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The influence of luminal pH on transport of neutral and charged dipeptides by rat small intestine, in vitro

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Abstract

Four hydrolysis-resistant dipeptides (D-phenylalanyl-L-alanine, D-phenylalanyl-L-glutamine, D-phenylalanyl-L-glutamate and D-phenylalanyl-L-lysine) were synthesized to investigate the effects of net charge on transmural dipeptide transport by isolated jejunal loops of rat small intestine. At a luminal pH of 7.4 and a concentration of 1 mM the two dipeptides with a net charge of -1 and $+1$ were transported at substantially slower rates (18 ± 1.3 and 8.4 ± 1.3 nmol min^{-1} (g dry wt.)$^{-1}$, respectively) than neutral D-phenylalanyl-L-alanine and D-phenylalanyl-L-glutamine (87 ± 0.2 and 197 ± 14 nmol min^{-1} (g dry wt.)$^{-1}$, respectively). We investigated the effects of luminal pH on dipeptide transport by varying the $NaHCO_3$ content of Krebs Ringer perfusate equilibrated with 95%O_2/5% CO_2. The pH changes did not affect water transport, but serosal glucose appearance increased significantly at pH 6.8. Transmural transport of D-phenylalanyl-L-alanine and D-phenylalanyl-L-glutamine at pH 6.8 was stimulated ($P < 0.01$) by 61% and 49%, respectively, whereas the lower pH increased the rate for negatively charged D-phenylalanyl-L-glutamate by 306% ($P < 0.01$) and decreased that for positively charged D-phenylalanyl-L-lysine by 46% ($P < 0.05$). Increasing luminal pH to 8.0 inhibited D-phenylalanyl-L-alanine transport by 60%, whereas D-phenylalanyl-L-lysine transport was 60% faster.

Keywords: Dipeptide; Transport; pH; Intestine; Rat

1. Introduction

The peptide transporter, PepT1, has only recently been cloned from rabbit intestine [1] and human intestine [2], but evidence has been available in the literature for some time to suggest that peptide transport in the small intestine is proton-dependent [3]. However, direct evidence for proton-dependent transport has mainly come from studies with atypical peptides such as glycyl-proline or glycyl-sarcosine, which have a net charge of 0 and are not hydrolysed by intestinal peptidases [4]. Other evidence [5] indicates that the proton-dependent peptide transporter has a high affinity for short peptides which include a hydrophobic amino acid.

Although the kidney has been shown to have a second peptide transporter, PepT2, [6], there are many similarities between peptide transport in the kidney

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and small intestine [7]. We have studied the effects of substrate charge on transport into renal apical membrane vesicles with radiolabelled versions of three hydrolysis-resistant dipeptides, D-phenylalanyl-L-alanine (D-Phe-L-Ala), D-phenylalanyl-L-glutamate (D-Phe-L-Glu) and D-phenylalanyl-L-lysine (D-Phe-L-Lys) [8], and shown that the driving force for peptide transport is the membrane potential rather than the proton gradient. Furthermore, although proton co-transport does occur with the neutral and negatively charged dipeptides, there is no evidence for proton transport together with positively charged D-Phe-L-Lys.

We have recently published the results of a study of the transmural transport and hydrolysis by the small intestine of all eight dipeptides combining the D- and L-forms of both Phe and Ala [9]. Although the highest rates of uptake were obtained with L-Phe at the N-terminus, N-terminal D-Phe gave the best resistance to hydrolysis and enabled us to show that D-Phe-L-Ala was accumulated within the mucosa against a concentration gradient. More recently we have found [10] that D-phenylalanyl-L-glutamine (D-Phe-L-Gln), which is also resistant to hydrolysis, shows a more rapid rate of transmural transport in the rat small intestine in agreement with data from Minami, Morse and Adibi [11]. D-Phe-L-Gln is accumulated within the mucosa to nearly 6 times the luminal concentration. Our study of the transmural transport of hydrolysis-resistant dipeptides with D-Phe at the N-terminus [9] clearly shows that D-Phe-L-Ala is crossing the intestinal epithelium by a transcellular route since its rate of serosal appearance was 10 times that for D-Phe-D-Ala. Since D-Phe-D-Ala was undetectable in the mucosal tissue [9], it could be considered to provide an upper limit for the rate of paracellular dipeptide transport in the isolated loop preparation.

The aim of the work presented in this paper is to use an intact preparation of rat small intestine where PepT1 has been shown to be the peptide transporter [12] to examine the influence of luminal pH on the transmural transport of hydrolysis-resistant dipeptides with a net charge of 0, −1 and +1. Transport studies with isolated loops of rat small intestine require perfusion with an oxidisable substrate in Krebs bicarbonate Ringer gassed with 95%O_2/5% CO_2 which gives a luminal pH of 7.4. However, by reducing the $NaHCO_3$ concentration to 5 mM or increasing it to 75 mM, we have been able to examine how dipeptide transport is altered when the luminal pH is decreased to 6.8 or increased to 8.0, respectively. A preliminary report of some of this work has been published [13].

2. Materials and methods

2.1. Materials

Reagents used for peptide synthesis were obtained from the Sigma Chemical Company Ltd, Dorset, UK. Sodium pentobarbitone was purchased from May and Baker Ltd, Dagenham, UK. Chemicals were all of analytical grade.

2.2. Animals

Male Wistar rats (250 g), obtained from Harlan Olac Ltd, Oxfordshire, UK, were fed ad libitum until they reached a weight of 280 g. For 18 h prior to the experiment the rats were deprived of food and allowed free access to 0.5% (w/v) D-glucose solution; intraperitoneal sodium pentobarbitone (10 mg/100 g body weight) was used to anaesthetize each animal.

2.3. Preparation and purification of the dipeptides

With the exception of D-Phe-L-Gln, all the dipeptides used in this investigation were synthesized as previously described [9]. The D-Phe-L-Gln was made from N-tertiary-butyloxycarbonyl-L-glutamine and N-benzyloxycarbonyl-D-phenylalanine. Briefly, the first stage was to convert the N-tertiary-butyloxycarbonyl-L-glutamine into its benzyl ester. After N-deprotecting the N-tertiary-butyloxycarbonyl-L-glutamine benzyl ester, it was reacted with N-benzyloxycarbonyl-D-phenylalanine dissolved in anhydrous dimethylformamide in the presence of 1,3-dichlorohexylcarbodiimide under an argon atmosphere. After removal of the dimethylformamide under a vacuum, the residue was taken up in a 1:1 mixture of methanol and dichloromethanol. The solution was filtered and washed with saturated $NaHCO_3$, citric acid, water

and a saturated solution of NaCl. The organic phase was dried over $MgSO_4$ and removal of the solvent left a solid, which was triturated with diethyl ether and bound to silica gel. This product was loaded onto a silica gel column and eluted with a 99:1 mixture of $CHCl_3$ and methanol. The fractions containing N-benzyloxycarbonyl-D-phenylalanine-L-glutamine benzyl ester were combined and concentrated. After removal of the protecting groups the residue was dissolved in water, filtered to remove organic impurities, concentrated and freeze-dried to give a white solid. The peptide product, which was at least 98% pure, was characterised by NMR spectroscopy, mass spectroscopy and checked for homogeneity by HPLC.

2.4. Perfusion technique

Isolated jejunal loops (15–20 cm in length) were perfused in single-pass mode for 2 h by the technique previously described [9]. The viability of the preparation was assessed by its ability to maintain a steady water flow and to transport D-glucose actively throughout the perfusion. The loops were suspended in liquid paraffin (specific gravity 0.83–0.86) at 37.5°C. The first 50 min was a control period during which the perfusate was Krebs bicarbonate Ringer gassed with 95% O_2/5% CO_2 to give a pH of 7.4 and containing 28 mM D-glucose. After 50 min the loop was perfused for a further 70 min with a second perfusate (which was identical to the first except for the presence of 1 mM dipeptide). Serosal secretion samples (collected every 10 min) and luminal perfusate samples (taken initially and at 20-min intervals) were deproteinised with 6% perchloric acid (P.C.A.). A measured segment of perfused intestine was blotted and dried to constant weight at 105°C. This gave the dry weight/cm which was used to calculate the total dry weight of the perfused segment. For the perfusions in which the pH of the luminal perfusate was reduced to 6.8, the $NaHCO_3$ concentration was reduced from 25 mM to 5 mM and the NaCl concentration was increased from 120 to 140 mM. In order to raise the pH of the luminal perfusate to 8.0, the $NaHCO_3$ concentration was increased to 75 mM and the NaCl concentration was reduced to 70 mM. For those perfusions in which the luminal pH was altered, the same luminal pH was used throughout the 2 h perfusion.

2.5. Preparation of serosal, luminal and tissue samples for HPLC analysis

The volumes of serosal samples were measured after centrifugation at $1800 \times g$ for 2 min to form a meniscus between any paraffin and the secretion. In order to measure dipeptide and amino acid concentrations in the mucosa at the end of the perfusion, a 4-cm portion of the perfused intestine was cut open lengthwise, gently blotted, the mucosa removed by scraping with a microscope slide, frozen in liquid nitrogen and weighed before being homogenized in 1 ml of 6% P.C.A. A separate measured portion of the perfused loop was cut open, gently blotted, the mucosa removed with a microscope slide, weighed (to determine the wet weight) and dried to constant weight at 105°C. This gave the dry weight:wet weight ratio which was used to calculate the water content of each tissue sample. No correction was made for extracellular space so that the tissue peptide concentrations in Table 3 which are above those in the lumen will be underestimates of the true tissue concentration.

Deproteinised serosal, luminal and tissue samples were centrifuged at $1800 \times g$ for 2 min; an aliquot of the supernatant was neutralized with 0.6 M KOH, rapidly frozen in liquid nitrogen, thawed, and recentrifuged at $1800 \times g$. Aliquots of these samples were analysed for peptide and free Phe by isocratic HPLC at 210 nm on a 5-μm ODS C18 column (Jones Chromatography, Hengoed, Glamorgan, UK). The mobile phase was 20% methanol/80% 21 mM KH_2PO_4 (pH 5).

2.6. Assay of D-glucose

The D-glucose concentrations in the serosal and luminal samples were determined by an automated method using a Cobas Mira autoanalyser (Roche, UK).

2.7. Calculation and expression of results

All results are expressed as mean $\pm$ standard error of the mean (SEM); $n = 4$ for each mean except where noted. Transport data are presented as cumulative rates of serosal appearance (e.g., nmol min^{-1} (g dry wt jejunal segment)$^{-1}$) calculated by covariance.

Statistical comparisons were carried out using 2-way analysis of variance to compare sets of concentration data, covariance analysis for cumulative serosal appearance and Student's *t*-test for the comparison of means.

3. Results

3.1. The influence of luminal pH on glucose and fluid transport

Perfusate bicarbonate concentrations of 75, 25 and 5 mM produced perfusate pH values of 8.0, 7.4 and 6.8, respectively, without destroying the buffering capacity of the perfusate or altering the Na^+ concentration. At all 3 pH values the rates of water transport and the transport of glucose against a concentration gradient were linear throughout the 2-h perfusion period. Table 1 compares the rates of serosal appearance of fluid and D-glucose at the 3 different perfusate pH values. Although there were small changes in the rate of serosal fluid appearance, these were not statistically significant ($P > 0.1$). The cumulative rate of glucose transport at pH 8.0 did not differ significantly from that at pH 7.4 ($P > 0.1$), but the rate of glucose appearance did rise significantly at pH 6.8 to 134% of the value at pH 7.4 ($P < 0.001$). Measurements of the rate of serosal lactate appearance showed that the reduction in luminal pH to 6.8 significantly inhibited the production of lactate ($P < 0.001$). The decrease in lactate formation accounted for more than half of the increase in the serosal glucose appearance rate and is in agreement with evidence from work on skeletal muscle [14] showing that a decrease in pH over this range inhibits the phosphofructokinase control enzyme in the glycolytic pathway. This enzyme has been shown to play a crucial role in the regulation of intestinal glucose metabolism so that the decrease in glucose metabolism was probably mainly a consequence of a pH effect on phosphofructokinase [15].

Table 1
Effect of luminal pH on the rates of glucose and fluid transport during a 2-h perfusion

Luminal pH	*n*	Rate of transmural transport	
		Glucose (μmol min^{-1} (g dry wt.)$^{-1}$)	Fluid (ml min^{-1} (g dry wt.)$^{-1}$)
6.8	16	19.74 ± 1.33 *	0.254 ± 0.0083
7.4	18	14.75 ± 0.73	0.270 ± 0.0096
8.0	8	13.29 ± 0.78	0.242 ± 0.0130

* $P < 0.001$ for the difference between the rate at pH 6.8 and pH 7.4; means ± SEM.

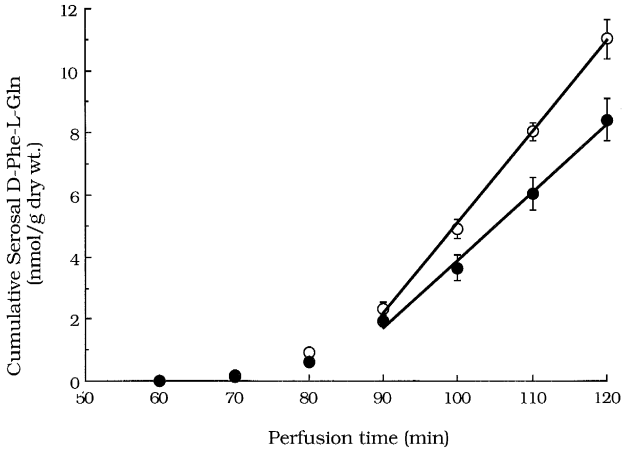

Fig. 1. The effects of reducing luminal pH on the transmural transport of D-Phe-L-Gln. ● Cumulative serosal D-Phe-L-Gln at pH 7.4, rate (90–120 min) = 197 ± 14 nmol min^{-1} (g dry wt.)$^{-1}$; ○ cumulative serosal D-Phe-L-Gln at pH 6.8, rate (90–120 min) = 293 ± 17 nmol min^{-1} (g dry wt.)$^{-1}$; $P < 0.01$ for the difference in rates. Means ± SEM ($n = 4$). Where no error bar is visible it falls within the symbol.

3.2. Effects of reducing luminal pH on transport of neutral and acidic hydrolysis-resistant dipeptides

Fig. 1 shows the cumulative serosal appearance of D-Phe-L-Gln perfused in single-pass mode through the lumen of isolated jejunal loops at 1 mM. At pH 7.4 the rate at which D-Phe-L-Gln appeared in the serosal secretions (197 ± 14 nmol min^{-1} (g dry wt.)$^{-1}$) is double that previously reported for D-Phe-L-Ala [9]. Reduction of the luminal pH to 6.8 increased the transmural transport rate of D-Phe-L-Gln by 49% ($P < 0.01$). Fig. 2 shows that at pH 7.4 the serosal appearance rate for D-Phe-L-Glu (18 ± 1.3 nmol min^{-1} (g dry wt.)$^{-1}$) was only 10% of the rate for D-Phe-L-Gln. However, when the luminal pH was reduced to 6.8 the transmural transport rate for D-Phe-L-Glu was more than 4 times as rapid.

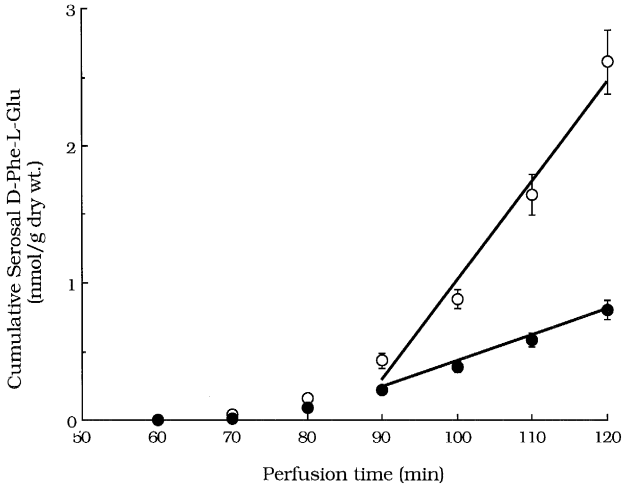

Fig. 2. The effects of reducing luminal pH on the transmural transport of D-Phe-L-Glu. ● Cumulative serosal D-Phe-L-Glu at pH 7.4, rate (90–120 min) = 18 ± 1.3 nmol min^{-1} (g dry wt.)$^{-1}$; ○ cumulative serosal D-Phe-L-Glu at pH 6.8, rate (90–120 min) = 73 ± 7 nmol min^{-1} (g dry wt.)$^{-1}$; $P < 0.01$ for the difference in rates. Means ± SEM ($n = 4$). Where no error bar is visible it falls within the symbol.

3.3. Effects of changes in luminal pH on transport of neutral and basic hydrolysis-resistant dipeptides

Table 2 shows that at pH 7.4 the basic dipeptide D-Phe-L-Lys was transported at only 10% of the rate found for D-Phe-L-Ala. Reducing the luminal pH to 6.8 increased the cumulative serosal appearance rate for D-Phe-L-Ala by 61% ($P < 0.01$). By contrast, the rate of D-Phe-L-Lys transport at pH 6.8 was reduced to 54% of the rate at pH 7.4 ($P < 0.05$). However, when the luminal pH was altered to 8.0 D-Phe-L-Ala transport was inhibited by 60% ($P < 0.01$), whereas the transport rate for D-Phe-L-Lys increased by 60% ($P < 0.05$).

Table 2
Effect of luminal pH on the rates of dipeptide transport

Luminal pH	Rate of transmural transport	
	D-Phe-L-Ala (nmol min^{-1} (g dry wt.)$^{-1}$)	D-Phe-L-Lys (nmol min^{-1} (g dry wt.)$^{-1}$)
6.8	140.8 ± 8.2 **	4.5 ± 0.2 *
7.4	87.0 ± 3.0	8.4 ± 1.3
8.0	35.0 ± 7.5 **	13.4 ± 0.8 *

Means ± SEM, $n = 4$; ** $P < 0.01$ with respect to the rate at pH 7.4; * $P < 0.05$ with respect to the rate at pH 7.4.

Table 3
Effect of luminal pH on the tissue dipeptide concentration at the end of the perfusion

Luminal peptide (1 mM)	Luminal pH:	Tissue concentration (mM)	
		6.8	7.4
D-Phe-L-Ala		2.72 ± 0.11 *	1.41 ± 0.11
D-Phe-L-Gln		6.17 ± 0.66	5.70 ± 0.41
D-Phe-L-Glu		5.85 ± 0.26 *	1.93 ± 0.39
D-Phe-L-Lys		0.46 ± 0.14	0.49 ± 0.14

Mean ± SEM, $n = 4$; * $P < 0.01$ with respect to the value for the same dipeptide at pH 7.4.

3.4. The influence of luminal pH on the accumulation of hydrolysis-resistant dipeptides

Table 3 shows that D-Phe-L-Ala, D-Phe-L-Glu and D-Phe-L-Gln accumulate in the mucosa against a concentration gradient at neutral pH and for D-Phe-L-Ala and D-Phe-L-Glu the peptide concentration in the mucosal tissue at the end of the 2-h perfusion was significantly higher ($P < 0.01$) at pH 6.8. The only hydrolysis-resistant dipeptide which was not concentrated by the mucosa at neutral or acid pH was D-Phe-L-Lys which may indicate a more rapid exit for the positively charged dipeptide on the basolateral side. At a luminal pH of 8.0 the mucosal tissue peptide concentration did not exceed that in the lumen for either D-Phe-L-Ala or D-Phe-L-Lys.

4. Discussion

4.1. The influence of luminal pH on transmural dipeptide transport in the intestine

The aim of the work reported in this paper was to investigate whether the transmural transport of neutral and charged dipeptides in the intact rat small intestine is consistent with the principles established for the renal brush-border membrane vesicles [8] and the results presented above indicate that this is the case. Transport of neutral and negatively charged dipeptides was stimulated by lowering the luminal pH to 6.8, whereas the reverse was true for positively charged D-Phe-L-Lys. Furthermore, as indicated in Table 2, increasing the luminal pH to 8.0 strongly

inhibited the transport of D-Phe-L-Ala but stimulated that of D-Phe-L-Lys.

4.2. Comparison of the effect of luminal pH on transport and accumulation of acidic and neutral dipeptides

D-Phe-L-Ala and D-Phe-L-Gln were accumulated against a concentration gradient (Table 3) and their transmural transport rates increased significantly at pH 6.8. These results are in agreement with the proton-dependent transport of neutral dipeptides by rabbit intestinal PepT1 [1]. However, the fact that at pH 6.8 the D-Phe-L-Glu transport rate increased to 4 times that at pH 7.4 is consistent with the requirement shown for renal membrane vesicles [8] where there was co-transport of two protons with a negatively charged dipeptide but only one proton with a neutral dipeptides.

4.3. The influence of luminal pH on transport of a basic dipeptide

The fact that D-Phe-L-Lys transport is substantially inhibited at a luminal pH of 6.8 shows that uptake of this dipeptide is not proton-dependent and may indicate that the increased proton concentration in the lumen is competing with the positively charged dipeptide for the binding site on the transporter. This conclusion is further supported by the finding that at pH 8.0 the transport of D-Phe-L-Lys was stimulated, whereas that of D-Phe-L-Ala was substantially reduced (Table 2).

4.4. Comparison of renal and intestinal dipeptide transport

In conclusion, the fact that the incorporation of D-Phe at the N-terminus of a dipeptide largely blocks hydrolysis has allowed us to demonstrate that the transmural transport of neutral and negatively charged dipeptides is linked to the co-transport of protons, whereas that of the positively charged D-Phe-L-Lys is not. Reducing the luminal pH to 6.8 stimulates D-Phe-L-Glu transport by an order of magnitude more than it stimulates transport of the neutral dipeptides and this is consistent with the co-transport of an additional proton with this negatively charged dipeptide as was the case for D-Phe-L-Glu transport by renal vesicles [8]. In both tissues the transport of each dipeptide is linked to the co-transport of sufficient protons to give the dipeptide a net charge of +1. Consequently it seems likely that dipeptide transport in the small intestine follows the model we have recently proposed for the kidney [16].

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