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Substrate specificity of the di/tripeptide transporter in human intestinal epithelia (Caco-2): identification of substrates that undergo H^+-coupled absorption

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1 pH-dependent transepithelial transport and intracellular accumulation of the hydrolysis-resistant dipeptide glycylsarcosine (Gly-Sar) have been demonstrated in the model human intestinal epithelial cell line, Caco-2.

2 Experiments with BCECF (2′,7′-*bis*(2-carboxyethyl)-5(6)-carboxyfluorescein)-loaded Caco-2 cells demonstrated that dipeptide (Gly-Sar) transport across the apical membrane is coupled to proton flow into the cell.

3 A range of postulated substrates for the intestinal di/tripeptide carrier were tested for their abilities to: (a) inhibit pH-dependent [^{14}C]Gly-Sar apical-to-basal transport and intracellular accumulation and (b) stimulate H^+ flow across the apical surface of BCECF-loaded Caco-2 cell monolayers.

4 A range of compounds (including Gly-Gly, Leu-Leu, Gly-Gly-Gly, cefadroxil and cephalexin) caused marked acidification of intracellular pH when perfused at the apical surface of Caco-2 cell monolayers. In contrast leucine and D-Leu-D-Leu failed to induce proton flow. The ability to induce proton-flow across the apical surface by these compounds, in this intestinal epithelium, was directly correlated to the relative inhibitory effects on [^{14}C]-Gly-Sar transport and accumulation.

5 The determination of substrate-induced intracellular pH change in the Caco-2 cell system may provide a useful rapid screen for candidate substrates for absorption via H^+-coupled transport mechanisms such as the intestinal di/tripeptide carrier in an appropriate physiological context.

Keywords: Proton-coupled transport; dipeptide; intestine; epithelium; Caco-2 cells; cell culture; cephalosporin; intracellular pH

Introduction

The initial barrier to drug delivery via the oral route is the relative ability of drug molecules to cross the intestinal epithelial cell wall. Absorption may occur by both passive (paracellular and transcellular) and active (transcellular) processes. An important membrane transporter involved in absorption via the transcellular route is the intestinal di/tripeptide carrier which plays a significant role in protein absorption (Matthews, 1975; Matthews & Adibi, 1976). This H^+-gradient driven carrier (Ganapathy & Leibach, 1985) is also thought to be primarily responsible for the oral absorption of a number of pharmaceuticals including some aminocephalosporin antibiotics (Nakashima *et al.*, 1984; Humphrey, 1986; Smith *et al.*, 1992). The driving force for this H^+-gradient driven absorptive mechanism is the acid microclimate (an area of low pH lying adjacent to the apical surface of the intestinal epithelium) which has been identified at the mucosal surface of the human jejunum both *in vivo* (Rawling *et al.*, 1987) and *in vitro* (Lucas *et al.*, 1978). Experiments aimed at identifying substrates that can access this H^+-driven transcellular transport route have generally utilized either whole tissue preparations (i.e. the hamster jejunum *in vitro*: Sleisenger *et al.*, 1976; Matthews & Burston, 1983) or brush-border membrane vesicles (BBMV) prepared from rat or rabbit small intestine (Ganapathy *et al.*, 1984; Ganapathy & Leibach, 1985; Okano *et al.*, 1986a,b; Thwaites *et al.*, 1993h). Until recently there had been relatively few studies at the cellular level (Dantzig & Bergin, 1990; Inui *et al.*, 1992; Thwaites *et al.*, 1993a,b,c).

The human intestinal epithelial cell line, Caco-2, expresses functional H^+-coupled dipeptide carriers at both apical and basolateral membranes (Thwaites *et al.*, 1993a,b). Transepithelial transport of the non-hydrolysable dipeptide Gly-Sar has been extensively characterized (Thwaites *et al.*, 1993a), whilst pH-dependent transport of the orally-absorbed cephalosporin, cephradine (Inui *et al.*, 1992), and the anti-cancer agent, bestatin (Saito & Inui, 1993) across epithelial monolayers of Caco-2 cells have been demonstrated. In addition, to expression of H^+-linked dipeptide transport, Caco-2 cells express a number of solute transporters including those for sugars (Blais *et al.*, 1987), amino acids (Hidalgo & Borchardt, 1990a) and bile acids (Hidalgo & Borchardt, 1990b) confirming the suitability of this human model system as an *in vitro* model of the human intestine.

Recently we have related the ability of a substrate to access the dipeptide transporter with its ability to induce H^+ flow across the apical surface of Caco-2 cell monolayers, as detected by changes in intracellular pH monitored using the pH-sensitive fluorescent dye, 2′,7′,-*bis*(2-carboxyethyl)-5(6)-carboxyfluorescein BCECF (Thwaites *et al.*, 1993a,b,c). Such a measure provides a novel method to discriminate between a non-transported inhibitor from a true substrate and allows assessment of the substrate-specificity of certain peptides, including those likely to undergo degradation at the epithelial surface (Thwaites *et al.*, 1993c). In addition, by comparison of the relative abilities of substrates to inhibit pH-dependent transepithelial transport (and cellular accumulation) of the dipeptide [^{14}C]-Gly-Sar with substrate-stimulated H^+-flow, it is possible to define potential inhibitors and to identify substrates accessing alternative H^+-linked carriers. Therefore, the aim of this investigation was to identify, by use of two complementary techniques, the range of substrates that access the human H^+-coupled small peptide transporter expressed in an appropriate physiological context, namely the human intestinal epithelial cell line, Caco-2.

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Methods

Cell culture

Caco-2 cells (passage number 94–116) were cultured in DMEM (with 4.5 g l^{-1} glucose), with 1% non-essential amino acids, 2 mM L-glutamine, 10% (v/v) foetal calf serum and gentamicin (60 μg ml^{-1}). Cell monolayers were prepared by seeding at high density (4.4–5.0 × 10^5 cells cm^{-2}) onto tissue culture inserts [Transwell (0.4 μm pore) polycarbonate filters (Costar), 24 mm (cat. no. 3412) or 12 mm (cat. no. 3401) in diameter]. Cell monolayers were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Media was replaced every 2–3 days. Cell confluence was estimated by microscopy and determination of transepithelial electrical resistance (R_T) using EVOM chopstick electrode STX-2 (WPI), measured at 37°C.

Transport experiments

Uptake and transport experiments with Gly-Sar and mannitol were performed 16–28 days after seeding and 18–24 h after media replacement. Transepithelial flux measurements were performed as described previously (Thwaites *et al.*, 1993a,b). Briefly, the cell monolayers (24 mm in diameter) were washed in 4 × 500 ml of modified Krebs buffer (all mM): NaCl 137, KCl 5.4, $CaCl_2$ 2.8, $MgSO_4$ 1.0, NaH_2PO_4 0.3, KH_2PO_4 0.3, glucose 10, HEPES/Tris 10 (pH 7.4) and were then placed in 6-well plates, each well containing 2 ml of modified Krebs (pH 7.4, 37°C). The pH 6.0/6.5 buffers were identical except that 10 mM MES replaced 10 mM HEPES. Aliquots of Krebs buffer (pH 7.4) were placed in the upper filter cup (apical solution) and the filters were incubated for 10 min at 37°C. The solutions in both apical and basal chambers were then replaced with the experimental buffers. The experimental composition of the buffers in the apical and basal chambers were identical except where stated otherwise. Radiolabelled Gly-Sar and mannitol (0.5 μCi ml^{-1}, 36 μM) were added to the apical chamber. In experiments involving high (10–20 mM) concentrations of substrates isoosmolarity was maintained by addition of mannitol to controls samples. Fluxes in the absorptive (apical-to-basal, J_{a-b}) direction were determined. Fluxes across the monolayers into the contralateral chamber are expressed as pmol.cm^{-2}.h^{-1} or (when stated) after subtraction of the paracellular (passive) component (as determined by mannitol flux) as % control (in the absence of inhibition) so that the stated values represent an estimate of the proportion of transcellular Gly-Sar transport only. However, since [^{3}H]-mannitol is used to estimated paracellular transport, it is likely that the passive component of transport is overestimated. Hence some dipeptides apparently reduce [^{14}C]-Gly-Sar transport to below zero. At the end of the incubation period, cell monolayers were washed in 4 × 500 ml volumes of Krebs buffer (pH 7.4) to remove any extracellular radiolabel, and removed from the insert. Cell monolayer-associated radiolabel was determined by scintillation counting. Cellular accumulation of Gly-Sar is expressed as μM or (after subtraction of filter-associated mannitol values) as % control (in the absence of inhibition). Cell height was determined by confocal microscopy and this value was used in the determination of intracellular volume (Thwaites *et al.*, 1993g).

Intracellular pH measurements

For intracellular pH (pH_i) measurements (Thwaites *et al.*, 1993a), Caco-2 cells grown to confluence (14–23 days after seeding) on 12 mm diameter Transwell polycarbonate filters (Costar) were loaded by incubation with BCECF-AM (5 μM), in both apical and basal chambers, for 40 min at 37°C. After loading, the 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}). All solutions were preheated to 37°C. For Na^+-free conditions the Krebs buffer was modified by replacement of NaCl with choline chloride and omission of NaH_2PO_4. Intracellular H^+ concentration was quantified by fluorescence (excitation at 440/490 nm and emission at 520 nm) from a small group of cells (5–10) using a photon counting system (Newcastle Photometric Systems). Intracellular BCECF fluorescence was converted to pH_i by comparison with values from an intracellular calibration curve using nigericin (10 μM) and high K^+ solutions (Thomas *et al.*, 1979; Watson *et al.*, 1991). Results are expressed as ΔpH_i or ΔpH_i min^{-1} (mean ± s.e.mean, *n*). The rate of change of intracellular pH (ΔpH_i min^{-1}) was calculated by linear regression using Photon Counter System 4.7 (Newcastle Photometric Systems). Changes in ΔpH_i min^{-1} (due to a change in the composition of the superfusate) were determined by linear regression, by comparison of the linear portions of the trace over 30–50 s (15–25 data points) periods before and after the change in composition. The specific change in ΔpH_i min^{-1} due to addition of a particular substrate can be obtained by subtraction of the ΔpH_i min^{-1} in the presence of apical acidity alone from the total ΔpH_i min^{-1} determined upon addition of a particular substrate to the superfusate.

Materials

[^{3}H]-mannitol (specific activity, 30 Ci $mmol^{-1}$) was obtained from NEN. [^{14}C]-Gly-Sar (L-glycyl[1-^{14}C]-sarcosine (specific activity, 14 mCi $mmol^{-1}$)) was from Amersham. Thyrotropin releasing hormone (TRH) was from Bachem (UK). Carnosine (β-alanine-L-histidine) was from Peptide Inst. Inc. (Japan). All other peptides and cephalosporins were from Sigma. BCECF, cell culture media, supplements and plastic were supplied by Life Technologies. All other chemicals were from Merck. All amino acids were the L-isomer unless stated otherwise.

Statistics

Results are expressed as mean ± s.e.mean (*n*). Statistical analysis was performed with Student's paired *t* test.

Results

It has been demonstrated previously that the transepithelial transport of [^{14}C]-Gly-Sar across Caco-2 cell monolayers is pH-dependent (Thwaites *et al.*, 1993a,b,c). In this study, apical-to-basal [^{14}C]-Gly-Sar transport in the presence of a pH gradient (apical pH 6.0, basolateral pH 7.4) was 479.6 ± 18.1 pmol cm^{-2} h^{-1} (n = 27), and was increased compared to mannitol transport (134.5 ± 8.8 pmol cm^{-2} h^{-1} (n = 27), $P<$ 0.001). The dipeptide Gly-Sar showed marked intracellular accumulation above medium levels (36 μM) at apical pH 6.0 (266.6 ± 14.2 μM, n = 33). Residual cell-associated mannitol (28.1 ± 2.9 μM (n = 33), $P<0.001$ compared to Gly-Sar) showed no apparent accumulation. In separate experiments, inclusion of 20 mM unlabelled Gly-Sar at the apical surface significantly reduced apical-to-basal [^{14}C]-Gly-Sar transport from 513.0 ± 23.3 pmol cm^{-2} h^{-1} (n = 13) to 142.0 ± 10.8 pmol cm^{-2} h^{-1} (n = 13, $P<0.001$). Under these conditions the [^{14}C]-Gly-Sar transport was not significantly different ($P>0.05$) from the paracellular flux estimated by mannitol transport. [^{14}C]-Gly-Sar intracellular accumulation was reduced by unlabelled Gly-Sar (20 mM) from 270.1 ± 23.5 μM (n = 14) to 92.3 ± 3.7 μM (n = 15, $P<0.001$).

Table 1 shows the effects of a wide range of substrates (each at 20 mM) on the pH-dependent apical-to-basal transport and intracellular accumulation of the model compound [^{14}C]-Gly-Sar. A variety of dipeptides (containing L-amino

Table 1 Effect of substrates (20 mM) on pH-dependent [^{14}C]-Gly-Sar transcellular transport and intracellular accumulation

Control	*Transport* 100%	*Uptake* 100%
Amino acid		
Leu	94.5 ± 8.2 (4) NS	92.9 ± 6.3 (4) NS
Dipeptides		
Gly-Gly	− 23.3 ± 1.5 (5)***	2.6 ± 0.3 (5)***
Gly-Phe	− 9.8 ± 0.2 (3)***	6.3 ± 0.3 (3)***
Gly-Pro	− 8.7 ± 0.4 (3)***	9.4 ± 0.3 (3)***
Pro-Gly	19.8 ± 5.6 (7)***	41.4 ± 6.1 (8)***
Gly-Leu	10.3 ± 5.8 (6)***	6.6 ± 1.1 (6)***
Leu-Gly	− 19.9 ± 4.2 (6)***	14.2 ± 0.7 (6)***
Leu-Leu	− 12.2 ± 0.7 (4)***	7.1 ± 0.2 (4)***
Ala-Ala	− 12.4 ± 1.3 (3)***	5.7 ± 0.2 (3)***
Carnosine	− 7.3 ± 1.2 (4)***	28.0 ± 4.0 (4)***
DAA-containing dipeptides		
Gly-D-Leu	22.1 ± 1.3 (4)***	60.3 ± 1.9 (5)***
D-Leu-Gly	42.9 ± 3.2 (4)***	61.5 ± 3.3 (4)***
D-Leu-D-Leu	87.5 ± 9.0 (4) NS	105.5 ± 9.9 (4) NS
D-Ala-D-Ala	90.9 ± 1.0 (3) NS	101.1 ± 14.2 (4) NS
Tripeptides		
3Gly	2.0 ± 1.4 (4)***	19.0 ± 0.9 (4)***
TRH	98.4 ± 1.5 (3) NS	109.2 ± 5.3 (3) NS
Tetrapeptides		
4Gly	−	84.8 ± 2.9 (4) NS
4Ala	− 15.9 ± 1.8 (5)***	6.8 ± 0.4 (5)***
Cephalosporins		
Cefadroxil	− 3.2 ± 0.9 (4)***	33.9 ± 0.7 (4)***
Cephalexin	26.5 ± 0.5 (4)***	63.3 ± 1.9 (4)***
Cephradine	21.4 ± 2.0 (4)***	63.7 ± 2.9 (4)***
Cefazolin	61.0 ± 5.5 (6)**	96.0 ± 6.6 (7) NS

Results are expressed as a percentage of the transcellular transport/accumulation observed in the absence of any competing substrates (20 mM), after subtraction of the paracellular (passive) component (estimated from mannitol flux).
***$P<0.001$ compared with control
**$P<0.01$
*$P<0.05$
NS $P<0.05$

acids in both N and C terminal positions) all significantly inhibited [^{14}C]-Gly-Sar transcellular transport and intracellular accumulation. For instance, the dipeptide, Leu-Leu, significantly reduced both [^{14}C]-Gly-Sar transport and accumulation (Table 1) whereas its constituent amino acid, leucine (20 mM) failed to inhibit either [^{14}C]-Gly-Sar transport or intracellular accumulation (Table 1). The position of the amino acids in the peptide is also important. The inhibitory effects of Pro-Gly on [^{14}C]-Gly-Sar transport and accumulation were significantly reduced compared to Gly-Pro ($P<0.05$ for transport, $P<0.01$ for uptake). Dipeptides containing one D-amino acid had a reduced effect, while dipeptides containing two D-amino acids had no significant effect on [^{14}C]-Gly-Sar transport and accumulation (Table 1). The tripeptide Gly-Gly-Gly significantly reduced transport and accumulation of [^{14}C]-Gly-Sar although a second tripeptide, the orally-active thyrotropin-releasing hormone (TRH; pGlu-His-Pro-NH_2), was without effect. The tetrapeptide tetraglycine caused no significant reduction although a second tetrapeptide (tetra-alanine) did significantly reduce both [^{14}C]-Gly-Sar transport and accumulation.

Dipeptide (Gly-Sar) transport across the apical membrane is associated with the movement of protons across the plasma membrane that can be detected by the resultant acidification of the intracellular environment (Thwaites *et al.*, 1993a). Figure 1 clearly indicates that after apical exposure to the dipeptide Gly-Sar at 20 mM (apical pH 6.5, basolateral pH 7.4) the rate of intracellular acidification (ΔpH_i min^{-1} 0.127 ± 0.021, $n=3$) due to substrate-induced H^+ flow into the cells is significantly greater ($P<0.001$) than in the presence of apical pH 6.5 alone (0.013 ± 0.010, $n=3$). A number of dipeptides including Gly-Pro, Gly-Leu, Gly-Phe, Gly-Gln, carnosine (not shown) and Val-Val (Thwaites *et al.*, 1993c) gave similar responses. The Gly-Pro induced intracellular acidification was significantly greater than that observed with Pro-Gly ($P<0.01$). Dipeptides containing D-amino acids (at both N and C terminals) such as D-Leu-D-Leu (Figure 1) and D-Ala-D-Ala (not shown) did not induce intracellular acidification when perfused (at 20 mM) at the apical membrane of Caco-2 cell monolayers. D-Leu-D-Leu (ΔpH_i min^{-1} 0.030 ± 0.007, $n=6$) failed to increase significantly ($P>0.05$) the rate of intracellular acidification observ-

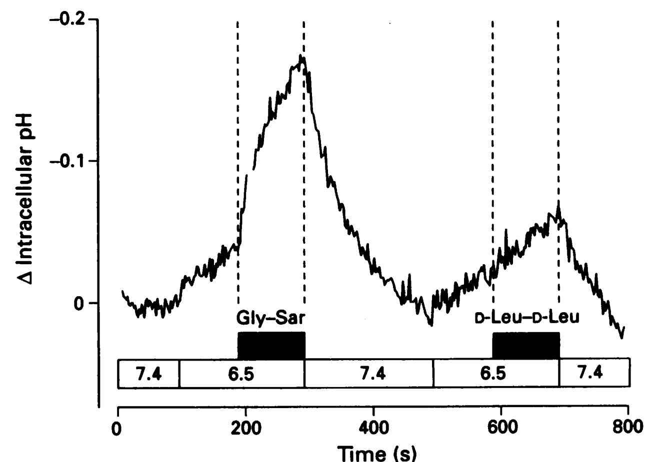

Figure 1 Intracellular pH measured in BCECF-loaded Caco-2 cell monolayers. The relative change in pH_i induced by apical superfusion of either 20 mM Gly-Sar or 20 mM D-Leu-D-Leu at apical pH 6.5 (basolateral pH 7.4). This figure is representative of four separate experiments.

ed with apical acidity alone (0.022 ± 0.004, $n = 7$), whilst the substrate-specific change with D-Leu-D-Leu (0.012 ± 0.004, $n = 6$) was minor compared to Gly-Sar (0.115 ± 0.018 ($n = 3$), $P < 0.001$).

Figure 2 demonstrates that intracellular acidification results from the action of the intact dipeptide (Leu-Leu) since the product of hydrolysis (leucine) does not produce cytosolic acidification. Leu-Leu significantly increased ($P < 0.01$) the rate of acidification (0.083 ± 0.018, $n = 7$) above the levels noted with apical acidity alone (0.016 ± 0.005, $n = 7$). This effect of Leu-Leu (0.068 ± 0.016, $n = 7$, above apical acidity alone) was significantly greater than the specific effects of leucine (0.014 ± 0.007, $n = 4$, $P < 0.05$) or D-Leu-D-Leu (0.012 ± 0.004, $n = 6$, $P < 0.05$). This confirms a previous study with the dipeptide Val-Val and its constituent amino acid valine (Thwaites *et al.*, 1993c).

Figure 3 indicates that tetrapeptides are relatively poor subtrates compared to di and tripeptides. The perfusion of either Gly-Gly or Gly-Gly-Gly at the apical surface of Caco-2 cell monolayers was associated with a significant increase in the rate of intracellular acidification compared with apical acidity alone (an increase above the rate with apical acidity alone of 0.123 ± 0.025 ($n = 5$) with Gly-Gly and 0.079 ± 0.018 ($n = 5$) with Gly-Gly-Gly, both $P < 0.001$ compared to apical acidity alone). Although an intracellular acidification is observed in the presence of tetraglycine (Figure 3; 0.028 ± 0.009, $n = 5$), the rate of acidification is significantly reduced compared to Gly-Gly and Gly-Gly-Gly (both $P < 0.01$). The relative magnitude of excursions in pH_i associated with each peptide are similar in nature to their relative inhibitory effects on [^{14}C]-Gly-Sar transport and accumulation (Table 1). Figure 3 demonstrates that, in contrast to leucine and valine, apical perfusion of glycine (the constituent amino acid of the three peptides used in Figure 3) is associated with intracellular acidification (0.121 ± 0.023, $n = 5$, $P < 0.001$ compared to apical acidity alone), of similar magnitude to that observed with the peptides Gly-Gly and Gly-Gly-Gly (both $P > 0.05$ compared to glycine). Glycine (20 mM) also caused a significant reduction in both [^{14}C]-Gly-Sar transport (49.9 ± 6.7% of control, $n = 14$, $P < 0.001$) and intracellular accumulation (52.4 ± 5.8% of control, $n = 14$, $P < 0.001$). However, the inhibitory effects of glycine on [^{14}C]-Gly-Sar transport and accumulation were significantly reduced compared to the effects of Gly-Gly ($P < 0.001$ for transport and uptake) and Gly-Gly-Gly ($P < 0.01$ for transport, $P < 0.05$ for uptake).

Three orally-active aminocephalosporin antibiotics (cephalexin, cefadroxil and cephradine) that have previously been identified as substrates for transport by the intestinal di/tripeptide carrier inhibited both transport and accumulation (Table 1). However, cefazolin had a reduced effect on [^{14}C]-Gly-Sar transport ($P < 0.01$) and no effect on intracellular accumulation ($P < 0.05$). Figure 4 compares the effects on pH_i of the orally-available cephalosporin antibiotic, cephalexin with the parenterally-administered, cefazolin. Both substrates (20 mM) were perfused at the apical surface of the Caco-2 cell monolayers in the presence of apical acidity. Clearly there is a difference in the responses to the two substrates. Cephalexin (CEX) significantly ($P < 0.05$) increases the rate of acidification (0.123 ± 0.029, $n = 5$ above apical acidity alone) and the intracellular environment is continuing to acidify at the end of the 100 s pulse (0.135 ± 0.20, $n = 5$). In contrast although there is also a rapid initial acidification when cefazolin (CEZ) is perfused at the apical surface (0.030 ± 0.021 ($n = 5$) above apical acidity alone) the response is significantly lower ($P < 0.05$) compared to the cephalexin-induced response, and reaches a plateau after approximately 20 s. At the end of the 100 s pulse the rate of acidification due to cefazolin (0.010 ± 0.038, $n = 5$) is significantly reduced compared to cephalexin ($P < 0.05$). When perfused at apical pH 6.5 (basolateral pH 7.4) at 20 mM both

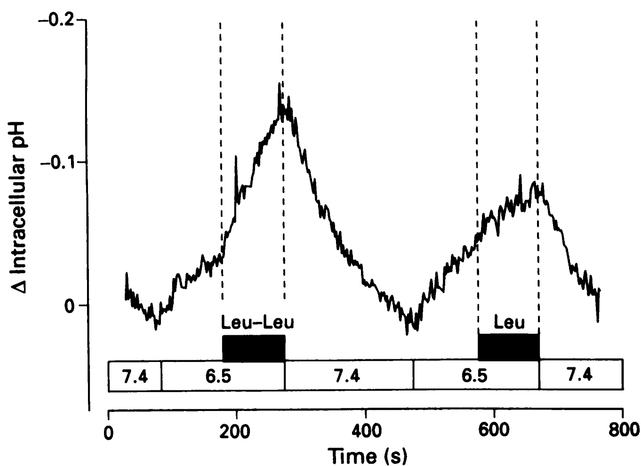

Figure 2 Intracellular pH measured in BCECF-loaded Caco-2 cell monolayers. The effect on intracellular pH of apical exposure to either 20 mM Leu-Leu or 20 mM leucine. Both substrates were superfused across the apical surface at pH 6.5. Basolateral pH was maintained at pH 7.4. The figure is a representative trace of three separate experiments.

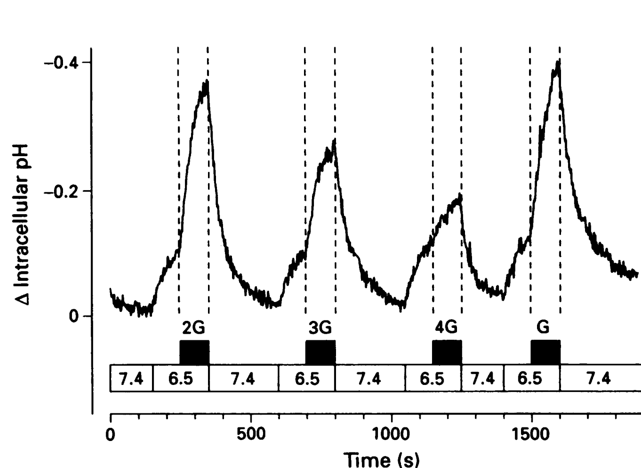

Figure 3 Intracellular pH measured in BCECF-loaded Caco-2 monolayers. The effects of apical exposure (all 10 mM, pH 6.5) to Gly-Gly (2G), Gly-Gly-Gly (3G), Gly-Gly-Gly-Gly (4G) and glycine (G). Basolateral pH was maintained at pH 7.4. The trace is representative of three separate experiments.

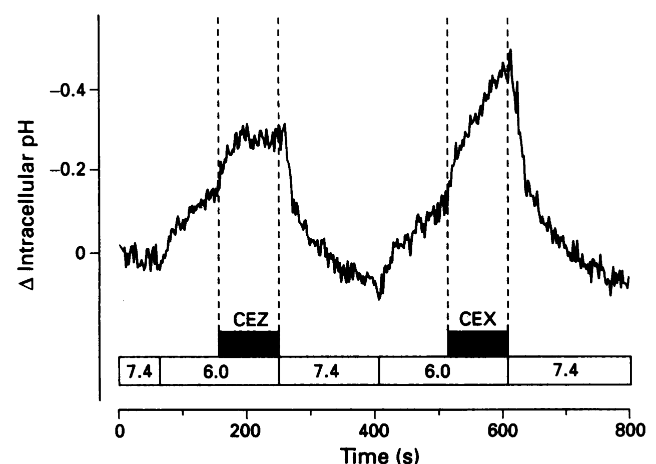

Figure 4 Intracellular pH measured in BCECF-loaded Caco-2 monolayers. The effect of 20 mM cephalexin (CEX) or 20 mM cefazolin (CEZ) on pH_i after exposure at the apical surface at pH 6.0. The basolateral pH was maintained at pH 7.4. This experiment is representative of four others.

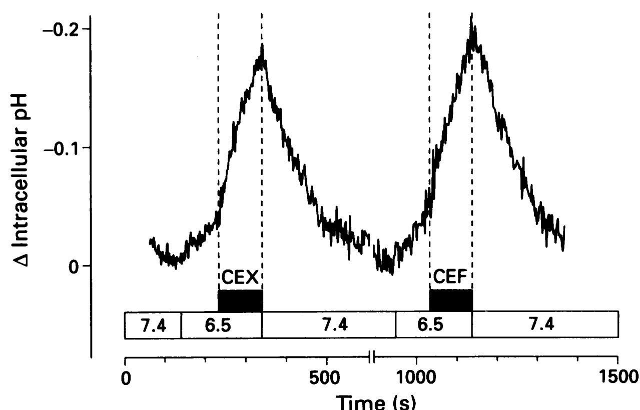

Figure 5 Intracellular pH changes in BCECF-loaded Caco-2 monolayers. The effect of cephalexin (CEX) and cefadroxil (CEF), when superfused (at 20 mM) at the apical surface at pH 6.5 (basolateral pH 7.4).

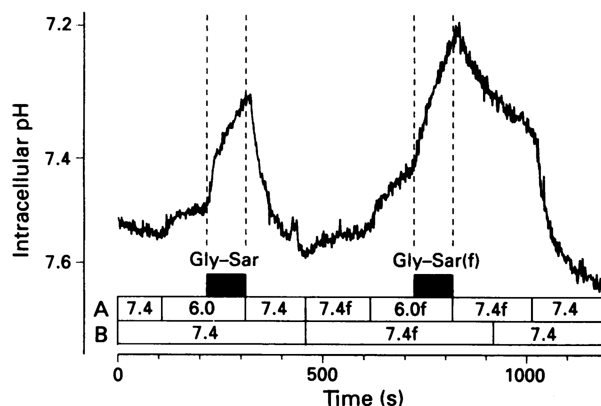

Figure 6 Intracellular pH changes in BCECF-loaded Caco-2 monolayers. The effect of Gly-Sar (20 mM) superfused at the apical surface (at pH 6.0) of Caco-2 cell monolayers in both Na^+-containing and Na^+-free conditions. (f) denotes Na^+-free conditions. Note that the Na^+-containing solutions are re-introduced sequentially to the basolateral (B) then apical (A) membranes.

cephalexin (CEX) and cefadroxil (CEF) are associated with intracellular acidification which is reversible upon removal of the two substrates from the apical chamber (Figure 5).

The experiment illustrated in Figure 6 demonstrates the Na^+-independent nature of the dipeptide-induced intracellular acidification. In a separate series of experiments the relative rates of dipeptide-induced acidification and the nature of the pH_i recovery mechanisms were determined in Na^+ and Na^+-free conditions. The cells were perfused for approximately 200 s until a relatively steady baseline was maintained (rate of acidification 0.001 ± 0.001, $n = 5$). When Na^+-free (pH 7.4) solutions were added to both apical and basal chambers the rate of acidification was 0.006 ± 0.004 ($n = 5$) which increased with apical pH 6.0 (Na^+-free) to 0.049 ± 0.009 ($n = 5$), and was further increased in the presence of apical Gly-Sar (0.158 ± 0.009, $n = 5$). When Gly-Sar was removed from the apical chamber but the transepithelial pH gradient maintained (apical pH 6.0 and basolateral pH 7.4, both Na^+-free) the rate of acidification decreased to 0.017 ± 0.012 ($n = 5$). When Na^+-free pH 7.4 buffer was returned to the apical chamber the intracellular pH recovery from the dipeptide-induced acid load was at a rate of 0.037 ± 0.011 ($n = 5$). Addition of Na^+ to the basolateral chamber had no effect (rate of recovery 0.029 ± 0.010, $n = 5$) whereas addition of Na^+ to the apical chamber markedly increased the rate of recovery (0.109 ± 0.024, $n = 5$). These data demonstrate that a Na^+/H^+ exchange mechanism is localized at the apical surface and confirms observations with the dipeptide Val-Val (Thwaites *et al.*, 1993c).

Discussion

The present study was designed to investigate the specificity of H^+-coupled transport in an epithelial context through the use of the human intestinal Caco-2 cell line. This cell line is known to express pH-dependent accumulation (Dantzig & Bergin, 1990) and transport of the orally-absorbed cephalosporins, cephalexin and cephradine (Inui *et al.*, 1992). These studies confirm earlier observations of pH-dependent aminocephalosporin uptake in rabbit brush-border membrane vesicle preparations (Okano *et al.*, 1986a). More recently our own studies have revealed the pH-dependent nature of dipeptide (Gly-Sar) transport and accumulation in this cell line (Thwaites *et al.*, 1993a). These data complement previous studies with the dipeptide Gly-Sar using rabbit intestinal brush border membrane vesicles (BBMV) (Ganapathy *et al.*, 1984; Ganapthy & Leibach, 1985; Thwaites *et al.*, 1993f). Importantly, we have demonstrated direct coupling of H^+ flow to dipeptide transport across the apical brush-border of these intestinal epithelial cells (Thwaites *et al.*, 1993a). This H^+-coupled dipeptide transport is electrogenic, stimulating inward I_{sc} in voltage-clamped Caco-2 cell monolayers (Thwaites *et al.*, 1993g) and is subject to regulation (inhibition) by protein kinase C (Brandsch *et al.*, 1994). Recently the cDNA encoding for a 707-amino-acid peptide transporter (PepT1) has been isolated from rabbit small intestine by expression cloning in *Xenopus laevis* oocytes (Fei *et al.*, 1994). *Xenopus* oocytes injected with PepT1 cRNA showed a 63 fold increase in [^{14}C]-Gly-Sar uptake when compared with control oocytes (Fei *et al.*, 1994). Furthermore, experiments in voltage-clamped oocytes (Fei *et al.*, 1994) indicate that transport of Gly-Sar is electrogenic, as demonstrated previously in Caco-2 cells (Thwaites *et al.*, 1993g). The Gly-Sar-evoked inward current in PepT1 cRNA-injected oocytes was associated with intracellular acidification (Fei *et al.*, 1994), demonstrating H^+/dipeptide symport, as had previously been demonstrated in Caco-2 cells (Thwaites *et al.*, 1993a,b,c).

Transepithelial transport and accumulation of the dipeptide Gly-Sar are inhibited by a range of other dipeptides including Gly-Pro, carnosine, Gly-Leu and Pro-Gly. Experiments with rabbit intestinal BBMV (Ganapathy *et al.*, 1984) show a similar inhibition pattern to that described here in Caco-2 cell monolayers. In both preparations Pro-Gly has the lowest inhibitory effect on Gly-Sar uptake/transport (Ganapathy *et al.*, 1984; Table 1). The ability of these compounds to inhibit Gly-Sar transport appears directly related to their ability to induce H^+ flow into the cells as determined by changes in intracellular pH (Figures 1–2). Gly-Pro, Gly-Leu, Gly-Phe, Gly-Gln, carnosine, Leu-Leu (Figure 2) and Val-Val (Thwaites *et al.*, 1993c) are all associated with intracellular acidification. Comparison of the effect on pH_i of Leu-Leu and its constituent amino acid (Figure 2) shows that the action is a dipeptide-specific effect. Leucine also failed to inhibit pH-stimulated [^{14}C]-Gly-Sar transport or accumulation (Table 1). This confirms a previous study in Caco-2 cells with the dipeptide Val-Val and the amino acid valine (Thwaites *et al.*, 1993c). Early experiments in the hamster jejunum *in vitro* demonstrated that the mucosal uptake of the dipeptide Leu-Leu was pH-dependent with a maximal uptake at apical pH 6.0 (Matthews & Burston, 1983).

The inhibitory effect of the tripeptide Gly-Gly-Gly suggests that tripeptides may also access this H^+-coupled transporter in Caco-2 epithelia, as demonstrated in other intestinal preparations (Matthews & Adibi, 1976). However, TRH (pGlu-His-Pro-NH_2) failed to inhibit Gly-Sar transport/accumulation. This confirms our previous studies of TRH transport in both rabbit and rat intestinal BBMV (Thwaites

et al., 1993h) and Caco-2 cell monolayers (Thwaites *et al.*, 1993d). It is thus likely that a passive paracellular route is the major pathway for the oral absorption of certain peptides such as TRH. The tetrapeptide, tetraglycine, failed to inhibit Gly-Sar accumulation confirming previous observations in the hamster jejunum *in vitro* (Matthews & Adibi, 1976; Sleisenger *et al.*, 1976) that transport by the intestinal small peptide carrier is restricted to substrates 2–3 amino acids in length. Adibi & Moore (1977) demonstrated (in studies in perfused human jejunum) that the rate of disappearance (and, therefore, absorption) of tetraglycine was slow when compared to Gly-Gly and Gly-Gly-Gly, linear up to 50 mM, and not inhibited by the dipeptide Gly-Leu, although Gly-Leu did inhibit Gly-Gly-Gly absorption. Gly-Leu also inhibits [^{14}C]-Gly-Sar transport in this study (Table 1). Several substrates that inhibit [^{14}C]-Gly-Sar transport (Table 1) and cause intracellular acidification in Caco-2 cells (including cefadroxil, cephalexin, Gly-Leu, Leu-Leu, carnosine, Gly-Gly and Gly-Gly-Gly) also evoked inward currents in PepT1 cRNA-injected oocytes whereas tetraglycine was without effect (Fei *et al.*, 1994). The amino acid glycine did not evoke inward current nor cause intracellular acidification in PepT1 cRNA-injected oocytes (Fei *et al.*, 1994). This observation (when considered with the relative magnitude of the pH_i changes in the presence of glycine, Gly-Gly and Gly-Gly-Gly in Caco-2 cells (Figure 3)), strengthens our hypothesis that the inhibitory effects of glycine on [^{14}C]-Gly-Sar transport are due to non-specific effects on the transepithelial pH gradient and that glycine transport is likely to be via another H^+-coupled carrier, most probably the H^+-coupled amino acid transporter in Caco-2 cells that plays a role in β-alanine (Thwaites *et al.*, 1993e), L-proline (Thwaites *et al.*, 1993f) and L-alanine (Thwaites *et al.*, 1994) transport. The inhibitory effects of tetra-alanine on [^{14}C]-Gly-Sar transport may suggest that the peptide carrier transports some tetrapeptides. However, this inhibitory effect could result from rapid hydrolysis to Ala-Ala (which also significantly reduces [^{14}C]-Gly-Sar transport; Table 1) or to L-alanine (Thwaites *et al.*, 1994) which could inhibit dipeptide transport by an effect on the driving force (the pH gradient) as suggested with glycine (see Discussion above). Thus the ability of peptide substrates (whose metabolism may yield true substrates for the dipeptide carrier or substrates for an alternative H^+-coupled transporter) to access the H^+/dipeptide carrier cannot be distinguished unequivocally in the Caco-2 cell system. In rabbit renal BBMV preparations, 1 mM tetra-alanine was completely hydrolysed to Ala-Ala in 5 s (Daniel *et al.*, 1992) whereas tetraglycine did not undergo hydrolysis.

The stereoselectivity (Matthews, 1975) of the transporter is indicated by the absence of inhibitory effects of D-Ala-D-Ala and D-Leu-D-Leu, and the reduced effects of Gly-D-Leu and D-Leu-Gly (Table 1). D-Leu-D-Leu (Figure 1) and D-Ala-D-Ala (not shown) also failed to affect intracellular pH when perfused at the apical surface of Caco-2 cells. These observations of stereoselectivity are consisent with previous studies in both human and rat intestine (Matthews, 1975). It is thought that introduction of D-amino acids into a dipeptide reduces affinity for the entry mechanism (Matthews, 1975).

Table 1 indicates that the inhibition profiles from this study and those with cephradine (Inui *et al.*, 1992) show a broadly similar pattern. In Caco-2 cell monolayers, pH-dependent cephradine transport and accumulation are inhibited by the dipeptides carnosine, Gly-Leu and Gly-Pro, and the cephalosporin cephalexin. Most recently pH-dependent transport of the anti-cancer agent, bestatin, has been demonstrated in Caco-2 cells and attributed to transport via the di/tripeptide carrier (Saito & Inui, 1993). The pH-dependent accumulation of bestatin across the apical surface of Caco-2 cells was inhibited by the dipeptides Gly-Sar, Gly-Leu, Gly-Pro and carnosine, and the cephalosporin, cephradine (Saito & Inui, 1993). Similar patterns of inhibition are indicated in Table 1. In studies with rabbit intestinal BBMV, uptake of cephradine, cephalexin and cefadroxil were enhanced by an inwardly-directed pH gradient whereas cefazolin uptake was low in comparison and was not stimulated by lowering extravesicular pH (Okano *et al.*, 1986a). This pH-dependent uptake of cephradine in rat intestinal BBMV was reduced in the presence of di and tripeptides (Okano *et al.*, 1986b). The orally-absorbed cephalosporins (cephradine, cefadroxil and cephalexin) all inhibit pH-stimulated [^{14}C]-Gly-Sar transport and intracellular accumulation in Caco-2 cell monolayers. In comparison cefazolin had a reduced effect which is reflected in its reduced effect on intracellular pH (Figure 4). This reduced affinity for the intestinal di/tripeptide transporter may account for the poorer oral absorption of this compound (Bergan, 1984). Cephalexin and cefadroxil both stimulated H^+ flow (Figure 4–5).

An additional feature of transport across an intact epithelial preparation is that it is also dependent upon dipeptide exit across the basolateral membrane. Although the majority of studies discussed above have concentrated on the role of the apical membrane in transepithelial transport, the importance of the basolateral membrane should not be underestimated. It is likely that this is the rate-limiting step in Gly-Sar transport (Thwaites *et al.*, 1993b). Gly-Sar and cephalexin have similar effects on pH_i when perfused at the apical surface of Caco-2 cells but show clear differences when perfused at the basolateral membrane (Thwaites *et al.*, 1993b). At basolateral pH 7.4, cephalexin caused a marked cytosolic acidification whereas Gly-Sar was without effect (Thwaites *et al.*, 1993b). Estimates of Gly-Sar and cephradine efflux in Caco-2 cell monolayers show that Gly-Sar leaves the cells predominantly across the apical surface (Thwaites *et al.*, 1993b) whereas cephradine leaves across the basolateral membrane (Inui *et al.*, 1992). These observations suggest that dipeptide and aminocephalosporin transport across the basolateral membrane of intestinal enterocytes may be via more than one mechanism.

There are likely to be several transport mechanisms in the gastrointestinal tract allowing nutrient absorption energized by the proton gradient present across the apical membrane of intestinal enterocyte. This area of acidity close to the apical surface has been demonstrated both *in vivo* (Rawlings *et al.*, 1987) and *in vitro* (Lucas *et al.*, 1978). The model proposed by Ganapathy & Leibach (1985) suggests that the pH gradient across the apical membrane of the intestinal enterocyte is maintained during H^+-coupled dipeptide transport by Na^+/H^+ exchange at the apical surface and Na^+ extrusion via the Na^+,K^+-ATPase at the basolateral membrane. The present data confirm the role of an apically-localized Na^+/H^+ exchanger in H^+-coupled dipeptide absorption.

The selectivity of the H^+-coupled small peptide carrier expressed at the apical surface of human intestinal epithelial Caco-2 cells has been established by competition studies with [^{14}C]-Gly-Sar and intracellular pH measurements with the pH-sensitive fluorescent dye BECEF. The carrier transports a range of di/tripeptides and cephalosporin antibiotics with an α-amino group. Affinity for the transporter is significantly reduced with peptides greater than three amino acids in length, peptides containing D-amino acids (at both N and C terminals) and cephalosporins without the α-amino group (cefazolin) as well as peptides with blocked N- and C-termini such as TRH. This specificity profile is similar to that observed in experiments with *Xenopus laevis* oocytes expressing the rabbit intestinal oligopeptide transporter (PepT1). A major advantage of the Caco-2 cell system (over oocytes expressing PepT1) is that alternative transport pathways (either H^+-linked transporters, Na^+-dependent transporters or the paracellular pathway) may be studied within the same cell system in an appropriate physiological context. Finally, the use of intracellular pH measurements to determine H^+ flow across either the apical or basolateral membranes provides a simple and rapid method for assessing the potential of novel drug compounds to undergo H^+-coupled intestinal absorption.

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