Structure–Transport Relationship for the Intestinal Small-Peptide Carrier: Is the Carbonyl Group of the Peptide Bond Relevant for Transport?

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Purpose. The objective of this study was to determine the influence of the peptide bond with emphasis on the carbonyl group on the interaction with and transport by the intestinal small-peptide carrier. Therefore enalapril, a known substrate for the small-peptide carrier, has been modified to an analogue with a reduced peptide bond, enamipril. The transport characteristics of both compounds have been determined.

Methods. The *in vitro* transport studies were performed using rat ileum in Ussing chambers. The transport of enalapril and enamipril were measured in a concentration range from 0.5–8 mM in both directions across the ileum, in the presence and absence of inhibitors. The interaction with the small-peptide carrier was studied by evaluating the ability of enalapril and its analogue enamipril to inhibit the transport rate of amoxycillin.

Results. Enalapril shows, besides passive diffusion (P_m 3.06 ± 0.14 · 10^{-6} cm/s), saturable transport kinetics (J_{max} = 16 ± 5 nmol/h·cm^2, K_m = 1.86 ± 0.64 mM) which can be inhibited with 10 mM cephalexin. The analogue with a reduced peptide bond does not show saturable transport from the mucosal to the serosal side, and cephalexin does not inhibit the flux of enamipril. However, the transport of enamipril from the serosal to mucosal side of the intestinal membrane is saturable and can be inhibited by 100 μM verapamil. Although enamipril is not a substrate for the small-peptide carrier in contrast to enalapril, both enalapril and enamipril are able to inhibit the active transport of amoxycillin with a K_i of 0.41 ± 0.24 mM and 0.24 ± 0.12 mM respectively.

Conclusions. The reduction of the peptide bond of enalapril results in a compound, enamipril, which does not show polarized and saturable transport from the mucosal to the serosal side of the intestinal tissue. Also because the transport of enamipril cannot be inhibited by cephalexin, the analogue with the reduced peptide bond is no longer a substrate for the intestinal small-peptide carrier. Therefore, it can be concluded that the carbonyl group is an essential structural requirement for transport by the small-peptide carrier. In contrast, the interaction with the small-peptide carrier is still present, shown by the inhibition of the fluxes of amoxycillin. Reduction of the peptide bond of enalapril resulted in a new substrate for the P-glycoprotein efflux pump.

KEY WORDS: intestinal small-peptide carrier; structure-transport relation; peptide bond; enalapril.

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INTRODUCTION

Most nutrients are absorbed in the intestine via carrier-mediated transport. Several transporters have been identified, among them are carriers for monocarboxylic acids, bile acids, amino acids, and di-/tripeptides (1). The absorption of di- and tripeptides in the intestine is mediated by a transmembrane protein, the small-peptide carrier (2). Besides transport of natural peptides, this carrier also plays a role in the absorption of several drugs, like cephalosporins (3–4), penicillins (5) and ACE-inhibitors (6–7). Among others, Chulavatnatol and Charles (8) investigated the importance of the small-peptide carrier in oral drug absorption and found that the absorption of amoxycillin was reduced to 19% when the dipeptide Gly-Pro was co-administered. The importance of the carrier in the absorption of peptide-like drugs justifies the question of which structural modifications are allowed in peptidomimetic drugs without loss of transport by the carrier.

Despite the isolation of the small-peptide carrier and the elucidation of the primary structure (9), no 3D-structure of this transport protein is available to provide information about structural requirements for transport by this carrier. Therefore, insight in the structure-activity relation of substrates must be obtained from *in vitro* transport studies. Several efforts have been made until now. Bai *et al.* (10) has shown the α-amino group in peptide analogues is not a structural feature of compounds which are transported by the small-peptide carrier. Studies of the transport of cephalosporins showed that no carrier-mediated transport occurs when the carboxylic acid group is not freely accessible (11). On the other hand, Swaan *et al.* (12) has shown that compounds with an additional free carboxylic acid group, like enalaprilat, have interaction with the carrier but are not transported.

Mapping of the pharmacophore of the small-peptide carrier resulted in two common structural features for transport by the intestinal small-peptide carrier: a free carboxylic acid group and the peptide bond (11). Until now, little is known about the precise function of the peptide bond for interaction with and transport by the small-peptide carrier. Recently, Enjoh *et al.* (13) reported the transport by the small-peptide carrier of arphamenine A, an analogue of the dipeptide, Arg-Phe. This compound contains a peptide bond in which the nitrogen atom is replaced by a methylene group to form a ketomethylene group. This suggests the nitrogen atom in the peptide bond is not essential for transport by the intestinal small-peptide carrier. However, the importance of the carbonyl group in the peptide bond is still not understood. Except for Temple *et al.* (14), nothing has been reported concerning the carbonyl group and transport by the small-peptide carrier in the gastro-intestinal tract. These authors claim the transport of 4-aminophenylacetic acid, a compound without a peptide bond, by the intestinal small-peptide carrier.

The aim of this study is to gain insight into the influence of the peptide bond, with emphasis on the carbonyl group, on the interaction with and transport by the small-peptide carrier. For this, an analogue of enalapril, a prodrug of the ACE-inhibitor enalaprilat and a known substrate for the intestinal small-peptide carrier (7,12), has been synthesized. This analogue with a so-called reduced peptide bond does not contain a carbonyl group in the peptide bond between the alanine and

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proline moiety of enalapril. In order to determine the necessity of the carbonyl group in the peptide bond for interaction with the intestinal small-peptide carrier, the influence of enalapril and the analogue on the transport of amoxycillin has been evaluated. The necessity for transport by the small-peptide carrier was studied by determining the concentration dependency of transport, the presence of polarized transport, and the ability of a known substrate for the small-peptide carrier, cephalexin (3,4) to interfere with the transport of both enalapril and the analogue.

MATERIALS AND METHODS

Materials

Enalapril maleate was a kind gift from MSD (Rahway, NJ). Cephalexin, amoxycillin trihydrate, verapamil, and other chemicals were obtained from commercial sources. All other reagents were of analytical grade.

Synthesis

The analogue of enalapril was synthesized *de novo* in 9 steps, starting from L-alanine which was coupled to the benzyl ester of proline. After the reduction of the resulting dipeptide and subsequent reductive amination, N-(ethoxycarbonyl-3-phenylpropyl)-L-alanyl[CH_2N]-L-proline was obtained with an overall yield of 13%. The extensive description of the synthesis is published elsewhere. The analogue is named enamipril because of the replacement of the peptide bond between the alanine and proline moiety by an amine group, as shown in Fig. 1b.

To prevent any possible interference of maleate, enalapril without maleate was made by preparing the benzyl ester of enalapril, purification by column chromatography followed by catalytic hydrogenation to remove the benzyl group. Enalapril, N-(ethoxycarbonyl-3-phenylpropyl)-L-alanyl-L-proline, was obtained in an overall yield of 76% (Fig. 1a).

Molecular Modelling

Molecular mechanical calculations of enalapril and enamipril were performed with the program Chem3D Pro. The TINKER minimiser was used for the energy minimisations *in vacuo*. The atoms of the phenyl group and the five-ring of proline of both compounds were superimposed to create a molecular overlay of the two structures. The fitting of both structures was terminated when a root mean square (RMS) of 0.005 was reached.

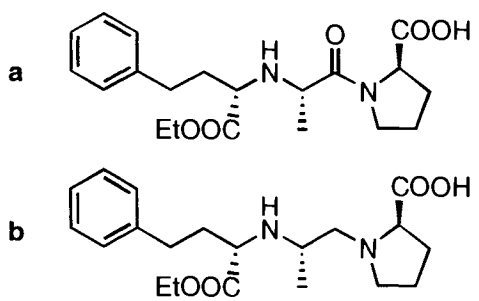

Fig. 1. Structural formulas of (a) enalapril and (b) enamipril.

Physicochemical Parameters

Solubility

The solubility of both compounds was estimated at ambient temperature by adding water, purified by reversed osmosis (RO-water), in steps of 50 μl to 25 mg compound in a 1.5 ml reaction tube. After each addition, the contents of the tube was vigorously mixed. From the amount of water resulting in complete dissolution (visually determined), the solubility was estimated.

Log D

To 1.0 ml of a 0.5 mM solution of enalapril or enamipril in bicarbonate-Ringer solution containing: Na^+, 140.6 mM; K^+, 5.0 mM; Ca^{2+}, 1.2 mM; Mg^{2+}, 1.2; Cl^- mM, 121.8 mM; HCO_3^-, 25 mM; HPO_4^{2-}, 1.6 mM; and $H_2PO_4^-$, 0.4 mM, pH of 7.4, 1.0 ml n-octanol, which was saturated with the buffer solution, was added. The dispersion was well shaken at 37°C for 3 h, after which the two phases were separated. The concentration of the compounds in the buffer phase before and after the distribution was determined by HPLC analysis. The partition coefficients were calculated using equation 1:

$$\log D = \log \frac{(C_{init} - C_{buf})}{C_{buf}} \quad (1)$$

D represents the partition coefficient between octanol and the buffer, C_{init} the concentration of the compound before the distribution and C_{buf} the concentration in the buffer phase. As a control, the procedure was repeated with 0.5 ml of the octanol phase which was extracted with an equal volume of a fresh buffer solution. The concentration of the compounds in the buffer layer was again determined by HPLC. The partition coefficients of the first and second distribution were equal within a range of 5%.

pK_a

Titration curves of enalapril and enamipril were determined with a Metrohm combititrator, equipped with a 614 Pulsomat. Solutions of ~0.16 mmol of the compounds in 6 ml 0.1 M HCl or NaOH were titrated with 0.5 M NaOH or HCl respectively.

Transport Studies

In vitro transport experiments of the compounds across living rat ileum were performed in Ussing chambers (15). Rat intestine was obtained from male Wistar rats (U:WU; 200–250 g) which had access to standard laboratory chow and tap water prior to experiments. After decapitation and laparotomy, the small intestine was quickly excised and placed into an ice-cold bicarbonate-Ringer solution pH 7.4, which composition is described in the log D determination. The ileum (5 cm proximal from the caecum) was taken, stripped from its underlying muscle layer and placed between two chambers. The tissue was bathed on both sides with bicarbonate-Ringer buffer containing 10 mM glucose on the serosal, and 10 mM mannitol on the mucosal side. The bathing solutions were kept at 38°C, rat body temperature, using thermostated water-jackets. During transport studies the integrity of the tissue was validated measuring the

permeability of the transport marker, fluorescein, with a donor concentration of 5.3 μM. Tissue viability was checked monitoring the electrical parameters of the tissue and measuring the response of the short circuited current to the mucosal addition of 500 μl 625 mM glucose at the end of each experiment.

The compounds were added to the donor side to a final concentration ranging from 0.5 to 8 mM. After 60 min, 500 μl samples were taken from the receiver side at 20 min intervals. To maintain a constant volume, 500 μl bicarbonate-Ringer buffer containing either 10 mM glucose or 10 mM mannitol was added after each sample. Effect of compound withdrawal was taken into account when calculating the fluxes.

Inhibition Studies

The influence of cephalexin, a substrate for the small-peptide carrier (3,4), on the transport rate of enalapril or enamipril (with a donor concentration of 2 mM) was studied according to the following procedure. The transport from the mucosal to the serosal side of enalapril or enamipril was monitored for 100 min as described above. After 140 min from the start of the experiment, an excess of cephalexin (with a final concentration of 10 mM) was added to the donor side to inhibit the carrier-mediated transport by the small-peptide carrier. Next, the transport of enalapril or enamipril was monitored with 20 min intervals.

To determine the possible involvement of the P-glycoprotein efflux pump in the transport of both enalapril and enamipril across the rat intestine, the effect of verapamil (16) was studied. The transport in both directions of enalapril or enamipril (at a donor concentration of 4 mM) was monitored before and after the addition of verapamil (at a final concentration of 100 μM) at both sides of the membrane.

Inhibition of amoxycillin transport by enalapril and enamipril was performed at amoxycillin concentrations of 0.25 to 8 mM. The flux from the mucosal to the serosal side was monitored for 140 min. Next, enalapril or enamipril (at a final concentration of 1 mM) was added to the mucosal side of the membrane, after which the flux was monitored for another 100 min.

Transport Data Analysis

Equation 2 was fitted to the measured fluxes of the compounds using non-linear regression:

$$J_{tot} = \frac{J_{max} \cdot C}{K_m + C} + P_m \cdot C \qquad (2)$$

Equation 2 is composed of a carrier mediated and passive transport component, in which J_{tot} represents the total measured flux, J_{max} the maximum flux of the substrate, K_m the apparent Michaelis-Menten constant, P_m the passive membrane permeability and C the concentration of the compound in the donor compartment. The transport rate across the rat ileum in the presence of an inhibitor can be described by equation 3:

$$J_{tot} = \frac{J_{max} \cdot C}{K_m + \frac{K_m \cdot I}{K_i} + C} + P_m \cdot C \qquad (3)$$

In the part of the formula describing the carrier-mediated transport an additional parameter, $K_m \cdot I/K_i$, was included in the denominator, with I as the concentration and K_i as the inhibition constant of the inhibitor.

Statistical Analysis

Significance tests on a mean of the flux were performed by using the paired Student t-test using a value for α of 0.05 (two sided).

HPLC Analysis

All compounds were determined by isocratic reversed-phase HPLC using a Waters 6000A pump (Waters Associates Inc., Milford, MA), a Kratos Spectroflow 773 variable wavelength UV detector (Kratos Analytical, Rotterdam, NL) at 220 nm, and a Basic Marathon autosampler (Spark, Emmen, NL). The column was a Merck LiChrosphere 100 RP-18 (5 μm) with a LiChroCART 4-4 (LiChrosphere) guard column (E. Merck Nederland BV, Amsterdam, NL). All mobile phases were filtered (Millipore 0.22 μm) and degassed before use; flow rate was 1.0 ml/min. The mobile phase for the amoxycillin analysis consisted of 95% 10 mM NH_4Ac and 5% acetonitril (v/v). The retention time of amoxycillin was 4.6 min, enalapril and enamipril had no mutual interference. A mixture of 20% methanol, 80% 5 mM $Bu_4NH_2PO_4$ (v/v) and 1 ml triethylamine for 1 l mobile phase with pH 2.5 was used for enalapril and enamipril. Both compounds showed a retention time of 5.0 min and neither cephalexin nor verapamil interfered in the analysis.

Fluorescein Measurements

The concentration of the integrity marker fluorescein at the receiver side was determined on a LS50 Luminescence spectrometer (Perkin Elmer, Buckinghamshire, UK) with an excitation wavelength of 486 nm (slid width 2.5 nm) and an emission wavelength of 516 nm (slid width 7.5 nm).

RESULTS AND DISCUSSION

Characterisation of Enalapril and Enamipril

For transport by the intestinal small-peptide carrier a free carboxylic acid group is essential. The reduction of the carbonyl group of the peptide bond might change the 3D-structure and thereby the accessibility of the carboxylic acid group for interaction with the carrier. Therefore, the influence of the removal of the carbonyl group from the peptide bond on the 3D-structure of enamipril compared to enalapril was evaluated with molecular modelling. The 3D-structures of enalapril and enamipril (Fig. 2) illustrate that enamipril still contains an accessible carboxylic acid group and that both compounds have a very similar conformation.

The effect of the reduction of the peptide bond on the physicochemical characteristics which might influence the transport across the intestinal tissue, was determined. Table I shows that both compounds can be classified as sparingly soluble (17). From the pK_a values (Table I) the overall charge of the molecules at pH 7.4 can be estimated: the ionic state of enalapril is 1^-, while enamipril is a zwitter-ion with an overall neutral charge, due to the replacement of the peptide bond by

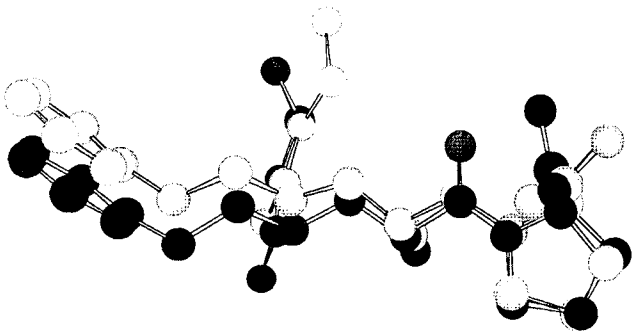

Fig. 2. Molecular overlay of 3D-structures of enalapril (black) and enamipril (white).

an amine group. In addition, the partition coefficients between n-octanol and bicarbonate-Ringer buffer of pH 7.4 (see log D values in Table I) show that enamipril is more lipophilic than enalapril. The found values for enalapril shown in Table I are in accordance with values reported in literature (18,19).

Transport Studies

The transport of enalapril and enamipril across the intestinal tissue was studied in both directions to obtain information about the mechanisms involved. In Fig. 3 the transport from the mucosal to the serosal side of both compounds is shown. Enalapril (closed triangles) shows saturable transport kinetics, which has been reported before (7,12). Using equation 2 to describe the transport of enalapril resulted in the following transport parameters: J_{max} 16 ± 5 nmol/h·cm^2, K_m 1.86 ± 0.64 mM and P_m 3.06 ± 0.14 · 10^{-6} cm/s. In contrast, the transport of enamipril is not saturable. Obviously, no active component in the mucosal to serosal transport for enamipril can be observed. The lower passive transport of enalapril compared to enamipril can be explained by the lower lipophilicity of enalapril (log D = −1.27) compared to enamipril (log D = 0.46).

To demonstrate the involvement of carrier-mediated transport of enalapril by means of the small-peptide carrier, the flux of 2 mM enalapril was determined before and after the addition of 10 mM cephalexin, a well known substrate for this carrier (3,4). As can be seen in Fig. 4, the flux of enalapril decreases to ~76% of the total flux which corresponds with the passive transport of enalapril across the rat ileum at this particular concentration. When in contrast the same amount of cephalexin was added to enamipril, no changes in the flux can be observed. Combining these results it can be concluded that the analogue with the reduced peptide bond, enamipril, is not a substrate for the intestinal small-peptide carrier in contrast to enalapril, and thus the carbonyl group in the peptide bond is indispensable for transport by this carrier.

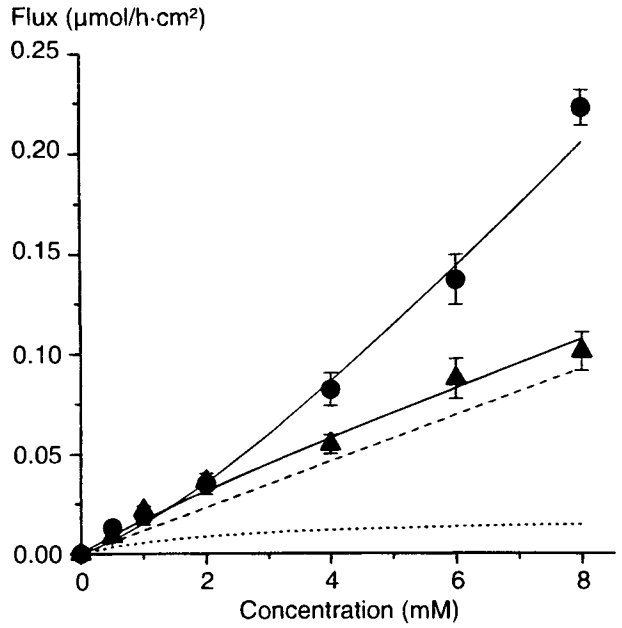

Fig. 3. Concentration-dependent transport from the mucosal to serosal side of enamipril (circles) and enalapril (triangles) across rat ileum. The dashed line represents the passive flux and the dotted line the active flux of enalapril based on the estimated transport parameters (n = 3–6, ± SEM).

Although enamipril is not transported by the small-peptide carrier, its ability to interact with the carrier is not known. To elucidate the influence of the removal of the carbonyl group on the interaction with the carrier, the effect of enalapril and enamipril on the transport rate of amoxycillin was investigated. The transport of amoxycillin, a reported substrate for the intestinal small-peptide carrier (5,8), was studied from the mucosal to serosal side in a concentration range up to 8 mM. Figure 5 shows the non-linear relation between the donor concentration and the corresponding flux of amoxycillin (closed squares). The transport can be described by equation 2, resulting in values for J_{max} of 60 ± 34 nmol/h·cm^2, K_m of 7.93 ± 3.23 mM and P_m of 1.72 ± 0.41 · 10^{-6} cm/s. The estimated passive (dashed line) and active process (dotted line) are drawn in Fig. 5. The addition of 1 mM enalapril (closed triangles) or 1 mM enamipril (closed circles) reduced the flux of amoxycillin to almost its passive component. Equation 3 was fitted to the obtained data, and resulted in a K_i = 0.41 ± 0.24 mM for enalapril and a K_i = 0.24 ± 0.12 mM for enamipril. This indicates both compounds can interact with the small-peptide carrier.

Table I. Physicochemical Parameters of Enalapril and Enamipril (n = 3, ± sd)

Compound	Molecular weight	Solubility (mg/ml H_2O)	Log D values	pK_a values
Enalapril	376.45	25	−1.27 ± 0.15[a]	2.85 ± 0.10 5.26 ± 0.11[b]
Enamipril	362.47	21	0.46 ± 0.04	3.81 ± 0.09 5.86 ± 0.08 9.37 ± 0.12

[a] log D = −1.15 at pH 7.0 (18).
[b] pK_a values are 2.97 and 5.35 respectively at 25°C (19).

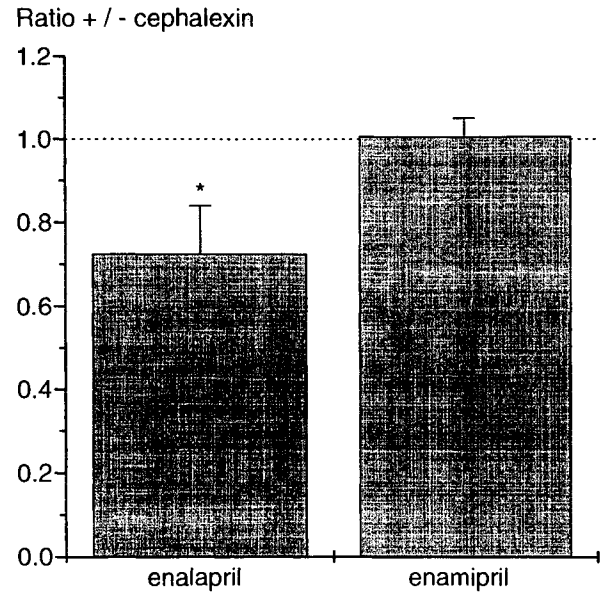

Fig. 4. Influence of 10 mM cephalexin on the flux of enalapril and enamipril at a donor concentration of 2 mM from the mucosal to serosal side. The results show the mean ratio of the flux after the addition of the inhibitor over the flux before the addition; * $p < 0.05$ (n = 3–5, ± sd).

To prove transport of a compound by the intestinal small-peptide carrier the following requirements have to be met: establishment of saturable transport kinetics of the compound, and inhibition of the transport with a known substrate for the small-peptide carrier, to exclude carrier-mediated transport by another carrier. Only inhibition of the transport of a peptide or any other substrate does not automatically mean that this compound will be transported by the carrier. However, this is often used as evidence for carrier-mediated transport itself, like for valacyclovir (20). In the case of 4-aminophenylacetic acid (14), the compound was claimed to be a substrate for the small-peptide carrier, although neither saturable transport kinetics nor inhibition of its transport by a known substrate for this carrier had been shown.

If a compound is transported by a carrier-mediated process, like the intestinal small-peptide carrier, the transport direction is also polarized. The transport of compounds is only mediated from the mucosal to the serosal side of the membrane. Therefore, transport of enalapril from the mucosal to the serosal side is composed of a passive part, linear with concentration plus an active component, which shows a non-linear relation with the concentration (Fig. 3). In the opposite direction (serosal to mucosal side) only passive transport of enalapril is expected. For enamipril, since it was shown not to be transported by the carrier, non-polarized transport was expected. Figure 6 shows the fluxes of both compounds from the serosal to the mucosal side. Indeed, the flux of enalapril increases linearly with the concentration. However, the flux is higher than the estimated passive flux from the mucosal to the serosal side. The flux of the integrity marker fluorescein also increased with the increasing concentration of enalapril, suggesting the increased enalapril flux is caused by the opening of the tight junctions by enalapril. This phenomenon was not studied further because it was beyond the scope of this study. In contrast to enalapril, enamipril shows a saturable transport profile, which can be divided in a passive (dashed line in Fig. 6) and an active process (dotted line in Fig. 6) using equation 2. The presence of an active component in

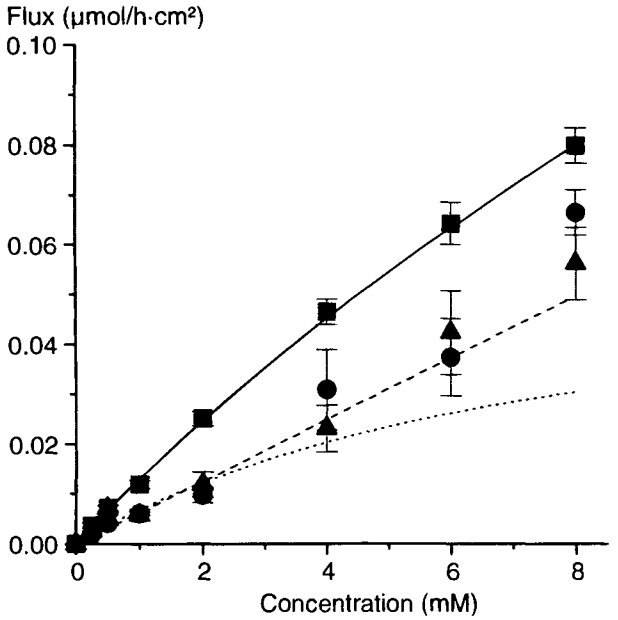

Fig. 5. Concentration-dependent transport of amoxycillin from mucosal to serosal side across rat ileum. Squares represent the fluxes of amoxycillin without inhibitor (n = 9–18), triangles with 1 mM enalapril (n = 3–6), and circles with 1 mM enamipril (n = 3–6). The dashed line indicates the calculated passive transport and the dotted line the active transport (data ± SEM).

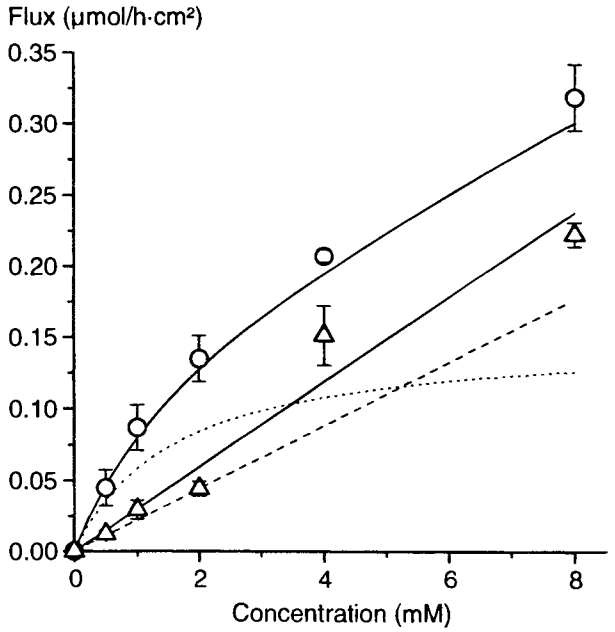

Fig. 6. Concentration-dependent transport from the serosal to mucosal side of enamipril (circles) and enalapril (triangles) across rat ileum. The dashed line represents the estimated passive flux and the dotted line the estimated active flux of enamipril (n = 3–6, ± SEM).

the serosal to mucosal transport of enamipril suggests an efflux mechanism is involved.

A well known secretory efflux system is the P-glycoprotein pump (21,22). This efflux system is responsible for drug excretion in cancer cells and several tissues like the intestine. The calcium channel blocker, verapamil, was one of the first described substrates for the P-glycoprotein pump (16). To determine whether the concentration-dependent efflux of enamipril was due to the involvement of the P-glycoprotein pump, the effect of verapamil on the flux of enamipril was studied. As shown in Fig. 7, verapamil increases the flux of enamipril from the mucosal to the serosal side and decreases the flux in the reverse direction. In contrast, verapamil did not influence the flux of enalapril in either of the two transport directions. These results indicate that enamipril in contrast to enalapril is a substrate for the P-glycoprotein efflux pump.

Combining the results from Figs. 3 and 7, the progressive flux of enamipril from the mucosal to the serosal side can be explained: with increasing donor concentrations of enamipril, the cellular concentration can become high enough to saturate the efflux pump, resulting in a higher flux.

CONCLUSIONS

This study shows the carbonyl group of the peptide bond is an essential structural feature for transport by the intestinal small-peptide carrier. The reduction of the peptide bond of a substrate results in a compound which is not transported by the small-peptide carrier anymore. First, by removing the carbonyl group in the peptide bond of enalapril, the resulting compound shows no longer saturable transport from the mucosal to the serosal side of the intestinal tissue. Second, the transport of this analogue cannot be inhibited by cephalexin, a substrate for the intestinal small-peptide carrier. In contrast, the interaction with the intestinal small-peptide carrier is still present: both enalapril and enamipril inhibit the active transport of amoxycillin. Surprisingly, the reduction of the peptide bond of enalapril, resulting in enamipril changes the affinity for the small-peptide carrier to an affinity for the P-glycoprotein efflux pump.

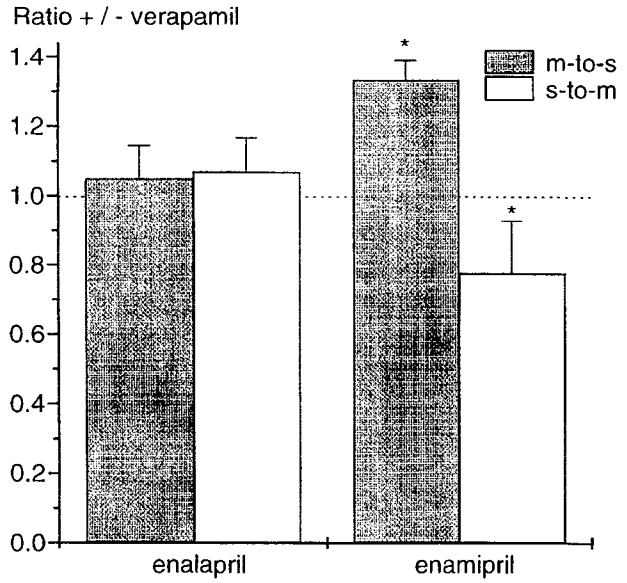

Fig. 7. Influence of 100 μM verapamil on the flux of 4 mM enalapril and enamipril from mucosal to serosal (grey) and serosal to mucosal side (white). The results show the mean ratio of the flux after the addition of the inhibitor over the flux before the addition; * $p < 0.05$ ($n = 3–5$, ± sd).

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