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H^+-coupled (Na^+-independent) proline transport in human intestinal (Caco-2) epithelial cell monolayers

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Previously, absorption of L-proline across the apical membrane of the intestinal enterocyte has been attributed to transport via the Na^+-dependent Imino system. However, net (absorptive) transport of proline across intact Caco-2 cell monolayers was enhanced by acidification of the apical environment, under both Na^+-containing and Na^+-free conditions. This Na^+-independent pH-dependent proline flux was associated with H^+ flow across the apical membrane as determined by continuous measurement of intracellular pH. H^+/proline symport was associated with an inward I_{sc} in voltage-clamped Caco-2 epithelial layers demonstrating the electrogenic nature of this transport process. In conclusion Caco-2 cells possess an apically-localised, Na^+-independent, electrogenic H^+/imino acid transporter which may play an important role in intestinal proline absorption.

H^+-coupled transport; Proline transport; Amino acid; Human intestine; Intestinal epithelium; Caco-2 cell

1. INTRODUCTION

Although H^+-coupling of solute transport is widely recognised in bacteria [1] the main focus for coupling of solute transfer secondarily to metabolic energy in mammalian cells is the transmembrane Na^+ electrochemical gradient [2]. It is generally accepted that absorption of amino acids across the intestinal enterocyte is a result of a number of Na^+-dependent and Na^+-independent carriers arranged in series at the apical and basolateral membranes [3,4]. For example, transepithelial absorption of L-proline is accomplished by transport via the Na^+-dependent Imino system at the apical membrane [5] and the Na^+-dependent system A at the basolateral membrane [3,4]. In humans, the hereditary malabsorption syndrome iminoglycinuria is associated with a defect in proline, hydroxyproline and glycine transport [6]. Patients show a variable pattern of defect expression, including differential effects on glycine and proline transport, as well as tissue specific effects (in some patients renal transport and intestinal absorption are both defective whereas in others only the renal defect is expressed) [6]. These observations suggest that multiple systems may be involved in proline transport/ absorption.

The Caco-2 cell system has considerable utility for intestinal absorption studies expressing a number of solute transporters including those for sugars [7], amino acids [8,9], bile acids [10], aminocephalosporins [11] and dipeptides [12]. Direct coupling of dipeptide transport to proton flux (by measurement of intracellular pH (pH_i) with the pH-sensitive fluorescent dye BCECF) in this human intestinal epithelial cell system [12,13] demonstrates the applicability of this model cell line to studies of H^+-coupled solute transport. Most recently, using this cell system, we have identified Na^+-independent, H^+-coupled transepithelial transport of β-alanine [14]. A previous investigation [9] characterized Na^+-dependent proline uptake in Caco-2 cells (grown on plastic) which on the basis of competition studies was attributed to transport via system A. Interestingly, proline uptake in Na^+-containing media was markedly stimulated in the presence of apical acidity [9]. The present investigation sought to determine whether this pH stimulated uptake of proline actually represents H^+-coupled transport similar to that for β-alanine [14].

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2. MATERIALS AND METHODS

2.1. *Materials*

L-[U-^{14}C]Proline (specific activity > 250 mCi/mmol) and L-[2,3-^{3}H]-proline (specific activity 20–40 Ci/mmol) were from Amersham. L-Proline was 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.

2.2. *Cell culture*

Caco-2 cells (passage number 98–116) 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 ($4.4–5.0 \times 10^5$ 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. Uptake, transport, pH_i and short-circuit

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current experiments were performed 22–25 days after seeding and 18–24 h after feeding.

2.3. *Transport experiments*

Transepithelial flux measurements were performed as described previously [14]. Briefly, the cell monolayers (24.5 mm in diameter) were washed in 4 × 500 ml of Na^+-containing or Na^+-free Krebs (pH 7.4) as appropriate, placed in 6-well plates, each well containing 2 ml of modified Krebs buffer (all mM), NaCl 137, KCl 5.4, $CaCl_2$ 2.8, $MgSO_4$ 1.0, NaH_2PO_4 0.3, KH_2PO_4 0.3, glucose 10, HEPES/Tris 10 (pH 7.4, 37°C) or Na^+-free Krebs (obtained by replacement of NaCl by choline Cl and omission of NaH_2PO_4). Buffers at pH 5.5, 6.0 and 6.5 were similar to those described above except that 10 mM MES replaced 10 mM HEPES, each was buffered to the required pH using Tris. Aliquots of the appropriate buffer were placed in the upper chamber (apical solution). [^{3}H]Proline (0.1–0.3 μCi/ml; 100 μM) and [^{14}C]proline (0.1–0.3 μCi/ml; 100 μM) were added to the apical and basal chambers, respectively. Fluxes in the absorptive (apical-to-basal, $J_{a\text{-}b}$) and secretory (basal-to-apical, $J_{b\text{-}a}$) directions were determined and are expressed as nmol · cm^{-2} · h^{-1}. 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 proline 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 ± S.E.M.

2.4. *Intracellular pH measurements*

For pH_i measurements [12], 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) and perfused at both apical and basolateral membranes [12]. 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 [15].

2.5. *Short-circuit current measurements*

Measurements of short-circuit current (I_{sc}) were made essentially as described previously [16]. Cultured epithelial layers (grown to confluence on 12 mm diameter Snapwell polycarbonate filters) were mounted in Ussing-type chambers maintained at 37°C, connected to an automatic voltage-clamp apparatus (World Precision Instruments) via KCl/agar salt-bridges and reversible electrodes (Ag/AgCl for current passage, calomel for voltage sensing) and measured in modified Krebs solutions (see above). The chemical flux equivalent of the I_{sc} is 1 μA · cm^{-2} = 36 nmol · cm^{-2} · h^{-1}.

3. RESULTS

Transepithelial transport of proline in both apical-to-basal ($J_{a\text{-}b}$) and basal-to-apical directions ($J_{b\text{-}a}$) was determined using monolayers of Caco-2 cells. In the absence of any transepithelial pH gradient (apical pH 7.4, basolateral pH 7.4) net absorptive transport (J_{net}) was small in both Na^+-containing (8.7 ± 3.5 nmol · cm^{-2} · h^{-1}) and Na^+-free (6.5 ± 3.1 nmol · cm^{-2} · h^{-1}) conditions (Fig. 1a). Acidification (pH 6.0) of the apical compartment increased J_{net} (Fig. 1a) in both Na^+-containing

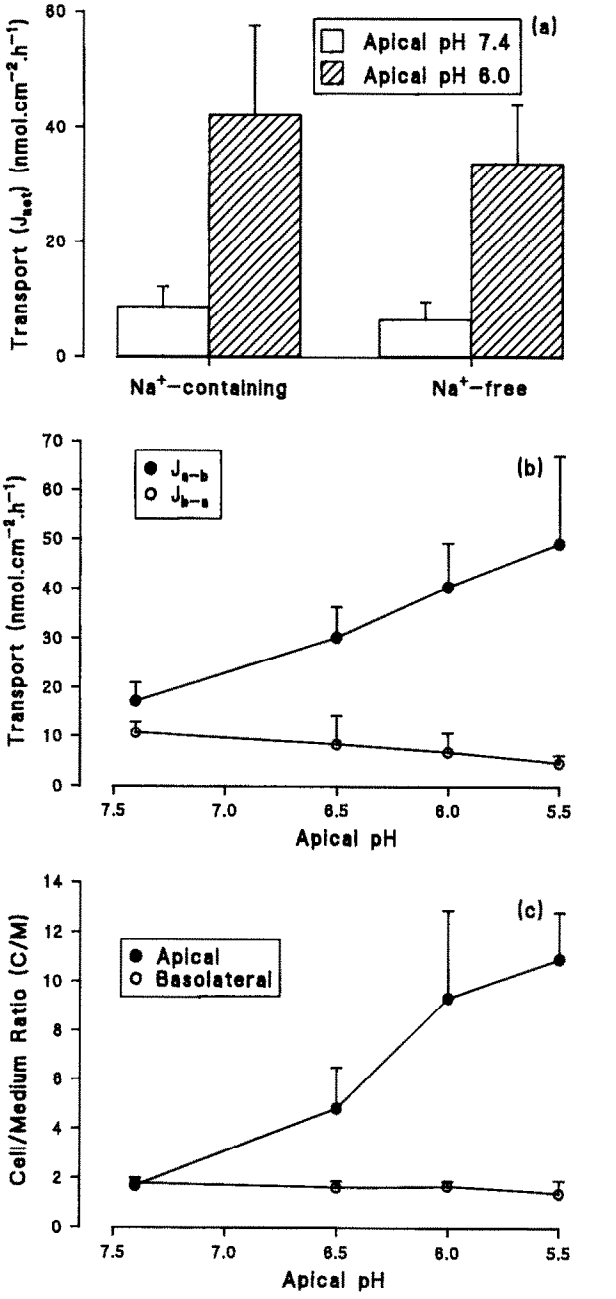

Fig. 1. (a) Net transepithelial transport (J_{net}) of proline across Caco-2 cell monolayers in the presence (striped columns) and absence (open columns) of a transepithelial pH gradient, in both Na^+-containing and Na^+-free conditions. (b) Effect of variation in apical pH on apical-to-basal ($J_{a\text{-}b}$, filled circles) and basal-to-apical ($J_{b\text{-}a}$, open circles) transport of proline in Na^+-free conditions. (c) Intracellular accumulation of proline in Caco-2 cell monolayers across the apical (filled circles) and basolateral (open circles) epithelial membranes in the absence of external Na^+. In all cases basolateral pH was maintained at pH 7.4, and L-proline was present in both apical and basal chambers (concentration 0.1 mM). Results are mean ± SEM, n = 4–5.

(42.2 ± 15.6 nmol · cm^{-2} · h^{-1}) and Na^+-free (33.6 ± 10.4 nmol · cm^{-2} · h^{-1}) conditions. The increase in J_{net} was due mainly to a pH-stimulated increase in $J_{a\text{-}b}$. With Na^+ present, apical acidity (pH 5.5) increased $J_{a\text{-}b}$ from

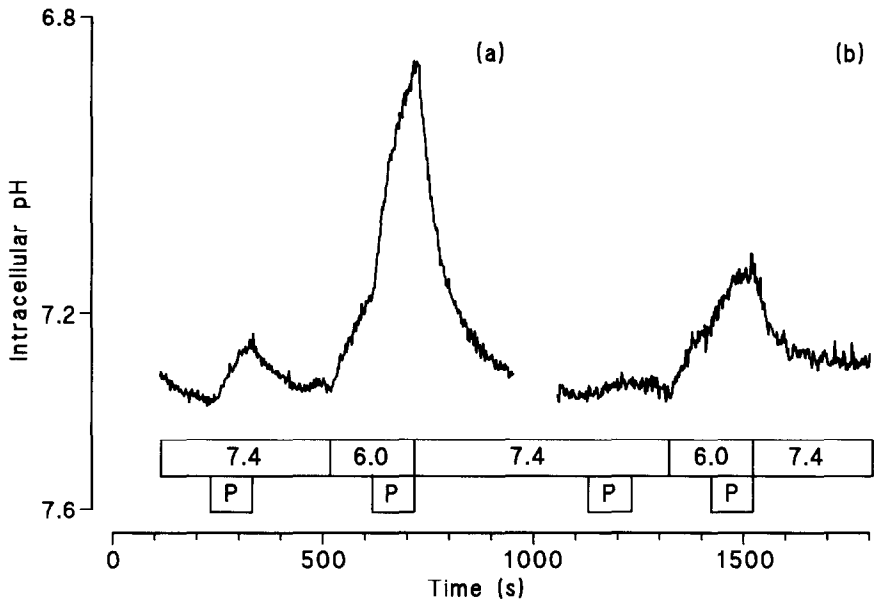

Fig. 2. The effect on intracellular pH of 20 mM proline (P) when perfused at both pH 7.4 and 6.0 at either the (a) apical (basolateral pH 7.4) or (b) basolateral (apical pH 7.4) membranes of BCECF-loaded Caco-2 cell monolayers. A single experiment representative of three separate experiments.

12.9 ± 2.5 nmol $\cdot$ cm^{-2} $\cdot$ h^{-1} ($n = 4$) to 62.3 ± 11.9 nmol $\cdot$ cm^{-2} $\cdot$ h^{-1} ($n = 4$), whereas $J_{b\text{-}a}$ was relatively unaffected (4.2 ± 2.1 nmol $\cdot$ cm^{-2} $\cdot$ h^{-1} and 4.0 ± 0.6 nmol $\cdot$ cm^{-2} $\cdot$ h^{-1} at apical pH 7.4 and 5.5, respectively, both $n = 4$). Similar data were obtained in Na^+-free conditions (Fig. 1b). Intracellular accumulation of proline across the apical membrane was increased by apical acidity. In the presence of external Na^+, accumulation across the apical membrane increased from 783.0 ± 220.8 μM ($n = 4$, C/M ratio 7.8) at apical pH 7.4, to 1453.0 ± 35.0 μM ($n = 4$, C/M ratio 14.5) at apical pH 6.0. In contrast, accumulation across the basolateral membrane (with Na^+ present) fell as a result of apical acidification from 854.5 ± 132.1 μM ($n = 4$, C/M ratio 8.5) to 192.3 ± 11.4 μM ($n = 4$, C/M ratio 1.9) at apical pH 7.4 and 5.5, respectively. Apical pH changes had an identical effect on intracellular proline accumulation in Na^+-free conditions (Fig. 1c).

Addition of proline (20 mM) to the apical perfusate of BCECF-loaded Caco-2 cell monolayers lead to acidification of the intracellular environment which was reversed upon removal of proline from the apical perfusate (Fig. 2a). When the pH of the apical perfusate was reduced to pH 6.0, an intracellular acidification was observed that was markedly enhanced in the presence of proline (20 mM) (Fig. 2a). When identical manipulations of the extracellular environment were performed at the basolateral membrane a different pattern emerged (Fig. 2b). At both basolateral pH 7.4 and 6.0 the responses to proline are markedly attenuated compared to responses at the apical surface.

Fig. 3 indicates that with the apical compartment held at pH 6.0 (basolateral pH 7.4) in Na^+-free conditions, the addition of proline to the apical surface of voltage-clamped Caco-2 cell monolayers is associated with a saturable (K_m 9.2 ± 0.9 mM) simulation of electrogenic transport and a maximal increase in I_{sc} of 378.0 ± 13.2 nmol $\cdot$ cm^{-2} $\cdot$ h^{-1}.

4. DISCUSSION

Historically the intestinal absorption of amino acids has been believed to be via a combination of passive transport and facilitated transport via the numerous Na^+-dependent and Na^+-independent amino acid carriers located at the apical and basolateral membranes of the intestinal enterocyte [3,4]. Until relatively recently [14] there has been no evidence to suggest a role for the transmembrane proton electrochemical gradient in the intestinal absorption of amino acids even though an area of low pH (the acid microclimate) has been shown to be present at the luminal surface of the intestinal epithelial wall as demonstrated both in vivo [17] and in vitro [18]. This transepithelial pH difference will provide a substantial proton gradient across the apical membrane which could be used as the energy source to drive net absorption of solutes as shown for di/tripeptides [12,19]. We have recently shown that the transepithelial transport of β-alanine in Caco-2 cell monolayers is driven by the proton electrochemical gradient in both the presence and absence of external Na^+ [14].

Using three complementary techniques (radiolabel fluxes, intracellular pH measurements, and short-circuit current measurements in voltage-clamped epithelial monolayers) we now show that transepithelial transport and intracellular accumulation of L-proline are coupled

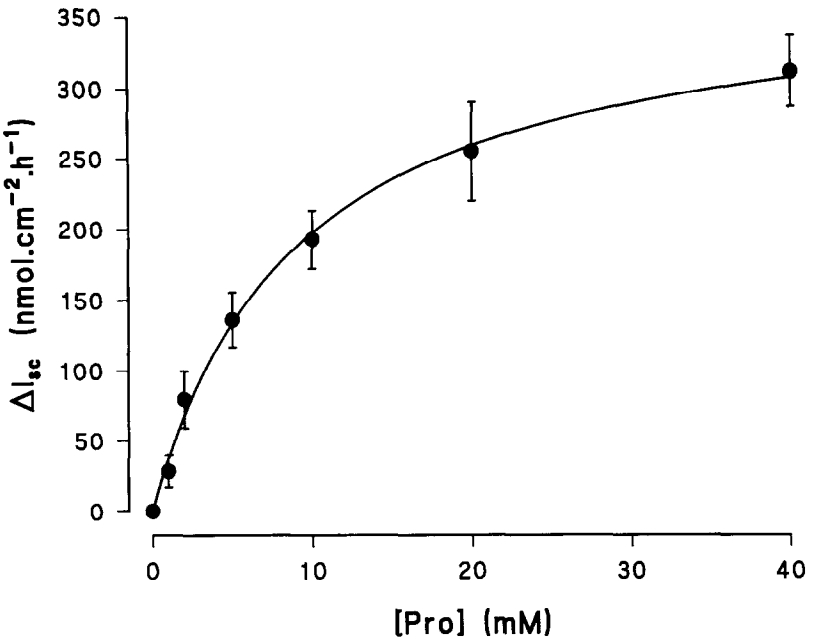

Fig. 3. Concentration dependent L-proline stimulation of electrogenic transport in voltage-clamped Caco-2 cell epithelial monolayers. Experiments were performed under Na^+-free conditions (apical pH 6.0, basolateral pH 7.4). L-Proline was added to the apical surface only. The rectangular hyperbola is the Michaelis–Menten fit for the data calculated by non-linear regression with the method of least-squares (Fig-P, Biosoft).

to the proton electrochemical gradient. Transepithelial transport and intracellular accumulation of proline are pH-dependent in both the presence and absence of external Na^+ (Fig. 1). This pH-dependency represents direct coupling of H^+ flow to proline transport across the apical membrane (Fig. 2) as demonstrated in BCECF-loaded Caco-2 cells. Addition of proline to the apical surface of voltage-clamped Caco-2 cell monolayers is associated with an inward I_{sc} in the absence of Na^+, consistent with electrogenic H^+-coupled proline transport (Fig. 3).

Clearly both β-alanine and L-proline can undergo H^+-coupled transport. Comparison of the I_{sc} measurements indicates that although the affinity for transport is similar (K_m 9.2 ± 0.9 and 8.1 ± 1.1 mM for L-proline and β-alanine, respectively) there appears to be a greater capacity for transport of L-proline (378.0 ± 13.2 nmol · cm^{-2} · h^{-1}, compared to 215.0 ± 10.0 nmol · cm^{-2} · h^{-1} for β-alanine). H^+-coupled β-alanine transport was inhibited by L-alanine [14] and L-proline (unpublished observation). In contrast, studies in rabbit intestinal BBMV demonstrated that NaCl-dependent β-alanine transport was unaffected by L-alanine or L-proline [20] and β-alanine is not an inhibitor of Na^+-dependent proline transport by the Imino carrier [5]. Although proline was a potent inhibitor of H^+-coupled β-alanine transport and accumulation the question of whether these two substrates are transported by a single H^+-coupled carrier is a difficult one to approach and remains to be answered. There are inherent difficulties in characterising the inhibitory actions of a number of substrates (amino acids) on a H^+-coupled transport process in intact cell systems. The inhibitory actions may arise as a consequence of two substrates (β-alanine and proline) competing directly for transport, or by interactions with the driving force (the proton electrochemical gradient) of transport.

Although the proton transmembrane electrochemical gradient is involved in intestinal absorption of certain solutes, such as dipeptides [12,13,19], and renal transport of some amino acids [21], a role in the intestinal absorption of other substrates such as amino acids has been disregarded. However, our recent demonstration of H^+-coupled β-alanine [14] and L-proline transport in monolayers of the human intestinal epithelial cell line Caco-2 suggest a more complex arrangement than previously recognised [3,4]. The presence of an H^+,K^+-ATPase [22] and Na^+/H^+ exchanger [13,23] in Caco-2 cells suggests that dissipative H^+ fluxes at the apical membrane may be directly extruded by an ATP-dependent pump, or indirectly coupled to metabolic energy via the Na^+ electrochemical gradient. In Na^+-containing media where pH_i maintenance is mainly dependent upon Na^+/H^+ exchange the existence of H^+/amino acid symport could easily be overlooked. These observations suggest an important role for the proton electrochemical gradient in the absorption of a range of solutes previously thought to be predominentely transported by Na^+-dependent mechanisms.

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