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H^+-coupled dipeptide (glycylsarcosine) transport across apical and basal borders of human intestinal Caco-2 cell monolayers display distinctive characteristics

David T. Thwaites, Colin D.A. Brown, Barry H. Hirst and Nicholas L. Simmons

Gastrointestinal Drug Delivery Research Centre, Department of Physiological Sciences, University of Newcastle upon Tyne, Medical School, Newcastle upon Tyne (UK)

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Transepithelial transport and intracellular accumulation of the dipeptide glycylsarcosine (Gly-Sar) were studied using intact monolayers of the human intestinal epithelial cell line, Caco-2. Gly-Sar transport was demonstrated in both absorptive (apical-to-basal) and secretory (basal-to-apical) directions. In both directions, transport and accumulation were enhanced in the presence of a pH gradient ($pH_o < pH_i$). Under conditions similar to those found at the intestinal membrane in vivo (apical pH 6.0, basolateral pH 7.4), net absorption (145.2 pmol/cm^2 per h) was observed, although experimental conditions could also be manipulated (apical pH 7.4, basolateral pH 6.0) so that net secretion was observed. Transport and accumulation (in both directions) were inhibited in the presence of either 20 mM (unlabelled) Gly-Sar or 20 mM cephalexin (an aminocephalosporin antibiotic). When added to either the apical or basolateral surface of BCECF (2',7',-bis(2-carboxyethyl)-5(6)-carboxyfluorescein)-loaded Caco-2 cell monolayers Gly-Sar (20 mM), at pH 6.0, caused a marked intracellular acidification, demonstrating that dipeptide absorption is accompanied by H^+-flow into the cells. Cephalexin (20 mM) had similar effects (as Gly-Sar) when presented at the apical surface but also caused a marked intracellular acidification when perfused into the basolateral chamber at pH 7.4. In contrast, addition of Gly-Sar (20 mM) to the basolateral chamber (at pH 7.4) had no effect. Transepithelial absorption of dipeptides (Gly-Sar) and β-lactam antibiotics (cephalexin) at low concentrations is predominately via a transcellular route mediated by carrier mechanisms located at both apical and basolateral membranes. Interestingly, Gly-Sar and cephalexin transport across the basolateral membrane (and, therefore, exit from the cell) display both common and distinct characteristics suggesting that more than one mechanism may be responsible for exit into the basolateral space.

Introduction

The Caco-2 cell system has been identified as an appropriate model for intestinal epithelial permeability studies [1] expressing a number of solute transporters including those for sugars [2–4], amino acids [5,6] and bile acids [7]. This cell line also expresses the transporter responsible for dipeptide and aminocephalosporin uptake [8]. Transepithelial transport of the orally-absorbed cephalosporin cephradine across epithelial monolayers of Caco-2 cells has subsequently been demonstrated by Inui et al. [9] confirming the validity of this human model system to determine fundamental properties associated with dipeptide/aminocephalosporin transport across both apical and basolateral membranes.

Studies with intestinal brush-border membrane vesicles (BBMV) have clearly demonstrated that the electrochemical gradient for H^+, rather than Na^+, energises di/tripeptide absorption [10]. The electrogenicity of dipeptide transport has been demonstrated in intact tissue [11]. Similarly, voltage clamping BBMV (inside negative) with a K^+ gradient (in the presence of valinomycin) accelerates dipeptide uptake [12–14] consistent with the electrogenic nature of dipeptide/H^+ transport. Recently using intact epithelial monolayers of Caco-2 cells we have demonstrated direct coupling of dipeptide transport to proton flux by measurement of pH_i with the pH-sensitive fluorescent dye BCECF [15].

In contrast to the apical membrane, the mechanism(s) involved in dipeptide exit across the basolateral

Correspondence to: D.T. Thwaites, Gastrointestinal Drug Delivery Research Centre, Department of Physiological Sciences, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK. Fax: +44 91 2226706.

membrane remains unclear, though recent evidence points to H^+-gradient acceleration of dipeptide uptake in rabbit intestinal enterocyte basolateral membrane vesicles [16]. Inui et al. [9] have directly measured cephradine efflux across both apical and basolateral membranes of Caco-2 cells and have confirmed that basolateral exit is mediated by a distinct *p*-chloromercuribenzene sulfonate-inhibitable transporter. Our own studies, on intact Caco-2 cell monolayers, provide evidence that Gly-Sar transport across the basolateral membrane face is also dependent upon a proton-gradient [15] which under certain circumstances leads to dipeptide-induced intracellular acidification.

The purpose of the present investigation has been to extend our observations of Gly-Sar accumulation and transport by epithelial monolayers of Caco-2 cells, specific attention being paid to the mechanism(s) of dipeptide and cephalosporin transport at the basolateral membrane.

Materials and Methods

Materials

[^{3}H]Mannitol (specific activity 30 Ci/mmol) was obtained from NEN. [^{14}C]Gly-Sar (L-glycyl[1-^{14}C]-sarcosine (specific activity 14 mCi/mmol)) was from Amersham. Gly-Sar, sarcosine and cephalexin were from Sigma. Acetonitrile (HPLC grade S) was supplied by Rathburn (UK). BCECF, cell culture media, supplements and plastic were supplied by Life Technologies. Thin-layer chromatography plates (silica gel and cellulose), acetic acid, n-butanol, ninhydrin spray and all other chemicals were from Merck and were of the highest quality available.

Cell culture

Caco-2 cells (passage number 94–101, 108 and 119–122) were cultured in DMEM (with 4.5 g/l glucose), with 1% non-essential amino acids, 2 mM L-glutamine, 10% (v/v) foetal calf serum and gentamicin (60 μg/ml). Cell monolayers were prepared by seeding at high density ($(4.4–5.0) \cdot 10^5$ cells/cm^2) onto tissue culture inserts (Transwell polycarbonate filters (Costar)). Cell monolayers were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Cell confluence was estimated by microscopy and determination of transepithelial electrical resistance (R_T), measured at 37°C.

Transport experiments

Uptake and transport experiments with Gly-Sar were performed 18–28 days after seeding (unless stated otherwise) and 18–24 h after feeding. Transepithelial flux measurements were performed as described previously [15]. Briefly, the cell monolayers (24.5 mm in diameter) were placed in 6-well plates, each well containing 2 ml of modified Krebs buffer (all mmol/l), 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, 37°C). The pH 6.0 buffer used in both transport and intracellular pH experiments was identical except that 10 mM Mes-Tris replaced 10 mM Hepes-Tris. A Na^+-free Krebs was obtained by replacement of NaCl by choline Cl and omission of NaH_2PO_4. 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. This procedure was repeated with fresh buffer (pH 7.4). The experimental composition of the buffers in the apical and basal chambers were identical except where stated otherwise. Radiolabelled Gly-Sar (0.5 μCi/ml; 36 μM) and mannitol (0.5 μCi/ml; 36 μM) were added to either the apical or basolateral chamber. In experiments involving high (20 mM) concentrations of Gly-Sar or cephalexin iso-osmolarity was maintained by addition of mannitol to control samples. Fluxes in both the absorptive (apical-to-basal, J_{a-b}) and secretory (basal-to-apical, J_{b-a}) directions were determined. Fluxes across the monolayers into the contralateral chamber are expressed as pmol/cm^2 per h, and in some cases (where stated) mannitol transport (passive/paracellular flux) is subtracted so that the stated values represent an estimate of transcellular Gly-Sar transport only. 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 loosely-associated radiolabel, and removed from the insert. Cell monolayer-associated radiolabel was determined by scintillation counting. Cellular accumulation of Gly-Sar (after subtraction of filter-associated mannitol values) is expressed as μM. Cell height was determined by confocal microscopy and this value was used in the determination of intracellular volume. Results are expressed as means $\pm$ S.E.

Efflux of [^{14}C]Gly-Sar across both apical and basolateral borders was measured by first loading monolayers with [^{14}C]Gly-Sar to equilibrium as indicated above (1 h). The monolayers were then washed (three times) with fresh Krebs. Loss of cellular [^{14}C]Gly-Sar into the apical and basolateral chambers (each containing 2 ml Krebs buffer, either pH 6.0 or 7.4) was determined after 30- and 60-min incubations. Remaining cellular [^{14}C]Gly-Sar was determined as described above.

Intracellular pH (pH_i) measurements

For intracellular pH (pH_i) measurements [15], Caco-2 cells grown to confluence 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) which allowed any combination of 6 apical and 6 basolateral solutions. Apical and basolateral bath volumes were 0.5 and 1 ml and bath contents could be completely changed in 6 or 12 s, respectively. All solutions were preheated to 37°C. 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 [17].

Thin-layer chromatography

The identity of the ^{14}C-label (either in the apical/basolateral chamber or that having crossed the cell monolayer into the contralateral chamber) at the end of the incubation period (up to 200 min) was determined. Two separate solvent systems were used consisting of methanol/acetonitrile/water (9:0.5:0.5, v/v [13]) and n-butanol/water/acetic acid (12:5:3, v/v). Using both systems the ^{14}C radioactivity was associated with the Gly-Sar standard only, as revealed by ninhydrin staining and scintillation counting.

Results

Transepithelial [^{14}C]Gly-Sar transport and cellular accumulation: the effect of extracellular pH

Transepithelial Gly-Sar transport in the apical-to-basal direction ($J_{a\text{-}b}$) was accelerated compared to the inert tracer mannitol (Fig. 1). After an initial lag phase transport was linear with respect to time up to 4 h. Transepithelial [^{14}C]Gly-Sar transport, from apical-to-basal bathing solutions (above those equivalent levels

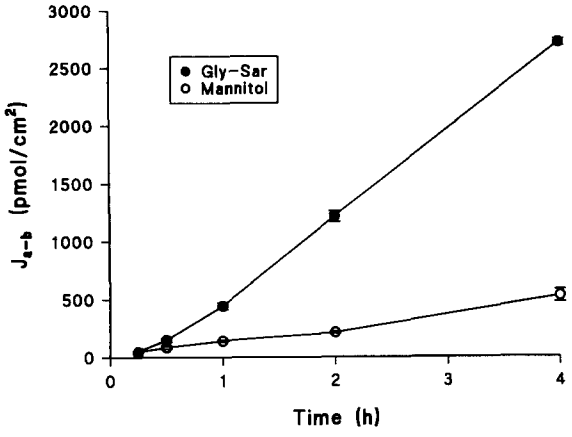

Fig. 1. Time-dependent transport of [^{14}C]Gly-Sar (filled circles) and [^3H]mannitol (open circles) from apical-to-basal surfaces of Caco-2 cell monolayers. Gly-Sar was initially absent from the basolateral chamber. Apical medium pH was 6.0 and basolateral pH 7.4. Apical Gly-Sar and mannitol concentrations were 36 μM. Data are the means ± S.E. of 3–5 separate monolayers.

for mannitol transport), were detectable as early as 7 days (post-seeding) in culture. At day 7, pH-dependent (apical pH 6.0, basolateral pH 7.4) dipeptide transport (106.4 ± 13.5 pmol/cm^2 per h, $n = 6$) and accumulation (553.2 ± 23.0 μM, $n = 6$) was detected. At pH 7.4, although there was intracellular accumulation (171.4 ± 6.3 μM, $n = 5$) transepithelial transport was low, being approximately 50 pmol/cm^2 per h lower than transport of the paracellular marker mannitol. By day 11 post-seeding, Gly-Sar transport (above mannitol levels) had increased at both pH 6.0 (196.5 ± 19.2 pmol/cm^2 per h, $n = 5$) and pH 7.4 (19.7 ± 5.5 pmol/cm^2 per h, $n = 3$). Accumulation at pH 6.0 remained steady (574.0 ± 16.5 μM, $n = 6$) but increased at pH 7.4 (247.6 ± 6.8 μM, $n = 5$). After 14 days in culture pH dependent transport (256.2 ± 11.9 pmol/cm^2 per h, $n = 4$) and accumulation (682.6 ± 20.0 μM, $n = 6$) were maximal and were maintained at 18 and 21 days in culture. Similarly, in the absence of a pH gradient, Gly-Sar transport (49.0 ± 9.1 pmol/cm^2 per h, $n = 3$) and accumulation (297.5 ± 20.7 μM, $n = 6$) were maximal after 14 days and maintained at 18 and 21 days post-seeding.

Fig. 2(a) highlights the effect of varying extracellular pH on transepithelial transport of Gly-Sar in both apical-to-basal ($J_{a\text{-}b}$) and basal-to-apical ($J_{b\text{-}a}$) directions. Even when both apical and basolateral pH were maintained at pH 7.4 it was evident that $J_{a\text{-}b}$ exceeds $J_{b\text{-}a}$. Thus net dipeptide absorption of 145.2 ± 10.8 pmol/cm^2 per h ($n = 6$) occurred in the absence of an externally applied pH gradient. Acidification of the apical bathing solution (whilst maintaining basolateral pH at 7.4) resulted in a marked increase in $J_{a\text{-}b}$ and reduction of $J_{b\text{-}a}$, i.e., net Gly-Sar absorption was increased to 403.2 pmol/cm^2 per h. When the apical pH was held at pH 7.4 and basolateral pH acidified to pH 6.0, Gly-Sar ($J_{b\text{-}a}$) flux was increased whilst $J_{a\text{-}b}$ fell only slightly. Importantly with a reversed transepithelial pH gradient from basal-to-apical surfaces, net Gly-Sar flux was reversed to become a net secretion of −211.7 ± 15.9 pmol/cm^2 per h. Fig. 2(b) shows the effects of these extracellular pH manipulations on intracellular accumulation of Gly-Sar from apical or basolateral solutions. With both bathing solutions at pH 7.4 there was a marked asymmetry in cellular accumulation, accumulation across the apical border (6.6-fold greater than in apical solution) exceeding that across the basolateral border by 5.5-fold. Cellular accumulation across the basolateral border was hardly more than equilibration (1.2-fold). Apical acidification increased accumulation of Gly-Sar to 8.1-fold greater than in the apical solution. In contrast basolateral acidification increased accumulation of Gly-Sar across the basolateral cell aspects to 2.8-fold greater than the basolateral solution concentration. However, even in the presence of basal acidification accumulation of

TABLE I

Transepithelial bidirectional fluxes of Gly-Sar ($J_{a\text{-}b}$, $J_{b\text{-}a}$) from either apical (a) or basolateral (b) surfaces together with calculated unidirectional fluxes across the apical ($J_{a\text{-}c}$, $J_{c\text{-}a}$) membrane to and from the cell (c) and across the basolateral membrane ($J_{b\text{-}c}$, $J_{c\text{-}b}$).

All fluxes are expressed in pmol/cm^2 per h. Apical and basal [Gly-Sar] was 36 μM. The ratio R is the relative accumulation of Gly-Sar (from apical surface)/(from basolateral surface).

Condition			$J_{a\text{-}b}$	$J_{b\text{-}a}$	J_{net}	R	$J_{a\text{-}c}$	$J_{c\text{-}a}$	$J_{c\text{-}b}$	$J_{b\text{-}c}$
apical	basal	n								
7.4	7.4	6	308.7± 9.0	163.5± 6.0	145.2±10.8	5.5	1206.3	1061.1	364.9	219.7
6.0	7.4	6	484.3±14.4	81.1± 8.3	390.6±16.6	16.4	1810.3	1407.1	501.3	110.7
7.4	6.0	6	243.3±11.4	455.0±11.1	−211.7±15.9	2.6	1426.3	1638.0	336.9	548.6

Gly-Sar across the apical border (6.8-fold greater than the Gly-Sar concentration in the apical solution) exceeded that across the basolateral border.

Since the fluxes shown in Fig. 2 were determined at steady state and since cellular accumulation of Gly-Sar together with the relative accumulation across both apical and basolateral surfaces has been determined (Fig. 2b) it is possible to estimate the unidirectional fluxes and permeabilities for Gly-Sar across both apical and basolateral membranes [18] as shown in Tables I and II. When a, b and c denote apical, basal and cell compartments, J the respective fluxes and R is the ratio of cellular accumulation of Gly-Sar across the apical or basolateral surfaces ((accumulation across the apical membrane)/(accumulation across the basolateral membrane)), then:

$$J_{a\text{-}c} = J_{b\text{-}a} \cdot R + J_{a\text{-}b},$$

$$J_{c\text{-}a} = J_{b\text{-}a} \cdot (1 + R),$$

$$J_{c\text{-}b} = J_{a\text{-}b} \cdot (1 + 1/R) \text{ and}$$

$$J_{b\text{-}c} = J_{b\text{-}a} + J_{a\text{-}b}/R.$$

The ratio of permeabilities for Gly-Sar at the apical membrane clearly showed a large asymmetry consistent with an 'active' component localised to this membrane domain. The exit permeability for Gly-Sar from cell to basolateral solution was low suggesting that this was the rate-limiting step for transepithelial Gly-Sar transport (Table II). Upon apical acidification, the unidirectional influx across the apical membrane was markedly stimulated consistent with a proton linked carrier at this membrane. The ratio of unidirectional permeabilities did not increase as markedly because the unidirectional efflux from cytoplasm to apical medium increased concurrently (Tables I and II). This may result from a decrease in cytoplasmic pH (see below). The ratio of permeabilities for Gly-Sar across the basolateral membrane was not unity suggesting that a facilitated step may also exist for dipeptide accumulation at the basolateral membrane. Acidification of the basal solution markedly increased flux of Gly-Sar across the basolateral membrane and the ratio of permeabilities increased from 4.7 under control conditions to 15.2 upon basal acidification, consistent with a proton-linked dipeptide transporter at the basolateral membrane. After Gly-Sar was accumulated within the cytosol, exit may be across either the apical or basolateral membranes. It was apparent that the exit permeability across the apical membrane was quantitatively more important ($P_{c\text{-}a} > P_{c\text{-}b}$, that is Gly-Sar will tend to recycle across the apical membrane), in all conditions (Table II). However, since both apical and basal medium acidification was associated with a small increase in permeability for Gly-Sar exit across the apical membrane, that across the basolateral membrane must be relatively insensitive to such manipulations.

TABLE II

Calculated unidirectional permeabilities for Gly-Sar across apical and basolateral membranes $P_{ij} = J_{ij}/C_i$ where ij represent the ipsilateral and contralateral flux compartment and P, J, and C are permeability, flux and concentration respectively. a, c and b denote apical, cell and basal compartments respectively.

Gly-Sar accumulations are those described in the Results section and are calculated as described in Methods.

Condition		$P_{a\text{-}c}$ (cm/h×10^3)	$P_{c\text{-}a}$ (cm/h×10^3)	Ratio $P_{a\text{-}c}/P_{c\text{-}a}$	$P_{c\text{-}b}$ (cm/h×10^3)	$P_{b\text{-}c}$ (cm/h×10^3)	Ratio $P_{b\text{-}c}/P_{c\text{-}b}$	Ratio $P_{c\text{-}a}/P_{c\text{-}b}$
apical	basal							
7.4	7.4	33.5	3.8	8.8	1.3	6.1	4.7	2.9
6.0	7.4	50.3	4.6	10.9	1.6	3.1	1.9	2.9
7.4	6.0	39.6	4.7	8.4	1.0	15.2	15.2	4.7

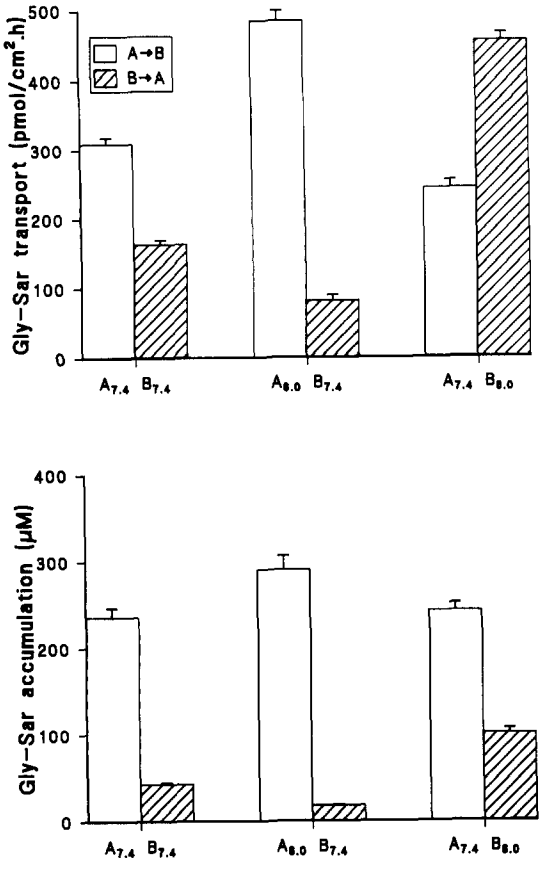

Fig. 2. (a) Transepithelial transport of [^{14}C]Gly-Sar across Caco-2 cell monolayers ($J_{a\text{-}b}$ (open columns) and $J_{b\text{-}a}$ (striped columns)). Apical (A) and basal (B) bathing solutions were as indicated and contained 36 μM Gly-Sar. Paired monolayers of the same cell batch were used to measure the bidirectional fluxes. Data are the means ± S.E. ($n = 4$–6) for each condition. (b) Cellular accumulation of Gly-Sar from apical (open columns) or basal (striped columns) bathing solutions at the end of the flux period. Other details as for Fig. 2(a).

[^{14}C]Gly-Sar efflux across apical and basolateral cell borders

The analysis of fluxes at steady state suggested that Gly-Sar exit across the basolateral membrane was rate limiting. We have, therefore, measured efflux of Gly-Sar directly. After cell loading from [^{14}C]Gly-Sar (72 μM) containing solutions, efflux was followed across both apical and basolateral cell borders into fresh solutions of varying pH. With bathing solutions of pH 7.4, efflux was predominately across the apical border, 59.8 ± 2.1% (8.8 ± 0.3 nmol/h, $n = 4$) of label exiting to the apical solution and only 19.2 ± 0.9% (2.6 ± 0.1 nmol/h, $n = 4$ (ratio 3.1:1)) to the basolateral bathing solution. This pattern was relatively insensitive to either apical (68.0 ± 0.4% (8.9 ± 0.2 nmol/h, $n = 4$) and 18.7 ± 0.3% (2.4 ± 0.0 nmol/h, $n = 4$) exiting across apical and basolateral cell borders, respectively, ratio 3.6:1) or basal acidification (70.0 ± 1.5% (10.6 ± 0.6 nmol/h, $n = 4$) and 10.5 ± 0.4% (1.6 ± 0.1 nmol/h, $n = 4$) exiting across apical and basolateral cell borders, respectively, ratio 6.7:1). These measurements are, therefore, in broad agreement to those made by calculation from steady-state data. It is apparent that alterations in cellular accumulation of Gly-Sar are largely a consequence of acid stimulated Gly-Sar influx across either apical or basolateral borders, respectively.

Independence of [^{14}C]Gly-Sar transport upon extracellular Na^+

Despite total omission of Na^+ from medium bathing both apical and basolateral surfaces the substantial net flux of dipeptide observed in Na^+-containing solutions was only marginally reduced (Fig. 3). This reduction was not a specific effect on the dipeptide transporter since epithelial conductance was decreased from 1.26 ± 0.05 mS/cm^2 ($n = 8$) to 0.71 ± 0.04 mS/cm^2 ($n = 9$) indicating that Na^+-free conditions have a general effect (reduction) on transepithelial conductive pathways. The effect on pH-dependent intracellular accumulation of dipeptide was more marked. Accumulation across the apical membrane decreased from 489.1 ± 23.4 ($n = 4$) to 160.7 ± 11.0 μM ($n = 5$) and across the basolateral surface from 151.9 ± 20.4 ($n = 4$) to 23.0 ± 1.8 μM ($n = 5$). In Na^+-free conditions this decrease in Gly-Sar accumulation will partly be due to a gradual intracellular acidification due to the absence of any pH_i regulation by the Na^+/H^+ exchanger.

Concentration dependence of [^{14}C]Gly-Sar transport

Fig. 4 shows Gly-Sar transport ($J_{a\text{-}b}$) with variation in Gly-Sar concentration in the apical chamber. Total transport at both pH 7.4 and 6.0 failed to saturate even at 20 mM Gly-Sar. It is evident that a saturable component of Gly-Sar transport co-exists with a linear flux component comprising diffusion, probably via a non-cellular paracellular route [19]. We have estimated the

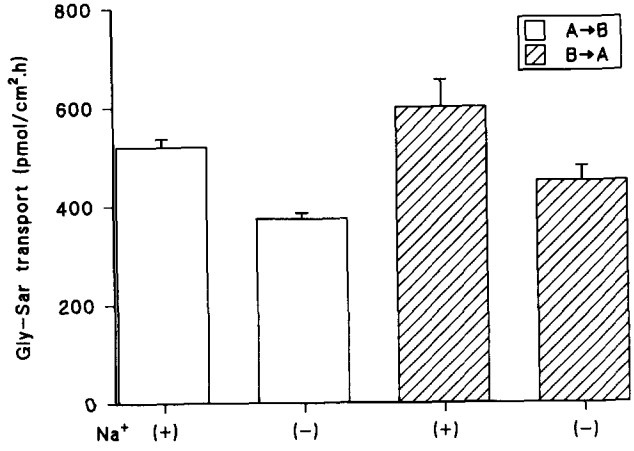

Fig. 3. Effect of Na^+-free conditions upon pH-dependent transepithelial [^{14}C]Gly-Sar fluxes ($J_{a\text{-}b}$ (open columns) and $J_{b\text{-}a}$ (striped columns)). The composition of the Na^+-free Krebs is described in Methods. Data are the means ± S.E. (four or five epithelial layers).

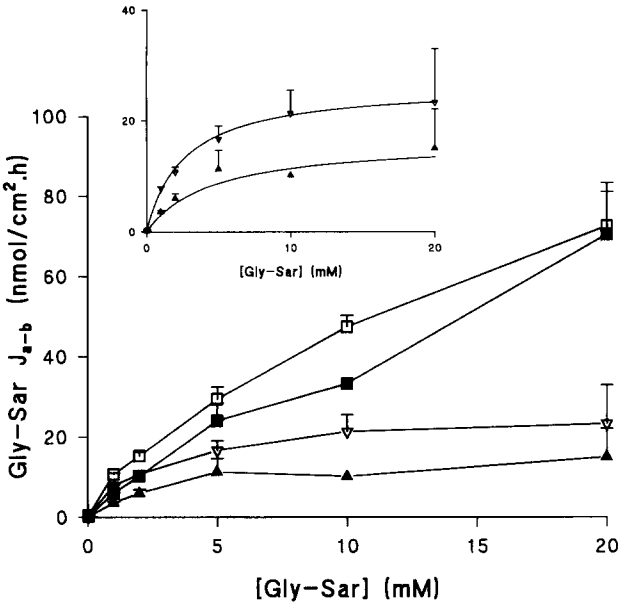

Fig. 4. Concentration dependence of transepithelial Gly-Sar transport from apical-to-basal bathing solutions. Gly-Sar was absent from the basolateral bathing solution. Transport was determined with basolateral pH 7.4 and apical pH either 6.0 (open symbols) or 7.4 (filled symbols). Inset illustrates Gly-Sar (J_{a-b}) flux at apical pH 6.0 (open inverted triangles) or 7.4 (filled triangles) after subtraction of a diffusive component estimated from mannitol (J_{a-b}) flux. Data are the means ± S.E. (4–6 epithelial layers).

likely magnitude of the linear component by using the passive leak permeability indicated by simultaneous determination of mannitol flux. Subtraction of this linear flux component, revealed the saturable nature of Gly-Sar transport. At pH 7.4, the calculated kinetic parameters were K_m 3.4 ± 1.3 mM, and the V_{max} was 16.5 ± 2.0 nmol/cm² per h. Upon apical acidification (pH 6.0), there was little change in the K_m (2.8 ± 0.2 mM) but the V_{max} increased to 26.7 ± 1.7 nmol/cm² per h.

The concentration dependence of Gly-Sar accumulation across the apical membrane at pH 6.0 is illustrated in Fig. 5. The K_m (6.5 mM) was in the same range to that observed for saturation of J_{a-b} (Fig. 4).

Competition of both [^{14}C]Gly-Sar transport and accumulation at apical and basolateral cell borders

Excess (20 mM) Gly-Sar and cephalexin were both inhibitors of [^{14}C]Gly-Sar transport (J_{a-b} and J_{b-a}) when measured under optimal conditions, i.e., apical or basal acidification (Fig. 6). Similarly, both Gly-Sar and cephalexin reduced intracellular accumulation of [^{14}C]Gly-Sar.

Intracellular pH (pH_i): the effect of apical or basolateral exposure to 20 mM Gly-Sar or cephalexin

The effect on pH_i of apical and basolateral exposure to Gly-Sar (20 mM) and cephalexin (20 mM),

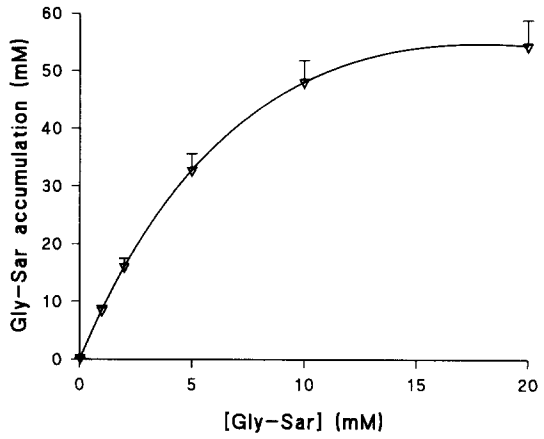

Fig. 5. Concentration dependence of Gly-Sar accumulation from the apical medium in the presence of an inwardly-directed pH gradient. Results are the means ± S.E. (n = 6).

when the media bathing the cell monolayers was pH 7.4 or 6.0, are compared in Fig. 7. At the apical membrane, both Gly-Sar and cephalexin were coupled

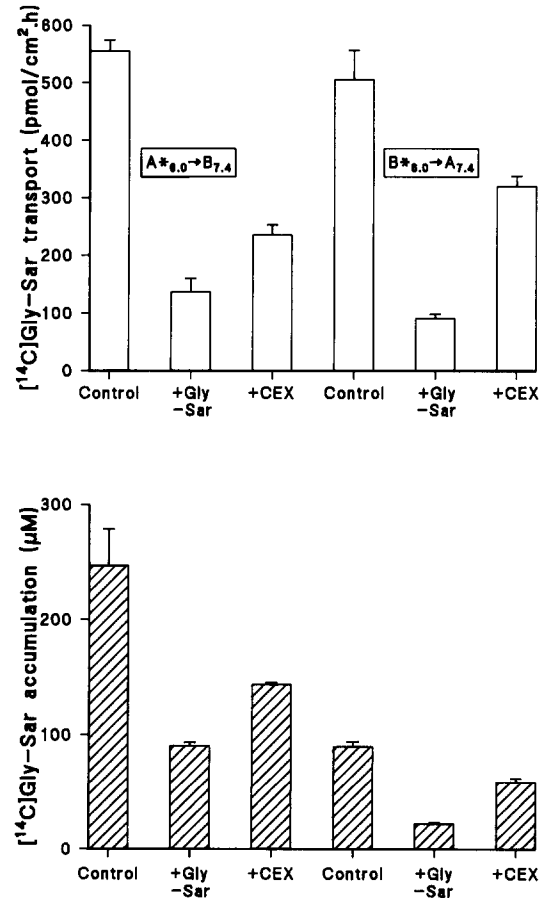

Fig. 6. Upper panel: Competition of apical-to-basal and basal-to-apical [^{14}C]Gly-Sar (36 μM) transport by Gly-Sar (20 mM) and cephalexin (20 mM) present in either apical or basal chambers, respectively. The contralateral chamber was without dipeptide/cephalosporin. Lower panel: Intracellular accumulation of [^{14}C]Gly-Sar from apical or basal bathing solutions. Competition by 20 mM Gly-Sar or 20 mM cephalexin. Data are the means ± S.E. (n = 4–6).

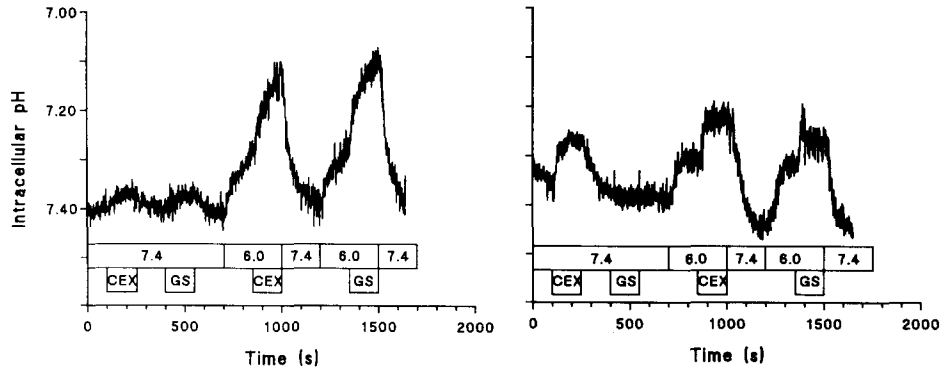

Fig. 7. Intracellular pH measured in BCECF-loaded Caco-2 monolayers. The effect of 20 mM Gly-Sar (GS) and 20 mM cephalexin (CEX) on pH_i after exposure to either the apical (left panel) or basolateral (right panel) membrane. Measurements were made at both pH 7.4 and 6.0. This experiment is representative of four others.

to proton influx and caused intracellular acidification. When the apical solution was acidified to pH 6.0, pH_i was decreased and both Gly-Sar and cephalexin enhanced this intracellular acidification (Fig. 7). In contrast, whereas cephalexin (20 mM) promoted intracellular acidification when present in the basolateral bathing solution (pH 7.4), Gly-Sar (20 mM) was without comparable effect. When basal pH was acidified to pH 6.0, pH_i was decreased, and this effect was enhanced by both cephalexin and Gly-Sar. The difference between cephalexin and Gly-Sar effects at the basolateral membrane contrast to the competitive action of cephalexin upon Gly-Sar transport and accumulation (above) and indicate common and dissimilar transport pathways for Gly-Sar and cephalexin at the basolateral membrane.

Discussion

Experiments aimed at understanding the fundamental properties of intestinal dipeptide and cephalosporin transport have invariably concentrated on utilising intestinal brush-border membrane vesicles (BBMV) [10,20]. In contrast to renal BBMV, although both proton-gradient and membrane-potential accelerations of dipeptide uptake (initial rates) are well documented in intestinal BBMV [10], dipeptide accumulation (dependent on the prevailing electrochemical gradient for protons) has only recently been convincingly demonstrated [14]. Little information is available regarding the mechanism of dipeptide exit across the basolateral membrane, although a single study has described H^+-dependent Gly-Pro uptake in basolateral membrane vesicles from rabbit intestine [16].

The present demonstration of pH-dependent apical-to-basal Gly-Sar transport across intestinal epithelial monolayers of Caco-2 cells is entirely consistent with the expression of the dipeptide transporter by these cells and is in agreement with observations of cephradine transport [9]. Gly-Sar transport and accumulation are inhibited by cephalexin at the apical membrane (present data) whilst cephradine transport and accumulation are inhibited by carnosine, Gly-Leu, Gly-Pro and Gly-Sar [9].

The direct demonstration of cytosolic acidification with apical Gly-Sar provides evidence for a direct coupling of protons with dipeptide transport. This effect is observed at both apical pH 7.4 and upon apical acidification (pH 6.0). This complements recent observations of intravesicular acidification of eel intestinal BBMV with glycylglycine [21]. Cephalexin also shows an identical pattern to Gly-Sar in promoting intracellular acidification across the apical membrane. Taken together, these observations suggest that dipeptides (Gly-Sar) and cephalosporins (cephalexin and cephradine) share a common transport system at the apical membrane of Caco-2 cells. The energy source for dipeptide/cephalosporin accumulation consists of both the pH gradient and transmembrane electrical potential difference. At a bulk apical pH of 7.4, it is likely that the pH gradient is minimal (Fig. 7). However, it is possible that an acid microclimate (an area of low pH adjacent to the apical membrane) may exist in the Caco-2 epithelium as has been demonstrated in the intestine both in vivo [22] and in vitro [23]. Alternatively, in the absence of a pH-microclimate, the observed accumulation of Gly-Sar must depend upon the transapical electrical potential difference. Dipeptide-induced membrane depolarisations have been observed in a number of species [11,13], these electrogenic responses being due to H^+-coupled dipeptide transport at the brush-border membrane.

We have provided evidence for specific transport of Gly-Sar at the basolateral membrane. Under certain conditions net transport of Gly-Sar can be reversed to become a net secretion. The physiological significance of dipeptide secretion by intact epithelial layers is not defined. However, in the absence of a significant lumi-

nal dipeptide concentration, carrier-mediated transfer (even at physiological basolateral pH values) in a secretory direction is clearly possible. Perhaps such a mediated transfer exists to clear dipeptides released by hydrolysis of bioactive peptides directed at the basolateral surface of intestinal cells in vivo. The present data also emphasise the importance of exit across the basolateral membrane to mediate absorptive transfer of dipeptides by the intact epithelium. Thus transepithelial Gly-Sar transport is likely to be rate-limited by $J_{c\text{-}b}$. The exit permeability across the basolateral membrane is not markedly affected by either alterations in apical or basal bathing solution pH, despite the observed change in intracellular pH (Fig 7). In addition it would appear that basolateral exit permeability is considerably smaller than that observed at the apical membrane. This contrasts to the data of Inui et al. [9] in Caco-2 cells where cephradine exit across the basolateral membrane exceeded that across the apical membrane.

The present data showing pH-dependent basal-to-apical Gly-Sar transport are consistent with a H^+-coupled dipeptide transport mechanism at the basolateral membrane. The asymmetry of Gly-Sar permeabilities across the basolateral membrane is similar to that observed at the apical membrane (Table II). With acidic basolateral solutions, additional cytosolic acidification with both Gly-Sar and cephalexin is direct evidence for proton coupling under these conditions. Cephalexin also reduces basal-to-apical Gly-Sar transport and accumulation across the basolateral membrane. These observations are consistent with a proton-linked carrier for Gly-Sar and cephalosporins at the basolateral membrane of Caco-2 cells and with studies with Gly-Pro using rabbit enterocyte basolateral-membrane vesicles [16].

At pH 7.4, marked cellular accumulation of Gly-Sar across the basolateral membrane does not occur. This arises from the smaller asymmetry in unidirectional permeabilities across the basolateral compared to the apical surface (Table II) and from the greater permeability of Gly-Sar exit across the apical compared to the basolateral membrane. Thus, it is possible that the properties of the H^+-coupled transporter at the basolateral face are different from the apical membrane. A Gly-Sar dependent acidification (or proton flux) across the basolateral membrane is not observed at basolateral pH 7.4. This contrasts with the effect of cephalexin at the basolateral border. Both cephalexin and Gly-Sar do result in cytosolic acidification if basolateral pH is acidified. Thus it may be concluded that though a common carrier for Gly-Sar and cephalosporins may exist at the basolateral membrane, distinct features of cephalexin transport, not shared by Gly-Sar are also present. These observations help explain the differences in efflux of Gly-Sar and cephradine noted in this study and the that of Inui et al. [9]. It is interesting to note that although both Gly-Sar and cephalexin are predominately zwitterionic at pH 6.0, at pH 7.4 approx. 25% of cephalexin is in the anionic form [24]. Thus cephalexin transport at the basolateral surface may involve multiple transport systems.

Small peptides (di/tripeptides) cross the intestinal wall by a number of routes. These include, breakdown to the constituent amino acids at the apical surface followed by amino acid absorption (by one of the many amino acid transporters present in the apical membrane), or passive transport of the intact peptide by the paracellular pathway [19]. In the present study we have used a hydrolysis resistant peptide Gly-Sar. The integrity of the transported species has been confirmed by thin-layer chromatography. The relative importance of the paracellular pathway varies. At relatively low Gly-Sar concentrations (36 μM), transepithelial transport of the paracellular marker mannitol is small compared to the dipeptide. Direct demonstration of H^+-coupled dipeptide transport (and intracellular accumulation) across both the apical and basolateral membranes also focuses on the importance of the transcellular route. However, at higher dipeptide concentrations (mM) the importance of the paracellular route is more significant. Thus at 20 mM Gly-Sar it is likely that the majority of peptide transfer across Caco-2 epithelium is passive.

Under physiological conditions dipeptides may cross the apical membrane via a H^+-coupled mechanism. Once inside the cells the fate of the peptide will depend on its relative resistance to hydrolysis. If resistant, the peptide may then access the (H^+-coupled) transporter(s) on the basolateral membrane which, in vivo, will mediate small peptide exit from the cytoplasm into the basolateral space. It is likely that the intracellular accumulation of dipeptides will favour exit, despite the existence of proton-coupled accumulative transport at the basolateral membrane.

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References

1 Hidalgo, I.J., Raub, T.J. and Borchardt, R.T. (1989) Gastroenterology 96, 736–749.
2 Blais, A., Bissonnette, A. and Berteloot, A. (1987) J. Membr. Biol. 99, 113–125.

3 Harris, D.S., Slot, J.W., Geuze, H.J. and James, D.E. (1992) Proc. Natl. Acad. Sci. USA 89, 7556–7560.
4 Mahraoui, L., Rousset, M., Dussaulx, E., Darmoul, D., Zweibaum, A. and Brot-Laroche, E. (1992) Am. J. Physiol. 263, G312–G318.
5 Hidalgo, I.J. and Borchardt, R.T. (1990) Biochim. Biophys. Acta 1028, 25–30.
6 Nicklin, P.L., Irwin, W.J., Hassan, I.F. and Mackay, M. (1992) Biochim. Biophys. Acta 1104, 283–292.
7 Hidalgo, I.J. and Borchardt, R.T. (1990) Biochim. Biophys. Acta 1035, 97–103.
8 Dantzig, A.H. and Bergin, L. (1990) Biochim. Biophys. Acta 1027, 211–217.
9 Inui, K.I., Yamamoto, M. and Saito, H. (1992) J. Pharmacol. Exp. Ther. 261, 195–201.
10 Ganapathy, V. and Leibach, F.H. (1985) Am. J. Physiol. 249, G153–G160.
11 Boyd, C.A.R. and Ward, M.R. (1982) J. Physiol. 324, 411–428.
12 Ganapathy, V. and Leibach, F.H. (1983) J. Biol. Chem. 258, 14189–14192.
13 Ganapathy, V., Burckhardt, G. and Leibach, F.H. (1984) J. Biol. Chem. 259, 8954–8959.
14 Thwaites, D.T., Simmons, N.L. and Hirst, B.H. (1993) Pharm. Res. 10, 667–673.
15 Thwaites, D.T., Brown, C.D.A., Hirst, B.H. and Simmons, N.L. (1993) J. Biol. Chem. 268, 7640–7642.
16 Dyer, J., Beechey, R.B., Gorvel, J.P., Smith, R.T., Wootton, R. and Shirazi-Beechey, S.P. (1990) Biochem. J. 269, 565–571.
17 Thomas, J.A., Bushbaum, R.N., Zimniak, A. and Racker, E. (1979) Biochemistry 18, 2230–2238.
18 Naftalin, R.J. and Curran, P.F. (1974) J. Membr. Biol. 16, 257–278.
19 Thwaites, D.T., Hirst, B.H. and Simmons, N.L. (1993) Pharm. Res. 10, 674–681.
20 Okano, T., Inui, K.I., Maegawa, H., Takano, M. and Hori, R. (1986) J. Biol. Chem. 261, 14130–14134.
21 Verri, T., Maffia, M. and Storelli, C. (1992) Biochim. Biophys. Acta 1110, 123–126.
22 Lucas, M. (1983) Gut 24, 734–739.
23 Lucas, M.L., Cooper, B.T., Lei, F.H., Johnson, I.T., Holmes, G.K.T., Blair, J.A. and Cooke, W.T. (1978) Gut 19, 735–742.
24 Purich, E.D., Colaizzi, J.L. and Poust, R.I. (1973) J. Pharm. Sci. 62, 545–549.