Evidence for tripeptide/H⁺ co-transport in rabbit renal brush-border membrane vesicles

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L-Phe-L-Pro-L-Ala is a tripeptide which is hydrolysable almost exclusively by dipeptidyl peptidase IV in rabbit renal brush-border membrane vesicles. In order to delineate the mechanism of the transport of an intact tripeptide across the brush-border membrane, we studied the characteristics of the uptake of [³H]Phe-Pro-Ala in membrane vesicles in which the activity of dipeptidylpeptidase IV was completely inhibited by treatment with di-isopropyl fluorophosphate. In these vesicles, uptake of radiolabel from the tripeptide was found to be Na⁺-independent, but was greatly stimulated by an inwardly directed H⁺ gradient. The H⁺-gradient-dependent radiolabel uptake appeared to be an active process, because the time course of uptake exhibited an overshoot phenomenon. The process was also electrogenic, being stimulated by an inside-negative membrane potential. Under the uptake-measurement conditions there was no detectable hydrolysis of [³H]Phe-Pro-Ala in the incubation medium when di-isopropyl fluorophosphate-treated membrane vesicles were used. Analysis of intravesicular contents revealed that the radiolabel inside the vesicles was predominantly (> 90 %) in the form of intact tripeptide. These data indicate that the uptake of radiolabel from [³H]Phe-Pro-Ala in the presence of an inwardly directed H⁺ gradient represents almost exclusively uptake of intact tripeptide. Uphill transport of the tripeptide was also demonstrable in the presence of an inwardly directed Na⁺ or K⁺ gradient, but only if nigericin was added to the medium. Under these conditions, nigericin, an ionophore for Na⁺, K⁺ and H⁺, was expected to generate a transmembrane H⁺ gradient. Uptake of Phe-Pro-Ala in the presence of a H⁺ gradient was inhibited by di- and tri-peptides, but not by free amino acids. It is concluded that tripeptide/H⁺ co-transport is the mechanism of Phe-Pro-Ala uptake in rabbit renal brush-border membrane vesicles.

INTRODUCTION

Active transport mechanisms for absorption of small peptides exist in mammalian small intestine and kidney (Matthews, 1975). The characteristics of peptide transport have been investigated in recent years using brush-border membrane vesicles isolated from these tissues (Ganapathy & Leibach, 1985, 1986; Hoshi, 1985). All these studies, however, have utilized dipeptides as test substrates for the peptide-transport system, even though competition experiments have clearly indicated that the transport system can also accept tripeptides as substrates. Transport of intact tripeptides has not yet been directly investigated in membrane vesicles. There is a single report by Addison *et al.* (1975) in which uptake of Gly-Sar-Sar and β -Ala-Gly-Gly was studied in everted rings of small intestine. The authors concluded that these two tripeptides were actively transported into the enterocytes by an Na⁺-dependent mechanism. Similar conclusions were drawn for dipeptide transport in small intestine from intact-tissue experiments, which suggested dipeptide/Na⁺ co-transport as the mechanism of peptide transport. However, recent investigations with purified brush-border membrane vesicles have convincingly shown that Na⁺ does not play a role in the transport of dipeptides. The driving force for dipeptide transport is a protonmotive force rather than a sodium-motive force (Ganapathy *et al.*, 1987a,b). Therefore the characteristics of tripeptide transport reported by Addison *et al.* (1975) may not reflect the actual mechanism of tripeptide transport across the brush-border membrane. It is clear from the foregoing discussion that more direct studies with tripeptide substrates are needed before definitive conclusions on tripeptide transport can be

drawn. An essential feature of these studies should be the demonstration of the transport of a tripeptide in intact form across the membrane. It would also be desirable if these studies are done in purified brush-border membrane vesicles rather than in intact tissues, because this experimental approach makes it feasible to determine the identity of the actual driving force for the transport.

Commercial non-availability of radiolabelled tripeptides and the potential hydrolysis of tripeptides by purified brush-border membranes were at least partly responsible for the lack of direct studies on tripeptide transport in vesicles. Here we describe an experimental approach which enabled us to determine the characteristics of transport of an intact tripeptide in renal brush-border membrane vesicles. The tripeptide selected for the study as a test substrate was Phe-Pro-Ala, which was custom-synthesized in radiolabelled form. Owing to the unique amino acid sequence of this peptide, it is hydrolysable only by one of the peptidases of the renal brush-border membrane, namely dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5). This enzyme is a serine peptidase and is highly sensitive to inhibition by di-isopropyl fluorophosphate (iPr₂P-F). DPP IV releases Xaa-Pro or Xaa-Ala type dipeptides from the N-terminus of larger peptides. A previous study from our laboratory (Miyamoto *et al.*, 1987) has shown that treatment of renal brush-border membrane vesicles with 1 mM-iPr₂P-F inhibits more than 99 % of DPP IV activity without interfering with the transport function of the vesicles. We therefore investigated the uptake of radiolabelled Phe-Pro-Ala in renal brush-border membrane vesicles that had been treated with 1 mM-iPr₂P-F. This approach has enabled us to demonstrate unequivocally that tripeptide/H⁺ co-

Abbreviations used: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine; Gly-Pro-pNA, Glycyl-L-prolyl *p*-nitroanilide; TFA, trifluoroacetic acid; Sar, sarcosine; DPP IV, dipeptidyl peptidase IV (EC 3.4.14.5); iPr₂P-F, di-isopropyl fluorophosphate; pH_i, internal pH; pH_o, (outside) external pH.

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transport is the mechanism of active transport of tripeptides in rabbit renal brush-border membrane vesicles.

EXPERIMENTAL

Materials

Unlabelled Phe-Pro-Ala was obtained from Bachem Bioscience, Philadelphia, PA, U.S.A. All other peptides, iPr_2P -F, FCCP and valinomycin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Glycyl-L-prolyl *p*-nitroanilide (Gly-Pro-pNA) was a gift from Professor Alfred Barth, Department of Biochemistry, Martin Luther University, Halle/Saale, German Democratic Republic. All other chemicals were of analytical grade.

[ring-4- 3H]Phe-Pro-Ala (sp. radioactivity 22 Ci/mmol) was custom-synthesized by Amersham International, Amersham, Bucks., U.K.

Methods

Preparation of brush-border membrane vesicles. Brush-border membrane vesicles from rabbit kidney were prepared by a Mg^{2+} -aggregation method as previously described (Miyamoto *et al.*, 1985; Takuwa *et al.*, 1985). Both cortical and medullary tissues were used for membrane preparation, because both portions of the kidney possess peptide-transport activity (Miyamoto *et al.*, 1988). Briefly, the tissue was homogenized in 10 vol. of homogenizing buffer (5 mM-EGTA/300 mM-mannitol/12 mM-Tris/NaOH, pH 7.5) for 1.5 min using an Ultra-Turrax instrument with the speed set at 50. An equal volume of ice-cold water was added to the homogenate. A stock solution of 1 M- $MgCl_2$ was added to the above suspension to a final concentration of 10 mM and the mixture was stirred for 1 min and left for 15 min at 4 °C. All subsequent steps were carried out at 4 °C. The mixture was centrifuged at 3000 *g* for 10 min and the resulting supernatant was centrifuged again at 38000 *g* for 30 min. The pellets containing brush-border membranes were suspended in suitable preloading buffer using a 25-gauge needle and washed twice with the buffer by dilution and centrifugation. In most cases the composition of the preloading buffer was 50 mM-Hepes/75 mM-Tris/100 mM- K_2SO_4 , pH 8.3. The protein concentration of the final membrane suspension was adjusted to 8 mg/ml and stored in small portions in liquid N_2 until use.

Treatment of membrane vesicles with iPr_2P -F. Membrane vesicles were treated with iPr_2P -F as described previously (Miyamoto *et al.*, 1987). iPr_2P -F was dissolved in ethanol and then added to the membrane suspension to a final concentration of 1 mM. The mixture was incubated at room temperature (22 °C) for 30 min and then used in uptake experiments. The concentration of ethanol during treatment was 1 %.

Uptake measurements. Uptake measurements were made at room temperature (22 °C) by using a rapid filtration technique (Ganapathy *et al.*, 1981). Millipore filters (DAWP type, 0.65 μ m pore size) were used. Uptake was initiated by rapidly mixing 40 μ l of the membrane suspension (320 μ g of protein) with 160 μ l of uptake buffer containing radiolabelled tripeptide. The composition of the uptake buffer varied depending upon the individual experiment, but in most cases it was 50 mM-Hepes/50 mM-Mes/25 mM-Tris/300 mM-mannitol, pH 6.0. Uptake was terminated by the addition of 3 ml of ice-cold stop buffer (2 mM-Hepes/Tris/210 mM-KCl, pH 7.5), followed by filtration. The filter was washed with 3 $\times$ 5 ml of the stop buffer and the radioactivity associated with the washed filter was counted.

DPP IV assay. The activity of DPP IV in purified brush-border membranes was assayed using Gly-Pro-pNA as the chromogenic substrate (Hama *et al.*, 1982). A 50 μ g portion of membrane protein was incubated at 22 °C with 2.5 mM-substrate in 3 ml of reaction buffer (125 mM-Tris/HCl, pH 8.0) and the generation of *p*-nitroaniline was continuously monitored at 380 nm for a period of 30 min with a Shimadzu model UV 160U recording spectrophotometer.

Analysis of [3H]Phe-Pro-Ala and its hydrolytic products. Total hydrolytic contents. Brush-border membrane vesicles (40 μ l, 8 mg of protein/ml) were incubated for 10 s with uptake buffer (160 μ l) containing [3H]Phe-Pro-Ala (final concn. 0.1 μ M). The pH of the incubation mixture was 6.7, and the temperature was 22 °C. Hydrolysis was stopped by the addition of 200 μ l of ice-cold 10 % (w/v) trifluoroacetic acid (TFA). The mixture was centrifuged to remove any precipitate, and the clear supernatant containing the peptide and its hydrolytic products was freeze-dried. The residue was dissolved in 200 μ l of 0.1 % TFA and applied on a PrepSep- C_{18} extraction column, followed by washing of the column with 0.1 % TFA. The bound peptides ([3H]Phe-Pro-Ala and [3H]Phe-Pro) were then eluted from the column with 6 ml of 60 % (v/v) methanol. The eluate was freeze-dried, and the residue was dissolved in 200 μ l of water and used for h.p.l.c. analysis.

Intravesicular contents. Brush-border membrane vesicles (40 μ l, 8 mg of protein/ml) were incubated with uptake buffer (160 μ l) containing [3H]Phe-Pro-Ala (final concn. 0.1 μ M). An inwardly directed H^+ gradient (pH_i 8.3; pH_o = 6.7) was present at the start of the experiment, and the incubation temperature was 22 °C. Uptake was terminated after 10 s by the addition of 3 ml of ice-cold stop buffer. The membrane vesicles were collected on a Millipore filter under vacuum. After washing with 3 $\times$ 5 ml of stop buffer, the filter was transferred to a tube containing 10 ml of ice-cold 5 % TFA. This was repeated 10 times, and all ten filters were collected in the test tube containing TFA. The tube was vortex-mixed vigorously for 5 min and the extract was centrifuged to remove any precipitate. The clear supernatant was freeze-dried and the residue was dissolved in 200 μ l of 0.1 % TFA. This solution was then prepared for h.p.l.c. analysis as described above for the total hydrolytic contents.

H.p.l.c. analysis. [3H]Phe-Pro-Ala and [3H]Phe-Pro were separated on a reverse-phase C_{18} column (4.6 mm $\times$ 250 mm) under isocratic conditions by h.p.l.c. (Beckman; Model System Gold). The mobile phase was methanol/tetrahydrofuran/water (8:1:11, by vol.) and the flow rate was 1.2 ml/min. The elution profile of Phe-Pro-Ala and Phe-Pro was previously determined by using standard peptides under similar conditions and the peptides were detected by measuring A_{214} . The retention time for Phe-Pro-Ala was 5.5 min and for Phe-Pro was 3.0 min. For the analysis of the experimental samples containing [3H]Phe-Pro-Ala and its hydrolytic products, 30 s fractions were collected immediately after sample injection and the radioactivity in each fraction was counted. The radioactivity associated with Phe-Pro-Ala and Phe-Pro was then quantified using the standard elution profiles for these peptides.

Calculations. Uptake measurements were routinely done in duplicate or triplicate, and the variation among the replicate values was always less than ± 10 % of the mean value. Each experiment was repeated with two or three different membrane preparations. Statistical significance was calculated by Student's *t* test and *P* < 0.05 was considered significant. The results are expressed as means $\pm$ S.D.

RESULTS

Effect of *iPr₂P-F* on DPP IV

We studied the effect of *iPr₂P-F* on the activity of DPP IV in renal brush-border membrane vesicles. DPP IV activity was monitored using Gly-Pro-pNA as the substrate. Hydrolysis of Gly-Pro-pNA was determined in control membrane vesicles and membrane vesicles which were treated with 1 mM-*iPr₂P-F* at 22 °C for 30 min (Fig. 1). Control membrane vesicles were able to generate *p*-nitroaniline, indicating the presence of DPP IV. By contrast, there was no detectable hydrolysis of Gly-Pro-pNA with *iPr₂P-F*-treated membrane vesicles. The results show that DPP IV activity is almost completely inhibited by 1 mM-*iPr₂P-F*. Therefore *iPr₂P-F*-treated membrane vesicles were used in subsequent experiments on Phe-Pro-Ala transport.

Fig. 1. Spectrophotometric scanning of Gly-Pro-pNA hydrolysis by control and *iPr₂P-F*-treated renal brush-border membrane vesicles

Table 1. Effects of an Na⁺ gradient and an H⁺ gradient on the uptake of radiolabel from [³H]Phe-Pro-Ala in *iPr₂P-F*-treated renal brush-border membrane vesicles

To investigate the effect of an Na⁺ gradient, the membrane vesicles were preloaded, after *iPr₂P-F* (1 mM) treatment, with 40 mM-Hepes/40 mM-Mes/45 mM-Tris/300 mM-mannitol, pH 6.7. Uptake was measured in a buffer containing 45 mM-Hepes/40 mM-Mes/40 mM-Tris, pH 6.7, and either 150 mM-NaCl or 150 mM-KCl. To investigate the effect of an H⁺ gradient, the membrane vesicles were preloaded, after *iPr₂P-F* (1 mM) treatment, with either 50 mM-Hepes/75 mM-Tris/100 mM-K₂SO₄, pH 8.3, or 45 mM-Hepes/40 mM-Mes/38.5 mM-Tris/100 mM-K₂SO₄, pH 6.7. Uptake was measured in a buffer containing either 50 mM-Hepes/50 mM-Mes/25 mM-Tris/300 mM-mannitol, pH 6.0, or 40 mM-Hepes/40 mM-Mes/45 mM-Tris/300 mM-mannitol, pH 6.7. In all cases, the concentration of [³H]Phe-Pro-Ala was 0.1 μM and the uptake was measured with a 10 s incubation period. The results are expressed as Phe-Pro-Ala equivalents (mean ± S.D., *n* = 6) and are from two different membrane preparations.

Gradient	Incubation conditions	Uptake of the Phe-Pro-Ala equivalent (pmol/10 s per mg of protein)
Na ⁺	[K ⁺] _o = 120 mM; [K ⁺] _i = 0 pH _o = pH _i = 6.7	0.05 ± 0.01
	[Na ⁺] _o = 120 mM; [Na ⁺] _i = 0 pH _o = pH _i = 6.7	0.10 ± 0.01
H ⁺	pH _o = pH _i = 6.7	0.08 ± 0.01
	pH _o = 6.7; pH _i = 8.3	2.38 ± 0.16

Fig. 2. Effects of an inwardly directed H⁺ gradient and a K⁺-diffusion potential (inside-negative) on the uptake of radiolabel from [³H]Phe-Pro-Ala

Membrane vesicles were preloaded with either 50 mM-Hepes/75 mM-Tris/100 mM-K₂SO₄, pH 8.3, or 40 mM-Mes/45 mM-Hepes/38.5 mM-Tris/100 mM-K₂SO₄, pH 6.7. The membrane vesicles were treated with 1 mM-*iPr₂P-F* at 22 °C for 30 min. Uptake of radiolabel from 0.1 μM-[³H]Phe-Pro-Ala was measured in either 50 mM-Mes/50 mM-Hepes/25 mM-Tris/300 mM-mannitol, pH 6.0, or 40 mM-Mes/40 mM-Hepes/45 mM-Tris/300 mM-mannitol, pH 6.7. Valinomycin was dissolved in ethanol and added to the uptake buffer. An equal amount of ethanol was added to the controls. The final concentration of valinomycin in the incubation medium was 10 μM. ●, pH_i = pH_o = 6.7; ○, pH_i = 8.3; pH_o = 6.7; ●, pH_i = 8.3, pH_o = 6.7, valinomycin.

Effects of an Na⁺ gradient and an H⁺ gradient on the uptake of radiolabel from [³H]Phe-Pro-Ala

The influence of an inwardly directed Na⁺ gradient ([Na⁺]_o = 120 mM; [Na⁺]_i = 0) and an inwardly directed H⁺ gradient (pH_o = 6.7; pH_i = 8.3) on the initial rates of radiolabel uptake from [³H]Phe-Pro-Ala (0.1 μM) was investigated in *iPr₂P-F*-treated brush-border membrane vesicles (Table 1). In the presence of an Na⁺ gradient, the uptake rate was 0.10 ± 0.01 pmol/10 s per mg of protein, which was significantly greater than the uptake rate in the presence of a K⁺ gradient (0.05 ± 0.01 pmol/10 s per mg of protein) (*P* < 0.001). When the uptake was measured in the absence of Na⁺, but in the presence of an H⁺ gradient, the rate was 2.38 ± 0.16 pmol/10 s per mg of protein, which was 24 times greater than the rate in the presence of an Na⁺ gradient. The value of pH_o was 6.7, both in the Na⁺-gradient and the H⁺-gradient experiments. We also measured the uptake rate at an extravesicular pH of 6.7, but in the

Fig. 3. H.p.l.c. analysis of [^3H]Phe-Pro-Ala hydrolysis by control and $\text{iPr}_2\text{P-F}$ -treated brush-border membrane vesicles

Details of the procedure are given in the Experimental section. (a) Total medium contents; (b) intravesicular contents; $\circ$, elution profile of radiolabel after incubation with control membrane vesicles; $\bullet$, elution profile of radiolabel after incubation with $\text{iPr}_2\text{P-F}$ -treated membrane vesicles; $\ominus$, elution profile for radiolabel before incubation with membrane vesicles.

absence of Na^+ and a transmembrane H^+ gradient. The uptake rate was again very low (0.08 ± 0.01 pmol/10 s per mg of protein). The results demonstrate that the uptake of radiolabel from the tripeptide is greatly stimulated by an inwardly directed H^+ gradient, even in the absence of Na^+ .

Time course of the H^+ -gradient-dependent uptake of the radiolabel

Fig. 2 describes the time course of radiolabel uptake from $0.1 \mu\text{M}$ -[^3H]Phe-Pro-Ala in $\text{iPr}_2\text{P-F}$ -treated brush-border membrane vesicles in the presence ($\text{pH}_o = 6.7$; $\text{pH}_i = 8.3$) or absence ($\text{pH}_o = \text{pH}_i = 6.7$) of an H^+ gradient. An outwardly directed K^+ gradient ($[\text{K}^+]_o = 40 \text{ mM}$; $[\text{K}^+]_i = 200 \text{ mM}$) was present in both cases. In the absence of an H^+ gradient, the uptake rate of the radiolabel was very slow, but increased with time and reached the equilibrium value at 60 min. On the other hand, the initial uptake rates were many-fold greater in the presence of an H^+ gradient than in its absence, and a transient accumulation of the radiolabel inside the vesicles against a concentration gradient was evident. The equilibrium values measured at 60 min were, however, not different in the presence or absence of an H^+ gradient. In the presence of an H^+ gradient, the intravesicular concentration of the radiolabel at the peak of the overshoot was 9 times greater than the equilibrium value.

Effect of an inside-negative membrane potential on the H^+ -gradient-dependent uptake of the radiolabel

Fig. 2 also describes the influence of a valinomycin-induced K^+ -diffusion potential (inside-negative) on the H^+ -gradient-dependent uptake of the radiolabel from $0.1 \mu\text{M}$ -[^3H]Phe-Pro-Ala. The initial rates of radiolabel uptake were stimulated more than

Fig. 4. Effect of nigericin on Phe-Pro-Ala uptake in the presence of an Na^+ or a K^+ gradient

Membrane vesicles were preloaded with 5 mM-Hepes/Tris buffer, pH 6.7, containing 418 mM-mannitol and then incubated with 1 mM- $\text{iPr}_2\text{P-F}$. Uptake of $0.1 \mu\text{M}$ -[^3H]Phe-Pro-Ala was measured in 5 mM-Hepes/Tris buffer, pH 6.7, containing either 209 mM- NaCl or 209 mM- KCl . Nigericin, dissolved in ethanol, was added to the uptake buffer, with the addition of ethanol alone to the controls. The final concentration of nigericin was $10 \mu\text{M}$. $\circ$, Na^+ gradient; $\bullet$, Na^+ gradient plus nigericin; $\square$, K^+ gradient; $\blacksquare$, K^+ gradient plus nigericin.

2-fold in the presence of an inside-negative membrane potential. These results strongly suggest that the uptake of radiolabel in the presence of an H^+ gradient is associated with the transfer of positive charge across the membrane.

Hydrolysis of [^3H]Phe-Pro-Ala by control and $\text{iPr}_2\text{P-F}$ -treated renal brush-border membrane vesicles

The above-described experiments demonstrate that the uptake of radiolabel from [^3H]Phe-Pro-Ala is stimulated by an H^+ gradient and is electrogenic in nature. These characteristics are similar to those of dipeptide uptake (Ganapathy *et al.*, 1987b). Before we ascribe these properties to tripeptide transport, the form in which the radiolabel is taken up into the vesicles should be determined. The radiolabel in [^3H]Phe-Pro-Ala is associated with the phenylalanine moiety. Hydrolysis of this peptide by DPP IV will generate the radiolabelled dipeptide, [^3H]Phe-Pro. However, since the uptake experiments were done in $\text{iPr}_2\text{P-F}$ -treated brush-border membrane vesicles, which had no detectable DPP IV activity, the radiolabelled tripeptide is expected to remain intact under the uptake conditions. In order to show that this indeed was the case, we determined the hydrolysis of [^3H]Phe-Pro-Ala by control and $\text{iPr}_2\text{P-F}$ -treated membrane vesicles.

We first analysed the hydrolytic products of [^3H]Phe-Pro-Ala in the total incubation medium. Control and $\text{iPr}_2\text{P-F}$ -treated brush-border membrane vesicles were incubated with $0.1 \mu\text{M}$ -[^3H]Phe-Pro-Ala under the uptake conditions ($\text{pH}_i = 8.3$; $\text{pH}_o = 6.7$; 320 μg of membrane protein; 22°C). A 10 s incubation was chosen because this was the time period used in most uptake

Table 2. Effect of FCCP on Phe-Pro-Ala uptake

Brush-border membrane vesicles were preloaded with 50 mM-Hepes/75 mM-Tris/100 mM-K₂SO₄, pH 8.3, and then treated with 1 mM-iPr₂P-F. Uptake of [³H]Phe-Pro-Ala (0.1 μM) was measured in a buffer containing 50 mM-Hepes, 50 mM-Mes, 22 mM-Tris and 100 mM-K₂SO₄, pH 6.0, using 10 s incubations. FCCP and valinomycin were dissolved in ethanol and added to the uptake buffer. The final concentration of FCCP and valinomycin was 20 μM. An equal amount of ethanol was added to the controls. The results are given as mean ± S.D. (n = 6) from two different membrane preparations.

Incubation conditions	Phe-Pro-Ala uptake (pmol/10 s per mg of protein)
pH _i = 8.3; pH _o = 6.7 [K ⁺] _i = [K ⁺] _o = 200 mM	1.39 ± 0.05
pH _i = 8.3; pH _o = 6.7 [K ⁺] _i = [K ⁺] _o = 200 mM; FCCP	0.07 ± 0.01
pH _i = 8.3; pH _o = 6.7 [K ⁺] _i = [K ⁺] _o = 200 mM; valinomycin	2.55 ± 0.05
pH _i = 8.3; pH _o = 6.7 [K ⁺] _i = [K ⁺] _o = 200 mM; FCCP + valinomycin	0.34 ± 0.08

experiments reported in the present study. The h.p.l.c. elution profile of the radiolabel in the total incubation medium before and after 10 s incubation of the labelled tripeptide with the membrane vesicles is given in Fig. 3(a). The radiolabel was exclusively associated with Phe-Pro-Ala before incubation with membrane vesicles. After incubation with control membrane vesicles, about 40 % of the radiolabel was in the form of Phe-Pro, indicating hydrolysis of the parent tripeptide by DPP IV. By contrast, there was no detectable formation of radiolabelled Phe-Pro when the tripeptide was incubated with iPr₂P-F-treated membrane vesicles, and all the radiolabel was still associated with Phe-Pro-Ala.

We also analysed the radiolabel in the intravesicular contents after 10 s uptake in control and iPr₂P-F-treated brush-border membrane vesicles, and the results are given in Fig. 3(b). In control membrane vesicles, most of the radiolabel inside the vesicles was in the form of Phe-Pro, with very little radiolabel associated with Phe-Pro-Ala. By contrast, more than 90 % of the radiolabel was in the form of Phe-Pro-Ala in the case of iPr₂P-F-treated membrane vesicles. These results clearly demonstrate that the uptake of radiolabel from [³H]Phe-Pro-Ala measured in iPr₂P-F-treated membrane vesicles predominantly represents the transport of tripeptide in the intact form.

Effect of nigericin on Phe-Pro-Ala uptake in the presence of an Na⁺ or a K⁺ gradient

To provide supporting evidence for the stimulation of tripeptide transport by an inwardly directed H⁺ gradient, we studied the effect of nigericin on the radiolabel uptake from [³H]Phe-Pro-Ala (0.1 μM) in the presence of an inwardly directed Na⁺ or K⁺ gradient, but in the absence of a transmembrane H⁺ gradient (pH_o = pH_i = 7.5) (Fig. 4). Nigericin is an ionophore for Na⁺, K⁺ and H⁺ and hence is expected to act as an Na⁺ (or K⁺)-H⁺ exchanger under the experimental conditions to generate an inwardly directed H⁺ gradient across the membrane (Balkovetz *et al.*, 1988). In the absence of nigericin, uptake of Phe-Pro-Ala was greater in the presence of an Na⁺ gradient than a K⁺ gradient, but there was no indication of transport against a concentration gradient. Addition of nigericin stimulated the uptake many-fold in the presence of an Na⁺ gradient as well as a

Table 3. Effect of amino acids and peptides on Phe-Pro-Ala uptake

Membrane vesicles were treated with 1 mM-iPr₂P-F. Uptake of 0.1 μM-[³H]Phe-Pro-Ala was measured with 10 s incubations in the presence of an H⁺ gradient (pH_i = 8.3; pH_o = 6.7). The final concentrations of amino acids and peptides were 0.5 mM. Values are means ± S.D. (n = 4) for two different membrane preparations.

Test compound	Phe-Pro-Ala uptake	
	(pmol/10 s per mg)	(%)
None	2.70 ± 0.17	100
Amino acids		
L-Alanine	2.29 ± 0.06	85
L-Proline	2.29 ± 0.04	85
L-Phenylalanine	2.62 ± 0.12	97
Dipeptides		
Phe-Pro	0.14 ± 0.01	5
Tyr-Pro	0.18 ± 0.01	7
Gly-Pro	0.22 ± 0.01	8
Ala-Pro	0.11 ± 0.01	4
Val-Pro	0.08 ± 0.01	3
Gly-Sar	0.39 ± 0.06	14
Tripeptides		
Ala-Pro-Gly	0.13 ± 0.01	5
Gly-Pro-Ala	0.43 ± 0.05	16
Val-Pro-Leu	0.03 ± 0.01	1
Phe-Pro-Gly	0.11 ± 0.02	4
Phe-Pro-Ala	0.09 ± 0.02	3

K⁺ gradient. Uptake against a concentration gradient was clearly evident in the presence of the ionophore. The results indicate that an inwardly directed H⁺ gradient was generated under these conditions which resulted in the stimulation of tripeptide uptake.

Effect of FCCP on Phe-Pro-Ala uptake

Table 2 describes the effect of FCCP, a proton ionophore, on the H⁺ gradient-dependent uptake of Phe-Pro-Ala in iPr₂P-F-treated vesicles. Uptake was monitored by measuring radiolabel uptake from 0.1 μM-[³H]Phe-Pro-Ala in the presence of an inwardly directed H⁺ gradient (pH_o = 6.7; pH_i = 8.3). K⁺ was present at equal concentrations (200 mM) on both sides of the membrane. Control uptake in the absence of the ionophore was 1.39 ± 0.05 pmol/10 s per mg of protein. Addition of FCCP reduced this uptake by 95 %. This reduction was primarily due to the FCCP-induced inside-positive H⁺-diffusion potential. When the membrane vesicles were voltage-clamped with valinomycin, the uptake nearly doubled compared with the control uptake. This suggests that, in control vesicles, the uptake of Phe-Pro-Ala generates an inside-positive membrane potential which reduces the subsequent uptake of the peptide. The inhibitory effect of the membrane potential is removed when the vesicles are voltage-clamped. Addition of FCCP to the voltage-clamped membrane vesicles reduced the uptake by 85 %. In contrast with the control membrane vesicles, the effect of FCCP in voltage-clamped membrane vesicles was not due to the H⁺-diffusion potential, but rather to the dissipation of the H⁺ gradient. This is because the FCCP-induced H⁺-diffusion potential is immediately nullified by valinomycin-mediated K⁺ movements, which will facilitate continued diffusion of H⁺ into the vesicles, resulting in the dissipation of the H⁺ gradient. It is clear from these studies that both an inwardly directed H⁺ gradient and an inside-negative membrane potential can provide the driving force for the active uptake of Phe-Pro-Ala.

Inhibition of Phe-Pro-Ala uptake by amino acids and peptides

The inhibitory effect of unlabelled amino acids and peptides

Fig. 5. Kinetics of Phe-Pro-Ala uptake

Membrane vesicles were preloaded with 50 mM-Hepes/75 mM-Tris/100 mM- K_2SO_4 , pH 8.3, and incubated with 1 mM- iPr_2P-F at 22 °C for 30 min. The composition of the uptake buffer was 50 mM-Mes/50 mM-Hepes/25 mM-Tris/300 mM-mannitol. Uptake rates were measured with 10 s incubations. The Phe-Pro-Ala concentration was varied from 1 to 200 μ M. Values are means $\pm$ S.D. for triplicate assays with two different membrane preparations. The data are presented as an Eadie-Hofstee plot (v/s versus v , where v is the rate of Phe-Pro-Ala uptake in nmol/10 s per mg of protein and s is the Phe-Pro-Ala concentration in mM).

on the uptake of radiolabel from [3H]Phe-Pro-Ala was investigated in iPr_2P-F -treated brush-border membrane vesicles. The concentration of the radiolabelled tripeptide was 0.1 μ M, and that of amino acids and peptides was 0.5 mM. The results are given in Table 3. Amino acids (phenylalanine, proline and alanine) had no or little effect on the uptake of Phe-Pro-Ala. By contrast, all of the peptides tested, di- as well as tri-peptides, markedly inhibited Phe-Pro-Ala uptake, and the inhibition ranged between 80 and 100%.

Kinetics of Phe-Pro-Ala uptake

The kinetic parameters of Phe-Pro-Ala uptake were determined by studying the effect of increasing concentration of the tripeptide on the rate of its uptake in iPr_2P-F -treated membrane vesicles. Uptake was measured in the presence of an inwardly directed H^+ gradient ($pH_o = 6.7$; $pH_i = 8.3$) and using a 10 s incubation period. The concentration of Phe-Pro-Ala was varied from 1 to 200 μ M. The results are given in Fig. 5 as an Eadie-Hofstee plot [(uptake rate/peptide concentration) versus uptake rate]. The plot was linear, indicating participation of a single transport system. The apparent dissociation constant, K_t , and the maximal velocity, V_{max} , were calculated by linear-regression analysis of the data. The K_t was $16 \pm 1 \mu$ M and the V_{max} was 0.42 ± 0.01 nmol/10 s per mg of protein. The uptake of radiolabel was very rapid, and the uptake rate measured with a 10 s incubation period did not strictly represent the initial rate. Therefore the kinetic constants determined from these experimental data represent only approximate values.

DISCUSSION

The purpose of the present investigation was to characterize the transport of an intact tripeptide in renal brush-border membrane vesicles. To our knowledge, renal transport of

tripeptides has not been studied previously, and the present report is the first to describe the properties of transmembrane transport of an intact tripeptide in the kidney. Even in the small intestine, for which voluminous information is available on peptide transport (Matthews, 1975, 1984; Matthews & Adibi, 1976), studies on intact tripeptide transport are nearly non-existent. Earlier studies have, however, shown that dipeptide transport in the intestine and kidney is inhibited by tripeptides, and this observation has led to the conclusion, though indirect, that tripeptide transport exhibits characteristics similar to those of dipeptide transport (Berteloot *et al.*, 1981, 1982; Rajendran *et al.*, 1984, 1985; Ganapathy & Leibach, 1982; Miyamoto *et al.*, 1987). But most of the tripeptides employed in the competition experiments were hydrolysable by brush-border peptidases, and therefore it was never certain whether the observed inhibition was due to intact tripeptides or their hydrolytic products. The present investigation provides, for the first time, unequivocal evidence that intact tripeptides are transported across the renal brush-border membrane. This project was made feasible by selecting Phe-Pro-Ala as the tripeptide substrate, which is hydrolysable by only one of the brush-border peptidases, namely DPP IV, and studying the uptake of the tripeptide in brush-border membrane vesicles under the conditions in which the enzyme was almost totally inhibited. iPr_2P-F was used as the specific inhibitor of DPP IV, and this compound had no effect on the transport function of the renal brush-border membrane.

We have demonstrated in the present study that (a) Phe-Pro-Ala is transported intact into the intravesicular space of the renal brush-border membrane vesicles; (b) imposition of an inwardly directed H^+ gradient stimulates and energizes the uptake of the tripeptide; (c) the H^+ gradient-dependent Phe-Pro-Ala uptake is electrogenic in nature; and (d) many di- and tri-peptides that are resistant to hydrolysis by brush-border membranes under the experimental conditions inhibit Phe-Pro-Ala uptake. Phe-Pro-Ala exists predominantly as a zwitterion at pH 6.7, and the electrogenicity of the uptake process indicates that a positively charged ion is co-transported with the tripeptide. Since the uptake is energized by an inwardly directed H^+ gradient, the co-transported cation is most likely H^+ . These results suggest that tripeptide/ H^+ co-transport is the mechanism of the active transport of an intact tripeptide in renal brush-border membrane vesicles.

H^+ -gradient-driven uphill transport of organic solutes is very common in microorganisms. The existence of similar transport systems in mammalian tissues has become recognized only in recent years. The organic solutes whose transport is energized by an H^+ gradient in the intestine and/or kidney include dipeptides (Ganapathy & Leibach, 1983; Ganapathy *et al.*, 1984, 1985; Skopicki *et al.*, 1988), folic acid (Schron *et al.*, 1985), aminocephalosporins (Okano *et al.*, 1986), amino acids (Roigaard-Peterson *et al.*, 1987; Rajendran *et al.*, 1987) and lactic acid (Tirupathi *et al.*, 1988). The present study shows that tripeptides also belong to this category.

Even though Na^+ does not play a direct role in the translocation step of the H^+ -coupled transport systems, this ion is expected to be involved in the transport process under conditions pertaining *in vivo*. In the small intestine and the kidney, an inwardly directed Na^+ gradient is generated and maintained across the brush-border membrane by the activity of the basal-lateral ($Na^+ + K^+$)-ATPase. The Na^+-H^+ exchanger localized in the brush-border membrane of these tissues (Murer *et al.*, 1976) catalyses the conversion of the Na^+ gradient to an H^+ gradient. Therefore the H^+ -gradient-driven transport systems are dependent upon Na^+ and ATP in intact tissues. In the present study, the uptake of Phe-Pro-Ala was significantly greater in the presence of an Na^+ gradient than a K^+ gradient. This was most likely due to the

conversion of the Na^+ gradient to an H^+ gradient by the brush-border Na^+-H^+ exchanger. The participation of Na^+-H^+ exchanger in the generation of the driving force for the H^+ -coupled peptide transport explains previously reported observations that uptake of di- and tri-peptides in intact intestinal cells is Na^+ -dependent (Matthews, 1975).

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