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Uptake and transepithelial transport of the orally absorbed cephalosporin cephalexin, in the human intestinal cell line, Caco-2

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Abstract

Cephalexin (CPX) uptake in cultures of the human colon adenocarcinoma cell lines, Caco-2 and HT-29 has been shown to involve the di-/tripeptide transporter (DPT). However, little is known about the mechanism mediating the transepithelial (TE) transport of CPX either in vivo or in cultured cells. In this study, uptake and TE transport of CPX were investigated in Caco-2 monolayers grown on microporous membranes. Caco-2 cells did not show net TE transport of CPX when the pH of both apical (AP) and basolateral (BL) bathing solutions was 7.4. When the pH of the AP bathing solution was decreased from 7.4 to 6.0, while maintaining the pH of the BL bathing solution at 7.4, AP-BL transport of CPX (0.1 mM) increased from 0.1 to 0.23% h^{-1} cm^{-2}. Reversal of the pH gradient across the monolayer (AP, pH 7.4; BL, pH 6.0) did not alter the BL-AP flux of CPX. Manipulation of the AP or BL pH between 5.5 and 7.4 affected neither the AP-BL nor the BL-AP flux of mannitol (both $\sim 0.1\%$ h^{-1} cm^{-2}), an internal marker of passive, paracellular diffusion. The pH-dependent AP uptake and AP-BL flux of CPX were time-, concentration- and temperature-dependent. Apparent half-maximal transport concentration (K_t) and maximal transport velocity (V_t) were 2.9 mM and 1.0 nmol min^{-1} mg $protein^{-1}$ for AP uptake, and 4.7 mM and 0.13 nmol min^{-1} cm^{-2} (0.30 nmol min^{-1} mg $protein^{-1}$) for AP-BL transport. The carrier-mediated AP uptake and AP-BL transport of CPX were inhibited by Gly-Pro, Pro-Gly, cephradine, cefadroxil, benzylpenicillin and ampicillin, but not by proline, glycine, valine, lysine or aspartic acid. In addition, CPX uptake was not inhibited by the nucleoside, adenosine, or the bile acid, taurocholic acid, suggesting that the uptake and TE transport of CPX involves the DPT but not other carriers present in the intestinal mucosa. We confirmed that the AP uptake of CPX involves mainly the DPT and conclude the following: (a) AP-BL transport of CPX is predominantly transcellular and involves a

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Abbreviations: HBSS, Hank's balanced salts solution; DR, distribution ratio; Gly-Pro, glycyl-L-proline; Pro-Gly, L-prolylglycine; ACE, angiotensin-converting enzyme; BSA, bovine serum albumin; TEER, transepithelial electrical resistance.

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carrier, probably the DPT; (b) the rate-limiting step in AP-BL transport of cephalexin appears to be BL efflux and not AP uptake; (c) interaction with the DPT alone may be a poor predictor of substrate transport via this carrier; and (c) the Caco-2 culture system is a good model for studying mucosal uptake and TE transport of small peptides and peptidomimetic drugs.

Key words: Peptide transporter; Caco-2; Cephalexin; Cephalosporin; Peptide

1. Introduction

The intestinal absorption of natural di- and tripeptides has been demonstrated to be a pH-dependent process, mediated by a di-/tripeptide carrier located on the apical membrane of small intestinal absorptive cells (Ganapathy and Leibach, 1985). The pH gradient required for the absorption of these molecules is generated by the combined action of a brush border membrane Na^+/H^+ exchanger and a basolateral membrane Na^+/K^+-ATPase (Ganapathy and Leibach, 1985). The di-/tripeptide carrier is also believed to mediate the transport of a number of structurally related molecules including β-lactam and cephalosporin antibiotics (Nakashima et al., 1984; Kramer et al., 1990), angiotensin-converting enzyme (ACE) inhibitors (Hu and Amidon, 1988), thyrotropin-releasing hormone (TRH) analogues (Yokohama et al., 1984) and renin inhibitors (Kleemann et al., 1992).

Much of the current information on peptide transporters has been obtained from studies with intestinal brush border membrane vesicles (BBMV) (Ganapathy and Leibach, 1985). This technique has been useful in elucidating some of the requirements for peptide uptake across the apical enterocyte plasma membrane. However, studies with BBMV provide no information on the mechanism involved in peptide efflux across the basolateral membrane of enterocytes. Thus, correlations between uptake into BBMV and luminal disappearance must assume that apical uptake is the rate-limiting step in transepithelial transport and do not take into consideration the mechanisms involved in basolateral efflux. For β-lactam antibiotics, there is evidence that luminal disappearance may greatly overestimate transepithelial transport (Sugawara et al., 1990). This limitation was addressed in a recent study utilizing inside-out basolateral membrane vesicles from rabbit intestine (Dyer et al., 1990). Uptake in this system (mimicking basolateral efflux) showed that apical uptake of [^{3}H] Gly-Pro was pH-dependent and carrier-mediated, suggesting the involvement of a peptide transporter functionally similar to that found on the apical membrane of enterocytes (Ganapathy and Leibach, 1985; Dyer et al., 1990). The role of a pH gradient on peptide efflux across the basolateral membrane in vivo is not known.

Additional efforts have focused on the development of cultured epithelial cell lines as in vitro models for drug and nutrient absorption (Audus et al., 1990; Wilson, 1990a). In particular, the human colon adenocarcinoma cell line Caco-2, has been characterized as a model transport system of polarized intestinal epithelium (Hidalgo et al., 1989; Wilson et al., 1990b), and shown to possess the di-/tripeptide carrier (Dantzig and Bergin, 1990). Two recent studies evaluated the apical uptake of cephalexin in Caco-2 and HT-29 cells (Dantzig and Bergin, 1988; 1990) and the transepithelial transport of cephradine in Caco-2 cells (Inui et al., 1992). Those reports demonstrated the presence of the peptide transporter in Caco-2 cells and suggested the utility of these cell systems to study peptide transport.

One important goal of in vitro transport model systems is to predict in vivo transport. Because peptide absorption involves apical uptake and basolateral efflux, greater understanding of transepithelial transport (in addition to apical uptake) in Caco-2 cells would further assess the utility of this system in predicting in vivo transport of peptide drugs. In this study, Caco-2 cells, grown on porous polycarbonate membranes, were utilized to probe requirements for uptake and transepithelial transport of the orally absorbed cephalosporin, cephalexin.

Apical-to-basolateral transport was evaluated to determine the contribution of the carrier-mediated process to the overall transepithelial transport of cephalexin in Caco-2 cell monolayers. The effects of age in culture, pH, temperature and concentration on cephalexin transport were investigated. The rate-limiting step in transepithelial transport of cephalexin was elucidated by comparing uptake parameters with transcellular transport parameters. Finally, the specificity of the transporter for cephalexin was examined by determining the effect of a number of compounds on the uptake and/or transepithelial transport of compound.

2. Materials and methods

2.1. Materials

D-[^{3}H]Cephalexin (4.1 Ci/mmol) was synthesized by the Department of Synthetic Chemistry, SmithKline Beecham (King of Prussia, PA). [^{14}C]Mannitol (49.3 mCi/mmol) was purchased from New England Nuclear Products (Boston, MA). Dulbecco's Modified Eagle's Medium (DMEM), MEM non-essential amino acids (NEAA) solution (100 ×), trypsin (0.25%)-EDTA (1 mM), penicillin (10000 U/ml) and streptomycin (10000 μg/ml) solution were purchased from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hazleton Laboratories (Lenexa, KS). Rat tail collagen (type I) was obtained from Collaborative Research (Bedford, MA). D-Cephalexin, Gly-Pro, Pro-Gly, proline, glycine, valine, aspartic acid, lysine, taurocholic acid, adenosine, cephradine, cefadroxil, ampicillin and penicillin G (benzylpenicillin) were purchased from Sigma Chemical Co. (St. Louis, MO). All buffers were also from Sigma.

2.2. Caco-2 cell cultures

Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD) and utilized between passages 80 and 102. Cells were grown in DMEM supplemented with 10% FBS, 1% glutamine, 1% NEAA, 100 U/ml of penicillin and 100 μg/ml streptomycin at 37°C in disposable T-75 culture flasks (Costar, Cambridge, MA) in a humidified air-5% CO_2 atmosphere. Cells were harvested with 0.25% (w/v) trypsin-1 mM EDTA and seeded onto collagen-coated 0.4 μm pore-Transwell filters (24 or 6.5 mm diameter, Costar) at a density of 6.3×10^4 cell/cm^2 as described previously (Hidalgo et al., 1989). Culture medium was replaced every 48 h for the first 6 days of culture and every 24 h thereafter as per manufacturer's instructions. Apical and basolateral reservoir volumes were maintained at 1.5 and 2.5 ml, respectively. Transepithelial electrical resistance (TEER) was measured using an EVOM voltohmmeter (World Precision Instruments, New Haven, CT). For some experiments, cells were seeded at the same initial density onto 0.4 μm pore-Snapwell filters (Costar). These cells were maintained as detailed above except that medium was replaced every 48 h for the duration of the culture with apical and basolateral chamber volumes maintained at 0.4 and 3.5 ml, repectively. Except where indicated, cells used routinely for transport experiments were cultured for 15–25 days and had TEER values of approx. 400–500 Ω cm^2.

2.3. Cephalexin transport studies

Transport experiments were carried out in Hank's balanced salts solution (HBSS) supplemented with 0.05% bovine serum albumin. Buffer pH was adjusted using either Mes, pH 5–6 or 10 mM Hepes, pH 6.5–7.4. The pH in the basolateral compartment was maintained at 7.4 while the apical pH was varied as specified by each experiment. 8 mM glucose plus 2 mM mannitol were routinely added to the basolateral reservoir and 10 mM mannitol added to the apical reservoir to balance osmolarity between the apical and basolateral sides of the monolayers.

2.3.1. Transwell experiments

Apical-to-basolateral and basolateral-to-apical flux experiments were conducted in quadrupli-

cate at 37°C with 100 μM cephalexin added to both reservoirs. [^{3}H]Cephalexin (0.5 μCi) was added to the donor side at the beginning of the flux period. [^{14}C]Mannitol (0.5 μCi) was routinely included as a marker of epithelial integrity. At each time point throughout the experiment, the content of the receiver side was removed and replaced with the same volume of fresh buffer. 10 μl aliquots were collected from the donor side at the beginning and end of each experiment for calculations of cumulative percent transport. All samples were mixed with 10 ml scintillation fluid (Ready-Safe, Beckman Instruments, Inc., Fullerton, CA) and radioactivity determined with a Beckman LS 9800 scintillation counter using the external channels ratio to correct for quenching. For these experiments, transport was expressed as an average rate (nmol h^{-1} cm^{-2} or % h^{-1} cm^{-2}) from quadruplicate determinations.

2.3.2. Diffusion chambers

Some transport experiments were conducted in side-by-side diffusion cells (Precision Instrument Design, Los Altos, CA) as described previously (Hidalgo et al., 1991). Stirring was achieved by gas lift (5% CO_2-95% O_2) and a constant temperature of 37°C was maintained by a water-heated jacket. Except where indicated, each compartment contained 5 ml HBSS/bovine serum albumin (BSA) with 10 mM mannitol added to the apical reservoir (pH 6) and 8 mM glucose plus 2 mM mannitol in the basolateral reservoir (pH 7.4) to adjust osmolarity. Cells grown on Snapwell culture filters were preincubated in the diffusion chambers for 15 min prior to each experiment. Transport experiments were conducted with 100 μM cephalexin added to both reservoirs. 1 μCi [^{3}H]cephalexin and 1 μCi [^{14}C]mannitol were added to the apical (donor) side at the beginning of the flux period. At the indicated times, 1 ml aliquots were withdrawn from the basolateral compartment and the volumes replaced with buffer. 100 μl aliquots were sampled from the donor side at the beginning and end of each flux study. All samples were mixed with 10 ml scintillation fluid and radioactivity determined as detailed above.

The concentration dependence of apical-basolateral transport of cephalexin was studied in diffusion chambers. Snapwell cultures were incubated with 100 μM cephalexin and 0.5 μCi each of [^{3}H]cephalexin and [^{14}C]mannitol in the absence or presence of 0.1, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0 or 25.0 mM unlabelled drug added to both apical and basolateral reservoirs. Samples were withdrawn from the basolateral chamber at 15, 30, 45, 60 and 90 min and radioactivity and transport rates determined as detailed in section 2.3.1.

2.4. Cephalexin uptake studies

2.4.1. Time course of cephalexin uptake

Cells cultured for 20 days were incubated in Transwell filters (6.5 mm diameter) with 100 μM cephalexin and 0.5 μCi [^{3}H]cephalexin in HBSS/BSA, pH 6 in the apical chamber. Basolateral pH was maintained at pH 7.4. Media in the respective reservoirs were supplemented with mannitol or glucose as detailed in Section 2.3.1. Incubations were carried out in quadruplicate for 2, 5, 10, 15, 30, 45, 60, 90 and 120 min at 37°C and 15, 30, 45, 60, 90 and 120 min at 4°C. At the end of each incubation, cells and polycarbonate membranes were cut out and washed extensively in ice-cold HBSS/BSA, pH 7.4. Cells and filters were solubilized in 10 ml scintillation fluid and radioactivity determined by liquid scintillation spectrometry. Cephalexin uptake rate is expressed as nmol/mg total protein. Total protein content of cells cultured on polycarbonate filters for 18 days was previously determined to be 0.425 mg protein/cm^2 (Hidalgo and Borchardt, 1990). The distribution ratio (DR), which equals the intracellular-to-extracellular drug concentration at equilibrium, is calculated based on a previously determined cellular volume for Caco-2 cells, 3.66 μl/mg protein (Burnham and Fondacaro, 1989).

2.4.2. Concentration dependence of cephalexin uptake

The uptake of 100 μM [^{3}H]cephalexin (0.5 μCi/well) was studied in 6.5 mm Transwells with equimolar concentrations of 0.1–25.0 mM drug added to each reservoir as detailed for transport experiments. Following a 15 min incubation, the

polycarbonate membranes were cut out from the plastic inserts and washed in cold HBSS/BSA, pH 7.4. Cells were solubilized in 10 ml scintillation fluid and radioactivity determined by liquid scintillation spectrometry. Cellular accumulation of cephalexin was expressed as a mean value (nmol min^{-1} mg $protein^{-1}$) from quadruplicate determinations.

2.4.3. Competition for cephalexin accumulation and transport by di-peptides, amino acids and β-lactam antibiotics

The effect of competing substrates on the uptake of 100 μM [^{3}H]cephalexin (0.5 μCi/well) was studied in 6.5-mm Transwells. Cells were incubated in the presence or absence of 25 mM concentrations of naturally occurring dipeptides, amino acids and β-lactam antibiotics as detailed in the Figures and legends. Incubations were carried out in quadruplicate for 15 min at 37°C. Cell monolayers and filters were washed and processed as described in section 2.4.2. Cellular uptake of cephalexin is expressed as nmol min^{-1} mg $protein^{-1}$. Similar experiments were carried out in diffusion chambers (see procedures detailed under Section 2.3.2) to determine the effect of 25.0 mM concentrations of these competitors on transcellular transport of cephalexin. Transport was expressed as an average rate (nmol min^{-1} cm^{-2}) from quadruplicate determinations.

2.5. Data analysis

Uptake rates were determined from 15 min incubation and transepithelial transport rates from the linear (steady-state) portion of the transport vs concentration. The best fit lines

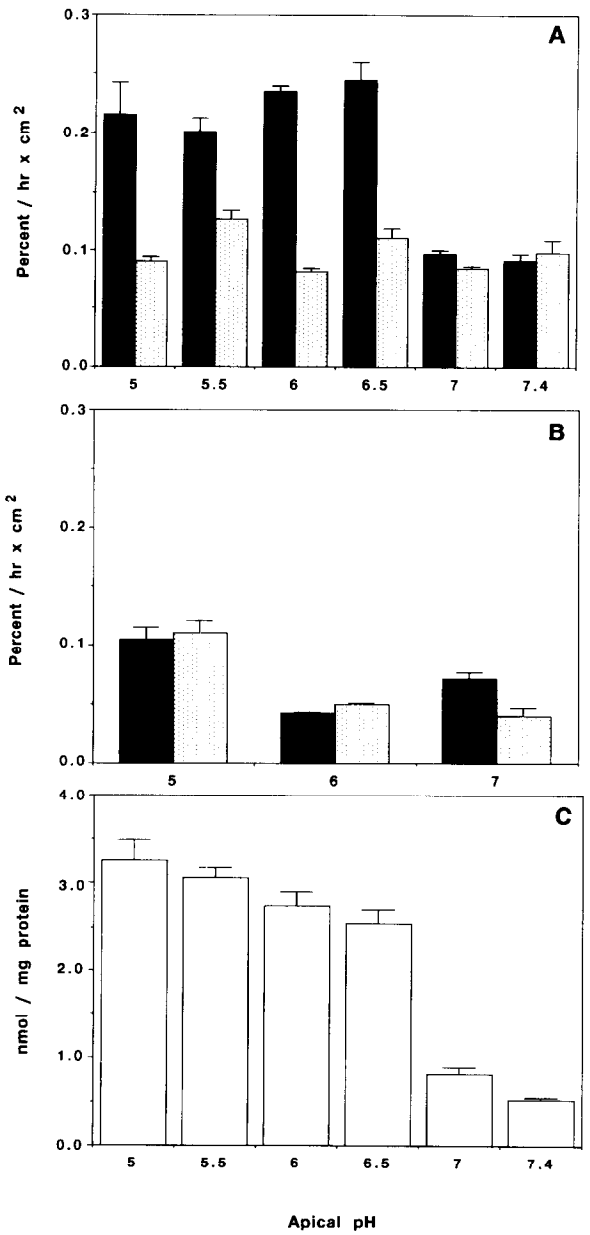

Fig. 1. pH dependence of cephalexin transport and cellular accumulation. (A) Apical-basolateral fluxes of 100 μM cephalexin (filled bars) and 10 mM mannitol (open bars) across Caco-2 monolayers with varying apical pH values during a 2 h time course. Basolateral pH was maintained at 7.4. Cephalexin and mannitol transport are expressed as % transport h^{-1} cm^{-2} (±SE) from quadruplicate determinations. (B) Basolateral-apical fluxes of 100 μM cephalexin (filled bars) and 10 mM mannitol (open bars) across Caco-2 monolayers measured at pH 5.0, 6.0 and 7.0 (apical reservoir) over a 2 h time course. Basolateral pH was maintained at pH 7.4. Cephalexin and mannitol transport are expressed as % transport h^{-1} cm^{-2} (±SE) from quadruplicate determinations. (C) At the end of the apical-basolateral flux experiments in panel A, cell-associated radioactivity was determined. Cephalexin accumulation as measured from cell-associated radioactivity is expressed as nmol/mg protein (±SE) from quadruplicate determinations.

through rate vs concentration points were obtained by using the nonlinear regression analysis program package, PCNONLIN (Metzler and Weiner, 1989). The equation fitted to the data is shown below:

$$V = \frac{V_t \cdot C}{K_t + C} + K_d \cdot C \quad (1)$$

where v is the uptake (nmol min^{-1} mg $protein^{-1}$) or transepithelial transport (nmol min^{-1} cm^{-2}) velocity, V_t, the maximal uptake (nmol min^{-1} mg $protein^{-1}$) or transepithelial transport (nmol min^{-1} cm^{-2}) velocity, K_t, half-maximal uptake or transport concentration (mM), C, cephalexin concentration (mM) and K_d the coefficient for non-mediated and passive uptake (nmol min^{-1} mg $protein^{-1}$ mM^{-1}) or transport (nmol min^{-1} cm^{-2} mM^{-1}) (Nakashima et al., 1984).

3. Results

3.1. Junctional integrity of cell monolayers

It was previously demonstrated that Caco-2 cells cultured on porous polycarbonate filters become confluent monolayers by day 7 (Hidalgo et al., 1989). The development of these tight junctions can be monitored by changes in transepithelial electrical resistance (TEER) and flux of a marker of passive, paracellular diffusion such as mannitol. TEER values and mannitol fluxes across Caco-2 monolayers between 9 and 27 days were investigated. TEER values increase 4-fold (137 ± 15 to 436 ± 23 Ω cm^2) with a corresponding 6-fold decrease in mannitol flux (5.47 ± 0.44 to $0.91 \pm 0.15\%$ h^{-1} cm^{-2}). Both indicators of junctional integrity remain stable between days 15 and 27.

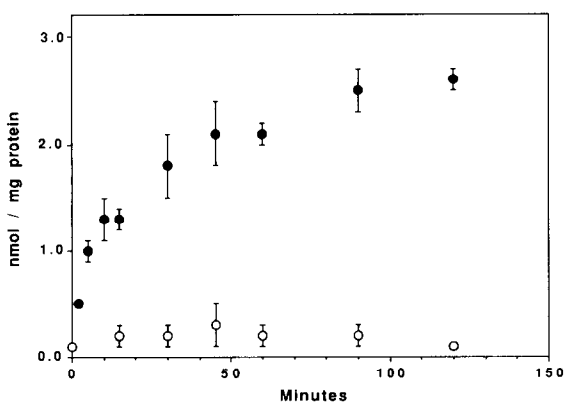

Fig. 2. Time course of cephalexin uptake by Caco-2 cells. The uptake of 100 μM cephalexin (apical pH 6.0) by Caco-2 cells cultured for 20 days was determined over a 2 h time course at 37°C (filled circles) and 4°C (open circles). At the indicated times, cephalexin uptake (cell-associated) radioactivity was determined. Results are mean nmol/mg protein ($\pm$SE) from quadruplicate determinations.

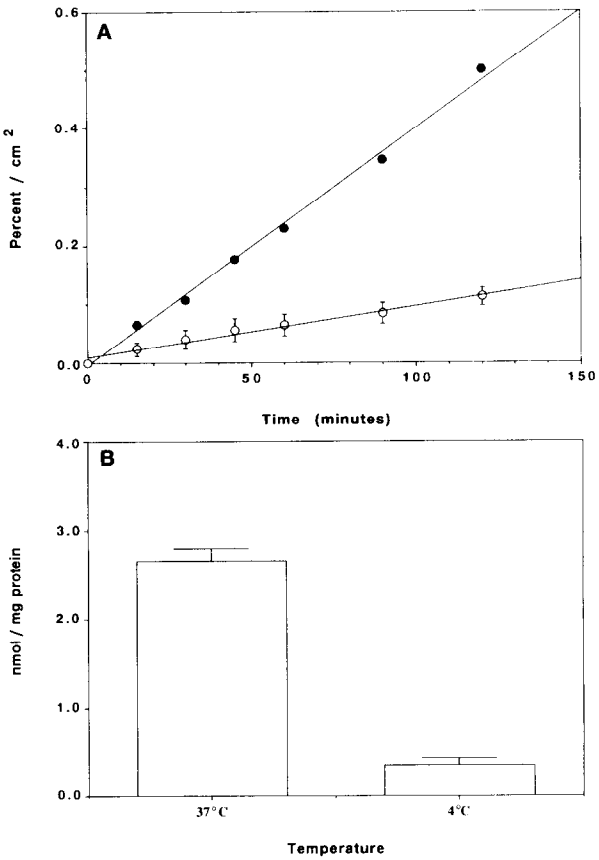

Fig. 3. Temperature dependence of transepithelial transport and accumulation of cephalexin by Caco-2 monolayers. (A) Transport of 100 μM cephalexin (apical pH 6.0) across cell monolayers at 37°C (closed circles) and 4°C (open circles). Transport is expressed as %/cm^2 ($\pm$SE) and values represent the average of quadruplicate determinations. (B) At the end of the flux experiments, in panel A, cephalexin accumulation was determined. Results are nmol/mg protein ($\pm$SE) from quadruplicate determinations.

3.2. pH dependence of cephalexin uptake and transport

The effect of varying apical pH from 5.0 to 7.4 on the transport of 100 μM cephalexin was examined in cells cultured for 20 days. The data in Fig. 1 demonstrate that cephalexin transport in the apical-basolateral direction is 2-fold greater at pH 5–6.5 (0.21–0.25% h^{-1} cm^{-2}) compared to pH 7–7.4 (0.09–0.11% h^{-1} cm^{-2}) (panel A). No significant differences were observed in mannitol flux, indicating that integrity of the monolayers was not altered by changes in apical pH. In contrast, basolateral-apical transport of cephalexin remained low at both acidic (pH 5–6) and neutral (pH 7) apical pH and was comparable to mannitol fluxes (Fig. 1B). A H^+ gradient directed in the basolateral-apical direction had no effect on the basolateral-apical cephalexin flux (data not shown). The steady-state cellular accumulation of cephalexin at 37°C was 3–4-fold greater in cells whose apical pH values were maintained between 5 and 6.5 (2.6–3.2 nmol/mg protein) compared to 7 or 7.4 (0.5–0.7 nmol/mg protein) (Fig. 1C).

3.3. Time course and temperature dependence of cephalexin uptake and transport

The time course of uptake for 100 μM cephalexin at 37 and 4°C was measured over 120 min at pH 6.0. The data in Fig. 2 indicate that cephalexin uptake is initially rapid and approaches a steady-state concentration of 2.02 ± 0.15 nmol/mg protein by 60 min. Maximal intracellular cephalexin accumulation at steady state (120 min) was 2.47 ± 0.05 nmol/mg protein with an intracellular/extracellular drug distribution ratio (DR) of 6.5 (not shown). At 4°C, cellular accumulation of cephalexin did not exceed 0.2 ± 0.05 nmol/mg protein with a calculated DR of 0.5 (not shown).

The data in Fig. 3 indicate that over a 2 h time course, cephalexin transport was 0.26% h^{-1} cm^{-2} at 37°C and 0.05% h^{-1} cm^{-2} at 4°C (panel A). Cell-associated cephalexin after the 2 h incuba-

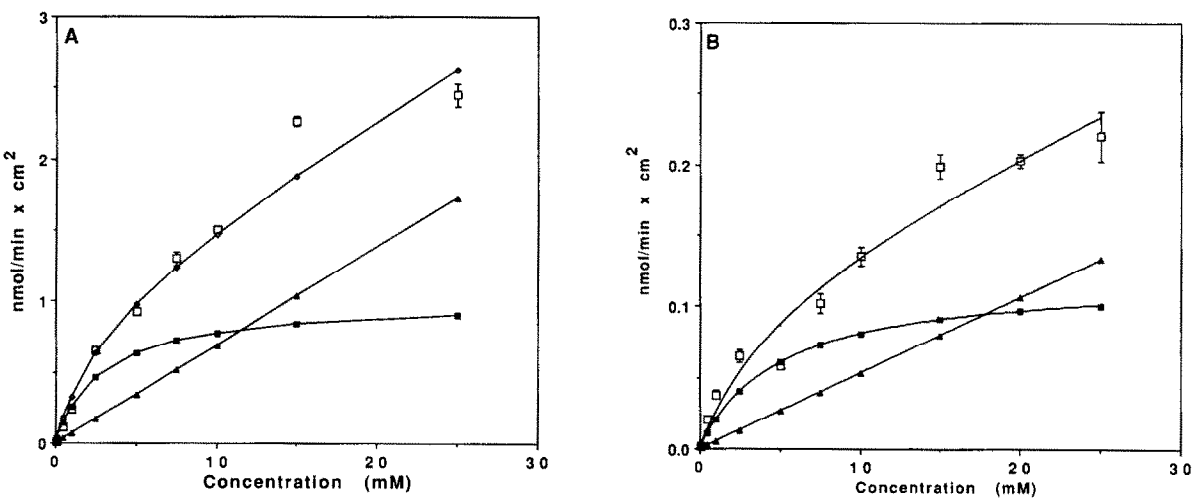

Fig. 4. Concentration dependence of cephalexin uptake and transport. (A) Cellular uptake of cephalexin by Caco-2 monolayers (apical pH 6.0) was carried out for 15 min at 37°C as detailed in Section 2. Drug uptake was calculated in nmol min^{-1} mg $protein^{-1}$ and then transformed to nmol min^{-1} cm^2 using the correction factor: 1 cm^2 = 0.425 mg protein (Hidalgo and Borchardt, 1991). Each value represents the average of quadruplicate determinations (±SE). (B) Transport of cephalexin (apical pH 6.0) across Caco-2 monolayers mounted in diffusion chambers determined over a 90 min time course as detailed in Section 2. Drug transport is expressed as nmol min^{-1} cm^2 (±SE) and each value represents the average of quadruplicate determinations. In both panels the line drawn through the open squares (observations) represents the best fit line using Eq. 1. The line through the squares and triangles represent the active and passive components, respectively from Eq. 1.

tion period was 2.7 nmol/mg protein at 37°C and 0.4 nmol/mg protein at 4°C (panel B). Thus, cellular accumulation of cephalexin correlated well with rates of transcellular transport.

3.4. Concentration dependence of cephalexin uptake and transport

The concentration dependence of cephalexin uptake and transport was examined over a concentration range of 0.1–25 mM. Data in Fig. 4A show that the uptake of cephalexin consists of a saturable process plus a nonsaturable one. Modelling the uptake data using Eq. 1 provided an apparent maximal transport velocity (V_t) of 1.0 nmol min^{-1} mg $protein^{-1}$, an apparent half-maximal uptake concentration of 2.9 mM for the saturable component, and a rate coefficient for the nonsaturable component, K_d, equal to 0.07 nmol min^{-1} mg $protein^{-1}$ mM^{-1} (Fig. 4A).

Cephalexin transport across cell monolayers also displays saturable kinetics. Transport rates increase rapidly at drug concentrations between 0.1 and 10 mM (Fig. 4B). Transport parameters, V_t, K_t and K_d were 0.13 nmol min^{-1} cm^{-2}, 5.0 mM, and 0.0042 nmol min^{-1} cm^{-2} mM^{-1}, respectively.

3.5. Directionality and temperature dependence of apical and basolateral efflux

The directionality of cephalexin efflux was also determined at 37 and 4°C. Of that cephalexin taken up from the apical side at 37°C, 45.7% underwent apical release and 35.6% basolateral release in 60 min at 37°C (Fig. 5A). At 4°C, apical and basolateral release were 17.8 and 1.7%, respectively (Fig. 5A). The release profiles at 37 and 4°C are consistent with the amount of the compound that remained in the cells at the end of the flux period (Fig. 5B).

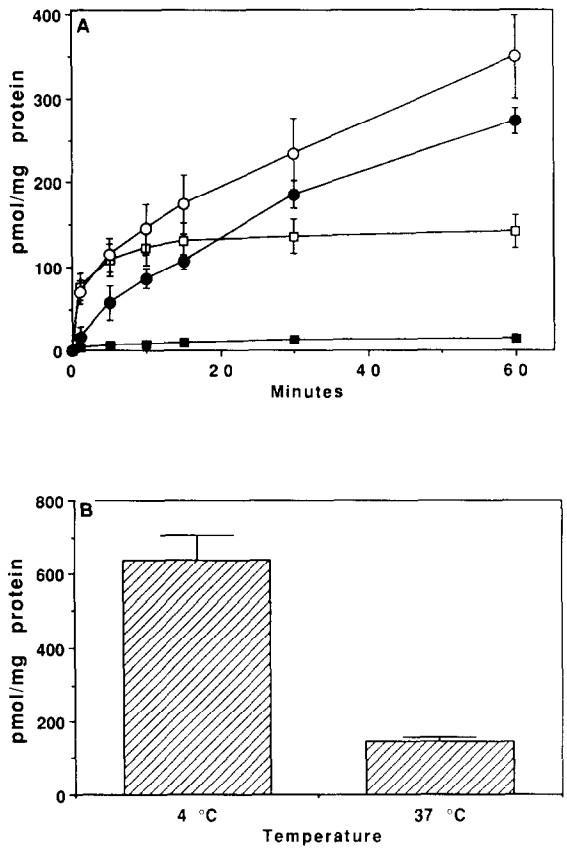

Fig. 5. Time and temperature dependence of apical and basolateral efflux. Cell monolayers (on polycarbonate filters with 4.71 cm^2 diameters) were incubated with 0.1 mM [^{3}H]cephalexin under standard uptake conditions (legend to Fig. 2) for 1 h at 37°C. At the end of the loading period the transwell inserts containing the cell monolayers were washed four times with ice-cold HBSS and transfered to different wells where they received the appropriate solutions on the apical and basolateral membrane pre-equilibrated at 37 or 4°C. Samples were obtained from the apical and basolateral sides at 1, 5, 10, 15, 30 and 60 min. At the end of the efflux period the filters containing the cells were cut and the cells dissolved as described under section 2. (A) Time courses of apical (open) and basolateral (filled) efflux at 37°C (circles) or 4°C (squares). (B) Amount of cephalexin that remained in the cells after the efflux period. Values shown are mean of four monolayers ± SD.

3.6. Effect of culture age on cephalexin transport

In order to investigate whether H^+-driven transporter activity is influenced by age in culture, transport of 100 μM cephalexin at pH 6 and pH 7.4 was examined in Caco-2 cells cultured from 10 to 25 days. The data in Fig. 6 indicate that cephalexin transport in the apical-basolateral direction is relatively high at day 10 at both pH 6 (panel A) and 7.4 (panel B). That mannitol transport is > 5-fold higher at day 10 compared to day

15–25 indicates a large paracellular component. By day 15, the profile of cephalexin transport indicates a significant difference between incubations at pH 6 vs 7.4. The reduction in drug transport rate at pH 7.4 occurs between day 10 and 15 and remains unchanged until day 25. At day 15 apical-to-basolateral cephalexin flux was 2-fold higher at apical pH 6 vs 7.4. This ratio of drug transport at the pH values tested was maintained until day 25. No significant difference was found in mannitol flux at pH 6 or 7.4. Cephalexin accumulation by Caco-2 cells was significantly higher at pH 6 compared to 7.4 as early as day 10 (Fig. 6C).

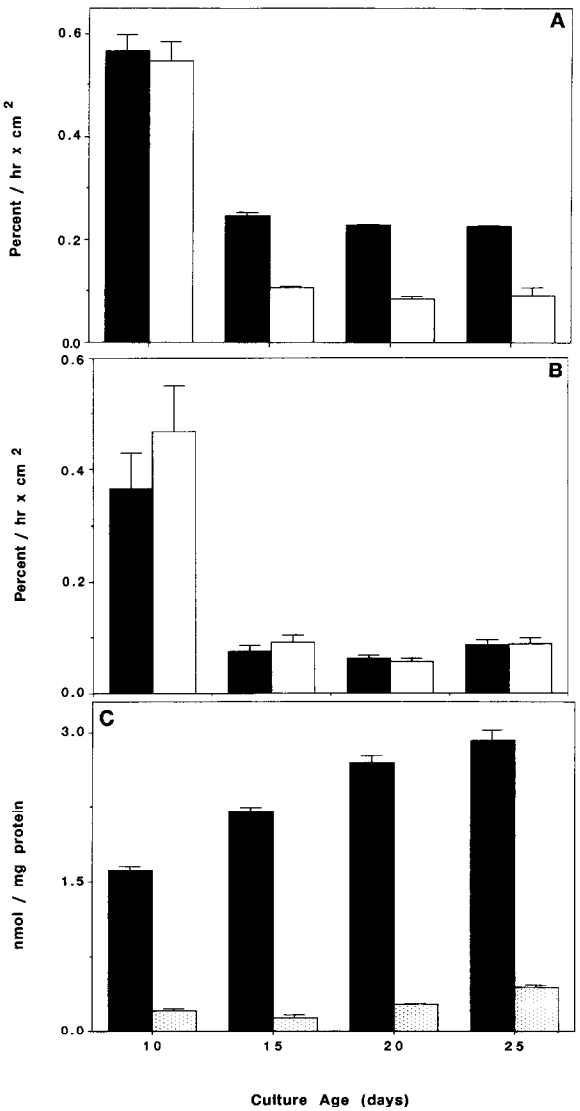

Fig. 6. Culture age dependence of cephalexin transport. (A) The apical-basolateral flux of 100 μM cephalexin (filled bars) and 10 mM mannitol (open bars) across Caco-2 cells at an apical pH 6 as detailed in section 2. (B) The apical-basolateral transport of 100 μM cephalexin and 10 mM mannitol across Caco-2 cells was carried out as detailed in panel A, with apical pH maintained at pH 7.4. Transport rate, expressed as $\%h^{-1}\ cm^{-2}$ ($\pm$SE), is an average of quadruplicate determinations. (C) At the end of each flux experiment, cephalexin accumulation was determined and is expresses as nmol/mg protein ($\pm$SE) from quadruplicate determinations.

3.7. Substrate specificity of cephalexin uptake and transport

To further characterize substrate specificity of the transporter in Caco-2 cells, selected dipeptides, amino acids and cephalosporins were tested for their potential as inhibitors of cephalexin uptake. 25 mM concentration of unlabeled cephalexin inhibited uptake of 100 μM radiolabelled drug by 74.8% (Fig. 7). The dipeptides Gly-Pro and Pro-Gly inhibited cephalexin uptake by 84 and 67%, respectively, but the single amino acids, Gly and Pro, did not alter the uptake of cephalexin significantly. Other amino acids bearing different electrical charges at pH 6.0 did not affect the uptake of cephalexin (Fig. 7). In addition, the bile acid, taurocholic acid, and the nucleoside, adenosine, had no effect on the uptake of cephalexin (Fig. 7). The orally absorbed cephalosporins, cephradine and cefadroxil, were as potent inhibitors of [^{3}H]cephalexin uptake as unlabeled cephalexin. The β-lactam antibiotics, ampicillin and penicillin G (benzylpenicillin) inhibited the uptake of cephalexin but less than the well absorbed cephalosporins. The data in Fig. 8A indicate that dipeptides and orally absorbed cephalosporins which effectively compete for cephalexin uptake also inhibit its transport across cell monolayers. However, while the trend of competition for transport is consistent with uptake results, apical-to-basolateral transport was less susceptible to inhibition than uptake.

Results also show that the residual (non-inhibited) component of apical-to-basolateral transport was the same as the pH-independent component (which occurred at pH 7.4) (Fig. 8). To

determine whether this pH-independent transport process was carrier-mediated, the transepithelial transport of radiolabeled cephalexin was examined in the presence or absence of 25 mM unlabeled cephalexin with and without a pH gradient. Results showing a transport component which is not inhibited even in the presence of an excess unlabelled cephalexin (Fig. 8B) suggest that transcellular transport of cephalexin involves at least one component that does not utilize the dipeptide carrier.

Panel C shows competition for proton-dependent transport for each treatment calculated by subtracting the proton-independent component from total transepithelial flux (panel A). The corrected data indicate a dramatic inhibition (> 90%) of H^+-dependent cephalexin transport by dipeptides and orally absorbed cephalosporins. Significant inhibition by ampicillin and penicillin G (54 and 82%, respectively) is also observed. As was determined for competition for uptake (Fig. 7), amino acids are poor inhibitors of cephalexin transport (21% or less).

Gly-Pro, cephradine and benzylpenicillin, three compounds which inhibited cephalexin uptake and transepithelial flux (Fig. 7 and 8), were determined to be competitive inhibitors of cephalexin uptake using Dixon plots (not shown), and K_i values were equal to 0.7, 5.8 and 5.5, respectively. Benzylpenicillin inhibited the apical uptake and apical-to-basolateral transport of cephalexin but itself did not undergo apical uptake into Caco-2 cell monolayers.

4. Discussion

We have studied both the uptake and transcellular transport of cephalexin in monolayers of Caco-2 cells to: (a) examine the relative contribution of paracellular and transcellular fluxes to transepithelial transport of cephalexin; (b) deter-

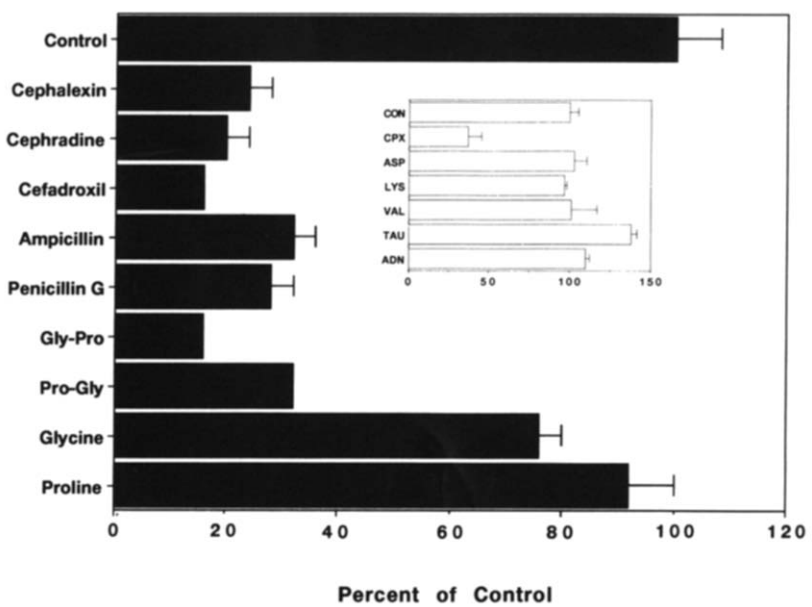

Fig. 7. Competition for cephalexin uptake by dipeptides, amino acids and other β-lactam antibiotics. Caco-2 cells were incubated for 15 min at 37°C (apical pH 6.0) with 100 μM cephalexin in the presence or absence of 25 mM concentrations of the inhibitors. Uptake of radioactive cephalexin in the absence of inhibitors was 83.4 ± 8.0 (±SE, $N = 4$) pmol/min^{-1} mg protein^{-1} and this is defined as 100% uptake. Uptake of radioactive cephalexin in the presence of inhibitors is expressed as % control (±SE, $N = 4$). The inset shows uptake in the absence (CON) and presence of a substrates for amino acid carrier and other carriers found in the small intestine and Caco-2 cells. CON, control; CPX, cephalexin (25 mM); ASP, aspartic acid (25 mM); LYS, lysine (25 mM); VAL, valine (25 mM); TAU, taurocholic acid (100 μM); ADN, adenosine (1 mM).

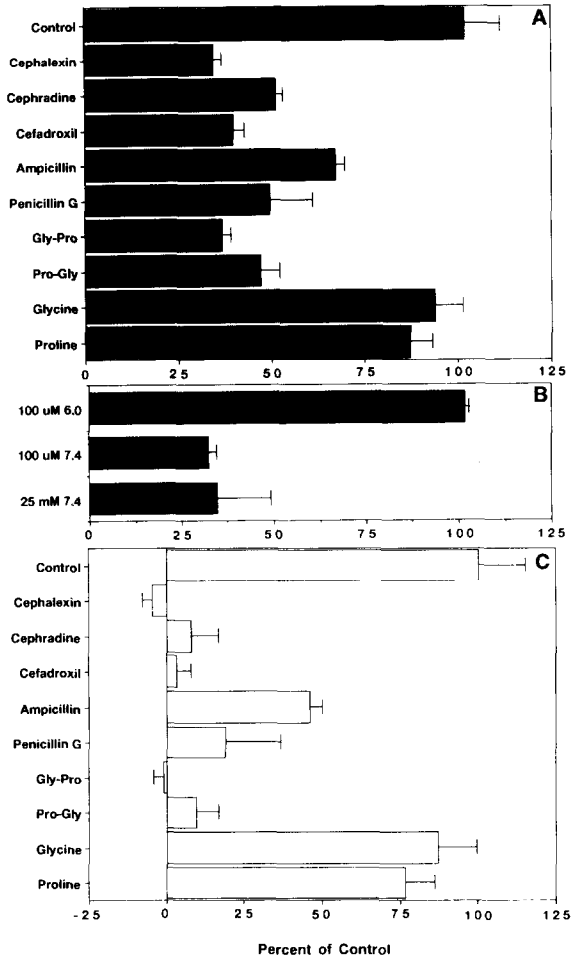

Fig. 8. Competition for cephalexin transport by dipeptides, amino acids and other β-lactam antibiotics. (A) Transport of 100 μM cephalexin across Caco-2 cell monolayers in the presence or absence of 25 mM concentrations of inhibitors was performed in diffusion chambers as detailed in section 2. Incubations were carried out at 37°C over a 90 min time course with an apical pH of 6. Transport in the control group was (4.75 pmol min^{-1} cm^{-2}) and this is defined as 100%. Cephalexin transport in the presence of inhibitors is expressed as % of control. Each value represents the average ($\pm$SE) of quadruplicate determinations. (B) Cephalexin (100 μM) transport was determined at apical pH 6.0 or 7.4 while maintaining the basolateral pH at 7.4. Cephalexin (100 μM) transport was also determined at pH 7.4 in the presence of 25 mM unlabeled cephalexin. Both apical and basolateral pH were 7.4 in the presence or absence of 25 mM cephalexin as described above. Values are % of control $\pm$ SE, $N = 4$. (C) Data represent transport values corrected for the H^+-independent component and were calculated by subtracting the transport rate observed in the presence of an excess unlabeled cephalexin from transport rates observed under other conditions.

mine the rate-limiting step (apical uptake or basolateral efflux) in the transepithelial transport of cephalexin; and (c) assess the utility of the Caco-2 cell monolayer system in evaluating interactions with the intestinal peptide carrier.

Results confirm that apical cephalexin uptake is mediated by a pH-dependent, carrier-mediated process, as reported previously (Dantzig and Bergin, 1988, 1990), and demonstrate the following: (a) the major component of transepithelial transport of cephalexin in Caco-2 cells is transcellular; (b) cellular uptake or accumulation of cephalexin shows good correlation with transepithelial transport; (c) as is the case for cellular uptake, transepithelial transport of cephalexin, in Caco-2 cells, occurs via a pH-dependent carrier-mediated mechanism; and (d) the ability of molecules to inhibit cephalexin uptake does not necessarily predict their transport via the di-/tripeptide carrier. These results agree with a previous study (Inui et al., 1992) which demonstrated that Caco-2 cells provide a useful model system for studying transport via the intestinal di- /tripeptide transporter.

4.1. Apical uptake versus transepithelial transport

Previous uptake studies employing Caco-2 and HT-29 cells found that cephalexin uptake is pH-dependent (Dantzig and Bergin, 1988, 1990). This compound has been shown to undergo transepithelial transport in vitro in the absence of an imposed pH gradient (Yamashita et al., 1986). However, there is little evidence for pH-dependent transepithelial transport of this compound as is the case for apical uptake (Hidalgo et al., 1991). Our results showing that both apical uptake and apical-to-basolateral transport of cephalexin were higher at pH 6.0 than at pH 7.4 are consistent with previous studies (Dantzig and Bergin, 1988, 1990). The present study utilized cell monolayers grown on filters to assess transepithelial transport. Thus, it was necessary to rule out the possibility that the apparent pH-dependent transport of cephalexin was a consequence of some nonspecific effect of this slightly acidic pH on the cephalexin molecule or on the permeability of the monolayers. A nonspecific effect of

the pH on the cephalexin molecule can be easily dismissed because cephalexin is zwitterionic over the entire pH range investigated (i.e., pH 5.5–7.4), and thus charge is not likely to be a determining factor on pH dependent uptake or transport. The increase in apical-to-basolateral flux at lower pH is most likely not due to some deleterious effect on the cells because: (a) the flux of mannitol was not altered by similar pH changes and (b) a pH gradient across the basolateral membrane did not alter the basolateral-to-apical transport of cephalexin or mannitol.

The lack of net cephalexin transport in the absence of a pH gradient in this study supports a recent observation on the transport of cephradine in Caco-2 cells (Inui et al., 1992) but disagrees with results in rabbit intestine (Hidalgo et al., 1991). In rabbit ileum and jejunum, mucosal-to-serosal fluxes were 3-times greater than serosal-to-mucosal fluxes (Hidalgo et al., 1991). The reason for this discrepancy is not known. One possible explanation is that Caco-2 cells may not be able to establish the microenvironment pH gradient required to drive peptide transport. This pH gradient has been measured in vivo (Lucas, 1983) and in intestinal mucosa in vitro (Daniel et al., 1985).

Although the intestinal peptide transporter has been found in a number of in vitro systems (Nakashima et al., 1984; Yamashita et al., 1986; Dantzig and Bergin, 1988, 1990; Kramer et al., 1990; Hidalgo et al., 1991; Inui et al., 1992) the role of this transporter on intestinal drug absorption in vivo remains to be demonstrated. In addition to having been characterized as transport model systems of the small intestinal epithelium (Hidalgo et al., 1989; Wilson et al., 1990), Caco-2 cells have been shown to express a peptide transporter functionally similar to that found in enterocytes (Dantzig and Bergin, 1990; Dantzig et al., 1992). Thus, we utilized the Caco-2 cell monolayer system to determine the magnitude of the contribution of the peptide transporter to the transepithelial transport of cephalexin. In other words, to answer the question: is the carrier-mediated transport of cephalexin a substantial component of the transepithelial transport of this compound or an interesting but minor pathway?

The fact that both apical uptake and apical-to-basolateral transport of cephalexin were pH, concentration- and temperature-dependent suggests that the peptide transporter is involved not only in the uptake but also in transepithelial transport of cephalexin in Caco-2 cells. This interpretation is supported by the similarity in K_t values for the carrier-mediated component of apical uptake (2.9 mM) and apical-to-basolateral transport (4.7 mM) found in this study. These K_t values are also comparable to those reported for uptake in Caco-2 cells (7.5 mM) (Dantzig and Bergin, 1990). Furthermore, the K_t for transepithelial transport in Caco-2 cells agrees with those reported for transepithelial transport in rabbit intestinal mucosa (1.4–2 mM) (Yamashita et al., 1986; Hidalgo et al., 1991) suggesting that the same transporter may be present in both systems.

Transcellular transport of peptides involves uptake across the apical membrane, diffusion through the cytoplasma and efflux across the basolateral membrane. While the peptide transporter present on the apical membrane of enterocytes has been widely investigated, there is little information on the processes involved in the basolateral efflux of di-/tripeptides. Dyer et al., (1990) have identified a dipeptide carrier in basolateral membrane vesicles from rabbit intestine and proposed that this carrier contributes to the transepithelial flux of small peptides. Determination of the existence of this basolateral transporter in Caco-2 cells warrants further investigation.

In general, correlation of uptake into brush border membrane vesicles with intestinal transport makes the assumption that apical uptake is the rate-limiting step in transepithelial transport and does not take into account the role of basolateral efflux in transepithelial transport (Hori et al., 1988). This assumption was evaluated in this study in the following manner: By definition, V_t is the maximum transport velocity of a carrier-mediated process. In addition, apical uptake is the first step in transepithelial transport. Thus, we hypothesized that if apical uptake was rate-limiting, V_t for apical uptake and V_t for apical-to-basolateral transport should be similar. On the other hand, if basolateral efflux is rate-limiting,

then the V_t for apical uptake should be greater than that for transepithelial transport, indicating a greater uptake capacity compared to efflux capacity. A previous study reported that the protein content of confluent Caco-2 cell monolayers is 0.425 mg protein/cm^2 (Hidalgo and Borchardt, 1990). Thus, the V_t value of 0.13 nmol min^{-1} cm^{-2} determined in the present study can be expressed as 0.3 nmol min^{-1} mg protein^{-1}. This value is 30% of the corresponding V_t for apical uptake (1.0 nmol min^{-1} mg protein^{-1}). It follows that basolateral efflux is probably the rate-limiting step in the transepithelial transport of cephalexin. The observation that at steady state, the ratio of intracellular to extracellular (medium) cephalexin concentration is 6.5 at 37°C but only 0.5 at 4°C lends support to this interpretation because for this magnitude of accumulation to take place uptake must exceed efflux.

These conclusions are not necessarily in disagreement with a recent study by Inui et al. (1992) in which similar rates for apical uptake and transepithelial transport of 1 mM cephalexin were reported. The rates of uptake and transepithelial transport may be comparable because 1 mM is below the K_m values for both processes. It is likely that at higher concentrations, the rate of carrier-mediated uptake would exceed the rate of carrier-mediated transepithelial transport because of the lower capacity of the basolateral efflux mechanism.

4.2. Transcellular versus paracellular transepithelial flux of cephalexin

To determine the relevance of the transporter in peptide absorption, we examined the processes involved in transepithelial transport. Results from temperature-dependent and pH-dependent transport across Caco-2 cell monolayers indicate that the predominant pathway for the transepithelial transport of cephalexin is transcellular. That both apical uptake and apical-to-basolateral transport exhibited the same pH-dependent profile indicates that the main component of transepithelial flux is transcellular. In addition, temperature-dependence of apical uptake and apical-to-basolateral transport would imply that transepithelial transport is mainly transcellular. The fact that at 4°C apical-to-basolateral cephalexin transport was reduced more than apical uptake suggests the involvement of a paracellular pathway in the transepithelial transport of cephalexin.

We suggested that apical uptake showed a greater inhibition than apical-to-basolateral transepithelial transport because the latter involves a paracellular component which is less sensitive to low temperature. This hypothesis was tested by determining apical and basolateral efflux at 37 and 4°C in cells preloaded at 37°C from the apical membrane (Fig. 5). Under the loading conditions used one might expect that release of cephalexin from the basolateral membrane reflects true basolateral efflux. On the other hand, release from the apical membrane may be a combination of true efflux plus dissociation from sites on the apical cell membrane.

The curves for apical release at 4 and 37°C were rapid and superimposable from 0 to 5 min suggesting that the cephalexin released in this period was most likely bound to the cells surface and not internalized. The absence of cephalexin release at 4°C after the initial 5 min and the lack of appreciable basolateral release through 1 h indicate that cephalexin transport across the cell membranes (apical or basolateral) is virtually shut down at 4°C (Fig. 5B). It appears that the amount of cephalexin released from the apical membrane after 5 min at 37°C constitutes true apical efflux.

Steady-state transepithelial transport rates of cephalexin (0.1 mM on the apical side) across cell monolayers were 1.67 and 4.27 pmol min^{-1} cm^{-2}, in the absence and presence of a pH gradient, respectively (Fig. 8B). This indicates that approx. 40% of the total transepithelial transport is pH-independent. The following lines of evidence suggest that this pH-independent transepithelial transport component is most likely passive paracellular diffusion. First, in the absence of a pH gradient, apical-to-basolateral cephalexin flux was: (a) identical to basolateral-to-apical cephalexin flux and equal to mannitol flux in both directions and (b) not inhibited by excess unlabeled cephalexin. Second, the transepithelial transport of radiolabeled cephalexin in the presence of inhibitors approached a constant value

(~ 30%) which is the same as that in the absence of a driving force (pH gradient) with or without 25 mM unlabeled cephalexin (Fig. 8).

4.3. Specificity of the peptide carrier

Competition with a number of compounds known to undergo carrier-mediated intestinal absorption provided some evidence for the uniqueness of the transport of cephalexin. For example, cephalexin uptake was inhibited by the dipeptides Gly-Pro and Pro-Gly but not by any of the amino acids tested (regardless of their electrical charge), the bile acid, taurocholic acid, or the nucleoside, adenosine, indicating that the cephalexin transporter is most likely the di-/tripeptide transporter. Although it is possible that there are multiple intestinal peptide transporters and that the cephalexin transporter may be a different subtype from the natural dipeptide transporter(s), the existing evidence supporting this possibility is too limited to make a clear determination (Rubino et al., 1971, Ganapathy et al., 1985; Inui, et al., 1988). Recently, a protein molecule with a molecular mass of 127 kDa has been isolated from rabbit intestine (Kramer et al., 1990). This protein is proposed to be a component of the di-/tripeptide transporter. The uptake of cephalexin in HT29 and Caco-2 cells and the accumulation and transport of cephradine in Caco-2 cells (Inui et al., 1992) suggest the presence of the transporter(s) in these cells. However, the presence of the protein molecule reported by Kramer et al. (1990) has not been demonstrated in Caco-2 or HT29 cells.

The goal of in vitro systems is to reveal information about the in vivo system being modeled. Therefore, very frequently, attempts are made to correlate results from in vitro systems with in vivo absorption (Hori et al., 1988). Our results with cephalexin demonstrate a good correlation between inhibition of uptake and inhibition of transepithelial transport. The observation that those compounds which inhibited apical uptake also inhibited apical-to-basolateral transport supports the argument that transepithelial transport of cephalexin in Caco-2 cells is mainly transcellular. The peptide transporter expressed by the colonic cell line, Caco-2, resembles its small intestinal counterpart (Dantzig and Bergin, 1988, 1990; Dantzig et al., 1992). But in the absence of established in vitro-in vivo correlations, extreme caution should be exercised in trying to predict transport on the basis of competition in this type of cell assays.

This limitation is illustrated by the results from inhibition experiments. The greater affinity of Gly-Pro ($K_i = 0.7$ mM) for the carrier compared to cephradine ($K_i = 5.8$ mM) could be expected because cephradine is not a true peptide and its size is closer to a tripeptide than to a dipeptide. However, results with bezylpenicillin cannot be interpreted clearly. Because existing evidence indicates that cephradine undergoes carrier-mediated transport (Inui et al., 1992) but benzylpenicillin does not (Ryan and Smith, 1989), one would have expected the affinity of benzylpenicillin to be lower than that of cephradine. What we found, however, was that the K_i of benzylpenicillin was undistinguishable from that of cephradine (5.5 vs 5.8 mM). Thus, we speculated that for benzylpenicillin neither mode of competition (e.g., competitive vs noncompetitive) nor the magnitude of the K_i values in this cell assay are good predictors of transepithelial transport via the di-/tripeptide transporter. To test this hypothesis we examined the uptake and transepithelial transport of benzylpenicillin in Caco-2 cell monolayers. The fact that the uptake of benzylpenicillin in Caco-2 cells is minimal and pH-independent agrees with a previous study which reported the absence of carrier-mediated transport of benzylpenicillin in rabbit intestinal mucosa in vitro (Ryan and Smith, 1989). Together, these results constitute evidence that although benzylpenicillin has the ability to interact with the peptide transporter, this interaction does not result in cellular uptake or transepithelial transport. This is supported by a recent study with a 127 kDa protein, suggested to be the peptide transporter (or a component of it) (Kramer et al., 1992). Following reconstitution of this protein was reconstituted into liposomes, both D- and L-cephalexin bound to the binding protein but only the D-isomer was taken up into the liposomes (Kramer et al., 1992), demonstrating that binding by the carrier does

not necessarily correlate with uptake or transport.

An additional variable that may complicate in vitro-in vivo correlations is the chemical/enzymatic liability of the inhibitor molecule either in the intestinal lumen or during presystemic absorption. Additional characterization with a large number of molecules and transport/absorption correlations will be required in order to establish the utility of Caco-2 cell monolayers in predicting absorption of peptidomimetic drugs.

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6. References

Audus, K.L., Bartel, R.L., Hidalgo, I.J. and Borchardt, R.T., The use of cultured epithelial and endothelial cells for drug transport and metabolism studies. *Pharm. Res.*, 7 (1990) 435–51.

Burnham, D.B. and Fondacaro, J.D., Secretagogue-induced protein phosphorylation and chloride transport in Caco-2 cells. *Am. J. Physiol.*, 256 (1989) G808-G816.

Daniel, H., Neugebauer, B., Kratz, A. and Rehner, G., Localization of acid microclimate along intestinal villi of rat jejunum. *Am. J. Physiol.* 248 (1985) G293-G298.

Dantzig, A.H. and Bergin, L., Uptake of the cephalosporin, cephalexin, by a dipeptide transport carrier in the human intestinal cell, Caco-2. *Biochim. Biophys. Acta*, 1027 (1990) 211–217.

Dantzig, A.H. and Bergin, L., Carrier-mediated uptake of cephalexin in human intestinal cells. *Biochem. Biophys. Res. Commun.*, 155 (1988) 1082–1087.

Dantzig, A.H., Tabas, L.B. and Bergin, L., Cefaclor uptake by the proton-dependent dipeptide transport carrier of human intestinal Caco-2 cells and comparison to cephalexin uptake. *Biochim. Biophys. Acta*, 1112 (1992) 167–173.

Dyer, J., Beechey, R.B., Gorvel, J.-P., Smith, R.T., Wootton, R. and Shirazi-Beechey, S.P., Glycyl-L-proline transport in rabbit enterocyte basolateral-membrane vesicles. *Biochem. J.*, 269 (1990) 565–571.

Ganapathy, V., Burckhardt, G., and Leibach, F., Peptide transport in rabbit intestinal brush-border membrane vesicles. *Biochim. Biophys. Acta*, 816 (1985) 234–240.

Ganapathy, V and Leibach, F.H., Is intestinal peptide transport energized by a proton gradient? *Am. J. Physiol.*, 249 (1985) G153-G160.

Hidalgo, I.J. and Borchardt, R.T., Transport of a large neutral amino acid (phenylalanine) in a human intestinal epithelial cell line: Caco-2. *Biochim. Biophys. Acta*, 1028 (1990) 25–30.

Hidalgo, I.J., Hillgren, K.M., Grass, G.M. and Borchardt, R.T., Characterization of the unstirred water layer in Caco-2 cell monolayers using a novel diffusion apparatus. *Pharm. Res.*, 8 (1991) 222–227.

Hidalgo, I.J., Raub, T.J. and Borchardt, R.T., Characterization of the human colon carcinoma cell line (Caco-2) as a model system for Intestinal epithelial permeability. *Gastroenterology*, 96 (1989) 736–749.

Hidalgo, I.J., Ryan, F.M., Marks, G. and Smith, P.L., Carrier-mediated transport of cephalexin (CPX) across intestinal mucosa. *Gastroenterology*, 100 (1991) A689 (Abstr.).

Hori, R., Okano, T., Kato, M. Maegawa, H. and Inui, K.-I., Intestinal absorption of cephalosporin antibiotics: correlation between intestinal absorption and brush border membrane transport. *J. Pharm. Pharmacol.*, 40 (1988) 646–647.

Hu, M and Amidon, G.L., Passive and carrier-mediated intestinal absorption components of Captopril. *J. Pharm. Sci.*, 77 (1988) 1007–1011.

Inui, K.-I., Okano, T., Maegawa, H., Kato, M., Takano, M. and Hori, R., H^+ coupled transport of p.o. cephalosporins via dipeptide carriers in rabbit intestinal brush-border membranes: Difference of transport characteristics between cefixime and cephradine. *J. Pharmacol. Exp. Ther.*, 247 (1988) 235–241.

Inui, K.-I., Yamamoto, M. and Saito, H., Transepithelial transport of oral cephalosporins by monolayers of intestinal epithelial cell line Caco-2: Specific transport systems in apical and basolateral membranes. *J. Pharmacol. Exper. Ther.*, 261 (1992) 195–201.

Kleemann, H.-W., Heitsch, H., Henning, R., Kramer, W., Kocher, W., Lerch, U., Linz, W., Nickel, W.-U., Ruppert, D., Urbach, H., Utz, R. Wagner, A., Weck, R. and Wiegand, F., Renin inhibitory pentols showing improved enteral bioavailability. *J. Med. Chem.*, 35 (1992) 559–567.

Kramer, W., Dechent, C., Girbig, F., Gutjahr, U. and Neubauer, H., Intestinal uptake of dipeptides and β-lactam antibiotics: I. The intestinal uptake system for dipeptides and β-lactam antibiotics is not part of a brush border membrane peptidase. *Biochim. Biophys. Acta*, 1030 (1990) 41–49.

Kramer, W., Girbig, F., Gutjahr, U., Kowalewski, S., Adam, F. and Schiebler, W., Ileal absorption of β-lactam antibiotics and oligopeptides. Functional and stereospecific reconstitution of the oligopeptide transport system from rabbit small intestine. *Eur. J. Biochem.*, 1030 (1992) 50–59.

Kramer, W., Gutjahr, U., Girbig, F. and Leipe, I., Intestinal absorption of dipeptides and β-lactam antibiotics: II. Pu-

rification of the protein for dipeptides and β-lactam antibiotics from rabbit small intestinal brush border membranes. *Biochim. Biophys. Acta*, 1030 (1990) 50–59.

Lucas, M., Determination of acid surface pH in vivo in rat proximal jejunum. *Gut*, 24 (1083) 734–739.

Metzler, C.M. and Weiner, D.L., PCNONLIN User Guide–Version 3.0, Statistical Consultants, Lexington, KY, 1989.

Nakashima, E., Tsuji, A., Mizuno, H. and Yamana, T., Kinetics and mechanism of in vitro uptake of amino-β-lactam antibiotics by rat small intestine and relation to the intact-peptide transport system. *Biochem. Pharmacol.*, 33 (1984) 3345–3352.

Rubino, A., Field, M. and Shwachman, H., Intestinal transport of amino acid residues of dipeptides. I. Influx of the glycine residue of glycyl-L-proline across mucosal border. *J. Biol. Chem.*, 246 (1971) 3542–3548.

Ryan, F.M. and Smith, P.L., Effects of altering fluid transport on the absorption of benzylpenicillin and mannitol across rat intestinal mucosal in vitro. *Pharm. Res.*, 6 (1989) S88.

Sugawara, M., Saitoh, H., Iseki, K., Miyazaki, K. and Arita, T., Contribution of passive transport mechanisms to the intestinal absorption of β-lactam antibiotics. *J. Pharm. Pharmacol.*, 42 (1990) 314–318.

Wilson, G., Cell culture techniques for the study of drug transport. *Eur. J. Drug Metab. Pharmacokinet.*, 15 (1990a) 159–163.

Wilson, G., Hassan, I.F., Dix, C.J., Williamson, I., Shah, R., Mackay, M. and Artursson, P., Transport and permeability properties of human Caco-2 cell monolayers. *J. Controlled Release*, 11 (1990b) 25–40.

Yamashita, S., Yamazaki, Y., Masada, M. Nadai, T., Kimura, T. and Sezaki, H., Investigations of influx of and accumulation processes of β-lactam antibiotics and their role in the transmural transfer across rat jejunum. *J. Pharmacobio-Dyn.*, 9 (1986) 368–374.

Yokohama, S., Yoshioka, T., Yamashita, K. and Kitamori, N., Intestinal absorption mechanisms of thyrotropin-releasing hormone. *J. Pharmacobio-Dyn.*, 7 (1984) 445–451.