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Direct assessment of dipeptide/H^+ symport in intact human intestinal (Caco-2) epithelium: a novel method utilising continuous intracellular pH measurement

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Direct demonstration of intact peptide transport by the intestinal H^+/dipeptide carrier is limited both by luminal/cytosolic hydrolysis and the availability of suitable radiolabelled substrates. Perfusion of Val-Val (20mM) at the apical surface of human intestinal epithelial (Caco-2) cell monolayers resulted in a marked intracellular acidification, due to dipeptide-induced H^+-flow across the apical membrane. Val-Val (20mM) also inhibited both the pH-dependent apical-to-basal transport and intracellular accumulation of [^{14}C]Gly-Sar. Valine (20mM) had no effect on [^{14}C]Gly-Sar transport (and intracellular accumulation) or pH_i. We conclude that this novel method for studying H^+-coupled transport clearly differentiates between the mechanisms responsible for absorption of an intact substrate and products of hydrolysis.

Protein digestion occurs by luminal and epithelial hydrolysis to form amino acids and small peptides (di/tripeptides) which are then absorbed across the intestinal epithelium via distinct mechanisms. It is generally accepted that amino acid absorption is mediated via a series of Na^+-dependent and independent carriers arranged in series at the apical and basolateral membranes of the intestinal enterocyte [1]. In contrast, the absorption of di/tripeptides is via pH-dependent H^+-coupled mechanisms also localised at both apical [2,3] and basolateral [3,4] membranes. Results of early investigations of di/tripeptide absorption using intact tissue preparations such as the hamster jejunal ring, *in vitro*, were difficult to interpret because of the hydrolysis of many peptides by the brush-border hydrolases to their constituent amino acids [5]. This problem has been partly overcome by use of substrates that are resistant to hydrolysis such as carnosine or Gly-Sar [2,3,5]. Recently the Caco-2 cell

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system has become popular in absorption studies since the system expresses a number of solute transporters including those for sugars [6-8], amino acids [9,10], bile acids [11], and aminocephalosporins [12] and dipeptides [3] making it a unique model system for intestinal epithelial permeability studies [13]. Using this system we have demonstrated direct coupling of dipeptide (Gly-Sar) transport to proton flux by measurement of intracellular pH (pH_i) with the pH-sensitive fluorescent dye BCECF [3]. Therefore, we can measure directly dipeptide-induced H^+ flow into the cells, and distinguish between substrates that access the H^+-coupled intestinal dipeptide transporter and others that are hydrolysed to amino acids whose absorption is coupled to gradients other than the proton-motive force. Furthermore, the mechanism of pH_i recovery after this substrate-induced acid load was investigated and identified as an apically-localised Na^+/H^+ exchanger.

MATERIALS AND METHODS

Materials. [^{14}C]Gly-Sar (L-glycyl[1-^{14}C]sarcosine (Specific activity 14 mCi/mmol)) was from Amersham. [^{3}H]Mannitol (Specific activity 30 Ci/mmol) was obtained from NEN. Gly-Sar, Val-Val and valine were from Sigma. BCECF (2',7'-bis(2-carboxyethyl-5(6)-carboxyfluorescein), cell culture media, supplements and plastic were supplied by Life Technologies. All other chemicals were from Merck and were of the highest quality available.

Cell culture. Caco-2 cells (passage number 103-115) were cultured in DMEM (with 4.5g/l glucose), with 1% non-essential amino acids, 2mM L-glutamine, 10% (v/v) foetal calf serum and gentamicin (60μg/ml). Cell monolayers were prepared by seeding at high density ($4.4\text{-}5.0 \times 10^5$ 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. [^{14}C]Gly-Sar uptake and transport experiments were performed 21-25 days after seeding (unless stated otherwise) and 18-24h after feeding. Transepithelial flux measurements were performed as described previously [3]. Briefly, the cell monolayers (24.5mm in diameter) were placed in 6-well plates, each well containing 2ml 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, 37°C). The pH 6.0 buffer used in both transport and intracellular pH experiments was identical except that 10mM MES/Tris replaced 10mM 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 chamber (apical solution) and the filters were incubated for 10min at 37°C. This procedure was repeated with fresh buffer (pH 7.4) before being replaced with the experimental buffers (apical pH 6.0, basolateral pH 7.4). Radiolabelled Gly-Sar (0.5μCi/ml; 36μM) and mannitol (0.5μCi/ml; 36μM) were added to the apical chamber. In experiments involving high dipeptide/amino acid concentrations (20mM) isoosmolarity was maintained by addition of mannitol. Fluxes in the absorptive (apical-to-basal) direction were determined and are expressed as pmol/cm^2.h^{-1}. At the end of the incubation period cell monolayers were washed in 4x500ml 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 or as a cell/medium (C/M) ratio. Cell height was

determined by confocal microscopy and this value was used in the determination of intracellular volume. Results are expressed as mean ± SEM.

Intracellular pH measurements. For pH_i measurements [3], Caco-2 cells grown to confluence on 12mm diameter Transwell polycarbonate filters (Costar) were loaded by incubation with BCECF-AM (5μM), in both apical and basal chambers, for 40min at 37°C. After loading, the inserts were placed in a 24mm 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 5ml/min) which allowed any combination of 6 apical and 6 basolateral solutions. Apical and basolateral bath volumes were 0.5 and 1ml and bath contents could be completely changed in 6 or 12s, respectively. All solutions were preheated to 37°C. Intracellular H^+ concentration was quantified by fluorescence (excitation at 440/490nm and emission at 520nm) 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 (10μM) and high K^+ solutions [14].

RESULTS

Transepithelial (apical-to-basal) transport and intracellular accumulation (across the apical membrane) of the dipeptide Gly-Sar were determined using monolayers of Caco-2 cells both in the presence and absence of excess (20mM) cold dipeptide (Val-Val) or amino acid (valine). Clearly, the dipeptide Val-Val caused a marked reduction in the pH-dependent, apical-to-basal transport of Gly-Sar, without altering transport of the paracellular marker mannitol (Fig. 1). Similarly, intracellular accumulation of [^{14}C]Gly-Sar was also reduced

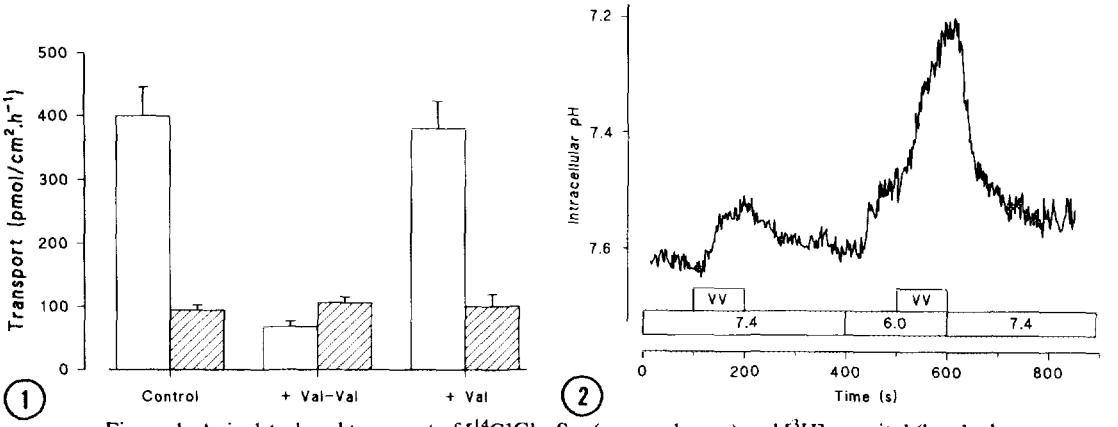

Figure 1. Apical-to-basal transport of [^{14}C]Gly-Sar (open columns) and [^{3}H]mannitol (hatched columns) across Caco-2 cell monolayers in the presence of a transepithelial pH gradient (apical pH 6.0, basolateral pH 7.4). Transport was determined under control conditions (in the absence of unlabelled dipeptide or amino acid) and in the presence of 20mM Val-Val or valine (Val). Results are the mean±SEM, n=3-4.

Figure 2. The effect on intracellular pH of 20mM Val-Val (VV) when perfused at the apical surface (at both pH 7.4 and 6.0) of BCECF-loaded Caco-2 cell monolayers. A single experiment representative of four separate experiments.

from 278.5±27.1 μM (n=4, C/M ratio 7.8) to 22.0±1.4 μM (n=4, C/M ratio 0.6). In contrast, valine (20mM) had no effect on either [^{14}C]Gly-Sar transport (Fig. 1) or accumulation (264.0±10.0 μM (C/M ratio 7.4) n=4).

Since dipeptides are absorbed by a H^+-coupled mechanism, movement of dipeptide across the apical membrane will be accompanied by H^+ flow into the cell and hence a lowering (acidification) of the intracellular pH. This effect is demonstrated in Fig. 2, where Val-Val causes marked acidification of the intracellular environment when perfused across the apical surface of Caco-2 cells with the apical medium held at either pH 7.4 or 6.0. Substrate-induced H^+ flow was also observed with a number of dipeptides which are relatively-resistant to hydrolysis including Gly-Pro, Gly-Phe, Gly-Gly, Pro-Gly and carnosine (not shown). The amino acid valine (20mM) had no effect (Fig. 3).

The Na^+-independence of dipeptide-induced H^+-flow (and hence intracellular acidification) across the apical surface is shown in Fig. 4. Note that the pH_i recovery from this dipeptide-induced acid load is dependent on the presence of Na^+ at the apical surface only.

DISCUSSION

Early studies of dipeptide absorption [5] suggested that dipeptides, like glucose and amino acids, were absorbed by a Na^+-dependent mechanism. These experiments were complicated by the stability of the substrate under examination since many dipeptides are highly susceptible to degradation to the constituent amino acids [5], followed by Na^+-dependent absorption. The choice of hydrolysis-resistant substrates such as carnosine and

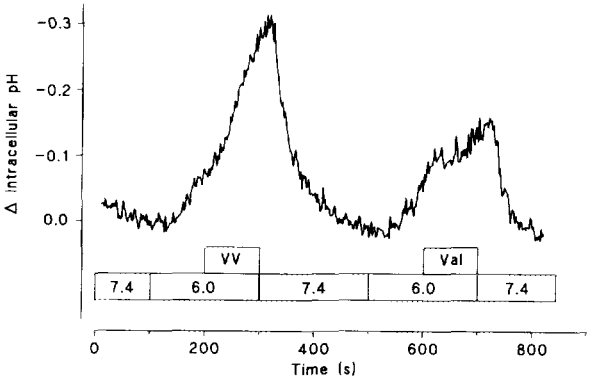

Figure 3. A comparison of the effects of 10mM Val-Val (VV) and 20mM valine (V) on intracellular pH when perfused at the apical surface at pH 6.0 (basolateral pH 7.4). A single trace representative of three others.

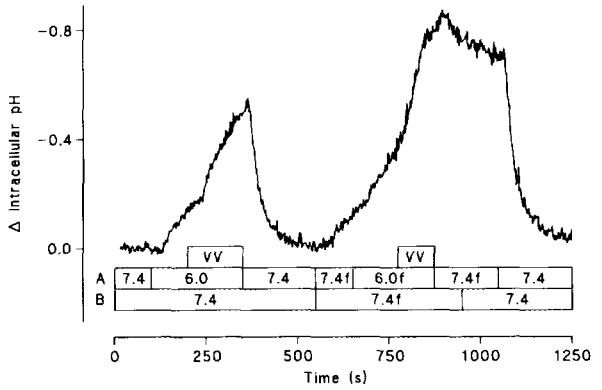

Figure 4. Dipeptide (Val-Val)-induced intracellular acidification in the presence and absence of extracellular Na^+ in both apical (A) and basolateral (B) chambers, f denotes Na^+ free conditions. Note that Na^+ is reintroduced sequentially to the basolateral then apical surface. This experiment is representative of three others.

Gly-Sar was a major development in these studies [2,3,5]. Experiments using stable dipeptides and brush-border membrane vesicle preparations have clearly established that dipeptides are absorbed across the apical membrane of the intestinal epithelium by a pH-dependent mechanism [2]. Furthermore, direct H^+-coupling to dipeptide transport has been demonstrated in both vesicle preparations [15] and Caco-2 cells [3].

Val-Val is a substrate that will undergo substantial degradation at the intestinal surface [16]. However, this dipeptide markedly decreases the pH-dependent transport and intracellular accumulation of the model substrate [^{14}C]Gly-Sar. Previous studies with hamster jejunal rings, *in vitro* [16] suggest that the transport of Val-Val is maximal at approximately pH 6.0-7.0, and at pH 6.0 the dipeptide is relatively stable (approximately 30% of the hydrolysis at pH 7.4). Interestingly, the pH adjacent to the intestinal surface both *in vivo* [17] and *in vitro* [18] has been shown to be more acidic than the luminal pH (the acid microclimate).

The direct demonstration of cytosolic acidification with apical Val-Val 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) (Fig. 2). Val-Val inhibits [^{14}C]Gly-Sar transport and shows an identical pattern to Gly-Sar in promoting intracellular acidification across the apical membrane. Taken together, these observations suggest that the dipeptides Gly-Sar and Val-Val share a common transport system at the apical membrane of Caco-2 cells. Valine had no effect on [^{14}C]Gly-Sar transport or accumulation and failed to cause acidification of the intracellular environment when perfused across the apical surface at either pH 6.0 (Fig. 3) or pH 7.4 (not shown). This demonstrates that the response observed upon

apical perfusion of Val-Val is an effect of intact peptide and obviates any possible effects of products of hydrolysis.

A further complication in dipeptide transport studies with intact tissues is that after H^+-coupled dipeptide transport across the apical membrane the protons are thought to recycle across the apical membrane via a Na^+/H^+ exchanger thus maintaining the acid microclimate at the apical surface. Clearly removal of extracellular Na^+ will decrease the ability of intact tissue to recycle protons and thus indirectly decrease dipeptide absorption and suggest that Na^+ is involved directly in coupling to dipeptide transport. This is an observation made many times [5]. This secondary response may now be explained because, as demonstrated in Fig. 4, not only does the dipeptide Val-Val cause intracellular acidification (and hence H^+ flow) in both Na^+-free and Na^+-containing conditions, it is also evident that the rate of pH_i recovery after dipeptide-induced acid load is dependent on the presence of Na^+ at the apical surface indicating the involvement of an apically-localised Na^+/H^+ exchanger. However, even in the absence of external Na^+ pH_i recovers (although at a slower rate) suggesting that an alternative transporter (i.e. H^+,K^+-ATPase [19]) is involved in pH_i regulation. It is likely that the relative roles (capacities) of these two mechanisms will vary depending upon the nutritional state of the intestinal contents, so that a balance is maintained between dissipative H^+ forces and pH_i regulation.

In conclusion, we describe a method for studying H^+-coupled transport which allows the separation of dipeptide transport across the apical membrane from constituent amino acids produced by peptide hydrolysis. This method will prove a useful tool in studies of H^+-coupled transport. Furthermore the demonstration of an apical Na^+/H^+ exchanger focusses attention on the mechanism of pH_i homeostasis in cells where dissipative H^+ transport plays an important role in normal functional solute absorption.

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