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Proline uptake by monolayers of human intestinal absorptive (Caco-2) cells in vitro

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Monolayers of the Caco-2 human intestinal cell line exhibit active and passive uptake systems for the imino acid L-proline. The active transport component is saturable and it is responsible for about two thirds of the observed flux over the nanomolar concentration range, at 37°C and pH 7.4. In contrast to L-phenylalanine, specific L-proline uptake has a high degree of sodium dependency and the efficiency of the carrier system is significantly reduced when protein synthesis (cycloheximide), Na^+/K^+-ATPase (ouabain) or cellular metabolism (sodium azide) are inhibited. The expression of the L-proline carrier by Caco-2 cells was under some degree of nutritional control. Glucose deficiency, over the time scale of the experiment, had no effect. The temperature-dependence of the specific uptake process followed the Arrhenius model with an apparent activation energy of 93.5 kJ $nmol^{-1}$. This pathway also displayed Michaelis-Menten concentration-dependence with a K_m^{sd} of 5.28 mM and a maximal transport flux (J_{max}^{sd}) of 835 pmol min^{-1} (10^6 cells)$^{-1}$. Although the passive component was unchanged, the pH of the donor phase exerted a profound effect on the active carrier component. Within the physiological pH range a local maximum efficiency was found at pH 7.4 but dramatic increases were noted as pH 5.0 was approached. In competition studies, with 100-fold excess of a second amino acid, strong inhibition of uptake was found with α-aminoisobutyric acid, L-alanine and L-serine whereas moderate inhibition was observed with glycine, D-proline and γ-aminoisobutyric acid. Aromatic and branched amino acids showed weak (L-valine) or no interaction (L-phenylalanine, L-leucine) with the carrier system. These data indicate that the carrier system for the uptake of L-proline has many features in common with the A system for amino acid transport.

Introduction

Amino acids traverse biological membranes by passive diffusion and a multiplicity of carrier systems. These carriers are classified as sodium-dependent or sodium-independent and sub-classified on the basis of kinetic parameters and cross-inhibition profiles for amino acid pairs. Distinct L-alanine-preferring and L-leucine-preferring amino acid carriers were first defined in the non-epithelial Ehrlich ascites tumour cells, and named the A and L systems, respectively [1]. At least twelve different amino acid carriers have subsequently been characterised in non-epithelial cells of vertebrates [2,3]. In addition to a number of these, epithelial cells possess a complement of carriers not present in non-epithelial cells [4].

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Delineation of intestinal epithelial amino acid transport is complicated by their overlapping carrier specificity, regional and inter-species variation [5] and the different models employed to study them. This situation is especially true for imino acids. Early reports described an exclusive imino acid transport system in the hamster small intestine [6–9] whereas in the rat, L-proline shares an uptake system with glycine [10–12] from which betaine (*N*-trimethylglycine) is excluded [11]. A later report suggested that the imino acid transporter of the rat small intestine resembled the A system [13]. This supposition was also postulated for the guinea pig ileum based on a 90% inhibition of L-proline uptake by α-(methylamino)isobutyric acid (MeAIB) into brush-border membrane vesicles [14], however, in the absence of a comprehensive amino acid cross-inhibition profile, the involvement of other systems cannot be discounted. Indeed, in rabbit jejunual brush-border membrane vesicles,the sodium-dependent L-proline uptake can be totally inhibited by

MeAIB but it is insensitive to L-alanine and to glycine and excludes β-alanine [15]. Therefore, uptake occurs via a system distinct from the A mediation and was denoted the IMINO carrier. An equivalent system has been described in intact epithelial preparations from the rabbit distal ileum [16]. However, a second sodium-dependent uptake pathway for imino acids which accepts neutral, cationic and non-α-amino acids present in the intact preparation [16] was not observed in brush-border membrane vesicles [15]. The intestinal transport of imino acids and non-α-amino acids has been linked in the rabbit and rat but each species has different characteristics [17]. A single study has shown L-proline uptake into adult human ileum is sodium-dependent but this was not extended to analysis of the Michaelis kinetic parameters or cross-inhibition profiles [18]. Recently, sodium-dependent carrier-mediated uptake of L-proline into brush-border membrane vesicles from human foetal small intestine has been demonstrated [19]. Uptake was attributed to the IMINO system, but this was not confirmed by inhibition studies.

The inter-species variation prevents extrapolation of animal data to the human situation and the lack of available tissue and rapid loss of viability on excision [20] has severely restricted human studies. Carrier-mediated uptake of L-alanine, L-phenylalanine [21] and dicarboxylic amino acids [22] into human jejunal brush-border membrane vesicles has been observed. The mechanism of L-proline uptake into the adult human small intestine has not be studied in detail to date. Considerable research effort has been involved in developing an in vitro model of the human gastrointestinal epithelium. Primary culture of small intestinal enterocytes has been limited by poor retention of anatomical and biochemical features in vivo [23]. Recently, attention has concentrated on colon adenocarcinoma cell lines which display properties similar to intestinal enterocytes. The human intestinal Caco-2 cell line [24] exhibits spontaneous enterocyte-like differentiation under standard culture conditions [25]. These columnar cells show morphological polarity having apical microvilli, with associated brush-border hydrolases [25,26], and basolaterally positioned nuclei. They form electrically tight monolayers and characteristically, for a functionally polar epithelium, form domes [25]. Initially, they were studied as a cellular model for differentiation of intestinal enterocytes [27]. More recently, however, carrier-mediated uptake and transport of nutrients by Caco-2 cells has been investigated and a similarity with many transporters normally present in the small intestine has been established. Transported ligands include methyl α-glucoside (glucose analogue) [28], bile-acids [29,30], cephalexin [31,32], folic acid [33], inorganic phosphate [34], L-phenylalanine [35,36] and vitamin B-12 [37]. Caco-2 monolayers have been proposed as a useful in vitro model of small intestinal epithelium [29,38].

This present study was undertaken to investigate carrier-mediated uptake of the imino acid, L-proline, by monolayers of human intestinal absorptive (Caco-2) cells.

Materials and Methods

Materials

The Caco-2 cell line was obtained from Professor Colin Hopkins, Imperial College, University of London and confirmed mycoplasma-negative using the Hoechst 33258 test [39]. Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS), glutamine, non-essential amino acids (NEAA) and penicillin-streptomycin (10000 iu ml^{-1} and 10 mg ml^{-1}) were obtained from Gibco (Paisley, Scotland, UK). Culture plastics were purchased from Sterilin (Hounslow, UK). L-[5-^{3}H]Proline (specific activity 26 mCi $mmol^{-1}$) and L-[^{3}H]phenylalanine (specific activity 106 Ci $mmol^{-1}$) were obtained from Amersham, UK and shown to be chromatographically homogeneous prior to use. Phosphate buffered saline (PBS) tablets were from Oxoid, UK. α-(Methylamino)isobutyric acid (MeAIB) was obtained from Aldrich Chemical Company (Gillingam, UK). All other chemicals were of cell culture grade or the highest purity available from Sigma Chemical Company (Poole, UK) unless otherwise indicated in the text.

Cell culture

Caco-2 cells were cultured in 150 cm^2 plastic T-flasks with DMEM containing 10% v/v FCS, 1% v/v NEAA and 1% v/v glutamine (maintenance medium). They were incubated at 37°C in a humidified atmosphere of 10% CO_2 in air. The culture medium was renewed on alternate days. Stock cultures were sub-cultured (1:3) weekly by trypsinisation with 0.25% trypsin and 0.2% disodium ethylenediamine tetraacetate in PBS. Cells were used between passages 94 and 112.

For uptake experiments' Caco-2 monolayers were cultured on six-well plates. The cells (2–3 d post-confluent) were trypsinised and resuspended in maintenance medium supplemented with 1% v/v penicillin and streptomycin (plate medium). The viable cell density was measured using a haemocytometer with trypan blue exclusion and reduced to $2.0 \cdot 10^5$ cells ml^{-1} by dilution with further plate medium. Each well was seeded with 5 ml of the diluted cell suspension ($1.0 \cdot 10^6$ cells). The six-well plates were incubated at 37°C in a humidified atmosphere of 10% CO_2 in air. Plate medium was renewed every 48 h and monolayers used for uptake studies after six days in culture.

Amino acid-free incubation media

Amino acid-free medium (AAFM) used for L-proline uptake experiments comprised 1% bovine serum albumin, 3.2 mM calcium chloride, 1.2 mM magnesium chloride, 4 mM potassium chloride, 150 mM sodium chloride and 5 mM glucose in double distilled water, buffered to pH 7.4 with 14 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes). A sodium-free amino acid-free medium was prepared by equimolar substitution of sodium chloride with choline chloride ($AAFM_{-NaCC}$) or lithium chloride ($AAFM_{-NaLi}$). A glucose-free medium ($AAFM_{-Glucose}$) was prepared by omitting glucose from the above formula. The osmolalities of these media were measured by freezing point depression (Knauer Kühlgerat, Temperatur-messgerät und Regler) and were identical to that of plate medium at 330 mosmol/kg.

Uptake experiments

L-[^{3}H]Proline uptake experiments were performed in the absence (total uptake) and presence (non-specific uptake) of a $1.0 \cdot 10^6$ molar excess of L-proline (50 mM), allowing specific uptake to be calculated by subtraction.

The plate medium was aspirated and the Caco-2 monolayer washed with PBS (2×5 ml $\times 5$ min) and finally with incubation medium (1×2 ml $\times 15$ min) to remove extracellular amino acids. The cells were incubated with 2.0 μCi of L-[^{3}H]proline in 2 ml (ie: 44 nM) of incubation medium, with or without excess (50 mM) L-proline, at 37°C for 30 min. Washing and incubation solutions were equilibrated to 37°C for 1 h prior to use. At the end of the incubation period the medium was removed from each well and added to 10 ml of scintillation cocktail (OptiPhase HiSafe 3, LKB). Each monolayer was rinsed carefully three times with ice-cold PBS-azide 0.05% w/v and solubilised with 2 ml of 0.1% v/v (aq) Triton X-100 (Aldrich Chemical Company, UK). The solubilised monolayers were added to 10 ml of scintillation cocktail. The tritium-content of each monolayer was determined using a Packard Tri-Carb 2000CA liquid scintillation analyser and corrected for the amount of L-[^{3}H]proline in the incubation chamber. A similar procedure was used for L-[^{3}H]phenylalanine uptake except the tritium-content was determined using Lumagel (Lumac, Netherlands) and a Beckman LS1801 liquid scintillation analyser.

Thin-layer chromatography (TLC) of the non-TCA precipitable Caco-2 cell fraction

Caco-2 monolayers were incubated with 44 nM L-[^{3}H]proline for 30 min at 37°C then the tritium-loaded cells were scraped off the bottom of each well and resuspended in double distilled water. The pooled cell suspensions were homogenised by 10 strokes in a Potter-Elvehjem homogeniser at 2000 rpm. The proteins in the homogenate were precipitated with trichloroacetic acid (TCA) and pelleted by centrifugation at $11\,600 \times g$ for 5 min (MSE minifuge). The supernatant was collected and lyophilised in an Edwards freeze dryer. The lyophilised residue was reconstituted and spotted onto a cellulose TLC plate (Whatman, Maidstone, UK). The chromatogram was developed with a mobile phase of butan-1-ol (60%), acetic acid (25%) and double distilled water (15%). At completion, the TLC plate was dried rapidly at 70°C and divided vertically into 1 cm blocks. Each block of cellulose was scraped into a scintillation vial together with 10 ml of OptiPhase Hisafe 3 and its tritium-content determined using a Packard Tri-Carb 2000CA liquid scintillation analyser. A reference sample was made by preparing a non-TCA precipitable Caco-2 cell fraction as described above but omitting radiolabel from the incubation stage. A tracer of L-[^{3}H]proline was added just before freeze drying.

Kinetics of L-proline uptake

L-Proline uptake into Caco-2 monolayers was determined, as described above, after 1.75, 2.5, 5, 10, 20, 30 and 45 min incubations. The distribution ratio (i.e. $[Pro]_{monolayer}/[Pro]_{incubation\ medium}$) after each incubation time was calculated. The monolayer volume used to calculate $[Pro]_{monolayer}$ was derived by multiplication of cell height from transmission electron microscopy (15 μm, unpublished data) and monolayer area (9.62 cm^2).

Inhibition of L-proline uptake by experimental conditions

Uptake experiments were performed as described above (control) or in the presence of inhibitors of the Na^+/K^+-ATPase (10 μM ouabain), oxidative phosphorylation (10 mM sodium azide) or protein synthesis (500 μM cycloheximide) and over a range of pH values (5.0 to 8.5). The sodium-dependence of L-proline uptake was further investigated by measuring uptake from normal and sodium-free incubation media. Precise experimental conditions for each study are given in the appropriate figure legends.

Sodium-dependence of L-proline uptake

Uptake of L-proline over a range of sodium ion concentrations (0, 10, 20, 30, 50, 70, 100 and 140 mM) was investigated. Each incubation medium was supplemented with choline chloride to give a combined concentration of 140 mM. Monolayers were washed (2×5 ml $\times 5$ min) with PBS followed by incubation medium with the appropriate sodium ion concentration. Uptake studies were performed over 20 min at 37°C as described above.

Concentration-dependence of L-proline uptake

Monolayers were incubated at 37°C, for 20 min, with a range of L-proline concentrations ($5 \cdot 10^{-4}$ M to

$5 \cdot 10^{-2}$ M) spiked with 2.0 μCi of L-[^{3}H]proline. Parallel experiments were performed as described above in the presence and absence of sodium. Total L-proline uptake was calculated by multiplication of L-[^{3}H]proline uptake with the dilution factor due to the addition of non-radioactive L-proline during preparation of each concentration. Kinetic parameters for uptake, K_m^{sd} and J_{max}^{sd}, were calculated using Enzpack 3 (Biosoft, Cambridge, UK).

Feeding regimen and L-proline uptake

Monolayers were fed with plate medium 48 h (usual regimen) and 12 h prior to uptake studies. Additionally, some monolayers were fed 24 h before uptake experiments with a protein-free amino acid-free medium comprising Hanks' balanced salt solution (HBSS) buffered to pH 7.4 with Hepes. Uptake studies were performed at 37°C over 20 min.

Results are presented as the mean values of one experiment ($n = 3$, unless otherwise stated) and experimental errors are given as standard deviations about that mean. Significance testing was performed using an unpaired Student's t-test.

Results

Seeding of $1.0 \cdot 10^6$ cells per well resulted in a confluent monolayer after 4 days of culture. After 6 days of culture there were $(3.4 \pm 0.3) \cdot 10^6$ ($n = 9$) cells per monolayer. Over the incubation period for all experimental conditions, no cell detachment or alteration of monolayer appearance was observed by phase-contrast microscopy.

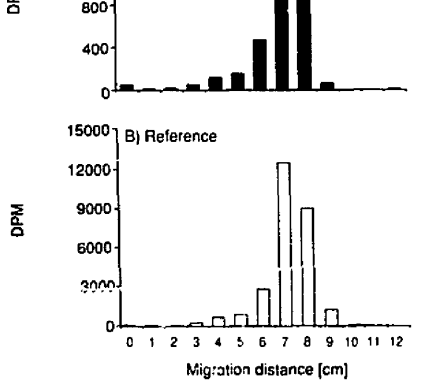

Fig. 1. Identification of L-[^{3}H]proline post-uptake. Thin-layer radiochromatograms of tritium-distribution in the non-TCA precipitable fraction of L-[^{3}H]proline loaded Caco-2 cells (A) and a reference L-[^{3}H]proline-spiked non-TCA precipitable cell fraction (B).

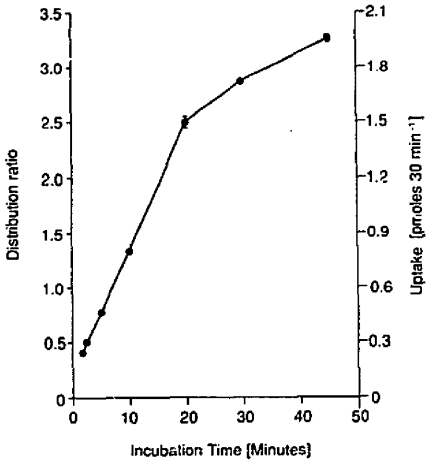

Fig. 2. Kinetics of L-proline uptake by Caco-2 monolayers. Monolayers were washed with PBS (2×5 ml×5 min) followed by AAFM (1×2 ml×15 min). Total L-[^{3}H]proline uptake was determined after 1.75, 2.5, 5, 10, 20, 30, and 45 min at 37°C. Data points represent the mean + S.D. of three samples.

Tritium entering the Caco-2 monolayers was positively identified as L-[^{3}H]proline by TLC of the non-TCA precipitable cell fraction (Fig. 1). Less than 1% of the tritium was removed by TCA precipitiation of the Caco-2 cell homogenate and this was abolished (> 80%) by a 30 min pre-incubation with an inhibitor of protein synthesis, 500 μM cycloheximide (results not shown). This indicates that only a small proportion of L-[^{3}H]proline is assimilated to protein by the cells over the experimental period.

The kinetic profile of L-[^{3}H]proline uptake (Fig. 2) shows an initial linear phase (uptake proportional to incubation time) for at least 20 min, thereafter, uptake tended to a plateau. A distribution ratio greater than unity (i.e. 3.26 ± 0.05 at 45 min) implies concentrative uptake. Therefore, L-proline uptake has an active component in addition to passive diffusion. This was confirmed by measuring uptake in the presence of 50 mM L-proline to estimate the passive uptake component. Typically, the unsaturable passive uptake component accounted for $32.2 \pm 1.9\%$ ($n = 18$) of the total uptake. It is the saturable, carrier-mediated, component which is considered further.

Parallel uptake studies in the presence and absence of sodium ions, showed carrier-mediated uptake to be significantly reduced by replacing sodium ions with choline chloride ($87.7 \pm 1.0\%$; $P < 0.001$) and lithium chloride ($77.0 \pm 1.2\%$; $P < 0.001$). Similarly, pre-incubation with 10 μM ouabain, an inhibitor of the Na^+/K^+-ATPase, reduced uptake by $82.6 \pm 2.8\%$ ($P < 0.001$, Fig. 3). In contrast, L-phenylalanine uptake from sodium-free (choline chloride) medium is not

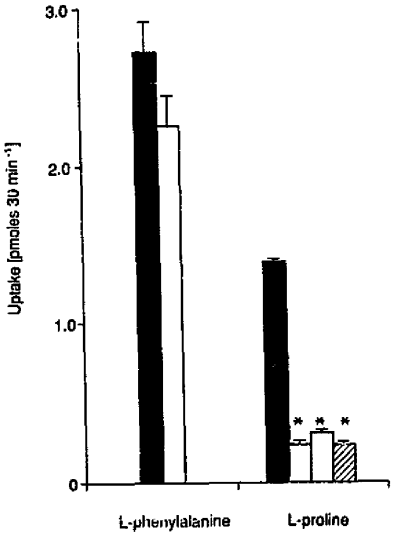

Fig. 3. Sodium-dependence of L-phenylalanine and L-proline uptake into Caco-2 monolayers. Monolayers were washed with PBS (2×5 ml×5 min) then AAFM (closed bar), AAFM $_{-NaCl}$ (open bar) or AAFM $_{-NaLi}$ (shaded bar, L-proline only) as appropriate. Uptake of L-[^{3}H]phenylalanine and L-[^{3}H]proline were determined from these media. In addition, monolayers were pre-incubated with 10 μM ouabain (1×5 ml×120 min) then washed with AAFM containing 10 μM ouabain (2×2 ml×5 min). Specific L-[^{3}H]proline uptake in the presence of 10 μM ouabain was determined (hatched bar, L-proline only). Data bars represent the mean ± S.D. of three samples. * denotes significant reduction, $P = 0.001$.

significantly different from control values. L-Proline uptake displays a sigmoidal relationship to sodium ion concentration. Uptake initially increased slowly at sodium ion concentrations below 30 mM before increasing rapidly between 30 and 50 mM but did not become maximal until much higher sodium concentrations (100 to 140 mM; Fig. 4).

Total L-proline uptake (J) in the presence of sodium ions is the sum of sodium-dependent (J^{sd}), sodium-independent (J^{si}) and diffusional (J^{d}) uptake mechanisms. In the absence of sodium ions it is the sum of (J^{si}) and (J^{d}) (Fig. 5A). Therefore, subtraction of concentration-dependent uptake curves performed in the presence and absence of sodium ions provides J^{sd} which depicts Michaelis-Menten saturation kinetics for the sodium-dependent uptake component (Fig. 5B). An Eadie-Hofstee plot of these data (Fig. 5C), according to the transformation shown below (Eqn. 1), was employed to calculate the following kinetic parameters; Michaelis constant, $K_m^{sd} = 5.28 \pm 0.83$ mM and maximal velocity, $J_{max}^{sd} = 835 \pm 83$ pmol min^{-1} (10^6 cells)$^{-1}$.

$$J^{sd} = -K_m^{sd} \times \frac{J^{sd}}{[Pro]} + J_{max}^{sd} \quad (1)$$

L-Proline uptake from glucose-free medium was not significantly different from control levels but was reduced by 10 mM sodium azide ($P < 0.02$; Table I). In addition, L-proline uptake was significantly ($P < 0.01$) reduced by decreasing the temperature below 37°C (77.5 ± 3.1 and 94.3 ± 0.3% reductions at 20 and 4°C, respectively; Fig. 6). An activation energy (E_a) for L-proline uptake of 95.3 kJ mol^{-1} was calculated by linear least-squares regression analysis of a plot of log k against $1/T$ according to the Arrhenius equation (Fig. 6 inset and Eqn. 2 below) for these data.

$$\log k = \log A - \frac{E_a}{2.303RT} \quad (2)$$

To explore the substrate selectivity of the L-proline carrier system, uptake of 50 μM L-[^{3}H]proline alone or in the presence of 5 mM concentrations of amino (including imino) acids and three synthetic analogues, α-, γ-aminoisobutyric acid and MeAIB, were compared (Table II). A differential ability to inhibit L-proline uptake was observed. L-Aspartic acid (−1.7 ± 5.2%), an acidic amino acid did not inhibit L-proline uptake. Similarly, aromatic amino acids (L-phenylalanire (3.0 ± 2.6%) and L-tryptophan (−5.1 ± 1.4%)) and the branched aliphatic side-chain amino acid L-leucine (−0.41 ± 6.4%) failed to significantly interfere with L-proline uptake. L-Valine (10.2 ± 1.9%) showed a small but significant inhibition. γ-Amino isobutyric acid (33.4 ± 13.0%), glycine (30.7 ± 2.0%), and the stereoisomer D-proline (36.9 ± 3.3%), only partially inhibited L-proline uptake. Moderate inhibition was ob-

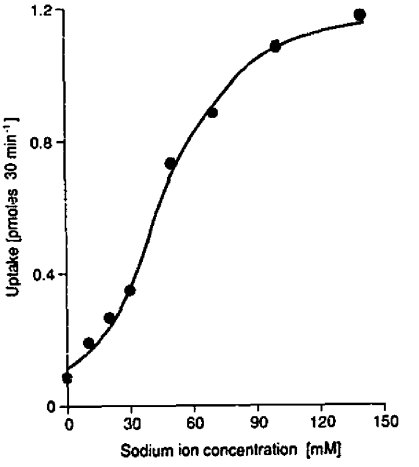

Fig. 4. L-proline uptake as a function of sodium ion concentration. Monolayers were washed (1×5 ml×15 min) with AAFM containing the appropriate sodium ion concentration. L-Proline uptake over 20 min at 37°C in the presence of 0, 10, 20, 30, 50, 70, 100 or 140 mM sodium ions. Data points represent the mean ± S.D. of three samples.

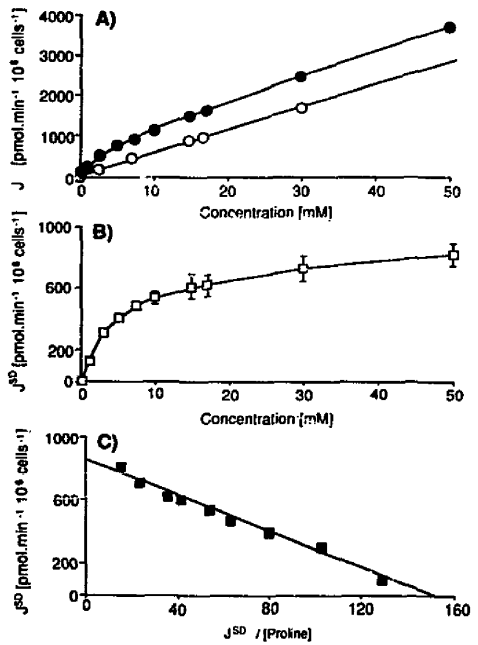

Fig. 5. Concentration-dependence of L-proline uptake. Monolayers were washed with PBS (2×5 ml×5 min) followed by AAFM or AAFM$_{-NaCl}$ (1×2 ml×15 min) as appropriate, then incubated for 20 min at 37°C with a range of L-[^{3}H]proline concentrations (0.5 mM to 50 mM) in the presence and absence of sodium ions. The concentration dependence of proline uptake in the presence (closed circles) and absence of sodium ions (open circles) is shown above (A). The difference between the two curves, as a function of concentration (open squares; B), was analysed using an Eadie-Hofstee plot (closed squares; C). Data points represent the mean ± S.D. of three samples.

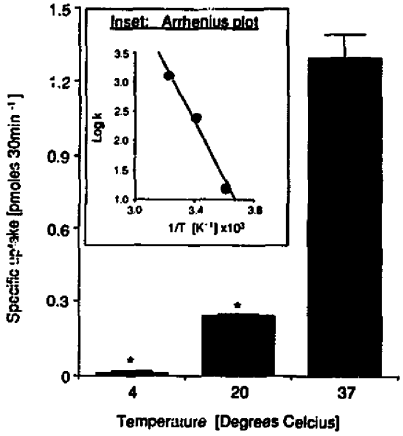

Fig. 6. Temperature dependence of L-proline uptake. Monolayers were washed with PBS (2×5 ml×5 min) followed by AAFM (1×2 ml×15 min) pre-equilibrated to 4, 20 or 37°C as appropriate. Specific L-[^{3}H]proline uptakes after 30 min incubation at 4, 20 and 37°C were determined. Data bars represent the mean ± S.D. of three samples and * denotes sigificant difference from uptake at 37°C, $P < 0.01$). The uptake activation energy, E_a was calculated from an Arrhenius plot (inset).

TABLE I

Effect of cycloheximide, glucose depletion and sodium azide on specific L-proline uptake

Cycloheximide: Monolayers were pre-incubated for 45 min with 500 μM cycloheximide, washed once with AAFM, and the specific uptake of L-[^{3}H]proline determined after 30 min at 37°C. Glucose depletion: Monolayers were washed with PBS (2×5 ml×5 min) followed by AAFM or AAFM$_{-Glucose}$ (1×2 ml×15 min) as appropriate. Specific uptake of L-[^{3}H]proline from AAFM and AAFM$_{-Glucose}$ as described above. Sodium azide: Monolayers were washed with PBS (2×5 ml×5 min) then pre-incubated with AAFM containing 10 mM sodium azide (1×2 ml×30 min). Specific L-[^{3}H]proline uptake in the presence of 10 mM sodium azide was determined as described above. The results are expressed as mean ± SD of three observations.

Condition	Specific uptake. % control (± S.D.)
+ Cycloheximide (500 μM)	58.5 (1.7) *
− Glucose	102.8 (20.6)
+ Sodium azide (10 mM)	41.4 (18.5) *

* $P < 0.02$.

served by amide, basic and sulphur containing amino acids as well as L-hydroxyproline (50.2 ± 6.2%). The most potent inhibitors were α-aminoisobutyric acid (75.2 ± 10.0%), L-alanine (69.3 ± 7.4%), MeAIB (58.3 ± 4.3%), L-serine (74.1 ± 1.7%) and L-proline (68.3 ± 0.3%) itself.

The expression of the L-proline carrier can be significantly up- and down-regulated by the monolayer feeding regimen (Table II). Uptake into monolayers that had experienced the normal feeding regimen (48 h; 942 ± 49 fmol (20 min)$^{-1}$) was significantly higher than after a recent replacement of maintenance medium (12 h; (844 ± 11 fmol (20 min)$^{-1}$). This suggests that up- and down-regulation of the L-proline carrier is under some degree of nutritional control. Similarly, a 24 h incubation in protein-free amino acid-free medium (HBSS + Hepes) produced a marked up-regulation of carrier-mediated uptake (1411 ± 69 fmol(20 min)$^{-1}$). This may again reflect nutritional control or a be caused by the absence of a specific 'down-regulating' component present in the maintenance medium. A significant inhibition of uptake (Table I; 41.5 ± 1.7%) occurred after a 45 min pre-incubation with an inhibitor of protein synthesis, 500 μM cycloheximide. One possible explanation for this is that the L-proline transporter is a protein with rapid catalytic and synthetic turnover.

L-Proline uptake exhibited a complex pH-dependence (Fig. 7). Specific uptake was pH-dependent whereas non-specific uptake remained relatively con-

TABLE II

Inhibition of L-proline uptake by amino acids and their analogues

Monolayers were washed with PBS (2×5 ml×5 min) followed by AAFM (1×2 ml×15 min). They were incubated with 50 μM L-[^{3}H]proline alone (control) or in the presence of a 100-fold excess of competing amino (imino) acid. The inhibition of L-[^{3}H]proline uptake is expressed as a percentage of control ± SD of three observations.

Side-chain type	Amino acid	% inhibition of specific uptake (± S.D.)
Acidic	L-aspartic acid	−1.7 (5.2)
Aliphatic	L-alanine	69.3 (7.4) ***
	L-alanine (50 mM)	101.4 (7.4) ***
	L-alanine (50 mM) + MeAIB (5 mM)	104.5 (3.1) ***
	β-alanine	43.5 (3.6) **
	glycine	30.7 (2.0) **
Aliphatic hydroxyl	L-serine	74.1 (1.7) ***
Amide	L-asparagine	51.4 (4.5) ***
Aromatic	L-phenylalanine	3.0 (2.6)
	L-tryptophan	−5.1 (1.4)
Basic	L-arginine	32.9 (1.2) **
	L-histidine	50.2 (9.9) **
Branched aliphatic	L-leucine	−0.4 (6.4)
	L-valine	10.2 (1.9) *
Imino	L-hydroxyproline	50.2 (6.2) **
	L-proline	68.3 (0.3) ***
	D-proline	36.9 (3.3) **
Sulphur containing	L-cysteine	48.7 (12.1) **
	L-methionine	53.4 (3.8) ***
Synthetic analogue	α-aminoisobutyric acid	75.2 (10.0) ***
	γ-aminoisobutyric acid	33.4 (13.0) **
	MeAIB	58.3 (4.3) ***

* $P = 0.05$, ** $P = 0.01$, *** $P < 0.001$.

TABLE III

Feeding regimen and L-proline uptake

Monolayers were fed with plate medium 48 h or 12 h or with HBSS + Hepes 24 h prior to experiments. They were washed with PBS (2×5 ml×5 min) followed by AAFM (1×2 ml×15 min) before uptake studies were performed as described above. The results are expressed as mean ± S.D. of three observations.

Last medium replacement; time lapse (type)	Specific uptake: mean (± S.D.) (fmol (20 min)$^{-1}$)
48 h (plate medium)	942 (49.4)
12 h (plate medium)	844 (11.3) *
24 h (HBSS + Hepes (14 mM))	1411 (69.6) *

* $P = 0.01$.

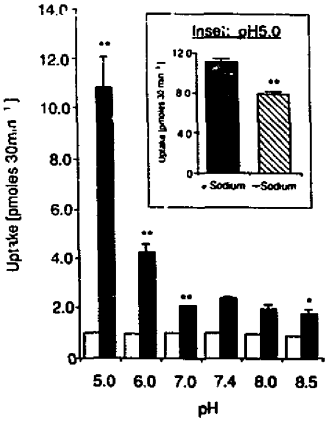

Fig. 7. The effect of pH on L-proline uptake. Monolayers were washed with PBS pH 7.4 (2×5 ml×5 min) followed by AAFM at pH 5.0, 6.0, 7.0, 7.4, 8.0 or 8.5 as appropriate (1×2 ml×15 min). L-[^{3}H]proline uptake was determined in the presence (non-specific, open bar) and absence (specific, closed bar) of 50 mM unlabelled L-proline at each pH value. (* and ** denote significantly different uptake to that at pH 7.4, $P = 0.05$ and $P = 0.01$, respectively). The inset shows the effect of sodium depletion on L-proline uptake at pH 5.0. Data bars represent the mean ± S.D. of three samples.

stant between pH 5.0 and 8.5. At pH values slightly higher or lower than 7.4, significant reductions in specific L-proline uptake ($P = 0.05$ and $P = 0.01$ at pH 8.5 and 7.0, respectively) were observed. Therefore, under neutral to mildly alkaline conditions specific uptake occurred maximally at pH 7.4. At acidic pH values, however, specific uptake was stimulated (4.6-fold at pH 5.0) but was only 29.4 ± 5.4% sodium-dependent at pH 5.0 compared to 87.7 ± 1.0% at pH 7.4.

Discussion

A high degree of coincidence between the L-[^{3}H]proline reference and post-uptake sample radio-TLC chromatograms shows that L-proline was not appreciably metabolised prior to, during or after uptake over the duration of the experiments. Therefore, uptake of tritium into Caco-2 monolayers overwhelmingly reflected intact L-[^{3}H]proline.

The results from the above experiments, designed to probe the mechanism(s) of L-proline entry into Caco-2 cells, suggest that uptake predominantly occurred (≈ 70%) via carrier-mediation. Firstly, uptake was concentrative, implying Caco-2 cells are able to actively accumulate L-proline beyond a concentration that can be explained by simple (passive) equilibration across their brush-border membranes. Secondly, uptake was dramatically reduced at temperatures below 37°C. The calculated activation energy of 95.3 kJ mol^{-1} for L-proline exceeds that anticipated for simple diffusion (<

16.8 kJ mol^{-1}) and is within the usual range for carrier-mediated processes (29.4 to 105.0 kJ mol^{-1}) [40]. Additionally, L-proline uptake was significantly reduced in the presence of 10 mM sodium azide suggesting a reliance on metabolic energy. The most likely explanation is that sodium azide interrupts the production of metabolic energy required to maintain the inward sodium ion electrochemical gradient which drives uptake (see later). Furthermore, L-proline uptake was concentration-dependent. This is consistent with the hypothesis that two modes of uptake into Caco-2 cells exist; (a) Saturable uptake via a finite number of 'carriers' and (b) non-saturable passive diffusion (uptake proportional to concentration). Experiments were performed on confluent monolayers which exhibit tight junctions between cells [25]. Therefore, the L-proline carrier described here is probably located at the brush border membrane. This is supported by L-proline transport experiments across Caco-2 monolayers, cultured on permeable supports, which show flux to be vectorial, occurring three times faster in the apical-to-basolateral direction (manuscript in preparation).

The activation energy for L-proline uptake is almost twice that described for L-phenylalanine transport across Caco-2 monolayers cultured on permeable supports [36]. Although the models used in these studies are not directly comparable, the different activation energies suggest that L-proline and L-phenylalanine are recognised by different carriers.

Amino (and imino) acid transporters are principally classified according to their reliance on sodium. The sodium-dependence of L-proline uptake was investigated by reducing the inward sodium ion gradient by performing uptake studies from sodium-free amino acid-free incubation medium or in the presence of 10 μM ouabain. Under both experimental conditions, the specific uptake of L-proline was reduced by approximately 85% and is therefore sodium-dependent. This agrees with previous work in other species [14,16] and in brush-border membrane vesicles prepared from adult [18] and foetal [19] human small intestine which showed that the initial uptake rate of L-proline was reduced in the absence of sodium ions. High extracellular sodium ion concentrations were required to drive L-proline uptake maximally which explains its striking sensitivity to ouabain. Interestingly, similar uptake experiments for L-[^{3}H]phenylalanine failed to demonstrate significant sodium-dependence. This contests the previously published observation that L-phenylalanine transport across Caco-2 monolayers cultured on permeable supports is reduced by 33% in the presence of 100 μM ouabain (implying sodium-dependence) [36]. Furthermore, an earlier report by the same workers found that L-phenylalanine transport was not reduced from sodium-free (choline chloride) incubation medium or by 2–5 mM ouabain, suggesting sodium-independent transport [35]. Their initial observation agrees with the results of L-phenylalanine transport experiments performed within our laboratories (manuscript in preparation).

Uptake was identical in the presence and absence of glucose indicating the intracellular carbohydrate pool is able to satisfy the energetic demands of the cells over the time-course of the experiment. Clearly, there is no direct interaction between glucose and the L-proline carrier. L-Phenylalanine transport was reduced in the absence of glucose [36] but whether this was a result of a reduced availability of metabolic energy or reduced solvent drag through the paracellular shunt pathway in the absence of glucose [41,42] was uncertain. Similarly, the transport of L-proline across Caco-2 monolayers cultured on permeable supports is reduced in the absence of glucose (results not shown).

The uptake of L-proline was performed over a range of concentrations (0.5 mM to 50 mM) in the presence and absence of sodium ions. The difference between the uptake profiles follows Michaelis-Menten saturation kinetics and the Eadie-Hofstee analysis yields a straight line suggesting a single agency is responsible for the sodium-dependent uptake of L-proline into Caco-2 cells. The Michaelis constant (K_m^{sd}) of 5.28 ± 0.83 mM is an order of magnitude higher than that for brush-border membrane vesicles prepared from guinea pig ileum ($K_m = 0.67$ mM) [14] and rabbit jejunum ($K_m = 0.55$ mM) [15]. This discrepancy may be an artefact of the different models used in these studies but more probably reflect functional differences between the uptake systems. Unfortunately, data concerning L-proline uptake into normal human tissues is limited and does not extend to the analysis of kinetic parameters for comparison [18,19].

The structural requirements of the carrier were investigated by observing the inhibition of carrier-mediated uptake of L-proline by a 100-fold excess of other amino (and imino) acids or synthetic analogues. The inhibition profile for L-proline uptake shows that aliphatic neutral amino acids can effectively compete but branching or aromatic content in the side chain dramatically reduced its ability to inhibit uptake. L-Proline uptake was inhibited by the non-α-amino acid β-alanine and γ-aminoisobutyric acid but to a lesser extent than by their α-analogues. Interestingly, D-proline significantly inhibited L-proline uptake (36.9 ± 3.3%) suggesting that the carrier involved in L-proline uptake has only moderate stereo-selectivity. The large neutral amino acid (LNAA) transporter in Caco-2 cells displays an opposite substrate specificity preferring neutral amino acids with bulky side chains and being very stereo-selective [36]. Additionally, glycine inhibits L-proline uptake but failed to influence L-phenylalanine transport across Caco-2 monolayers [36]. It is

noteworthy that L-phenylalanine did not significantly inhibit L-proline uptake ($3.0 \pm 2.6\%$) confirming they are recognised by different carriers in the Caco-2 cell line. These two discrete mediations share the properties of being reduced by basic but not acidic amino acids.

To our knowledge, this is the first detailed characterisation of L-proline uptake into an adult human intestinal preparation, therefore, a comparison of the cross-inhibition profile in Caco-2 cells with those described for other species is merited. In non-epithelial cell types and at the basolateral surface of enterocytes a sodium-dependent, and highly stereo-specific ASC system which excludes MeAIB can serve L-proline [43]. The moderate stereo-selectivity,its strong inhibition by MeAIB and α-aminoisobutyric acid and the inability of lithium ions to substitute for sodium ions [44] indicates the ASC system is not responsible for L-proline uptake in Caco-2 cells. Similarly, uptake is not mediated by the IMINO system which serves L-proline and MeAIB but excludes β-alanine and is insensitive to L-alanine and glycine. This was confirmed by the fact that MeAIB could not inhibit L-proline uptake beyond the L-alanine-sensitive component. In agreement with observations in the rat small intestine, the cross-inhibition uptake profile for L-proline resembles the A system. Firstly, uptake is sodium-dependent and secondly,it is strongly inhibited by α-aminoisobutyric acid, MeAIB and small aliphatic neutral amino acids (e.g. L-alanine, L-serine) but not by branched or aromatic neutral amino acids (e.g. L-leucine, L-phenylalanine, L-tryptophan, L-valine). However, this profile is dissimilar in that the inhibition potency of non-α-amino acids is lower than their α-analogues according to the sequence $\gamma > \beta \gg \alpha$. This is clearly not the case in Caco-2 cells where the inhibition potency of L-alanine is greater than thgt of β-alanine and α-aminoisobutyric acid is greater than α-aminoisobutyric acid. The rabbit small intestine possesses a high affinity, sodium-dependent carrier of neutral and cationic amino acids which serves imino acids and accepts non-α-amino acids according to the sequence $\alpha > \beta > \gamma$ but is more sensitive to L-leucine than the Caco-2 system. The involvement of the A system in L-proline uptake was also postulated for the guinea pig ileum [14], however, in the absence of cross-inhibition studies, other systems cannot be rejected and its Michaelis kinetic parameters were very similar to the IMINO system of the rabbit jejunum [15]. Variance with observations for imino acid uptake by other species may reflect inter-species heterogenicity or that this system is artificially induced in this tumour cell line. The precise nature of this discrepancy awaits experiments in normal adult human small intestinal preparations.

Under neutral to mildly alkaline conditions, L-proline uptake occurs optimally at pH 7.4. This local pH optimum is identical to that of the A system. The ionisation state of L-proline is pH-dependent and is largely cationic at pH values below its isoelectric point, $pI = 6.3$ [45]. The large stimulation of L-proline uptake may be a result of enhanced carrier efficiency, however, the concomitant reduction in sodium-dependence and predicted change in the ionisation state of L-proline suggests another uptake mechanism may prevail at acidic pH values.

We have characterised an amino acid uptake carrier that serves L-proline and which is distinct from the LNAA (L-phenylalanine) transporter previously reported in Caco-2 cells [36]. In two earlier reports, L-alanine uptake into [46] and transport across [47] Caco-2 cells were suspected of being carrier-mediated but were not studied in detail. Our work probably represents a detailed description of that system which serves both L-alanine and L-proline.

Caco-2 monolayers have been proposed as a useful predictive model of the adult human small intestinal epithelium [29,38]. However, the major barrier to evaluating this hypothesis is the lack of data for the normal human small intestine, and comparison with other species is inappropriate due to inter-species variation. At best, Caco-2 monolayers only partially explain the intestinal uptake mechanism of imino acids in the normal adult human small intestine. This can be deduced indirectly from our knowledge of clinical disorders of amino acid absorption. Hartnup's disease results from a congenital defect in the absorption of neutral amino acids in which imino acids do not become clinically deficient [48]. Therefore, at least one uptake carrier, selective for imino acids and excluding neutral amino acids, must be present in the human small intestine. The predicted exclusive imino acid pathway was not observed in Caco-2 cells. This study, therefore, highlights the caution which should be employed when inferring 'normal' uptake phenomena from the Caco-2 in vitro model of the human small intestine.

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