Peptide Mimics as Substrates for the Intestinal Peptide Transporter*

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4-Aminophenylacetic acid (4-APAA), a peptide mimic lacking a peptide bond, has been shown to interact with a proton-coupled oligopeptide transporter using a number of different experimental approaches. In addition to inhibiting transport of labeled peptides, these studies show that 4-APAA is itself translocated.

4-APAA transport across the rat intact intestine was stimulated 18-fold by luminal acidification (to pH 6.8) as determined by high performance liquid chromatography (HPLC); in enterocytes isolated from mouse small intestine the intracellular pH was reduced on application of 4-APAA, as shown fluorimetrically with the pH indicator carboxy-SNARF; 4-APAA trans-stimulated radiolabeled peptide transport in brush-border membrane vesicles isolated from rat renal cortex; and in Xenopus oocytes expressing PepT1, 4-APAA produced trans-stimulation of radiolabeled peptide efflux, and as determined by HPLC, was a substrate for translocation by this transporter.

These results with 4-APAA show for the first time that the presence of a peptide bond is not a requirement for rapid translocation through the proton-linked oligopeptide transporter (PepT1). Further investigation will be needed to determine the minimal structural requirements for a molecule to be a substrate for this transporter.

The rapid uptake of intact small peptides across the brush-border membrane of the small intestinal epithelium is the major route for absorption of dietary protein α-amino nitrogen (1). Hitherto, it has been thought that a number of chemical features, for example free amino and carboxyl termini, are essential in contributing to substrate interaction with, and translocation through, the intestinal peptide transporter.

These features include the presence of a peptide bond within the substrate molecules. Indeed a major review (1) states that "it is the presence of peptide bonds which make di- and tripeptide acceptable to the peptide transport systems." Although previous work (e.g. Ref. 2) has shown that molecules lacking this feature can inhibit transport of peptides (presumably by substrate binding), we describe here for the first time rapid translocation of a small totally non-peptidic substrate through the intestinal peptide transporter. The substrate, 4-aminophenylacetic acid (4-APAA), selected on the basis of its chemical structure, it being a potential mimic of a dipeptide (D-Phe-L-Ala) (Fig. 1) which previously we have shown to be an excellent substrate for epithelial peptide transport (3, 4).

EXPERIMENTAL PROCEDURES

Rat renal brush-border membrane vesicles were prepared as described previously (5), and initial rates of labeled peptide transport (influx, efflux) were determined by rapid filtration (4, 6). Rat intestinal loops in vitro and vascu larly perfused small intestine in situ were used to measure transepithelial fluxes in the intact small intestine as described previously (3, 7). Luminal pH was changed using a previously published protocol (8). Isolated murine enterocytes were prepared by enzymatic digestion using haluronidase, and intracellular pH was determined fluorimetrically using carboxy-SNARF (9). Isolated cells were exposed to 20 mM HEPES-buffered Krebs solution containing either the dipeptide (Phe-Ala) or 4-APAA. The intracellular ratiometric signal (540 nm excitation, 590 nm/640 nm emission) was calibrated after the experiments by superfusing solutions of 140 mM KCl containing 10 μM nigericin and buffered to pH 5.5, 7.5, and 9.5 with PIPES, HEPES, and CAPS, respectively. Xenopus oocyte expression of PepT1 cRNA was as described (10, 11) with subsequent HPLC detection (3) of transported substrate and correction for transport in water-injected controls. For efflux assays 4.6 nl (110 fmol) of radiolabeled dipeptide (D-Phe-L-Gln, 0.1 μCi/μl) was injected into each oocyte. The assay was started after the oocytes were washed, 15 min after injection.

Inhibition constants (K_i) were determined as described previously (6). Briefly radiolabeled dipeptide influx was corrected for non-mediated transport by subtracting the transport of radiolabel seen in the presence of saturating unlabeled peptide (10 μM). The ENZFITTER program was then used to calculate the K_i for mediated transport only.

RESULTS

As part of a screening assay for potential substrates for epithelial peptide transport we used a preparation from rat renal cortex of apical brush-border membrane vesicles (5). Fig. 2 shows, using this assay, that 4-APAA is capable of cis-inhibition and trans-stimulation of labeled D-Phe-L-Glu influx and efflux, respectively. Because in the efflux assay there is no proton electrochemical gradient (the membrane being voltage-clamped using valinomycin and high K⁺) in both intracellular and extravesicular compartments and there being no pH gradient across the membrane) the ability of 4-APAA to trans-stimulate efflux of peptide must mean that it is translocated as a substrate (12). Based on this initial observation, three types of experiments were performed which functionally measure transport of this molecule in relation to intestinal absorption.

Using isolated rat small intestine in vitro, 4-APAA was shown using HPLC detection to be transported rapidly across the intact epithelium at a rate which was very substantially increased by external acidification (pH of lumen decreased from 7.4 to 6.8)

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characteristic of proton-coupled peptide transport (Table I). Moreover associated with this stimulation of transepithelial transport there was “active accumulation” of 4-APAA within the tissue (to a final concentration 3.7 times greater than that present in the lumen).

Intestinal absorption (nanomoles/min/g dry wt) of peptide (d-Phe-L-Gln) and 4-APAA was measured by the vascularly perfused rat small intestine (7). The luminal pH was 6.8, and substrates were perfused through the lumen (single pass) at a concentration of 1 mM. For inhibition studies, a steady state rate of transport was established, at 20 min the indicated inhibitor (10 mM) was added to the lumen and the new steady-state rate of transport was determined. Transport of substrates was determined by HPLC analysis of the portal venous effluent.

TABLE I
Transport of peptides and 4-APAA across intact small intestinal epithelium (rat)

| Condition                          | Transport rate (nmol/min/g dry wt) | Vascular appearance rate (nmol/min/g dry wt) |
|------------------------------------|-----------------------------------|-----------------------------------------------|
| Luminal pH 7.4                     | 53.2 ± 6.9                        | 218 ± 21                                      |
| Luminal pH 6.8                     | 880 ± 83                          | 314 ± 16                                      |
| Tissue accumulation (nmol)         |                                   |                                               |
| Luminal pH 7.4                     | 0.24 ± 0.16                       | 5.7 ± 0.41                                    |
| Luminal pH 6.8                     | 3.70 ± 0.13                       | 4.5 ± 0.35                                    |
| Vascular appearance rate           |                                   |                                               |
| Control                            | 329 ± 20                          | 253 ± 4.5                                     |
| + 4-APAA (10 mM)                   | 136 ± 2.3                         |                                               |
| + d-Phe-L-Gln (10 mM)              | 165 ± 10                          |                                               |

preparation transport of 4-APAA was inhibited significantly by concomitant addition of 10 mM d-Phe-L-Gln. Conversely, transport of d-Phe-L-Gln was inhibited significantly by the addition of 10 mM 4-APAA (Table I).

When isolated intestinal enterocytes were superfused with a solution containing Phe-Ala the peptide caused intracellular acidification (as expected for a substrate for a proton-coupled transporter (10)) (Fig. 3); applied separately, the isostere 4-APAA (2 mM) had the same effect. However in the presence of supramaximal concentration (20 mM) of dipeptide no further acidification was induced by the addition of (2 mM) 4-APAA, although on its own 4-APAA caused (reversible) acidification. At higher concentrations (20 mM) 4-APAA acted as a weak acid producing transient intracellular acidification and alkalinization following, respectively, its addition to and removal from the superfusate. In the presence of 20 mM 4-APAA, dipeptide failed to elicit additional acidification.
ing transient intracellular acidification and alkalinization following, respectively, its addition to and removal from the superfusate.)

Fig. 4 directly confirms that 4-APAA is a substrate for the intestinal peptide transporter PepT1 when this protein is expressed in Xenopus oocytes. This figure shows that 4-APAA (measured by HPLC) is transported into PepT1-expressing oocytes, that 4-APAA inhibits peptide (D-Phe-L-Gln) uptake into PepT1-expressing oocytes, and that radiolabeled peptide (D-Phe-L-Gln) efflux from Xenopus oocytes expressing PepT1 in the presence and absence of external substrate (D-Phe-L-Gln or 4-APAA). The external pH was 6.0. Data points are mean ± S.E. (n = 5). The data are significantly different from control (*, p < 0.002 for the peptide and for the 4-APAA in PepT1-injected (cross-hatched bars) but not in water-injected (open bars) controls. Injected oocytes were studied 3 days after cRNA injection. The concentrations of peptide (10 mM) and 4-APAA (20 mM) were chosen to be saturating (peptide) and approximately twice the K_i (4-APAA) for cis-inhibition of influx (cf. panel B).

**DISCUSSION**

The results from these transport assays demonstrate unambiguously that the presence of a peptide bond is not necessary for the rapid translocation of a substrate through the intestinal peptide transporter (PepT1). Our study leaves open the question as to the minimum requirements for a molecule to be a substrate. In the case of 4-APAA the fact that this molecule has both amino- and carboxyl-terminals separated by 5 carbon atoms as well as being planar (through its possession of an aromatic ring) may be pertinent. Nevertheless the findings reported here offer scope for chemical design of drugs which, despite lacking a peptide bond, will be absorbed rapidly from the small intestine via this transporter.

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