Competitive Inhibition of Glycylsarcosine Transport by Enalapril in Rabbit Renal Brush Border Membrane Vesicles: Interaction of ACE Inhibitors with High-Affinity H^+/Peptide Symporter

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Purpose. To examine the inhibitory potential of enalapril [and other angiotensin converting enzyme (ACE) inhibitors] on glycylsarcosine (GlySar) transport by the high-affinity renal peptide transporter.
Methods. Studies were performed in rabbit renal brush border membrane vesicles in which the uptake of radiolabeled GlySar was examined in the absence and presence of captopril, enalapril, enalaprilat, fosinopril, lisinopril, quinapril, quinaprilat, ramipril and zofenopril.
Results. Kinetic analyses demonstrated that enalapril inhibited the uptake of GlySar in a competitive manner (Ki $\approx$ 6 mM). Fosinopril and zofenopril had the greatest inhibitory potency (IC50 values of 55 and 81 μM, respectively) while the other ACE inhibitors exhibited low-affinity interactions with the renal peptide transporter. With respect to structure-function, ACE inhibitor affinity was strongly correlated with drug lipophilicity ($r = 0.944$, $p < 0.001$ for all ACE inhibitors; $r = 0.983$, $p < 0.001$ without enalaprilat, quinaprilat and quinapril).
Conclusions. The data suggest that enalapril and GlySar compete for the same substrate-binding site on the high-affinity peptide transporter in kidney, and that ACE inhibitors can interact with the renal carrier and inhibit dipeptide transport.

KEY WORDS: renal peptide transporter; glycylsarcosine; ACE inhibitors; competitive inhibition; lipophilicity.

INTRODUCTION

Although peptide transport across the luminal membrane appears to be functionally similar in the intestine and kidney, the renal peptide transporter PepT2 shows a much higher affinity for the same substrates and a different substrate specificity as compared to the intestinal transporter PepT1 (1–4). In particular, it has been reported that while angiotensin converting enzyme (ACE) inhibitors without an α-amino group are not recognized by the substrate binding site of the renal peptide/H^+ symporter in rabbits (5), no such limitation is observed for its intestinal homologue (6). However, it was subsequently found (7) that dipeptide uptake could be significantly inhibited and in a noncompetitive manner by quinapril (an ACE inhibitor peptidomimetic) in rabbit renal brush border membrane vesicles (BBMV).

In view of this finding, and because of the close structural similarity between enalapril and quinapril, the primary purpose of this study was to characterize the inhibitory potential of enalapril on glycylsarcosine transport, and its mechanism of inhibition. Upon completing these studies, other ACE inhibitors (Fig. 1) with varying degrees of lipophilicity were subsequently tested for their inhibitory effect on dipeptide tranport. Overall, we demonstrate for the first time that enalapril can competitively inhibit dipeptide transport, suggesting that enalapril and glycylsarcosine share a common binding site. Our results are also novel in demonstrating a highly significant correlation between ACE inhibitor affinities for the renal peptide transporter and their experimentally-determined lipophilicities.

METHODS

Materials

[^{14}C]Glycylsarcosine (GlySar; 119 mCi/mmol) was purchased from Amersham (Chicago, IL). Enalapril and enalaprilat were gifts from Merck (Rahway, NJ), quinapril and quinaprilat from Parke-Davis (Ann Arbor, MI), fosinopril and zofenopril from Bristol-Myers Squibb (Princeton, NJ) and ramipril from Hoechst Marion Roussel (Cincinnati, OH). Captopril and lisinopril were obtained from Sigma Chemical Co (St. Louis, MO). Other chemicals were obtained from standard sources and were of the highest quality available.

Renal Membrane Vesicles

Brush border membrane vesicles (BBMV) were isolated using a divalent cation precipitation method coupled to differential centrifugation, as described previously (7). In these experiments, the vesicles were prepared from whole cortex plus outer medulla of male New Zealand white rabbits (2–3 kg). Typically, about 14 g of tissue per rabbit were obtained and from this about 2.7 mg BBMV per g of tissue. The entire procedure was carried out on ice or at 4°C. BBMV preparations were prepared the day before use, stored at 4°C overnight and used within 3 days.

Marker Enzyme and Protein Assays

Alkaline phosphatase (ALP; a marker for brush border membranes) and Na^+/K^+-ATPase (a marker for basolateral membranes) activities were used to determine the purity and cross-contamination, respectively, of the BBMV preparations. Alkaline phosphatase was determined using a commercially available kit (Sigma Diagnostics, Kit #245, St. Louis, MO) and Na^+/K^+-ATPase was assessed by Jørgensen and Skou (8) with phosphate determined by the method of Fiske and Subbarow (9). Protein was assayed according to the method of Bradford (10) using γ-globulin as standard.

Uptake Experiments

Transport measurements were performed at 22°C using a rapid filtration technique (11). BBMV preparations were suspended in loading buffer to give a final protein concentration

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ABBREVIATIONS: ACE, angiotensin converting enzyme; BBMV, brush border membrane vesicles; GlySar, glycylsarcosine; Tris, tris(hydroxymethyl)aminomethane; Hepes, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid); ALP, alkaline phosphatase; Mes, 2-(N-morpholino)ethanesulfonic acid.

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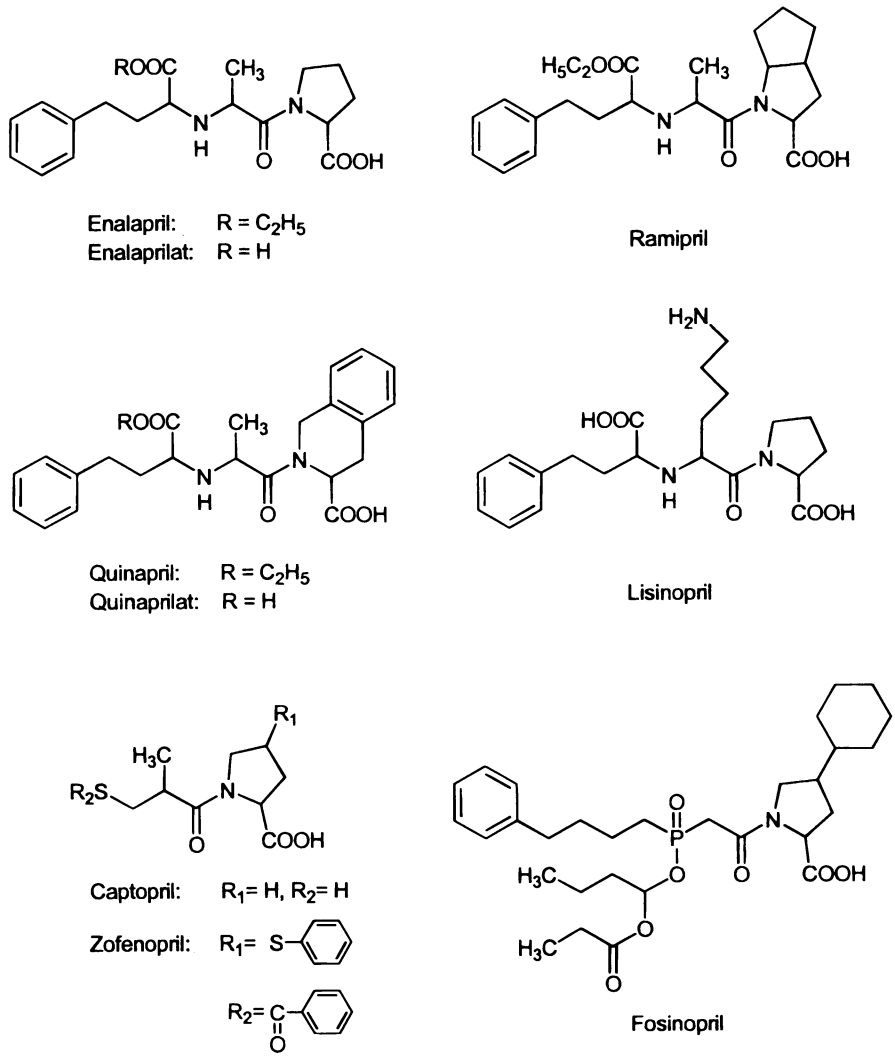

Fig. 1. Chemical structures of angiotensin converting enzyme inhibitors.

of 5–10 mg/ml and incubated for 1 hour at room temperature before uptake measurements. Uptake studies were initiated by adding 80 μl of reaction buffer containing radiolabeled GlySar into 20 μl of BBMV. At appropriate times, the reaction was stopped by adding 2 ml of ice-cold stop solution (2 mM Hepes and 210 mM KCl, pH 7.5). The mixture was immediately filtered under vacuum through a prewetted Millipore filter (PHWP, 0.3 μm) using a 10-place filtering manifold (Hoefer Scientific Instruments, San Francisco, CA). The membrane filter was then washed six times with 2 ml of ice-cold stop solution. The filters were dissolved in 8 ml of scintillation cocktail (Ready Protein; Beckman Instruments, Fullerton, CA) and radioactivity of each sample was determined by liquid scintillation counting using an external standard method for quench correction. Nonspecific binding was assessed by running a zero-time uptake in the presence of vesicles in which radiolabeled substrate and stop solution were added simultaneously. This value was subtracted from the overall uptake, and corrected uptake data were expressed as the amount of substrate taken up per milligram of protein. Since glycylsarcosine does not bind to membrane vesicles (12), radiolabeled GlySar retained on the filters after blank correction represents intravesicular drug. It should also be noted that GlySar is a model substrate due to its resistance to enzymatic hydrolysis in purified BBMV (13).

For cis-inhibition studies, the uptake of radiolabeled GlySar was conducted in the presence of an inwardly-directed H^+ gradient. Thus, vesicles were suspended in loading buffer containing 50 mM Hepes, 75 mM Tris and 100 mM K_2SO_4 (pH 8.4). The uptake buffer contained varying concentrations of radiolabeled GlySar (± inhibitors) in 50 mM Mes, 50 mM Hepes, 25 mM Tris and 300 mM mannitol (pH 6.1). Under these conditions, the final incubation was pH 6.7.

Determination of Lipophilicity

The octanol-water distribution coefficients of the ACE inhibitors captopril, enalapril, enalaprilat, fosinopril, lisinopril, quinapril, quinaprilat, ramipril and zofenopril were determined at pH 6.7 using the procedures described by Dearden and Vresnen (14) and Ranadive et al. (15) with minor modifications. The aqueous media was prepared using 8.3 mM phosphate buffer (pH 7.2), adjusted to pH 6.7 with 14.8 M (i.e., 85% w/w) phosphoric acid in the presence of test compounds. Prior to use, the octanol and aqueous phases were saturated with

each other by constant stirring for at least 12 hours. A known amount of ACE inhibitor was dissolved in the aqueous phase (3–5 different concentrations were tested) prior to equilibration with octanol, and equilibration was achieved by shaking the two phases at 180 times/min for 2 hours in a 15-ml polypropylene conical tube with screw cap. The mixture was then centrifuged at 2000 rpm for 1 hour, and 0.9 ml aliquots of the aqueous phase were removed for drug analysis.

ACE inhibitor concentrations was determined by adapting the HPLC methods of Kugler et al. (16) and Ranadive et al. (15). Samples were analyzed by an HPLC system consisting of a solvent delivery system (Spectroflow 400; ABI Kratos Analytical Division, Ramsey, NJ), a photodiode array detector with NEC APC IV data acquisition capabilities (Waters 990, Waters Corp., Milford, MA), an autosampler (ISS-100; Perkin-Elmer, Norwalk, CT) and a C8 column (5 μm, 250 × 4.6 mm, Econosil; Alltech Associates, Deerfield, IL). Samples were eluted at ambient temperature by a mobile phase consisting of 65% acetonitrile, 35% water, 0.02% triethylamine and 0.13% phosphoric acid (pH 2.4) and a flow rate of 1.0 ml/min, except for enalapril (55% acetonitrile, 45% water, 0.02% triethylamine, 0.13% phosphoric acid; pH 2.4) and lisinopril (65% methanol, 35% water, 0.02% triethylamine, 0.26% phosphoric acid; pH 2.6). The detector was set at 220 nm, and cephalothin (0.12–0.72 mM) was used as the internal standard for all analyses except for that of captopril in which cephradine (0.76 mM) was the internal standard. ACE inhibitor concentrations varied from 0.01 to 7.03 mM, depending upon the drug being analyzed, and all standard curves had coefficients of determination of ≥0.994.

Data Analysis

Unless otherwise specified, data are reported as mean ±SD of 3 to 5 different membrane preparations, with each preparation conducted in triplicate. Statistical differences among treatment groups were evaluated by analysis of variance (ANOVA) and pairwise comparisons were made using Tukey's test ($\alpha = 0.05$). All statistical computations were performed using SYSTAT v5.03 (Systat, Inc., Evanston, IL). Nonlinear as well as linear regression analyses were conducted using Scientist v2.01 (MicroMath Scientific Software, Salt Lake City, UT) and a weighting factor of unity. The quality of the fit was determined by evaluating the coefficient of determination (r^2), the standard deviation of parameter estimates, and by visual inspection of the residuals.

For dose-response studies in which drug uptake can be abolished, the inhibitory effect is described by the model:

$$E = Eo \cdot \left(\frac{IC50^S}{IC50^S + I^S}\right) \quad (1)$$

where E is the observed uptake, Eo is the uptake in the absence of inhibitor, I is the inhibitor concentration, IC50 is the concentration that causes 50% inhibition of the maximal drug effect, and s is the slope factor. The parameters, IC50 and s, were estimated for each membrane preparation by fitting the data to Equation (1) using nonlinear regression.

For kinetic studies, the concentration-dependent uptake of substrate can be expressed by:

$$v = \frac{Vmax \cdot C}{Km + C} \quad (2)$$

where v represents the uptake rate, Vmax is the maximal rate of uptake, Km is the Michaelis constant, and C is the substrate (GlySar) concentration. It is also convenient to transform the Michaelis-Menten equation into one that gives a straight line. This can be done by taking the reciprocal of both sides of the equation to give:

$$\frac{1}{v} = \frac{1}{Vmax} + \frac{Km}{Vmax} \cdot \frac{1}{C} \quad (3)$$

From a plot of 1/v vs. 1/C, Vmax and Km could then be estimated by linear regression analysis.

Enalapril inhibited the uptake of GlySar in a competitive manner (see Results section) and, as a result, the kinetics become:

$$v = \frac{Vmax \cdot C}{Km \cdot \left(1 + \frac{I}{Ki}\right) + C} \quad (4)$$

where Ki is the inhibition constant. Lineweaver-Burk transformation of Equation (4) gives:

$$\frac{1}{v} = \frac{1}{Vmax} + \frac{Km \cdot \left(1 + \frac{I}{Ki}\right)}{Vmax} \cdot \frac{1}{C} \quad (5)$$

In the presence of a competitive inhibitor, the apparent value (Km.app) is equal to Km multiplied by (1 + I/Ki). As can be seen from Equation (5), the slope of the plot equals Km · (1 + I/Ki)/Vm. By plotting the slopes vs. inhibitor concentrations (I), the x-intercept will be equal to −Ki.

The reciprocal equation for competitive inhibition can also be rearranged to that of a Dixon plot:

$$\frac{1}{v} = \frac{Km}{Vmax \cdot Ki \cdot C} \cdot I + \frac{1}{Vmax} \cdot \left(1 + \frac{Km}{C}\right) \quad (6)$$

By plotting 1/v vs. inhibitor concentrations (I), a slope of Km/(Vm · Ki · C) will be obtained. A further plot of the slopes vs. reciprocal substrate concentrations (1/C) will result in a line that passes through the origin. From the slope of this line (and knowing the Vm and Km values), Ki can be estimated.

The Ki of enalapril was determined by three different approaches: i) by linear regression of the Lineweaver-Burk plots expressed by Equations (3) and (5), ii) by linear regression of the Dixon plots expressed by Equation (6) and iii) according to the method of Cheng and Prusoff (17) in which Ki is equivalent to IC50/(1 + C/Km) for a competitive inhibitor.

For a drug initially dissolved in the aqueous phase, the distribution coefficient (D) can be calculated using the equation:

$$D = \frac{Vw \cdot (Cwi - Cwf)}{Vo \cdot Cwf} \quad (7)$$

where Vw and Vo represent the volumes of the aqueous and octanol phases, respectively, and Cwi and Cwf represent the respective initial and final concentrations of drug in the aqueous phase. Since self-association may occur as the concentration of test compound increases, the distribution coefficient was determined at infinitely dilute concentration where the activity coefficient of compound is equal to one. To accomplish this end, the log D values were reported as the y-intercept of a plot of log D vs. initial aqueous concentration for each ACE inhibitor.

In these studies, GlySar uptake (± ACE inhibitors) was functionally dominated by the high-affinity renal peptide transporter even though both PepT1 and PepT2 are present in kidney. This occurs because of the relatively low expression levels of PepT1 in kidney (3,4,18) and because we have optimized our kinetic conditions, as demonstrated previously (7). Thus, all experiments were performed at low substrate concentrations since, under linear conditions, over 90% of the flux for GlySar in rabbit renal BBMV was accounted for by the high-affinity, low-capacity transporter (19). In this regard, dose-response studies were performed with GlySar at 15 μM, and kinetic studies were performed with GlySar at 39–400 μM (Lineweaver-Burk plots) and 12–200 μM (Dixon plots). More importantly, no systematic deviations were observed in any of the linear models tested (Eqs. 3, 5 and 6) and all Ki estimates were comparable for enalapril, regardless of method, as was its nature of inhibition (i.e., competitive).

RESULTS

Membrane Purity

A total of 30 rabbit renal BBMV preparations were evaluated (whole cortex plus outer medulla). We found that ALP activity was highly enriched (12.5 ± 2.2-fold) in the final membrane preparation as compared to the initial homogenate, with no enrichment of Na^+,K^+-ATPase activity (0.63 ± 0.37-fold). These results are comparable with those reported in the literature (20–23) and demonstrate that the isolated BBMV were highly purified with negligible cross-contamination.

Kinetic Studies with Enalapril

The inhibitory effect of enalapril on H^+-dependent GlySar uptake was initially evaluated by the 10-sec uptake of radiolabeled GlySar (15 μM) in the presence of increasing concentrations of enalapril (0.1–25 mM). As shown in Figure 2, enalapril substantially inhibited the uptake of GlySar and in a concentration-dependent manner (<15% of control values). To further investigate the mechanism of this interaction, the 10-sec uptake kinetics of 12 μM radiolabeled GlySar (plus unlabeled substrate, total concentration 39–400 μM) was measured in the absence or presence of 2 and 4 mM of enalapril during an inwardly-directed proton gradient. As shown by the Lineweaver-Burk plot in Figure 3 (top panel), a change was observed in the slope and x-intercept of the curve, but not the y-intercept in the presence of inhibitor. As a result, kinetic analysis (Table I) revealed an increase in apparent Km values for GlySar as the concentrations of enalapril increased (150 μM for control vs. 193 and 259 μM in the presence of 2 and 4 mM enalapril, respectively; $p < 0.05$). On the other hand, apparent Vm values for GlySar did not change significantly when studied with enalapril (335 to 350 pmol/mg/10sec for all three treatments). These results demonstrate that the interaction between enalapril and GlySar on the high-affinity carrier system was competitive. This mechanism was further confirmed by the Dixon plots in Figure 4. As shown, the curves (1/uptake vs. inhibitor) intersect above the x-axis (top panel) whereas the slopes of these same curves vs. 1/GlySar form a line that goes right through the origin (bottom panel). Regardless of the method used (i.e., Lineweaver Burk, Fig. 3 (bottom panel), Dixon or dose-response; Table I), the Ki values of enalapril were comparable

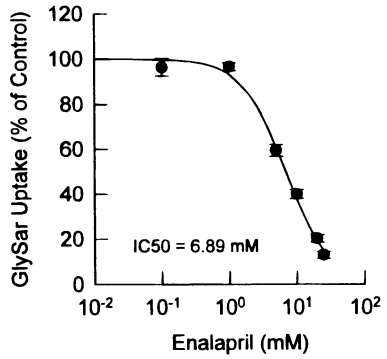

Fig. 2. Dose-response effect of enalapril on GlySar uptake in BBMV. Membrane vesicles were suspended in buffer, pH 8.4 (50 mM Hepes, 75 mM Tris and 100 mM K_2SO_4) and uptake was initiated in buffer, pH 6.1 (50 mM Mes, 50 mM Hepes, 25 mM Tris and 300 mM mannitol; final incubation pH 6.7). The 10-sec uptake of 15 μM [^{14}C]GlySar was measured as a function of increasing concentrations of unlabeled enalapril (0.1–25 mM). Data are mean ±SE from 3 separate membrane preparations. Lines were generated using fitted mean parameters (see Table II), as determined by nonlinear regression analysis.

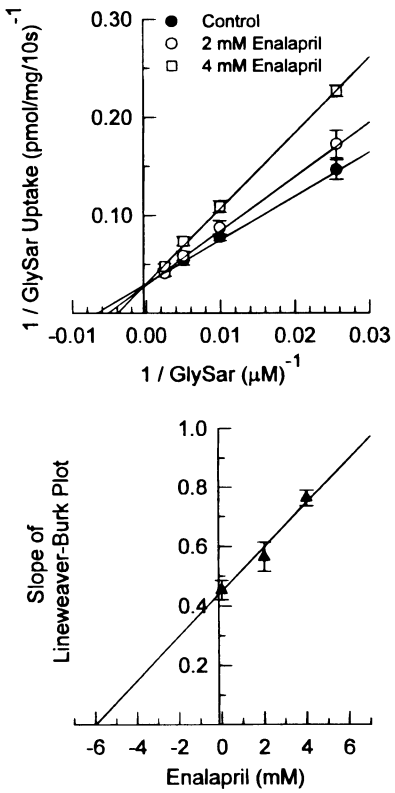

Fig. 3. Lineweaver-Burk plots of the effect of enalapril on the kinetics of GlySar uptake in BBMV. Membrane vesicles were suspended in buffer, pH 8.4 (50 mM Hepes, 75 mM Tris and 100 mM K_2SO_4) and uptake was initiated in buffer, pH 6.1 (50 mM Mes, 50 mM Hepes, 25 mM Tris and 300 mM mannitol; final incubation pH 6.7). The 10-sec uptake of 39–400 μM [^{14}C]GlySar was measured alone and in the presence of enalapril (2 and 4 mM). Data are mean ± SE from 5 separate membrane preparations. Lines were generated using fitted mean parameters (see Table I), as determined by linear regression analysis.

Table I. Effect of Enalapril Inhibition on GlySar Uptake in Rabbit Renal BBMV[a]

GlySar Parameters	Vmax (pmol/mg/10 sec)	Km (μM)
Lineweaver-Burk		
GlySar (Control)	335 ± 74	150 ± 30 A
+ Enalapril 2 mM	350 ± 55	193 ± 24
+ Enalapril 4 mM	336 ± 115	259 ± 98 A
Significance[b]	p = 0.954	p = 0.046
		Ki
Enalapril parameters		(mM)
Lineweaver-Burk		5.96 ± 2.00
Dixon		6.23 ± 1.74
Dose-response		6.26 ± 0.37

[a] Values are mean ± SD from 3–5 separate membrane preparations.
[b] P values were determined by ANOVA. For a given parameter, mean values with the same capital letter are significantly different (Tukey's test; α = 0.05).

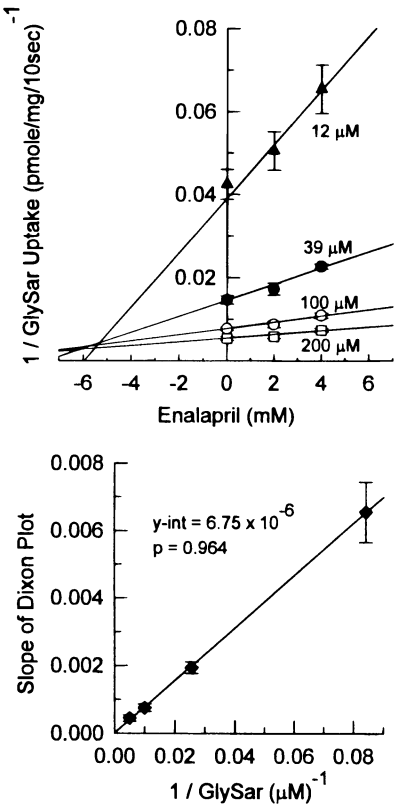

Fig. 4. Dixon plots of the effect of enalapril on the kinetics of GlySar uptake in BBMV. Membrane vesicles were suspended in buffer, pH 8.4 (50 mM Hepes, 75 mM Tris and 100 mM K_2SO_4) and uptake was initiated in buffer, pH 6.1 (50 mM Mes, 50 mM Hepes, 25 mM Tris and 300 mM mannitol; final incubation pH 6.7). The 10-sec uptake of 12–200 μM [^{14}C]GlySar was measured alone and in the presence of enalapril (2 and 4 mM). Data are mean ±SE from 5 separate membrane preparations. Lines were generated using fitted mean parameters (see Table I), as determined by linear regression analysis.

Table II. Dose-Response Parameters of ACE Inhibitors in Rabbit Renal BBMV[a]

ACE Inhibitor	IC50 (mM)	s (slope factor)
Captopril	19.3 ± 0.3	1.23 ± 0.22
Enalapril	6.89 ± 0.40	1.35 ± 0.15
Enalaprilat	52.2 ± 6.7	1.73 ± 1.14
Fosinopril	0.055 ± 0.006	1.36 ± 0.11
Lisinopril	21.7 ± 2.4	1.00 ± 0.05
Quinapril	0.89 ± 0.06	1.17 ± 0.15
Quinaprilat	4.73 ± 0.88	1.30 ± 0.45
Ramipril	0.89 ± 0.14	1.54 ± 0.04
Zofenopril	0.081 ± 0.008	0.73 ± 0.04
Significance[b]	p < 0.001	p = 0.253

[a] Values are mean ± SD from 3 separate membrane preparations.
[b] P values were determined by ANOVA.

at about 6 mM. It should also be noted that the competitive inhibition of GlySar by enalapril was specific since there was no significant change in the equilibrium uptakes of GlySar (as measured at 2 hours).

Dose-Response Studies with ACE Inhibitors

As shown previously (Fig. 2), the inhibitory potency of enalapril was low with an IC50 of 6.89 ± 0.40 mM. Similar analyses were then performed with several other ACE inhibitors (Table II; for all analyses, the coefficient of determination, r^2, was ≥0.943). Only fosinopril and zofenopril presented IC50 values of less than 100 μM (i.e., 55 μM and 81 μM, respectively). The other ACE inhibitors exhibited low-affinity interactions with the renal peptide transporter of which enalaprilat's interaction was the weakest (IC50 of 52 mM) toward GlySar uptake. Overall, the inhibitory potency of ACE inhibitors was as follows: fosinopril > zofenopril > ramipril ≈ quinapril > quinaprilat > enalapril > captopril > lisinopril > enalaprilat.

ACE Inhibitor-Lipophilicity Studies

The octanol-water distribution coefficients of ACE inhibitors (i.e., captopril, enalapril, enalaprilat, fosinopril, lisinopril, quinapril, quinaprilat, ramipril and zofenopril) are displayed in Table III. As observed, lisinopril was the least lipophilic (log

Table III. Octanol-Water Distribution Coefficients (D) of ACE Inhibitors at pH 6.7[a]

ACE inhibitor	D	log D
Captopril	0.082 ± 0.021	−1.10 ± 0.13
Enalapril	0.110 ± 0.016	−0.96 ± 0.06
Enalaprilat	0.085 ± 0.011	−1.07 ± 0.06
Fosinopril	389 ± 8	2.59 ± 0.01
Lisinopril	0.033 ± 0.012	−1.50 ± 0.14
Quinapril	1.88 ± 0.02	0.27 ± 0.01
Quinaprilat	0.049 ± 0.008	−1.31 ± 0.07
Ramipril	1.89 ± 0.05	0.28 ± 0.01
Zofenopril	40.2 ± 0.6	1.60 ± 0.01
Significance[b]	p < 0.001	

[a] Values are mean ± SD from 3 separate experiments.
[b] P values were determined by ANOVA.

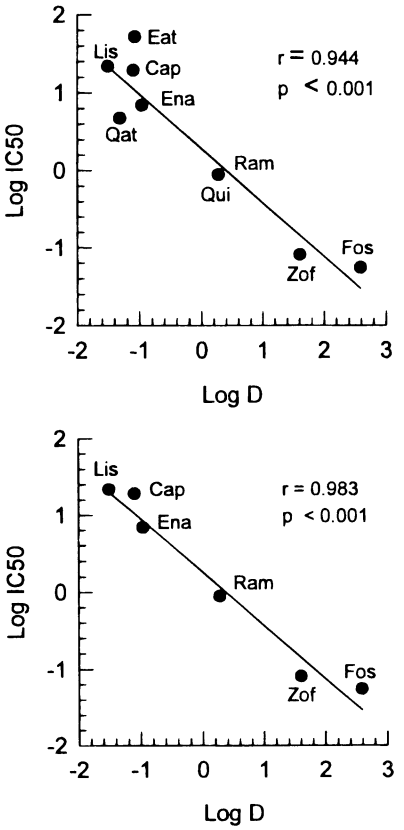

Fig. 5. Relationship between inhibitory potency (expressed as log IC50) of angiotensin converting enzyme inhibitors in BBMV and lipophilicity (expressed as log D). Data are mean ± SE (see Tables II and III). Lines represent the best fit of data as determined by linear regression analysis. Cap, captopril; Ena, enalapril; Eat, enalaprilat; Fos, fosinopril; Lis, lisinopril; Qui, quinapril; Qat, quinaprilat; Ram, ramipril; Zof, zofenopril.

D = −1.50) and fosinopril the most lipophilic (log D = 2.59) of the ACE inhibitors tested. In comparing the distribution coefficients between prodrugs and metabolites of the carboxyalkyl-type ACE inhibitors, the formation of an ester prodrug increased the distribution coefficient by 1.3-fold for enalapril vs. enalaprilat and by 38-fold for quinapril vs. quinaprilat. Substitutions of a phenylthio group and S-benzoyl ester on captopril resulted in a 500-fold increase in the lipophilicity of zofenopril. In addition, replacement of the proline residue by a bicyclic ring made quinapril and ramipril 17-times more lipophilic than enalapril. Overall, the lipophilicity of ACE inhibitors was as follows: fosinopril > zofenopril > ramipril ≈ quinapril > enalapril > enalaprilat ≈ captopril > quinaprilat > lisinopril. With respect to structure-function relationships, there was a strong negative correlation between the log IC50 values and log distribution coefficients of ACE inhibitors (Fig. 5; $r = 0.944$, $p < 0.001$ for top panel and $r = 0.983$, $p < 0.001$ for bottom panel). As observed in these figures, affinity for the renal peptide transporter increased (i.e., lower IC50) as lipophilicity of the ACE inhibitor increased (higher D).

DISCUSSION

It has been reported that contrary to the intestinal peptide/H^+ symporter PepT1 (6), peptide-like drugs lacking an α-amino group appear not to interact with the substrate-binding site of the renal protein PepT2 (5). As a result, the renal peptide transporter is more selective in that it does not transport ACE inhibitors. This conclusion was based on the fact that 5 mM captopril or enalapril failed to inhibit the pH-dependent uptake of radiolabeled cefadroxil in rabbit PepT2 cRNA-injected Xenopus oocytes. In contrast, a significant inhibition of cefadroxil by 10 mM enalapril or captopril, (but not lisinopril) in pH 6.5 buffer was observed in oocytes injected with rabbit PepT1 cRNA. Electrophysiology studies confirmed that captopril was taken up by rabbit PepT1-injected oocytes via an electrogenic transport mechanism. Recently, we reported that quinapril was capable of noncompetitively inhibiting the uptake of GlySar by the high-affinity peptide transporter in rabbit renal BBMV (7). In view of this apparent contradiction with other investigators (5), our initial studies were extended to enalapril and then to other ACE inhibitors.

In the present study, kinetic analyses demonstrated that GlySar uptake was substantially inhibited by enalapril and in a competitive manner ($K_i \approx 6$ mM). This finding suggests that enalapril and GlySar compete for the same substrate-binding site on PepT2, and that enalapril may be transported by the renal carrier protein. This contention is supported by preliminary results using a two-electrode voltage-clamped (electrophysiologic) technique in which inwardly-directed currents were observed for enalapril in Xenopus oocytes injected with the cRNA of rat PepT2 (24). Since enalapril and quinapril are very similar in structure, it is interesting that one ACE inhibitor (i.e., enalapril in this study) exhibits a competitive inhibition of dipeptide uptake while the other ACE inhbitor (i.e., quinapril) exhibits a noncompetitive inhibition of the same dipeptide (7). In this regard, quinapril differs from enalapril in having an isoquinolone substitution of the proline moeity at the carboxyl end of the peptidomimetic structure (Fig. 1). Although speculative, this substituion may result in additional steric or lipophilic interactions such that quinapril binds to the renal peptide transporter but is not, in itself, transported.

Substantial effort has been applied in trying to define the structural requirements for substrate recognition by the high-affinity renal peptide transporter (4,25–27). In general, the following substrate features appear to be important for effective interactions with the renal carrier: i) both free amino and carboxyl termini, ii) a free amino group located in the α-position, iii) a trans peptide bond rather than a cis configuration, iv) a preference for L-amino acids in both amino and carboxyl termini, and v) peptides containing 2 or 3 amino acid residues. In addition, a striking finding was that hydrophobicity of side chains was the primary determinant of substrate affinity for the renal peptide transporter. With this in mind, a model containing hydrophobic pockets has been proposed for the renal carrier (4).

Our results support this tenet and demonstrate a strong correlation between the lipophilicity of ACE inhibitors and their affinity for the high-affinity peptide transporter in kidney. As shown in Figure 5, lipophilicity can account for about 90% of the variability in IC50 values among the ACE inhibitors tested (top panel, $r^2 = 0.891$). However, it has been reported that although dicarboxylic acid metabolites (eg., enalaprilat and quinaprilat) are capable of binding to the intestinal peptide transporter, they are not actively transported themselves (28,29). Lisinopril, on the other hand, can still be transported since the unfavorable effect of a second carboxylic group is neutralized

by intramolecular stabilization. Finally, quinapril is assumed not to be transported given its noncompetitive inhibition of GlySar in a similar system (7). As a result, when the data were reanalyzed without enalaprilat, quinaprilat and quinapril, over 96% of the variability in substrate affinity could be explained by differences in ACE inhibitor lipophilicity (Fig. 5; bottom panel, $r^2 = 0.966$). It should be appreciated that from inhibition experiments alone, one cannot conclude, a priori, that all compounds showing an interaction with the renal peptide carrier are indeed transported across the brush border membrane. Notwithstanding this uncertainty, the IC50 values (which are roughly equivalent to Ki, regardless of mechanism) still allow for a description of the inhibitory potency (i.e., affinity) of ACE inhibitors, and the influence of drug lipophilicity on recognition by the renal peptide/H^+ symporter.

In summary, our studies in rabbit renal BBMV argue against a free α-amino group as being an absolute requirement for substrate recognition by the high-affinity renal peptide transporter. Contrary to current opinion, we conclusively demonstrate that ACE inhibitors (lacking a free amino group in the α-position) can interact with the renal carrier and inhibit dipeptide transport. In this regard, the inhibitory potency of ACE inhibitors for the renal peptide transporter was strongly correlated with drug lipophilicity. In addition, GlySar uptake by the high-affinity H^+/peptide symporter was inhibited by enalapril in a competitive manner, suggesting that enalapril and glycylsarcosine share a common binding site. Although this finding implies that enalapril may also be a substrate for the carrier, more definitive studies will be required to reach this conclusion. Our current endeavors are aimed at providing such direct evidence for enalapril (and other ACE inhibitor) transport via PepT2.

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