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The Role of the Proton Electrochemical Gradient in the Transepithelial Absorption of Amino Acids by Human Intestinal Caco-2 Cell Monolayers

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Abstract. We determined the extent of Na^+-independent, proton-driven amino acid transport in human intestinal epithelia (Caco-2). In Na^+-free conditions, acidification of the apical medium (apical pH 6.0, basolateral pH 7.4) is associated with a saturable net absorption of glycine. With Na^+-free media and apical pH set at 6.0, (basolateral pH 7.4), competition studies with glycine indicate that proline, hydroxyproline, sarcosine, betaine, taurine, β-alanine, α-aminoisobutyric acid (AIB), α-methylaminoisobutyric acid (MeAIB), τ-amino-n-butyric acid and L-alanine are likely substrates for pH-dependent transport in the brush border of Caco-2 cells. Both D-serine and D-alanine were also substrates. In contrast leucine, isoleucine, valine, phenylalanine, methionine, threonine, cysteine, asparagine, glutamine, histidine, arginine, lysine, glutamate and D-aspartate were not effective substrates. Perfusion of those amino acids capable of inhibition of acid-stimulated net glycine transport at the brush-border surface of Caco-2 cell monolayers loaded with the pH-sensitive dye 2′,7′-bis(2-carboxyethyl-5(6)-carboxyfluorescein) (BCECF) caused cytosolic acidification consistent with proton/amino acid symport. In addition, these amino acids stimulate an inward short-circuit current (I_{sc}) in voltage-clamped Caco-2 cell monolayers in Na^+-free media (pH 6.0). Other amino acids such as leucine, isoleucine, phenylalanine, tryptophan, methionine, valine, serine, glutamine, asparagine, D-aspartic acid, glutamic acid, cysteine, lysine, arginine and histidine were without effect on both pH_i and inward I_{sc}. In conclusion, Caco-2 cells express a Na^+-independent, H^+-coupled, rheogenic amino acid transporter at the apical brush-border membrane which plays an important role in the transepithelial transport of a range of amino acids across this human intestinal epithelium.

Key words: Amino acid — Protons — Human intestinal cells — Absorption — Caco-2

Introduction

The ability of the proton electrochemical gradient to energize the net uptake of certain solutes such as di- and tripeptides across the brush border of mammalian small intestine has been recognized for several years (Ganapathy & Leibach, 1985). The occurrence of other substrate transport linked to the proton rather than the Na^+-electrochemical gradient has received comparatively little attention. Recently, we have demonstrated in human intestinal Caco-2 cells (exhibiting enterocytic differentiation), that in addition to Na^+-coupled mechanisms at both the apical and basolateral cell membranes, the transport and cellular accumulation across the apical membrane of certain amino acids (i.e., β-alanine, L-proline, L-alanine and MeAIB) may be energized by extracellular acidification, even in Na^+-free conditions (Thwaites et al., 1993*b;* 1993*c;* 1994; 1995). By analogy to proton/substrate transport in bacteria (West & Mitchell, 1973) we have demonstrated directly that the transport of β-alanine and proline across the apical membrane is coupled with proton inflow since apical perfusion of these amino acids causes an intracellular acidification in BCECF-loaded Caco-2 cell monolayers (Thwaites et al., 1993*b;* 1993*c*). Proton cotransport with either β-alanine or proline across the apical membrane of voltage-clamped Caco-2 cell monolayers is also rheogenic, being associated with an inward short-circuit current, I_{sc} (Thwaites et al., 1993*b;* 1993*c*). Thus using three independent techniques applied to the same cell system, data consistent

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with proton-coupled amino acid transport have been presented. Competition experiments (Thwaites et al., 1993*b;* Thwaites et al., 1994) in Caco-2 cells suggest that the substrate specificity of the proton-linked amino acid transport may not be restricted to β-alanine, L-alanine and proline. Glycine inhibits the net absorptive transport and cellular accumulation (across the apical membrane) of both β-alanine (Thwaites et al., 1993*b*) and L-alanine (Thwaites et al., 1994). The purpose of the present study was to determine the full extent of proton-linked transport of amino acids by human intestinal Caco-2 cells. Substrates were examined for their effects of pH-stimulated glycine transport, their ability to stimulate intracellular acidification and I_{sc} when perfused at the apical surface (Thwaites et al., 1993*b;* 1993*c;* 1994).

Materials and Methods

Materials

[U-^{14}C]glycine (specific activity 100 mCi/mmol), [2-^{3}H]glycine (specific activity 60 Ci/mmol), [1,2-^{14}C]taurine (specific activity 109 mCi/mmol) and D-[1-^{3}H(N)]mannitol (specific activity 30 Ci/mmol) were obtained from Du Pont-New England Nuclear. 2-amino[1-^{14}C]isobutyric acid (specific activity 60 mCi/mmol) was from Amersham. All amino acids were from Sigma. All amino acids were the L-isomer unless stated otherwise. All other chemicals were from Merck. Cell culture consumables were from Life Technologies.

Cell Culture

Caco-2 cells (passage number 104–121) were cultured as described previously (Thwaites et al., 1993*a*). Cell monolayers were prepared by seeding at high density ($4.4–5.0 \times 10^5$ cells · cm^2) onto 24.5 mm diameter tissue culture inserts [polycarbonate filters (Costar)]. Cell monolayers were maintained at 37°C in an atmosphere of 5% CO_2 in air. Cell confluence was estimated by microscopy and determination of transepithelial resistance. Radiolabelled flux, pH_i and conductance experiments were performed 14–25 days after seeding and 18–24 hr after feeding.

Transport Determinations

Bidirectional transepithelial flux measurements were performed essentially as described previously (Thwaites et al., 1993*b;* Simmons, 1990). Briefly, cell monolayers were washed (4 × in 500 ml of modified Krebs (of composition all mmol/l, NaCl 140, KCl 5.4, $CaCl_2$ 2.8, $MgSO_4$ 1.2, NaH_2PO_4 0.3, KH_2PO_4 0.3, HEPES 10, glucose 10 (pH to 7.4 at 37°C with Tris base))) or Na^+-free Krebs where appropriate (as above but choline Cl replaced NaCl and NaH_2PO_4 omitted), and placed in fresh 6-well plates, each well containing 2 ml of prewarmed modified Krebs or Na^+-free Krebs buffer (pH 7.4). Aliquots of fresh Krebs buffer or Na^+-free Krebs (pH 7.4) were then placed in the apical chamber. Choline media did not contain detectable Na^+ (<0.1 mM). In addition, in experimental media following incubation with cell layers no Na^+ could be detected. For the pH 5.5–6.5 buffers, 10 mM MES was used to replace HEPES, and the required pH was obtained by addition of Tris base. Radiolabelled glycine was used at tracer concentrations (0.1–0.3 μCi/ml) with glycine added to give a final concentration (unless stated) of 100 μM; [^{3}H]label was used to trace apical-to-basal glycine flux (J_{a-b}) and [^{14}C]label to simultaneously trace basal-to-apical glycine flux (J_{b-a}). Aliquots (200 μl) of fluid from each chamber were removed for dual-label scintillation counting at 20 and 80 min. Fluxes across the monolayers into the contralateral chamber are expressed as nmol · cm^{-2} · hr^{-1}. At the end of the incubation period cell monolayers were washed in 4 × 500 ml volumes of ice-cold Krebs (or Na^+-free 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 glycine is expressed as mM (or μM) or as a cell to medium (C/M) ratio. When the transepithelial flux and accumulation of taurine and AIB were determined the experimental procedures were essentially the same as those described above except that only the [^{14}C]-form of each isotope was available (hence only unidirectional fluxes were determined across each individual monolayer). In these experiments [^{3}H]mannitol was included in the incubation media to assess passive (paracellular) transport. Cell height was determined by confocal imaging of intact cell layers and this value was used to estimate intracellular water. No allowance was made for osmotically inactive space, thus intracellular concentrations are likely to be underestimated.

Intracellular pH Determinations

For pH_i measurements, Caco-2 cells (on 12 mm diameter polycarbonate filters) were loaded by incubation with BCECF-AM (5μM), 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 basal chambers was accomplished as described previously (Thwaites et al., 1993*a;* 1993*b*). 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 Photometrics). 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). Results are expressed as pH_i or ΔpH_i.

Electrophysiological Determinations

Measurements of short-circuit current (I_{sc}) were made essentially as described previously (Thwaites et al., 1993*b;* Simmons, 1990). Cultured epithelial layers (on 10 mm-diameter polycarbonate filters) were mounted in Ussing type chambers (Precision Instrument Design) maintained at 37°C, connected to an automatic voltage current clamp (WPI DVC 1000) via KCl/agar salt-bridges and reversible electrodes (Ag/AgCl for current passage, calomel for voltage sensing). Measurements of open-circuit electrical p.d., transepithelial resistance (R_T) and short-circuit current (I_{sc}) were made in modified and Na^+-free Krebs solutions (*see above*). The chemical flux equivalent of the I_{sc} is 1 μamp · cm^{-2} = 36 nmol · cm^{-2} · hr^{-1}.

Statistics

Statistical comparison of mean values were made using a Students' paired *t*-test. Constants for Michaelis-Menton kinetics were calculated by nonlinear regression with the method of least-squares (FIG-P, Biosoft).

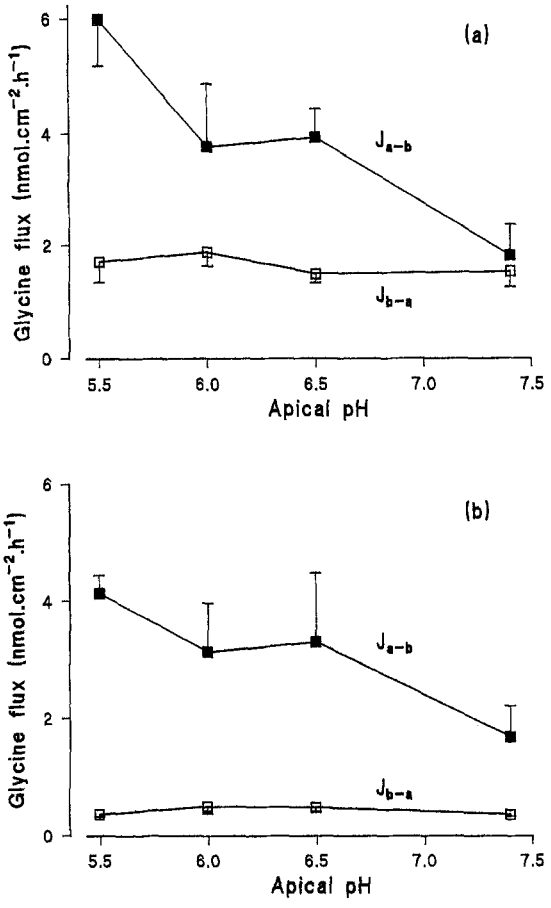

Fig. 1. pH-dependent transport of glycine across Caco-2 cell monolayers. Bidirectional transepithelial fluxes of glycine (■, J_{a-b}, apical-to-basal transport; □, J_{b-a}, basal-to-apical transport) were measured simultaneously at steady state across Caco-2 cell monolayers. Glycine (100 μM) was present in both apical and basolateral solutions. Net glycine fluxes ($J_{net} = J_{a-b} - J_{b-a}$) are given by the difference between the bidirectional fluxes. Fluxes in (*a*) Na^+-containing and (*b*) Na^+-free media. Apical medium pH was varied between 5.5 and 7.4 while the basal medium pH was maintained at pH 7.4. Results are illustrated as mean ± SEM (n = 4–5).

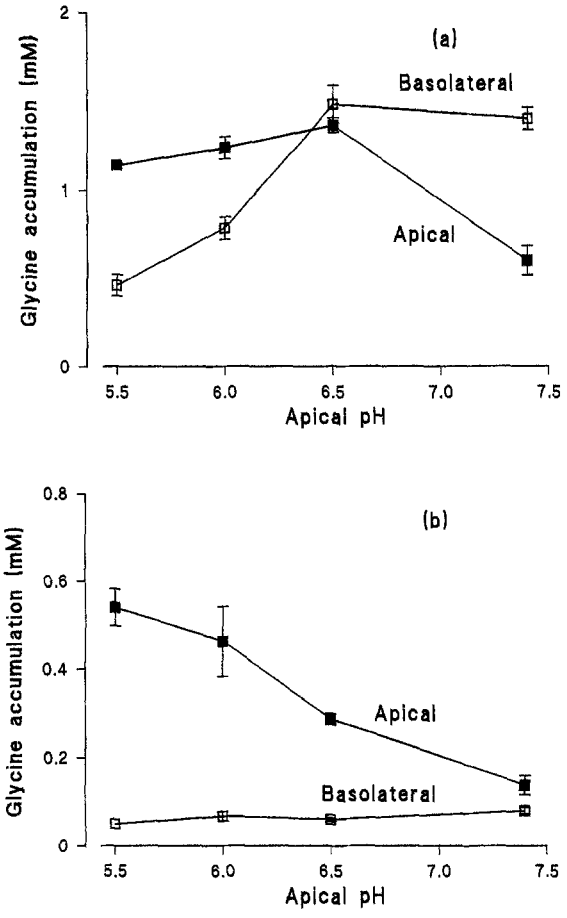

Fig. 2. pH-dependent cellular accumulation of glycine. Cellular glycine was measured at steady state. Glycine accumulation from the apical (■) and basolateral (□) media in (*a*) Na^+-containing and (*b*) Na^+-free media. Other details as for Fig. 1.

Results

Figure 1 shows that acidification at the apical surface results in a stimulation of net glycine absorption across the human intestinal Caco-2 epithelium primarily by increasing the apical-to-basal transport (J_{a-b}) both in Na^+-rich (Fig. 1*a*) and Na^+-free (Fig. 1*b*) conditions. In Na^+-free media, though the level of bidirectional fluxes are decreased (particularly J_{b-a}) the magnitude of net glycine absorption with apical acidity is equivalent to that observed in Na^+-rich media. At apical pH 5.5 (basolateral pH 7.4), J_{net} was 4.3 ± 1.8 (n = 5) nmol · cm^{-2} · hr^{-1} in Na^+-containing media and 3.8 ± 0.7 (n = 5) nmol · cm^{-2} · hr^{-1} in Na^+-free media ($P > 0.05$). In Na^+-containing conditions (apical and basolateral pH 7.4), the net absorption of glycine is limited (0.3 ± 1.1 (n = 5) nmol · cm^{-2} · hr^{-1}) and does not differ significantly from zero (Fig. 1*a*). It is, therefore, apparent that the imposition of an apical acidic environment is crucial in the development of net transepithelial glycine flux in this cultured human intestinal system. In Na^+-containing media (both apical and basolateral pH 7.4) glycine (100 μM) is accumulated avidly across both cell membranes (Fig. 2*a*, C/M ratio is 18.7 for total uptake). The apparent accumulation across the basal border is particularly striking (C/M ratio 14.0). Acidification of the apical medium depresses total cellular accumulation of glycine (C/M ratio 15.9) due to reduction of glycine accumulation at the basal border (Fig. 2*a*, C/M ratio 4.5). In contrast, apical medium acidification increases cellular accumulation of glycine across the apical membrane (Fig. 2*a*, C/M ratio 5.9 to 11.4 at apical pH 7.4 and pH 5.5, respectively). In Na^+-free conditions (Fig. 2*b*), the accumulation of glycine at the basal cell border is eliminated (C/M ratio 0.8) and there is no dependence upon apical pH. In contrast, though the level of cellular accumulation at the apical border is depressed in Na^+-free condi-

tions, the stimulation of cellular glycine accumulation by apical acidity is still evident (Fig. 2*b*). It may be concluded that both Na^+-dependent and Na^+-independent systems for glycine transport and accumulation exist at the apical and basal membranes of Caco-2 cells. Lowering apical pH stimulates activity of an accumulative transporter at the apical membrane even in Na^+-free conditions. However, apical acidity markedly depresses the activity of a Na^+-dependent system in the basolateral membrane.

The transepithelial transport and accumulation of AIB and taurine also showed marked pH-dependency. In both Na^+-containing and Na^+-free conditions net absorption of AIB was dependent upon apical acidity. In the absence of external Na^+, J_{net} was increased from 13 ± 40 (n = 5) pmol · cm^{-2} · hr^{-1} to 293 ± 26 (n = 5) pmol · cm^{-2} · hr^{-1} when apical pH was reduced from pH 7.4 to 6.0. Similarly, accumulation across the apical membrane was increased from C/M ratio of 0.59 ± 0.06 to 6.12 ± 0.32 when apical pH was reduced from pH 7.4 to 6.0. In Na^+-free conditions, uptake of AIB across the basolateral membrane was low and unaffected by apical pH (C/M ratio of 0.61 ± 0.07 (n = 5) at pH 7.4 and 0.72 ± 0.11 (n = 5) at pH 6.0). However, in the presence of external Na^+, AIB accumulation across the basolateral membrane was marked (C/M ratio 6.01 ± 1.01, n = 4) and reduced at apical pH 6.0 (C/M ratio 2.41 ± 0.36, n = 5). Transepithelial transport and cellular accumulation of taurine showed a broadly similar pattern to that observed with glycine and AIB. In the presence or absence of external Na^+, only with apical acidity (apical pH 6.0, basolateral pH 7.4) was net absorption observed (332 ± 42 (n = 5) pmol · cm^{-2} · hr^{-1} in Na^+-free conditions). Cellular accumulation (in Na^+-free conditions) was increased from a C/M ratio of 1.68 to 0.11 (n = 4) to 7.46 ± 0.71 (n = 5) when apical pH was lowered from pH 7.4 to 6.0. Basolateral uptake was low and unaffected by apical pH in the absence of external Na^+ (C/M ratio of 0.10 ± 0.03 (n = 4) and 0.18 ± 0.10 (n = 5) at apical pH 7.4 and 6.0, respectively).

Net transepithelial absorptive transport of glycine in Na^+-free conditions (apical pH 6.0, basolateral pH 7.4) displays saturation kinetics (Fig. 3*a*). The apparent K_m was 10.5 ± 4.0 mM and V_{max} of 460 ± 84 nmol · cm^{-2} · hr^{-1}. In contrast, J_{b-a} shows a linear dependence upon glycine concentration (Fig. 3*a*). Figure 3*b* shows the concentration dependence of cellular glycine accumulation with the relative contributions from either the apical or basal surface. In contrast to accumulation across the basal surface, which shows a linear dependence upon extracellular glycine concentrations and no concentrative uptake, uptake of glycine across the apical membrane in Na^+-free acidic (pH 6.0) conditions shows net concentrative accumulation above extracellular levels and a degree of saturation.

Net absorptive transport and cellular accumulation

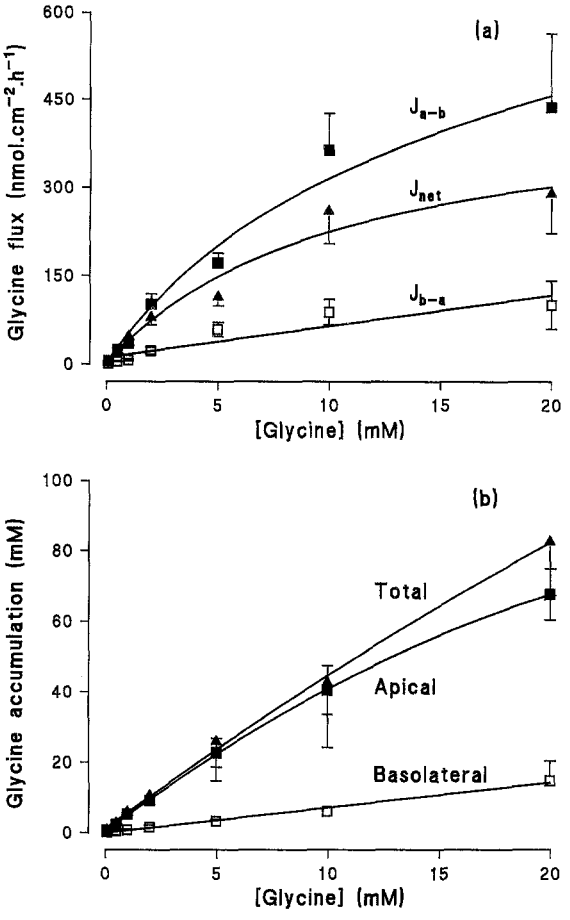

Fig. 3. Concentration-dependent transport of glycine. (*a*) Concentration dependence of glycine J_{a-b} (■), J_{b-a} (□), and J_{net} (filled triangles) in Na^+-free (choline) media with apical pH 6.0 and basolateral pH 7.4. Glycine concentrations were equal in both apical and basal solutions. Results are mean ± SEM, n = 3 cell layers per concentration. The rectangular hyperbola shown for J_{net} is the Michaelis-Menton fit for the data (*see* text). The line through J_{b-a} data is a linear regression (J_{b-a} = 5.3 · [glycine] + 10.8, r = 0.92, n = 21, $P < 0.001$). (*b*) Concentration dependence of glycine intracellular accumulation (total (filled triangles), and relative contributions across apical (■) and basolateral (□) membranes). Results are mean ± SEM, n = 3 per data point.

of 100 μM glycine driven by apical acidity (pH 6.0) in Na^+-free conditions shows selective inhibition by other amino acids (Table). Two groups of amino acids emerge when inhibition of net glycine transport is considered. First, those amino acids capable of inhibition of J_{net} and apical uptake, namely glycine, sarcosine, betaine, proline, hydroxy-proline, MeAIB, AIB, GABA, β-alanine and taurine. L-alanine, together with the D-isomers, D-alanine and D-serine were effective substrates (all 20 mM). Note that L-alanine inhibition of glycine J_{net} did not reach statistical significance due to data scatter. L-serine, though capable of inhibition of net glycine transport had only a small inhibitory effect on glycine accumulation at the apical membrane. Secondly, those amino

Table. Specificity of H^+/amino acid symport

	J_{net}	Cellular accumulation			I_{sc}	
		A	B	n	μamps · cm^{-2}	n
Control	100 ± 6	100 ± 2	100 ± 3	40–51		
Gly	29 ± 6*	45 ± 2*	40 ± 3*	5	4.8 ± 1.0	6
Sar	49 ± 6*	46 ± 1*	94 ± 4ns	9–13	4.8 ± 1.0	4
Bet	25 ± 6*	50 ± 1*	72 ± 3*	8–9	3.9 ± 0.4	4
Pro	15 ± 11*	32 ± 2*	52 ± 4*	10–14	5.2 ± 1.5	4
HO-Pro	58 ± 9*	47 ± 3*	66 ± 3*	8	2.9 ± 0.5	4
MeAIB	14 ± 12*	41 ± 2*	87 ± 6ns	3–4	7.1 ± 0.9	4
AIB	63 ± 12*	63 ± 5*	61 ± 5*	12–16	1.9 ± 0.1	5
GABA	37 ± 9*	31 ± 1*	90 ± 5ns	6–7	4.8 ± 0.7	4
β-Ala	41 ± 14*	54 ± 2*	83 ± 4*	9	10.1 ± 1.2	7
Tau	58 ± 10*	57 ± 3*	73 ± 4*	9–13	4.1 ± 0.8	5
Ala	67 ± 17ns	38 ± 1*	41 ± 4*	8	4.0 ± 0.5	3
D-Ala	31 ± 7*	56 ± 3*	64 ± 7*	4–8	3.2 ± 0.6	4
Ser	67 ± 6*	82 ± 4*	52 ± 5*	10–14	0.9 ± 0.2	10
D-Ser	60 ± 9*	65 ± 3*	47 ± 3*	9	4.2 ± 0.5	10
Thr	122 ± 24ns	93 ± 7ns	62 ± 5*	4–8	0.0 ± 0.1	4
Leu	95 ± 19ns	114 ± 5*	67 ± 8*	9	0.1 ± 0.3	3
Ile	95 ± 11ns	115 ± 7ns	59 ± 4*	5–9	0.3 ± 0.2	3
Val	178 ± 44ns	93 ± 6ns	54 ± 3*	7–8	0.2 ± 0.1	3
Phe	106 ± 15ns	134 ± 3*	60 ± 12*	3–7	0.4 ± 0.5	3
Met	86 ± 17ns	82 ± 7ns	48 ± 3*	5–9	−0.1 ± 0.1	3
Cys	65 ± 11ns	110 ± 8ns	50 ± 5*	7–14	0.3 ± 0.2	3
Gln	96 ± 21ns	103 ± 8ns	57 ± 8*	4–8	0.2 ± 0.3	4
Asn	87 ± 8ns	94 ± 2ns	49 ± 5*	3–8	0.1 ± 0.1	3
His	104 ± 21ns	79 ± 3*	42 ± 3*	8–9	−0.1 ± 0.1	3
Arg	151 ± 29ns	71 ± 7*	51 ± 6*	4	−4.9 ± 0.5	3
Lys	144 ± 28ns	82 ± 4*	74 ± 10*	7	−5.5 ± 0.5	4
Glu	100 ± 17ns	96 ± 5ns	91 ± 8ns	5	0.8 ± 0.3	4
D-Asp	127 ± 9ns	101 ± 6ns	83 ± 7ns	5	1.1 ± 0.7	5

Inhibition of Na^+-independent (apical pH 6.0, basolateral pH 7.4) glycine (100 μM) net absorptive transport (J_{net})and glycine cellular accumulation across the (A) apical and (B) basolateral cell membranes by various amino acids and derivatives (all at 20 mM). Results are expressed as a percentage (mean ± SEM) of control (transport and accumulation in the absence of inhibitors). Inward I_{sc} in response to apical additions of amino acids in Na^+-free media (pH 6.0). Data are expressed as the mean ± SEM of the change observed after 2–3 min.

acids that fail to significantly reduce J_{net}, but may or may not inhibit glycine uptake at the apical surface. These include threonine, leucine, isoleucine, valine, phenylalanine, methionine, cysteine, glutamine, asparagine, histidine, arginine, lysine, glutamate and D-aspartate. Histidine, arginine and lysine produce a small but significant reduction in accumulation of glycine across the apical membrane. It should be noted that the inhibitory profile for amino acid equilibration across the basal membrane may be considered in relation to the Na^+-independent transporters present; sarcosine, MeAIB, GABA, glutamate and D-aspartate had no effect on cellular glycine accumulation from the basolateral surface (*see* Discussion). The ability of various amino acids to inhibit the proton-driven accumulation of glycine is distinct from known properties of Na^+-independent transport at the brush-border membrane and may be considered to reflect the substrate specificity of the proton/amino acid symport at this membrane (*see* Discussion).

The inhibitory effects of several amino acids (all at 20 mM) on the pH-dependent cellular accumulation of AIB (Fig. 4*a*) and taurine (Fig. 4*b*) across the apical membrane of Caco-2 cells is similar to the inhibition profile noted with glycine (Table). With glycine, AIB, or taurine as the test compound (Table; Fig. 4) the rank order of inhibitory effects on pH-stimulated uptake across the apical membrane was proline > glycine > serine > valine.

If the existence of an acid-stimulated net glycine flux and inhibitory profile is due to the existence of a proton/amino acid symport at the apical membrane, measurement of intracellular pH during amino acid superfusion at the apical membrane should be associated with intracellular acidification (Thwaites et al., 1993*a;* 1993*b;* 1993*c*). Figure 5*a* shows a typical trace of pH_i, measured in epithelial layers of Caco-2 cells loaded with the intracellular pH-sensitive fluorochrome BCECF, following apical exposure to 20 mM glycine. A rapid revers-

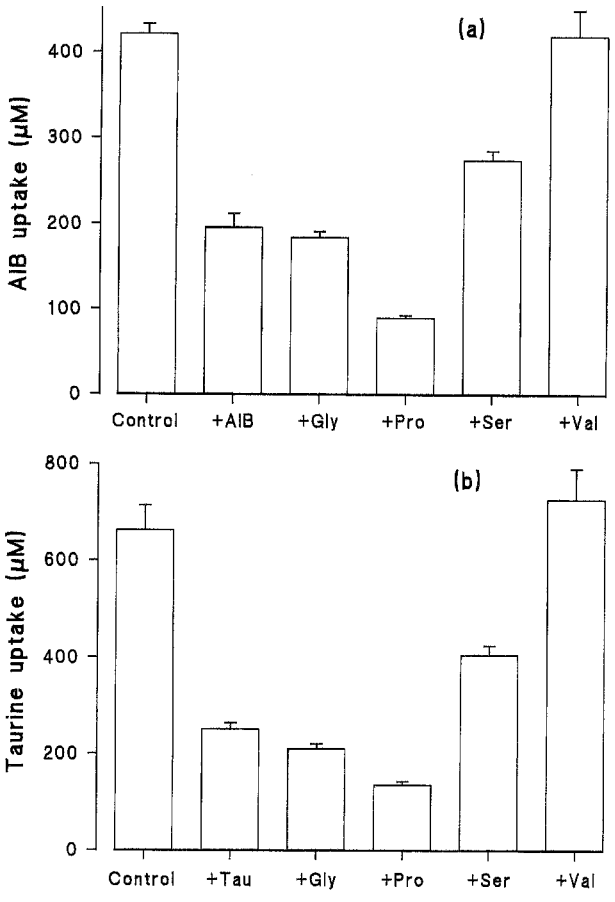

Fig. 4. Inhibition of Na^+-independent, pH-stimulated (*a*) AIB and (*b*) taurine uptake across the apical membrane of Caco-2 cell monolayers by a range of amino acids (all 20 mM). Experiments were performed in Na^+-free conditions (apical pH 6.0, basolateral pH 7.4). AIB (*a*) and taurine (*b*) were present in both apical and basolateral compartments at 100 μM. Results are mean ± SEM (n = 6).

ible, intracellular acidification was observed upon superfusion with glycine at the apical membrane which is enhanced upon acidification of the apical medium superfusate. In contrast, addition of glycine (20 mM) to the basolateral chamber had no substantial effect on pH_i (Fig. 5*b*) even with acidic basal medium superfusate. It is most likely that the proton/glycine symport is expressed solely at the apical brush-border membrane. The ability of a number of amino acids (all 20 mM) to promote intracellular acidification (consistent with the ability to act as substrates for proton/amino acid transport) when perfused at the apical membrane (pH 6.0–6.5, basolateral pH 7.4) of Caco-2 cells has been tested (Fig. 6). Glycine was included in each experiment to act as a control for interexperiment variation (Fig. 6). AIB, taurine and sarcosine (Fig. 6*a*), betaine (Fig. 6*c*), and GABA and hydroxyproline (Fig. 6*e*) generate intracellular pH perturbations of similar magnitude to those observed with glycine. Additional amino acids giving responses similar to glycine were L-alanine (Thwaites et al., 1994), β-alanine (Thwaites et al., 1993*b*), MeAIB (Thwaites et al., 1995) and proline (Thwaites et al., 1993*c*). D-Serine (Fig. 6*c*) and D-alanine (*not shown*) were also associated with intracellular acidification but the response was reduced compared to glycine. In contrast, a number of other amino acids had only minimal effects (Fig. 6), including methionine and isoleucine (Fig. 6*b*), serine (Fig. 6*c*), histidine, arginine and cysteine (Fig. 6*d*), and D-aspartic acid and glutamic acid (Fig. 6*f*), and may be clearly differentiated from glycine. Valine (Thwaites et al., 1994), leucine and phenylalanine (Thwaites et al., 1995), threonine, tryptophan, glutamine, asparagine and lysine (*not shown*) were also without effect upon pH_i. Thus those amino acids capable of pronounced inhibition of pH-stimulated glycine J_{net} and cellular uptake across the apical membrane, generate similar intracellular acidification compared to glycine upon superfusion in acidic media, whereas those amino acids not capable of marked inhibition of net glycine flux or accumulation at the apical surface do not generate marked intracellular acidification.

Proton/dipeptide transport is linked to the Na^+-electrochemical gradient in enterocytes in an indirect manner via the operation of Na^+/H^+ exchange (Ganapathy & Leibach, 1985). Fig. 7 shows that in Na^+-free conditions superfusion of glycine (20 mM) at the apical surface (pH 6.0) causes enhanced intracellular acidification compared to Na^+-rich media. Recovery of pH_i following substrate-induced acid load was slow in the absence of extracellular Na^+ (Fig. 7) and was unaltered by reintroduction of Na^+ to the basolateral superfusate. In contrast, after reintroduction of Na^+ to the apical superfusate, pH_i rapidly returned towards control values, indicating that a Na^+/H^+ exchanger is most likely localised to the apical membrane (Fig. 7). Similar observations have been made with alanine (Thwaites et al., 1994) and MeAIB (Thwaites et al., 1995). It seems likely that amino acid/proton symport may be linked indirectly to the Na^+ electrochemical gradient in Na^+-rich media via the operation of an apical Na^+/H^+ exchanger.

We have demonstrated previously that proton/β-alanine transport is rheogenic since apical β-alanine is capable of generating an inward I_{sc} in Na^+-free acidic media in voltage-clamped epithelial layers of Caco-2 cells in Ussing chambers (Thwaites et al., 1993*b*). In Na^+-free conditions, with the apical pH held at 6.0, glycine (20 mM) induced a rapid and reversible inward short-circuit current (I_{sc}), similar to that reported for β-alanine (Fig. 8*a*). The glycine stimulated increment in inwards I_{sc} was saturable with half-maximal activation of I_{sc} observed at 5.3 ± 0.4 mM (Fig. 8*b*). The maximal increase in I_{sc} was 593 ± 15 $nmol \cdot cm^{-2} \cdot hr^{-1}$. The Table shows that there is congruence between the ability of various amino acids to inhibit net glycine transport, cellular accumulation of glycine across the apical membrane and the ability of the various amino acids to stim-

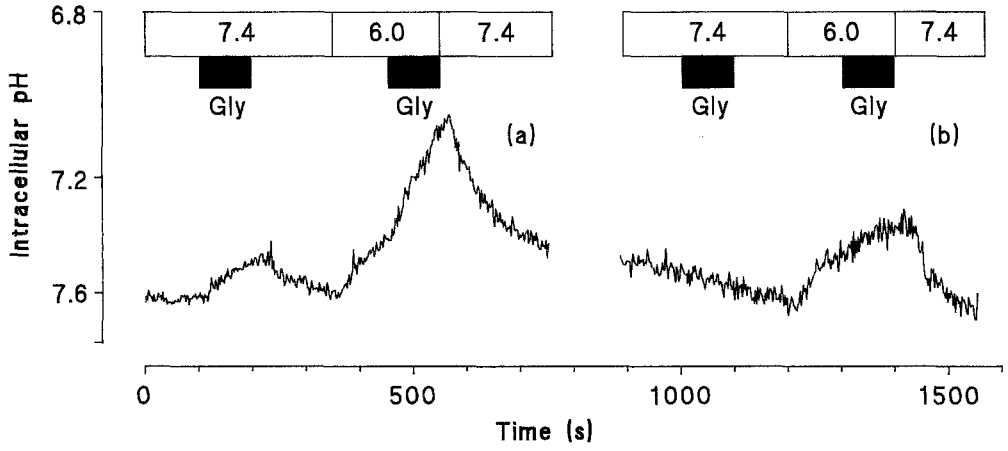

Fig. 5. Glycine-induced intracellular acidification in Caco-2 cell monolayers. pH_i was measured in monolayers of Caco-2 cells loaded with the pH sensitive fluorochrome BCECF. (*a*) The effect of apical exposure to glycine (20 mM) with apical pH at 7.4 or 6.0 (basolateral pH 7.4). (*b*) The effect of basolateral exposure to glycine (20 mM) with basolateral pH at 7.4 or 6.0 (apical pH 7.4). A single trace representative of three separate experiments.

ulate an inward I_{sc}; notably both D-serine and D-alanine were capable of stimulation of inwards I_{sc} and inhibition of glycine transport/accumulation whereas threonine and leucine were ineffective. The basic amino acids, lysine and arginine resulted in a stimulation of outward I_{sc} and were ineffective in inhibition of glycine transport and accumulation.

Discussion

Absorptive transport of glycine across the brush border of the intestinal enterocyte has been thought previously to result from the action of the Na^+-dependent system B (formerly known as NBB and which transports most neutral amino acids) and the Na^+-independent system L (Barker & Ellory, 1990; Stevens, Kaunitz & Wright, 1984). In addition, transepithelial transport of glycine is accomplished by exit across the basolateral membrane via Na^+-dependent systems A and ASC as well as the Na^+-independent systems L and asc (Lash & Jones, 1984; Stevens et al., 1984; Barker & Ellory, 1990). However, the possible role (in mediation of part of the apical or basolateral flux) of other transporters such as the Cl^-/HCO_3^- exchanger or simple diffusion cannot be excluded (Stevens et al., 1984; Barker & Ellory, 1990). Na^+-dependent and Na^+-independent components of the bidirectional fluxes and accumulation of glycine across either the apical or basolateral membranes of human Caco-2 intestinal cells are demonstrated (Figs. 1 and 2). A striking feature is the large Na^+-dependent accumulation of glycine across the basolateral membrane of the Caco-2 intestinal cell (Fig. 2*a*). As the apical pH is lowered in the presence of glycine (100 μM) it is likely that pH_i will acidify. Note the inhibition of the Na^+-dependent basolateral accumulation of glycine as apical pH is lowered from pH 7.4 to 5.5 (Fig. 2*a*). System A is characterized by both its Na^+-dependence and its reduced activity at low pH (Barker & Ellory, 1990). Thus it seems likely that system A is present in the basolateral membrane of the Caco-2 intestinal cell. In Na^+-free conditions, equilibration of glycine across the basolateral membrane is inhibited by glycine L-alanine, proline, AIB, leucine, valine, serine, phenylalanine, methionine, threonine, isoleucine, cysteine, glutamine, asparagine and histidine (Table). These are all substrates for the Na^+-independent systems L and/or asc (Shotwell et al., 1981; Lash & Jones, 1984; Barker & Ellory, 1990). Notably MeAIB, GABA and sarcosine (Table) had relatively little effect on basolateral uptake of glycine in Na^+-free conditions (Table). These substrates are unlikely to be transported by systems L and asc (Stevens et al., 1984; Barker & Ellory, 1990). Thus this pattern of inhibition suggests that systems L, asc and A coexist at the basolateral membrane of Caco-2 cells. Thus within the present data we can define the existence of Na^+-dependent and Na^+-independent transport systems at either brush-border or basolateral membrane of the Caco-2 epithelial cells with properties similar to those recorded for membrane vesicles isolated from mammalian gut (Lash & Jones, 1984; Stevens et al., 1984).

An interesting feature of the expression of Na^+-dependent accumulative transporters at both apical and basolateral membranes is the inability of this intact epithelium to generate significant net glycine absorptive fluxes with equal concentrations of glycine present in apical and basolateral media. The relative levels of transporter expression in human Caco-2 intestinal epithelia ensure cellular accumulation without net transepithelial transport. It is only with the imposition of an external pH gradient across the brush-border membrane of the Caco-2 epithelium that significant net glycine flux

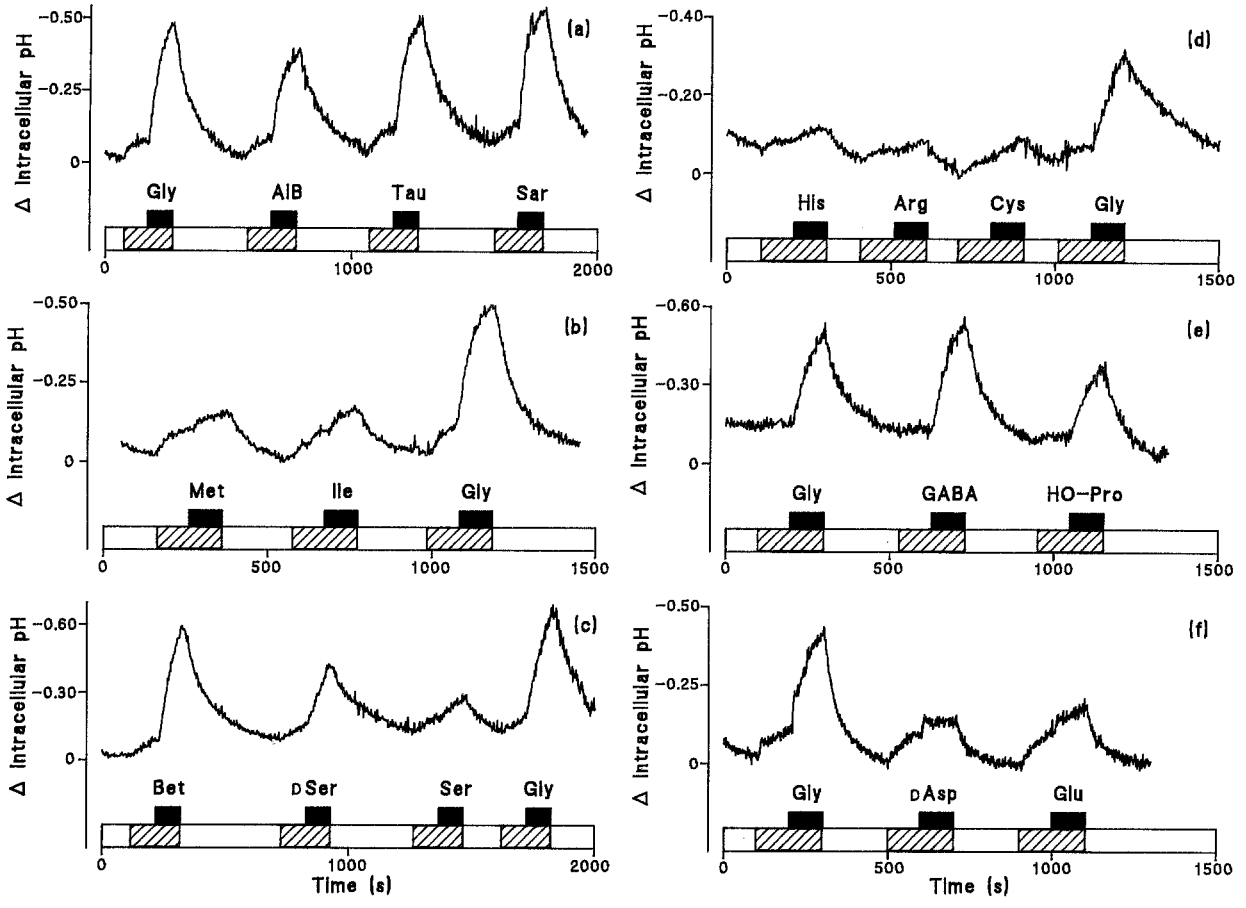

Fig. 6. The ability of amino acids to promote intracellular acidification when perfused at the apical surface of Caco-2 cell monolayers. Sequential perfusion of media of differing apical composition was made as indicated by the boxed area above the time axis (open boxes, apical pH 7.4; striped boxes, apical pH 6.0 or 6.5; solid boxes, amino acid). Basolateral pH was pH 7.4. All amino acid concentrations were 20 mM. In each experiment glycine (*Gly*) was included as a standard. Other amino acids examined were (*a*) AIB, taurine (*Tau*) and sarcosine (*Sar*); (*b*) methionine (*Met*) and isoleucine (*Ile*); (*c*) betaine (*Bet*), D-serine (*DSer*) and serine (*Ser*); (*d*) histidine (*His*), arginine (*Arg*) and cysteine (*Cys*); (*e*) GABA and hydroxyproline (*HO-Pro*); (*f*) D-aspartic acid (*DAsp*) and glutamic acid (*Glu*).

is observed. A similar observation is made with both AIB and taurine.

We have reported previously the partial characterization of a proton/amino acid symport mediating net transport of β-alanine, L-alanine and proline in Caco-2 epithelium (Thwaites et al., 1993*b*; 1993*c*; 1994). The present data extend these observations to provide a comprehensive characterization of H^+/amino acid transport. As with β-alanine (Thwaites et al., 1993*b*) and for the cloned H^+/di-tripeptide transporter expressed in oocytes (Fei et al., 1994) there are three independent lines of experimental measurement to investigate the possibility that the transepithelial transport of glycine may be energized by both the H^+ and Na^+-electrochemical gradients. Glycine fluxes have been measured in both Na^+-containing and Na^+-free (choline) media with the apical solution at pH 7.4, 6.5, 6.0 and 5.5. It is apparent that in Na^+-free conditions the net flux and cellular accumulation of glycine is dependent upon the proton gradient across the brush-border membrane. If the pH-dependence of such glycine transport across the apical membrane is due to coupling of amino acid influx to protons, transport should be associated with intracellular acidification. In BCECF-loaded epithelial layers of Caco-2 cells, apical superfusion with glycine is associated with an intracellular acidification, enhanced when the apical perfusate is held at pH 6.0. Finally, since amino acids exist predominately as zwitterions at the pH values used in the present study (Cohn & Edsall, 1943), proton/amino acid symport activity should be electrogenic. In Na^+-free acidic conditions, glycine generates an inward short-circuit current entirely consistent with apical brush-border proton/glycine symport.

On the basis of selective inhibition of net glycine transport and the ability of amino acids to generate intracellular acidification we have defined those amino acids capable of proton symport as glycine, L-alanine, β-alanine, proline, hydroxyproline, AIB, MeAIB,

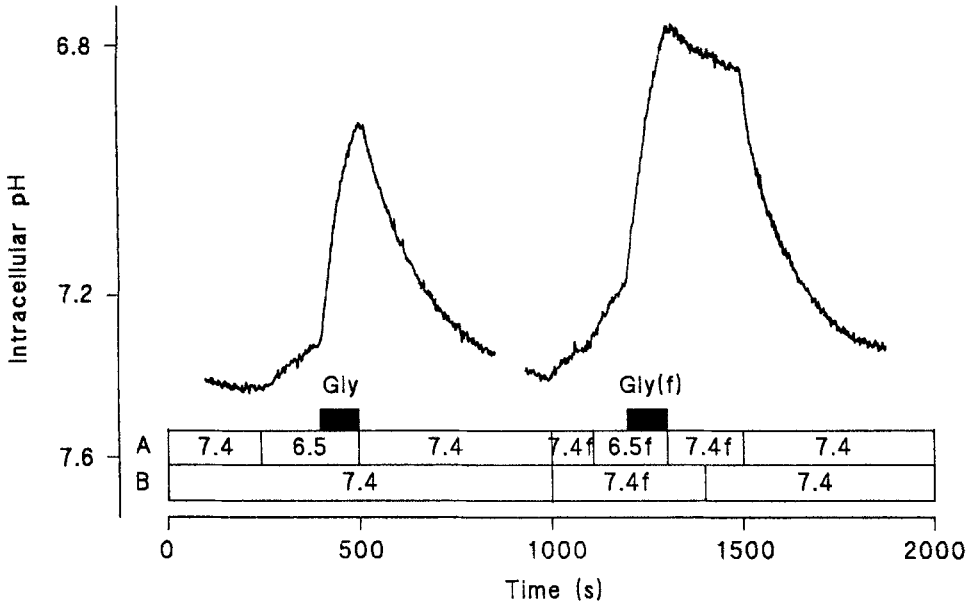

Fig. 7. The effect of extracellular Na^+ on glycine-stimulated intracellular acidification. The effect of apical perfusion of glycine (20 mM) in Na^+-containing and Na^+-free (*f*) conditions. A single trace representative of three separate experiments. Apical (*A*) or basolateral (*B*) media composition are denoted by the boxes above the time axis. Glycine was perfused at the apical membrane only. Note that after glycine-induced intracellular acidification in Na^+-free conditions, Na^+-containing media is reintroduced sequentially to the basolateral then apical bathing solutions. Note that substantial recovery of pH_i occurs only after Na^+ is reintroduced to the apical bathing solution.

GABA, taurine, betaine and sarcosine. The D-isomers of alanine and serine are also weak substrates suggesting the steroselectivity is poor. The proton-dependent, Na^+-independent system described here in the brush border of Caco-2 cells is not related to the known brush-border amino acid transport systems (Barker & Ellory, 1990). However, there are a number of amino acid transporters sensitive to pH. The activity of the Na^+-dependent system A which is restricted to the basolateral membrane in intestinal epithelia (Barker & Ellory, 1990) decreases as pH is lowered. In Chinese hamster ovary cells, uptake of AIB via system A is reduced by approximately 80% when pH is lowered from pH 7.5 to 6.0 (Shotwell et al., 1981). A similar effect is noted in Caco-2 cells where a marked decrease in uptake of AIB and glycine (Fig. 2) across the basolateral membrane is observed. This suggests that Na^+-dependent transport of glycine and AIB across the basolateral membrane of Caco-2 cells is mediated in part by system A and that the pH-sensitive site of the carrier may be on the internal face. In contrast to system A, the Na^+-independent system L is stimulated by lowering pH (Shotwell et al., 1981). However, the H^+-coupled amino acid carrier described in this study is unlikely to be system L since leucine failed to stimulate intracellular acidification (Thwaites et al., 1995), or inhibit pH-stimulated glycine (Table) or MeAIB (Thwaites et al., 1994*a*) uptake at the apical membrane of Caco-2 cells. Furthermore, MeAIB and β-alanine are excluded from system L (Stevens et al., 1984; Barker & Ellory, 1990). The Na^+-dependent system X^-_{AG} is also effected by pH. However, the X^-_{AG} specific substrate D-aspartic acid did not induce intracellular acidification when perfused at the apical membrane of Caco-2 cells (Fig. 6*f*) nor did it inhibit pH-dependent glycine uptake (Table). In rabbit jejunal brush-border membrane vesicles uptake of D-aspartic acid at pH 6.0 was not inhibited by glycine, alanine, MeAIB, proline or hydroxyproline (Maenz et al., 1992), all substrates for H^+-coupled transport in Caco-2 cells. pH-dependent transport of MeAIB (Thwaites et al., 1995) and β-alanine (Thwaites et al., 1993*b*) in Caco-2 cells suggests no relationship to the Na^+-dependent brush-border membrane system B (Stevens et al., 1984). The relatively broad specificity of substrates that access this H^+-coupled carrier in Caco-2 cells most closely resembles the specificity of the Na^+-dependent (Cl^--independent) imino carrier characterized in rat small intestine (Munck et al., 1994). The rat imino carrier transports proline, β-alanine, taurine, sarcosine and GABA (all substrates for H^+-coupled transport in Caco-2 cells) and also shows preference for the D- rather than L-form of serine. However, the main difference in specificity between the rat imino carrier and the transporter described in this study is their relative affinity for L-alanine. Evidence suggests that L-alanine is a non-transported substrate in rat intestine (Munck et al., 1994) but is transported in Caco-2 cells (Thwaites et al., 1994). Another broad specificity amino acid carrier is system $B^{0,+}$ characterized in mouse blastocysts (Van

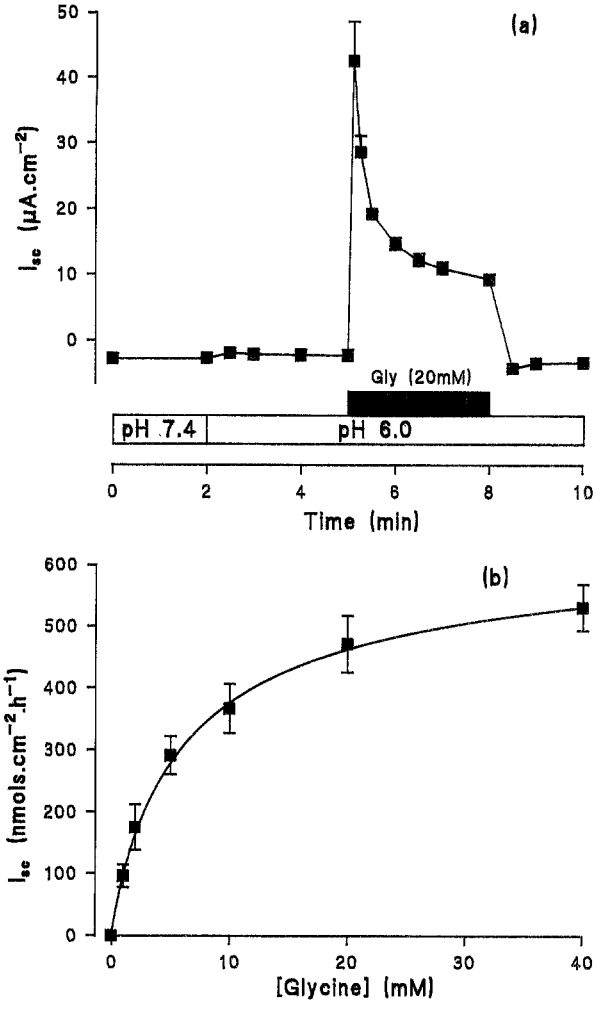

Fig. 8. Glycine transport in short-circuited Caco-2 epithelial monolayers is rheogenic. Epithelial monolayers of Caco-2 cells were continuously short-circuited in Na^+-free media and the short-circuit current (I_{sc}) response determined. Basolateral pH was pH 7.4 (*a*) Time dependence of inward I_{sc} after incubation in apical media at pH 6.0 and exposure to glycine (20 mM). Data are the mean ± SEM (n = 6). (*b*) Concentration dependence of the steady-state I_{sc} (2–3 min of exposure) to glycine in Na^+-free media at pH 6.0. Data are the mean ± SEM (n = 5).

Winkle, Christensen & Campione, 1985). Although $B^{0,+}$ transports L-alanine the inhibition profile is different from that described in Caco-2 cells. L-Alanine transport in mouse blastocysts is inhibited by lysine, arginine and threonine but not by proline, hydroxyproline, β-alanine, taurine or MeAIB (Van Winkle et al., 1985). However, it should be noted that the inhibitory effect of lysine on L-alanine transport was markedly reduced at pH 6.1 (Van Winkle et al., 1985).

In red cells glycine transport has been partitioned between several tranporters (Young, Jones & Ellory, 1981; Barker & Ellory, 1990; King & Gunn, 1991). Interestingly the anionic form of the amino acid may undergo exchange with hydroxyl ions (or H^+ cotransport) via the stillbene–sensitive Band 3 exchanger. Our present data do not allow us to distinguish between H^+ symport or OH^- antiport, raising the possibility that an apical anion exchanger may mediate the acid-stimulated glycine flux reported here. Three lines of evidence lead us to believe that the anion-exchanger is not involved. First, incubation with 100 μM DIDS does not modify the pH_i response to MeAIB (*data not shown*). Secondly DIDS-sensitive glycine transport in red cells is reduced as medium pH is made acidic due to a decrease in the glycine anion concentration (in Caco-2 cells J_{net} increases as the apical pH is made acidic). Finally, transport via Band 3 is selective. Glycine, serine and cysteine are transported whereas alanine, proline, valine and threonine are not (Young et al., 1981). This profile clearly does not match the selectivity data reported in this study.

In addition to the radiolabel flux data presented here for glycine, AIB and taurine, we have also demonstrated by radioisotopic flux determinations the Na^+-independent acid stimulated net transport of β-alanine (Thwaites et al., 1993*b*), proline (Thwaites et al., 1993*c*), MeAIB (Thwaites et al., 1995) and L-alanine (Thwaites et al., 1994). Furthermore, Na^+-independent leucine and phenylalanine transport across Caco-2 epithelia were not stimulated by apical acidity (*unpublished observations*). Cross-inhibition data for β-alanine (Thwaites et al., 1993*b*), MeAIB (Thwaites et al., 1995) and L-alanine (Thwaites et al., 1994) is consistent with the existence of a single transport system. Furthermore, the similar excursions of intracellular pH following apical superfusion with differing amino acids measured in a single experiment (Fig. 6*a*) is also suggestive of a single transport system. Since cross inhibition of amino acid fluxes may occur by alteration of the proton motive force across the apical membrane, more rigorous experimentation to address this point is required where additivity of responses to pH_i and rheogenic current are measured. For the cloned H^+/di-tripeptide transporter (PepT1) expressed in oocytes there is no evidence that glycine can act as a substrate (Fei et al., 1994). The question whether those amino acids are common substrates for a single proton/amino acid symport (PAT), or whether they utilize distinct transporters linked to the H^+-electrochemical gradient requires molecular identification. Multiple pH-dependent transporters for amino acids have been described in sugar beet leaves (Li & Bush, 1990). Also the SAF family of alanine/glycine transporters includes proteins (e.g., that from the thermophilic bacterium PS3), that show H^+/amino acid symport (Reizer, Reizer & Saier, 1994). By analogy with other mammalian amino acid transporters (e.g., glutamate transporters; McGivan & Pastor-Anglada, 1994), it is likely that system PAT will be one of a family of transporters.

The ability of cultured human intestinal cell layers to utilize the transmembrane proton gradient rather than the Na^+ electrochemical gradient to energize the transepithelial transport of substrates such as dipeptides and amino

acids focuses attention on the establishment, maintenance and regulation of the brush-border proton electrochemical gradient. Under physiological conditions in the human small intestine an acid microclimate (an area of low pH adjacent to the apical membrane) has been demonstrated in vitro (Lucas et al., 1978) and in vivo (Rawlings, Lucas & Russel, 1987). An apical Na^+/H^+ exchange mechanism (Fig. 7) will allow coupling of proton/amino acid symport to Na^+ in an indirect way. In addition, there is evidence for Na^+-independent pH_i recovery which may well reflect the operation of an H^+, K^+-ATPase (Abrahamse, Bindels & van Os, 1992). In the physiological situation (in the presence of extracellular Na^+) electrogenic H^+/amino acid symport at the apical membrane will occur simultaneously with Na^+/amino acid symport, and where the two gradients are linked via the transmembrane p.d. together with the Na^+/H^+ exchanger, it is possible to speculate on the relative advantage of such an arrangement. Is solute reabsorptive capacity maximized? Na^+-coupled transport capacity is ultimately dependent upon the extrusion capacity of Na^+ by the Na^+, K^+-ATPase at the basolateral membrane, whereas H^+-coupled transport can not only use the energy-transduction capacity of the Na^+, K^+-ATPase but also the H^+, K^+-ATpase. In addition, intracellular proton load may be buffered by the intracellular chemical buffers in a way that is not possible for intracellular Na^+. Thus it is possible that the H^+/solute symports may be of functional significance to allow rapid assimilation of bolus delivery of nutrient at the absorptive surface. This emphasizes the potential importance of the acidic microclimate (Lucas et al., 1978; Rawlings et al., 1987) to normal intestinal absorptive function.

H^+/dipeptide symport is observed not only in the small intestine but also in the renal proximal tubule (Ganapathy & Leibach, 1986; Silbernagl, 1988). In renal brush-border membrane vesicles, a H^+- and Na^+-independent electrogenic transport of glycine has been reported (Rajendran et al., 1987). This renal transport was similar to that reported here for human intestinal Caco-2 cells, that is H^+/glycine transport was inhibited by proline, sarcosine, β-alanine but not by leucine and lysine (Rajendran et al., 1987). In addition, proton driven L-alanine and AIB transport have been demonstrated (Vorum et al., 1988; Jessen et al., 1991). Thus in several respects including data on the specificity of this transport, the data obtained in rabbit renal brush-border membrane vesicles are similar to those described here for the brush border of human intestinal cells. It thus seems likely that H^+-coupled amino acid transporters may occur in both the renal and intestinal epithelia. Whether there is full molecular identity will first require cloning and sequencing of the carrier(s) involved.

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