cephalexin uptake. The uptake rate is expressed as nmol/min per mg protein. Each point is the mean of quadriplicate points determined from two independent experiments. The upper curve representing the total uptake rate measured at 37 °C, and the bottom curve representing non-saturable uptake measured at 4 °C, are based on a least squares computer fit of the data to Eqn. 1 (see text). The parameter estimates are given in Results. Inset shows an Eadie-Hofstee plot of the uptake rates after correction for the non-saturable component. The line was drawn by the method of least squares (correlation coefficient = 0.94) and the estimated V_{max} was 6.7 nmol/min per mg protein and K_m was 8.5 mM.

in sodium-free buffer at 4 °C to estimate non-saturable uptake, and at 37 °C. Drug uptake was linear over the concentration range when the cells were incubated at 4 °C (Fig. 4). By contrast, when the cells were incubated at 37 °C the uptake rate was enhanced and tended to saturate as the cephalexin concentration increased. An Eadie-Hofstee plot of the uptake rates after correction for the non-saturable uptake gave no indication of multiple transport systems (inset Fig. 4). Consequently, the data were fitted by computer to a single Michaelis-Menten term plus a non-saturable term:

$$v = \frac{V_{max}[S]}{K_m + [S]} + K_d[S] \quad (1)$$

The kinetic parameters for the cephalexin transport carrier were: V_{max} of 6.5 ± 0.9 nmol/min per mg protein, K_m of 7.5 ± 2.8 mM, and K_d of 0.18 ± 0.01 nmol/min per mg protein per mM. Thus, a sodium-independent transport carrier with a low affinity for cephalexin is responsible for uptake into Caco-2 cells in this concentration range.

Effect of dipeptides, amino acids and other agents on drug uptake

Cephalexin uptake is mediated by a dipeptide carrier located in the intestine and the kidney [13–27]. In the

TABLE II

Effect of dipeptides, amino acids, organic anions, and cephalosporins on the uptake rate of 1 mM cephalexin

The uptake rate of 1 mM D-[9-^{14}C]cephalexin in the absence of inhibitors was 1.68 nmol/min per mg protein in sodium-free flux buffer. Values represent the means ± S.D. of triplicate samples.

Compound	Concn. (mM)	% Inhibition
Dipeptides		
L-Phe-Gly	10	84 ± 2 [a]
Gly-L-Pro	10	84 ± 4 [a]
L-Carnosine	10	82 ± 6 [a]
L-Pro-Gly	10	47 ± 2 [a]
Amino acids		
Glycine	10	5 ± 5
L-Proline	10	2 ± 12
L-Phenylalanine	10	4 ± 13
Cephalosporins		
Cefaclor	30	76 ± 2 [a]
Cephalexin	35	69 ± 1 [a]
Organic anions		
p-Aminohippuric acid	1	6 ± 3
Furosemide	1	4 ± 9
Bumetanide	1	19 ± 8
Biotin	1	12 ± 2
Inhibitors		
SITS	1	−14 ± 6
DIDS	1	−6 ± 5

[a] Data were analyzed by Student's *t*-test ($P < 0.001$).

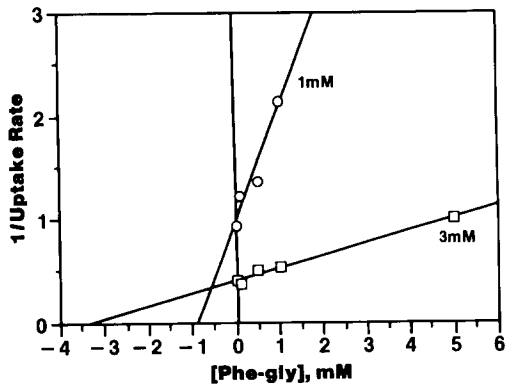

Fig. 5. Dixon-Webb plot of the uptake of D-[9-^{14}C]cephalexin in the presence of L-phenylalanylglycine. The rate of cephalexin uptake was measured at 1 and 3 mM in sodium-free flux buffer in the presence of increasing concentrations of the dipeptide. Lines were drawn by the method of least squares. The intersection of the lines were used to calculate $-K_i$. The units of the ordinate are nmol^{-1}·min·mg protein. The plot is representative of two experiments.

kidney, cephalosporins may also be taken up by an organic anion transporter [38] and by the H^+/organic cation antiporter [39]. To examine the properties of the transporter responsible for uptake into Caco-2 cells, the effect of addition of dipeptides, amino acids, and several organic anions on the uptake rate of 1 mM cephalexin at pH 6.0 was examined (Table II). The presence of 10 mM glycine, proline, phenylalanine, 1 mM of several organic anions or two compounds that inhibit the renal organic anion transporter, SITS and DIDS [38], had little to no effect on uptake. By contrast, drug uptake was reduced by 48 to 86% in the presence of 10 mM dipeptides, 35 mM cephalexin, or 30 mM cefaclor, a close structural analog (Fig. 1).

Substrate specificity of the carrier

The affinity of the transport carrier for the dipeptides and cephalexin was determined. The uptake rates of 1 mM and 3 mM cephalexin in the presence of increasing concentrations of the test compound were used to calculate the K_i from a Dixon-Webb plot. As illustrated in Fig. 5 for the dipeptide L-Phe-Gly, the substrates were competitive inhibitors. The K_i values are summarized in Table III. The transport carrier exhibited an 11-fold preference for the L- over the D-isomer of cephalexin, and had a higher affinity for the dipeptides, Gly-L-Pro and L-Phe-Gly than for L-carnosine or L-Pro-Gly. Taken together, these data indicate that cephalexin uptake is mediated by a dipeptide transport carrier and not by an amino acid transport carrier or an organic anion transporter.

TABLE III

Affinity of the transport carrier for selected dipeptides and cephalexin

Uptake rates of 1 mM and 3 mM D-[9-^{14}C]cephalexin were measured in the presence of increasing concentrations of the compound in sodium-free flux buffer. The K_i was calculated from a Dixon-Webb plot as described in the legend of Fig. 5.

Compound	K_i (mM)
Gly-L-Pro	0.7
L-Phe-Gly	0.6
L-Carnosine	4.6
L-Pro-Gly	20.6
D-Cephalexin	9.2
L-Cephalexin	0.8

Discussion

The present study demonstrates that the human intestinal epithelial cell line Caco-2 possesses a transport carrier that mediates the uptake of the orally absorbed antibiotic, cephalexin. This transport carrier shares several characteristics with a dipeptide transport carrier present in the intestine: (a) The transporter is sodium-independent [12–27]. (b) Uptake at pH 6.0 is stimulated by 2.8-fold by an inward-directed proton gradient [16,17,20,22,24,25,40,41]. (Although a study of peptide transport in brush border vesicles indicated that an inward-directed proton gradient did not stimulate uptake [18]). (c) Uptake of 1 mM cephalexin is energy-dependent and the drug is concentrated 3.5-fold intracellularly [41,42]. (d) The affinity of the carrier for cephalexin (K_m of 7.5 ± 2.8 mM) is within the range reported for the rat transport carrier (K_m of 2.2 to 19 mM; Refs. 26, 27, 42). (e) The carrier shows stereospecificity for the L-isomer over the D-isomer of cephalexin (K_i of 0.8 mM versus 9.2 mM) consistent with studies conducted with the rat indicating that the L-isomer of cephalexin is preferred [43]. (f) Cephalexin uptake into Caco-2 cells is competitively inhibited by dipeptides. The transporter has an affinity for Gly-L-Pro (K_i of 0.7 mM) similar to the dipeptide transporter of the rabbit (K_m of 0.9 mM; Refs. 12, 44) and the human (4.1 mM; Ref. 14). Therefore, the cephalexin transporter of Caco-2 cells closely resembles the proton-dependent dipeptide transport carrier of the intestine. The presence of the dipeptide transporter provides additional support that cultured Caco-2 cells exhibit enterocyte-like properties of the small intestine. In addition the cells provide an in vitro model for examining the regulation of the dipeptide transport carrier in response to the presence of nutrients and hormones in the growth medium and as a function of the differentiation of the cells.

Multiple transporters for the uptake of dipeptides and oral cephalosporins has been suggested [13,45–47]. The report that several polypeptides in intestinal brush border vesicles are labeled with photoaffinity probes of

cephalexin and Gly-L-Pro may indicate that more than one carrier is present [48]. Although the present investigation shows that Caco-2 cells take up cephalexin by a single saturable transport system operating in the concentration range of 0.5 to 35 mM, we cannot rule out the possibility that these cells may have more than one transport carrier for cephalexin and dipeptides. For example, the non-saturable portion of cephalexin uptake may be due in part to the contribution of a very low affinity carrier, besides the diffusion component. Furthermore, the presence of a dipeptide transport carrier with a low capacity and high affinity in the nanomolar range, such as has been described in renal tissue [46], would not have been detected. Whether multiple dipeptide transporters are present and whether they are differentially expressed by Caco-2 cells as a function of their growth and differentiation needs to be determined. It should be noted that in an earlier study from our laboratory conducted with another human intestinal enterocyte-like cell line, HT-29-A1, cephalexin uptake was mediated by an energy-independent transporter [28] and uptake was not concentrative (unpublished data). The finding that Caco-2 and HT-29-A1 take up cephalexin by similar transport carriers that differ in their energy requirements indicates that more than one transport system may be observed in cultured intestinal epithelial cells.

The Na^+/H^+-antiporter is thought to play a major role in maintaining the proton gradient across the luminal membrane of intestinal epithelial cells [49,50]. The antiporter has been proposed to indirectly energize the uptake of dipeptides [and cephalosporins] by the proton-dependent dipeptide transporter in the intestine and to drive their uphill transport [51]. Caco-2 cells possess a Na^+/H^+-antiporter [52]; however, drug uptake into Caco-2 cells is not stimulated by the presence of sodium in the flux buffer when the cells are grown for 14 days (Fig. 2). The finding that Caco-2 cells concentrate cephalexin intracellularly 3.5-fold in sodium-free buffer is unexpected. Sodium-independent mechanisms must be responsible for maintaining the pH gradient across the plasma membrane of Caco-2 cells, and for indirectly energizing uptake via the dipeptide carrier. Further studies are needed to examine these mechanisms.

Established cell lines, such as Caco-2 and HT-29, with properties of a human intestinal enterocyte offer an alternate model to study absorption processes in the intestine. One clear advantage is that the cells stay viable throughout the transport studies. Also, a monolayer of cells may be grown on a porous support to represent an intact epithelium. Vectorial transport through the monolayer may be studied to dissect paracellular pathways and transport mechanisms present on the apical and basolateral membranes [53,54]. These cell lines will provide a unique in vitro intestinal model with the advantages inherent to cell culture.

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