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Rapid report

Proton-coupled oligopeptide transport by rat renal cortical brush border membrane vesicles: a functional analysis using ACE inhibitors to determine the isoform of the transporter

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Abstract

We demonstrate that the angiotensin-converting enzyme inhibitors enalapril and captopril inhibit the transport of D-Phe-L-Gln into PepT1-expressing *Xenopus* oocytes and into rat renal cortical brush border membrane vesicles (BBMV). The kinetics of inhibition are competitive. Enalapril and captopril are not substrates for PepT2 (Boll et al., Proc. Natl. Acad. Sci. 93 (1996) 284–289). Therefore we conclude that in rat renal cortical BBMV this neutral dipeptide is transported via PepT1. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: PepT1; Angiotensin-converting enzyme inhibitor; Oocyte; Renal brush border membrane vesicle; Oligopeptide transport; (*Xenopus*)

PepT1 and PepT2 are proton-coupled oligopeptide transporters responsible for the transport of di- and tripeptides, in particular across the brush border membrane of certain epithelia. The lower affinity PepT1 isoform was originally cloned from rabbit small intestine [2], the higher affinity isoform PepT2 from human kidney [3]. Northern blot analysis using rat tissue has shown that, as well as being expressed in the small intestine, PepT1 message is also found in the kidney cortex [4]. In contrast, Northern blot analysis with rat PepT2 has shown its mRNA to be located predominantly in renal medulla, to a markedly lesser extent in renal cortex and not in the intestine [5].

A unique property of these peptide transporters is their extremely broad substrate specificity. As well as transporting all naturally occurring di- and tripeptides (potentially 20^2 dipeptides and 20^3 tripeptides) a number of therapeutically active molecules which mimic the structure of an oligopeptide are also substrates, e.g. β-lactam antibiotics [2] and the angiotensin-converting enzyme (ACE) inhibitors [6]. Understanding the mechanism of the absorption of these molecules both in the small intestine and kidney has obvious implications in the oral absorption and clearance of these drugs.

The ACE inhibitor captopril was first demonstrated to be a substrate for the intestinal peptide transporter by Hu and Amidon [6]; subsequently enalapril was also shown to be a substrate [7]. It is

Abbreviations: ACE, angiotensin-converting enzyme; BBMV, brush border membrane vesicle(s); HEPES, (*N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulphonic acid]; MES, 2-[*N*-morpholino]ethanesulphonic acid; Tris, Tris(hydroxymethyl)aminomethane hydrochloride

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not surprising that these molecules are substrates for the peptide transporter as chemically they resemble the structure of a di- and tripeptide respectively (Fig. 1a). More recently Boll et al. [8] have shown that 10 mM captopril and enalapril inhibit the transport of the β-lactam antibiotic cephadroxil into *Xenopus laevis* oocytes expressing the cloned rabbit transporter PepT1. However, of considerable interest was their later finding that in a similar experiment using PepT2 expressing *X. laevis* oocytes they did not observe an interaction between this isoform of the peptide transporter and the ACE inhibitors; cephadroxil transport into PepT2 expressing oocytes was not inhibited by either 5 mM enalapril or captopril [1].

In our laboratory we routinely use a preparation of brush border membrane vesicles (BBMV) obtained from rat renal cortex as a model system in which to study proton-coupled oligopeptide transport. Our kinetic data [9] are consistent with peptide transport through a single saturable system that obeys Michaelis-Menten kinetics. However, the question has often arisen as to whether this system is PepT1 or PepT2. In this study we have exploited the different recognition of the ACE inhibitors by PepT1 and PepT2 to address this question. The ability of the ACE inhibitors enalapril maleate (here after referred to as enalapril) and captopril to inhibit dipeptide transport in rat renal BBMV and *X. laevis* oocytes expressing rabbit PepT1 has been examined.

Brush border membrane vesicles were obtained from rat renal cortex by standard double magnesium precipitation [10]. Transport studies were performed using radiolabelled hydrolysis resistant dipeptides (custom synthesised by Cambridge Research Biochemicals, UK) under initial rate conditions (2 s or 4 s) at 37°C using standard rapid filtration as described [9,11]. Briefly membrane vesicles were resuspended in a high potassium solution containing the potassium ionophore valinomycin (100 mM K-gluconate, 20 mM D-mannitol, 0.1 mM $MgSO_4$, 80 mM Tris-HEPES pH 7.4, valinomycin 20 μg/mg protein) and the transport reaction commenced by the addition of 20 μl of vesicles (12.5 mg/ml) to 80 μl of incubation medium (100 mM Na-gluconate, 20 mM D-mannitol, 0.1 mM $MgSO_4$, 80 mM Tris-MES pH 5.5) containing the labelled dipeptide (0.42 μM) and the appropriate concentration of ACE inhibitor (0–20 mM). The composition of the media was such that on mixing, an inwardly directed pH gradient (pH_{out}=5.5, pH_{in}=7.4) was imposed with membrane potential clamped inside negative. The reaction was terminated after the appropriate time by the addition of 5 ml of ice-cold stop solution (150 mM NaCl, 50 mM $MgSO_4$, 30 mM D-mannitol, 5 mM Tris-MES pH 5.5) followed by rapid filtration and 2×5 ml further washes. The radiolabel associated with the filters was measured by liquid scintillation counting.

X. laevis oocytes were obtained from mature female frogs and collagenased (Type 1A, Sigma) in a nominally calcium free solution (80 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES pH 7.6) for 30 min before being manually defolliculated and maintained in modified Barth's solution at 17°C. Oocytes were injected the day after isolation with 25 ng rabbit PepT1 cRNA or an equivalent volume of water and transport studies were performed 3–5 days post injection as described previously [12]. Briefly oocytes (five) were placed in 100 μl of medium (95 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM Tris-HEPES pH 5.5) containing radiolabelled dipeptide (0.42 μM) and the appropriate concentration of ACE inhibitor (0–20 mM). After 1 h oocytes were removed and washed with 5×1 ml ice-cold Barth's solution. Radiolabel associated with individual oocytes was measured by liquid scintillation counting after disruption with 2% sodium lauryl sulphate.

Transport of the neutral dipeptide D-Phe-L-Gln into PepT1 expressing *Xenopus* oocytes is clearly inhibited by enalapril and captopril (Fig. 1a) at a single high concentration of 20 mM thus confirming the findings of Boll et al. [8]. When this experiment was repeated using rat renal BBMV an identical observation was made (Fig. 1b) thus providing evidence for the first time that enalapril and captopril interact with the isoform of the proton-coupled oligopeptide transporter present in these renal cortical BBMV.

This inhibition was explored further to establish the affinity for binding of captopril and enalapril in the two experimental systems. The concentration dependence of inhibition of D-Phe-L-Gln was studied over the concentration range 0–20 mM in both the BBMV and the PepT1 expressing oocyte preparations. An example of the data obtained is shown in Fig. 2 and from Michaelis-Menten kinetics the affinity constant K_i could be calculated (Table 1). Kinetic

analysis of data by Michaelis-Menten kinetics for a single system was performed using Inplot. The data analysed were mediated transport only, i.e. after subtraction of non-mediated transport (which is defined by uptake of labelled peptide remaining in the presence of 20 mM unlabelled dipeptide). The affinity constants for captopril and enalapril are 4.0 ± 1.0 mM and 1.1 ± 0.3 mM respectively in renal BBMV and 8.7 ± 0.9 mM and 4.3 ± 0.2 mM in PepT1 expressing *Xenopus* oocytes. These values for renal cortical BBMV fall within the range seen previously using small intestine (e.g. [6,13–15]). In each system enalapril binds with higher affinity to the oligopeptide transporter than captopril which is consistent with other published data using intestinal BBMV [14].

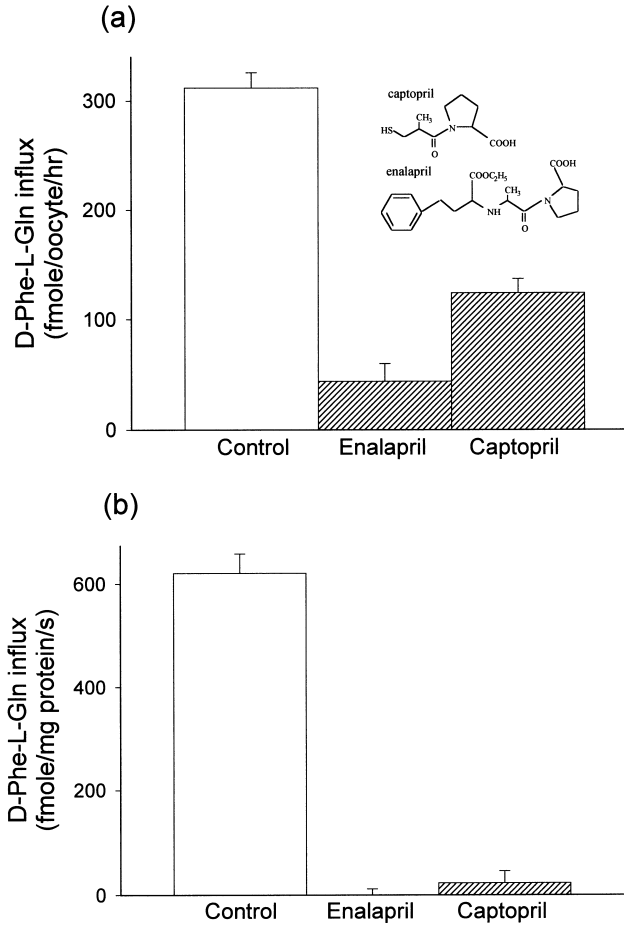

Fig. 1. Enalapril and captopril inhibition of tracer dipeptide influx. Control (no ACE inhibitor present), enalapril and captopril (20 mM ACE inhibitor present). (a) PepT1 expressing *X. laevis* oocytes, $pH_{out} = 5.5$. Data points are mean ± S.E.M. (two preparations of oocytes, five oocytes per preparation) where transport into control (H_2O-injected) oocytes has been subtracted. (b) Rat renal BBMV, $pH_{out} = 5.5$, $pH_{in} = 7.4$ and membrane potential clamped inside negative. Data points are mean ± S.E.M. (two vesicle preparations, three estimates per preparation) corrected for non-mediated transport (approx. 10%) by subtraction of labelled transport remaining in the presence of 20 mM unlabelled dipeptide.

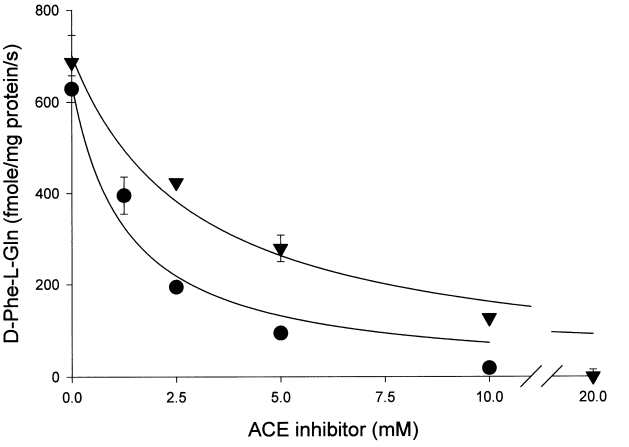

Fig. 2. Concentration dependence of enalapril and captopril inhibition of mediated D-Phe-L-Gln influx into renal BBMV. Enalapril (0–10 mM, circle), captopril (0–20 mM, triangle). Data points are mean ± S.E.M. (triplicate estimates from representative vesicle preparations). Solid line is best fit of data to Michaelis-Menten kinetics. Calculated inhibition constants (K_i) for enalapril and captopril are 1.3 mM and 3.0 mM respectively.

It is of interest that for a given ACE inhibitor the K_i for inhibition of peptide transport is always somewhat smaller when the measurement is made in BBMV compared to the *Xenopus* oocyte expression system; the difference for enalapril and captopril is 2–4-fold and we have made similar observations previously using other substrates [16]. Possible explanations include differences in the imposed electrochemical driving force in the two experimental systems and thermodynamic effects resulting from transport being studied at different temperatures. The possibility that in the BBMV another system may be working in parallel with PepT1, e.g. PepT2, is unlikely as an explanation since the measured difference in affinity between the two systems (2–4-fold) is over an order of magnitude smaller than the difference in measured substrate affinity observed previously for cloned PepT1 and PepT2 (50-fold); thus the K_m for cephadroxil transport in PepT1 expressing *Xenopus* oocytes is 1.1 mM [8] while for translocation of the same substrate through PepT2 it is 24 μM [1]. Con-

Table 1
Inhibition constants (K_i, mM) and apparent inhibition constants ($K_{i\,app}$, mM) for enalapril and captopril inhibition of tracer D-Phe-L-Gln influx into renal BBMV and PepT1 expressing oocytes

	Unlabelled D-Phe-L-Gln (mM)		Enalapril	Captopril
Renal BBMV	0	K_i	1.1 ± 0.3	4.0 ± 1.0
	0.21	$K_{i\,app}$	1.7 ± 0.1	8.1 ± 1.0
PepT1 expressing oocytes	0	K_i	4.3 ± 0.2	8.7 ± 0.9
	1	$K_{i\,app}$	6.5 ± 0.2	15.2 ± 0

K_i values calculated in the absence (tracer labelled dipeptide only) of unlabelled dipeptide. $K_{i\,app}$ values were calculated in the presence of unlabelled D-Phe-L-Gln (with tracer labelled dipeptide) at the indicated concentrations (equivalent to the K_m measured previously for the dipeptide in each system). K_i and $K_{i\,app}$ values were calculated from Michaelis-Menten kinetics as described in the text. Data points are mean ± S.E.M. ($n = 2$).

sistent with the small difference in affinity we observe between our two experimental systems are the data of Terada et al. [17] and Saito et al. [4]. In each study the transport of β-lactam antibiotics through cloned rat PepT1 was measured and depending on the expression system used (*Xenopus* oocytes or LLC-PK_1 cells) a 2-fold difference in affinity was observed.

In this study the K_i measurements in each system were also performed in the presence of unlabelled D-Phe-L-Gln ($K_{i\,app}$, Table 1) in order to investigate the nature of the inhibition. It can be seen for each ACE inhibitor, in both experimental systems, that the K_i in the presence of unlabelled dipeptide ($K_{i\,app}$) was greater than in the absence of unlabelled dipeptide (K_i, Table 1), e.g. in PepT1-expressing oocytes the K_i (in the absence of unlabelled D-Phe-L-Gln) was 8.7 ± 0.9 mM and increased to 15.2 ± 0 mM in the presence of 1 mM unlabelled D-Phe-L-Gln ($K_{i\,app}$). Qualitatively a shift of the K_i in this direction is characteristic of competitive inhibition [18]. Note that the shift in the observed K_i, in each system, for each ACE inhibitor, was in the range 1.5–2-fold. The concentration of unlabelled peptide used in each case was equivalent to the previously determined K_m so quantitatively if the mechanism of inhibition were competitive a doubling of the observed K_i is predicted. We conclude that our data support this mechanism and indicate that ACE inhibitor and dipeptide compete for the same binding site on PepT1. The competitive inhibition seen in this study supports the finding of Swaan et al. [15] (who observed that enalapril competitively inhibited cephalexin transport in an intestinal Ussing chamber preparation) but is in contrast to the published data of Yuasa et al. [13] (who found that enalapril non-competitively inhibited the transport of the aminocephalosporin cephradine in rabbit intestinal BBMV).

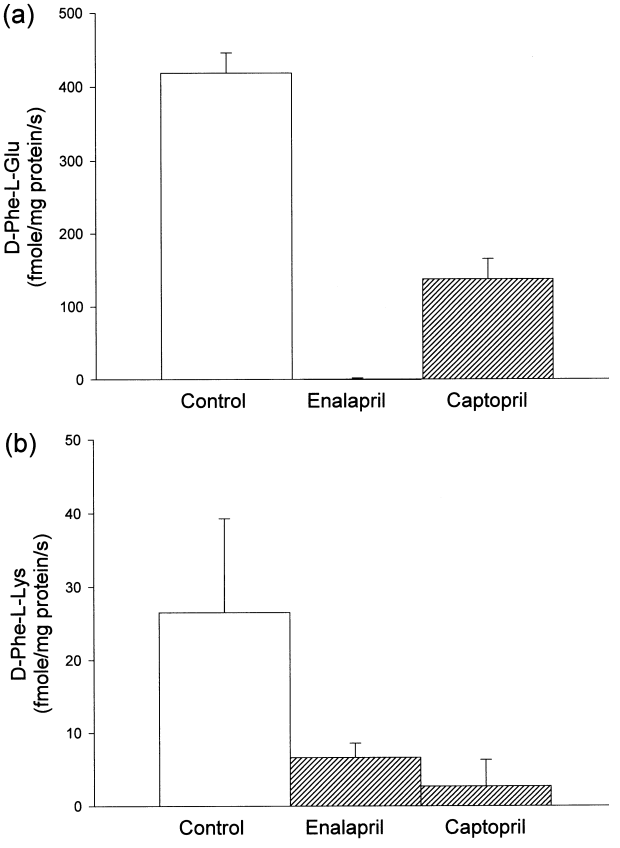

Fig. 3. Enalapril and captopril inhibition of charged tracer dipeptide influx into renal BBMV: (a) D-Phe-L-Glu (anionic) and (b) D-Phe-L-Lys (cationic). Control (no ACE inhibitor present), enalapril and captopril (20 mM ACE inhibitor present). Data points are mean ± S.E.M. (triplicate estimates from representative vesicle preparations) corrected for non-mediated transport by subtraction of labelled transport remaining in the presence of 20 mM unlabelled substrate dipeptide.

Interest has arisen recently regarding the mechanism whereby PepT1 is able to transport differently charged dipeptides. We previously demonstrated that the stoichiometry of proton-peptide cotransport in rat renal BBMV was variable and depended on the net charge carried by the substrate, i.e. the proton-peptide coupling ratio for anionic (D-Phe-L-Glu), neutral (D-Phe-L-Ala) and cationic (D-Phe-L-Lys) dipeptides was 2:1, 1:1 and 0:1 respectively [11]. Subsequently the stoichiometry for neutral (Gly-Ala) and anionic (Gly-Glu) dipeptides has been confirmed in PepT1-expressing *Xenopus* oocytes [19]. However, in this latter paper the transport of the cationic dipeptide (Gly-Lys) was found to be pH dependent with a coupling ratio of 1:1. One possible explanation for the difference observed is that in renal BBMV D-Phe-L-Lys is transported through a different system (e.g. PepT2). However, Fig. 3 shows that the transport of both the anionic dipeptide D-Phe-L-Glu and the cationic dipeptide D-Phe-L-Lys in renal BBMV is inhibited by both enalapril and captopril providing evidence that in this system both these charged peptides are transported by PepT1. The difference in the estimated coupling ratio for D-Phe-L-Lys in renal cortical BBMV and Gly-Lys in PepT1-expressing *Xenopus* oocytes therefore awaits explanation.

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