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Interaction of renin inhibitors with the intestinal uptake system for oligopeptides and β-lactam antibiotics

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The interaction of two renin inhibitors, S 86 2033 and S 86 3390, with the uptake system for β-lactam antibiotics and small peptides in the brush border membrane of enterocytes from rabbit small intestine was investigated using brush border membrane vesicles. Both renin inhibitors inhibited the uptake of the orally active cephalosporin cephalexin into brush border membrane vesicles from rabbit small intestine in a concentration-dependent manner. 1.1 mM of S 86 3390 and 2.5 mM of S 86 2033 led to a half-maximal inhibition of the H^+-dependent uptake of cephalexin. Both renin inhibitors were stable against peptidases of the brush border membrane. The uptake of cephalexin into brush border membrane vesicles (1 min of incubation) was competitively inhibited by S 86 2033 and S 86 3390 suggesting a direct interaction of these compounds with the intestinal peptide uptake system. The renin inhibitors are transported across the brush border membrane into the intravesicular space as was shown by equilibrium uptake studies dependent upon the medium osmolarity. The uptake of S 86 3390 was stimulated by an inwardly directed H^+-gradient and occurred with a transient accumulation against a concentration gradient (overshoot phenomenon). The renin inhibitors S 86 2033 and 86 3390 also caused a concentration-dependent inhibition in the extent of photoaffinity labeling of the putative peptide transport protein of apparent M_r 127000 in the brush border membrane of small intestinal enterocytes. In conclusion, these studies show that renin inhibitors specifically interact with the intestinal uptake system shared by small peptides and β-lactam antibiotics.

Introduction

The aspartic proteinase renin (EC 3.4.23.15) is the first and rate-limiting enzyme in the renin-angiotensin cascade. The renin-angiotensin system is significantly involved in the regulation of blood pressure and fluid volume. Inhibitors of human renin as agents for the treatment of essential hypertension and congestive heart failure are extensively under research [1–7]. Renin inhibitors have been reported to be active in vitro, but their therapeutic efficacy up to now was insufficient mainly due to a lack of oral activity or stability [8]. Therefore the search for orally active renin inhibitors remains a challenge. The most potent renin inhibitors are, as analogues of the natural substrate angiotensinogen, peptide derivatives where the Leu-Val bond in angiotensinogen was replaced.

Small peptides are taken up in the small intestine by a carrier-mediated uptake process [9–12]. The uptake of small peptides is stimulated by an inwardly directed H^+-gradient [13,14] which is generated by the combined action of a Na^+/H^+-exchanger in the brush border membrane and a Na^+/K^+-ATPase in the basolateral membrane [15]. This transport system for oligopeptides is shared by orally active α-amino-β-lactam antibiotics [16–18]. The protein components of this physiologically and pharmacologically important transport system have been characterized recently in our laboratory by photoaffinity labeling techniques. With radioactively labeled photolabile derivatives of penicillins, cephalosporins and dipeptides an integral membrane protein with an apparent molecular weight of 127000 was identified as (a component of) the peptide transporter in enterocytes from rabbit, rat and pig [19–22]. The peptide transporter specifically binds β-lactam antibiotics and small

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peptides and has no affinity for amino acids, bile acids or sugars. Since renin inhibitors are peptide-derived drugs, it seems possible that orally active renin inhibitors interact with the intestinal peptide transporter and are taken up from the intestinal lumen by this transport system. In order to evaluate the structure-activity relationships of renin inhibitors for intestinal absorption we investigated in the present study whether the renin inhibitors S 86 2033 and S 86 3390 interact with the intestinal uptake system for small peptides and β-lactam antibiotics.

Materials and Methods

Materials. Photoaffinity labeling was performed with [^{3}H]benzylpenicillin (specific radioactivity of 8–18 Ci/mmol, obtained from Amersham Buchler GmbH Braunschweig, F.R.G.). D-[U-^{14}C]Glucose (specific radioactivity 258.5 mCi/mmol) was from NEN (Du Pont de Nemours, Dreieich, F.R.G.). Cephalexin and molecular weight markers for electrophoresis were from Sigma (München, F.R.G.). Hepes, acrylamide and *N,N'*-methylenebisacrylamide were purchased from Serva (Heidelberg, F.R.G.), scintillator Quickszint 501 and solubilizer Biolute S were from Zinsser Analytik GmbH (Frankfurt, F.R.G.). Cellulose nitrate filters (type HAWP 0.45 μm, 25 mm) for the transport studies were from Millipore (Eschborn, F.R.G.). Solvents for HPLC and HPTLC thin-layer plates were from Merck (Darmstadt, F.R.G.). Solvents for HPLC and HPTLC thin-layer plates were from Merck (Darmstadt, F.R.G.). The renin inhibitors S 86 3390 and S 86 2033 (see Fig. 1) were synthesized as described [23]. All other solvents were from the commercial sources and of analytical grade.

Preparation of brush border membrane vesicles. Brush border membrane vesicles were prepared from the small intestine of male white rabbits (3–3.5 kg, Tierzucht Kastengrund, Hoechst Aktiengesellschaft, F.R.G.) maintained on standard diets and tap water ad libitum by the Mg^{2+}-precipitation method as described previously [18,21,22]. Protein was determined according to Bradford [24] using the Bio-Rad kit (München, F.R.G.).

Uptake studies. Transport studies were performed at 30 °C using the membrane filtration method [25,26] as described [18,21,22,27,28]. The determination of cephalexin or S 86 3390 taken up by the vesicles was performed by reversed phase HPLC using a Bischoff 250 × 4 mm column (Bischoff, Leonberg, F.R.G.) filled with LiChrosorb RP 18 (7 μm, Merck Darmstadt, F.R.G.) using acetonitrile/30 mM sodium phosphate buffer (pH 7.0) (16 : 84, v/v) as eluent [18]. Retention time for cephalexin under these conditions was 4.16 min and 7.44 min for S 86 3390. For uptake studies in dependency on the osmolarity of the medium, brush border membrane vesicles equilibrated with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol were incubated at 20 °C for 60 min with a 2 mM solution of the renin inhibitor in 20 mM citrate-Tris buffer (pH 6.0)/140 mM KCl containing appropriate concentrations of cellobiose. For uptake measurements with D-[U-^{14}C]glucose the cellulose nitrate filters were dissolved in 4 ml of scintillator Quickszint 361 (Zinsser Analytic GmbH, Frankfurt, F.R.G.) and radioactivity was measured by liquid scintillation counting. Each transport experiment was performed at least three times with different membrane preparations. Each measurement was performed in triplicate. All values are given as mean ± S.D.

Metabolic stability of renin inhibitors against brush border membrane enzymes. In order to determine the stability of S 86 2033 and S 86 3390 against the action of brush border membrane bound peptidases, membrane vesicles (100 μg, 20 μl) were mixed with 180 μl of a 2 mM solution of the corresponding renin inhibitor in 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol. After 0, 5, 10, 20, 30, 60 and 120 min of incubation at 20 °C, 20-μl aliquots were removed and immediately mixed with 40 μl of dioxane to stop enzymatic reactions. After centrifugation the supernatants were applied to HPTLC thin-layer plates (10 × 20 cm) and chromatograms were developed in *n*-butanol/water/acetic acid (9 : 2 : 1, v/v/v) as eluent [29]. After drying the individual tracks were scanned at 220 nm with a densitometer CD 50 (DESAGA, Heidelberg, F.R.G.).

Photoaffinity labeling. For photoaffinity labeling with benzylpenicillin [22,27] brush border membrane vesicles (200 μg) loaded with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol were incubated with 3 μCi [^{3}H]benzylpenicillin in 200 μl of 10 mM citrate-Tris buffer (pH 6.0)/140 mM KCl either in the absence or in the presence of the indicated concentrations of renin inhibitors in the dark. Subsequently the vesicles were irradiated at 254 nm for 120 s in a Rayonet RPR 100 photochemical reactor (The Southern Ultraviolet Company, Hamden, CT) equipped with 16 RPR 2537 Å lamps at a distance of 15 cm from the lamps. After dilution with 1.5 ml of ice-cold 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol/4 mM PMSF/4 mM EDTA/4 mM iodoacetamide the membranes were collected by centrifugation at 48 000 × *g* for 30 min. After resuspension in 200 μl of water protein was precipitated according to Wessel and Flügge [30].

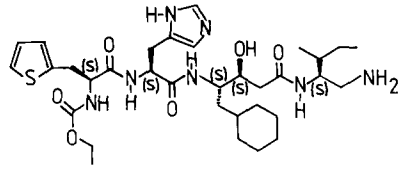
Sodium dodecylsulfate polyacrylamide gel electrophoresis. Membrane proteins were dissolved in 70 μl of 62.5 mM Tris-HCl buffer (pH 6.8)/2% sodium dodecylsulfate/5% 2-mercaptoethanol/0.005% Bromophenol blue. After centrifugation, the clear supernatants were applied onto discontinuous vertical sodium dodecylsulfate slab gels (20 × 15 × 0.15 cm) with a total acrylamide concentration of 7.5% at a ratio of acrylamide/bisacrylamide of 97.2 : 2.8. Fixing, staining, scanning and count-

ing of radioactivity in the gels was performed as described previously [21,22,27,31].

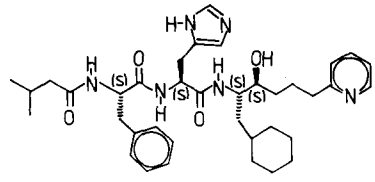
Results and Discussion

Interaction of the renin inhibitors S 86 2033 and S 86 3390 with the intestinal peptide transport system

In order to characterize possible interactions of the renin inhibitors S 86 2033 and S 86 3390 (Fig. 1) with the intestinal uptake system for peptides and β-lactam



S 86 2033



S 86 3390

Fig. 1. Structural formulas of the renin inhibitors S 86 2033 and S 86 3390.

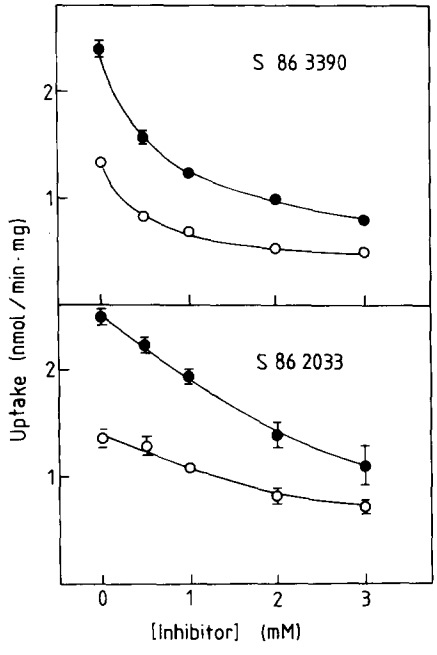


Fig. 2. Concentration dependent inhibition of cephalexin uptake into brush border membrane vesicles by the renin inhibitors S 86 2033 and S 86 3390. Brush border membrane vesicles (100 μg, 10 μl) from rabbit small intestine preloaded with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol were incubated with 90 μl of 10 mM citrate-Tris buffer (pH 6.0)/140 mM KCl/3% DMSO containing 1 mM (○) or 2 mM (●) of cephalexin and 0, 0.4, 1, 2 or 3 mM of S 86 2033 or S 86 3390. Initial uptake of cephalexin was measured for 1 min.

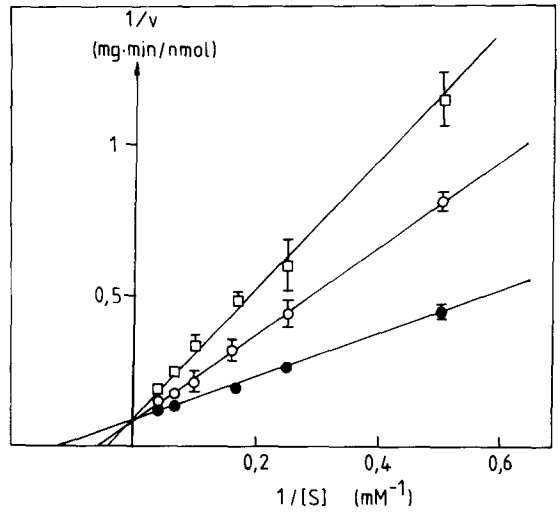


Fig. 3. Kinetics of the inhibition of cephalexin uptake into brush border membrane vesicles by the renin inhibitors S 86 2033 and S 86 3390. Brush border membrane vesicles (100 μg, 10 μl) from rabbit small intestine preloaded with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol were incubated with 90 μl of citrate-Tris buffer (pH 6.0)/140 mM KCl/3% DMSO containing 2, 4, 6, 10, 15 or 25 mM cephalexin either in the absence (●) or in the presence of 3 mM of S 86 2033 (○) or S 86 3390 (□). Initial uptake was measured for 1 min.

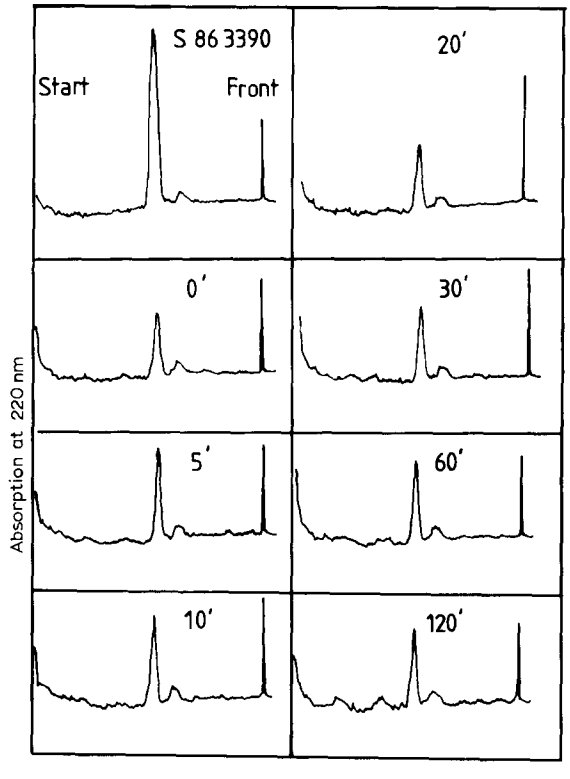


Fig. 4. Effect of intestinal brush border membrane vesicles on S 86 3390. A 2 mM solution of S 86 3390 in 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol was incubated with 100 μg of brush border membrane protein at 20 °C. After 0, 5, 10, 20, 30, 60 and 120 min of incubation aliquots were removed and protein was precipitated with dioxane. After centrifugation the clear supernatants were put on HPTLC plates. The chromatograms were developed in *n*-butanol/water/acetic acid (9:2:1, v/v/v) and S 86 3390 was detected by densitometry at 220 nm.

antibiotics, we investigated in a first series of experiments the influence of increasing concentrations of these derivatives on the H^+-dependent uptake of the orally active α-aminocephalosporin cephalexin into brush border membrane vesicles. The H^+-dependent uptake of cephalexin was measured in the presence of an inwardly directed H^+-gradient ($pH_{out} = 6.0$, $pH_{in} = 7.4$) reflecting the physiological situation with an acidic microclimate at the surface of the intestinal absorptive cell [32]. Usually the uptake of cephalexin was measured for 1 min, since under the conditions chosen cephalexin uptake into brush border membrane vesicles was nearly linear up to 1 min of incubation as described earlier [18]. The uptake rate of cephalexin was measured at cephalexin concentrations of 1 mM and 2 mM both in the absence and in the presence of the renin inhibitors S 86 2033 and S 86 3390. Fig. 2 shows that both renin inhibitors led to a concentration-dependent inhibition of cephalexin uptake into rabbit small intestinal brush border membrane vesicles. A half-maximal inhibition of cephalexin uptake (IC_{50}) at a concentration of 2 mM cephalexin was achieved with 1.1 mM S 88 3390 and 2.5 mM S 88 2033, respectively. These inhibition constants in the millimolar range are similar to the K_m value of cephalexin for the intestinal peptide uptake system [18] indicating affinities of the renin inhibitors to the transport system comparable to those of orally active α-amino-β-lactam antibiotics. In a second series of experiments we determined the type of inhibition of cephalexin uptake by the renin inhibitors. Fig. 3 shows that both, S 86 2033 and S 86 3390 led to a competitive inhibition of cephalexin uptake into the brush border membrane vesicles. This competitive inhibition indicates that both renin inhibitors compete with cephalexin for binding to the transport site of the intestinal peptide transport system.

Since S 86 2033 and S 86 3390 are peptide derivatives, the possibility that the abovementioned competitive inhibition of cephalexin uptake by these compounds is caused by proteolytic smaller peptide fragments rather than by the intact renin inhibitor molecule had to be excluded. Therefore, we determined the stability of both compounds against peptide hydrolyzing enzymes of the brush border membrane. Brush border membrane vesicles were incubated with solutions of the renin inhibitors and after definite times the composition of the medium was analyzed by thin-layer chromatography. Fig. 4 shows that S 86 3390 was completely resistant against hydrolysis by brush border membrane enzymes and remained unchanged up to an incubation period of 2 h. Similar results were found with S 86 2033 (data not shown).

From these studies it was clear that both renin inhibitors interact in intact form with the intestinal peptide transport system. It can not concluded, however, from these studies that S 86 2033 and S 86 3390 can cross the brush border membrane and reach the intravesicular space. If the renin inhibitors are transported across the brush border membrane into the intravesicular space, the equilibrium uptake values should be inversely corre-

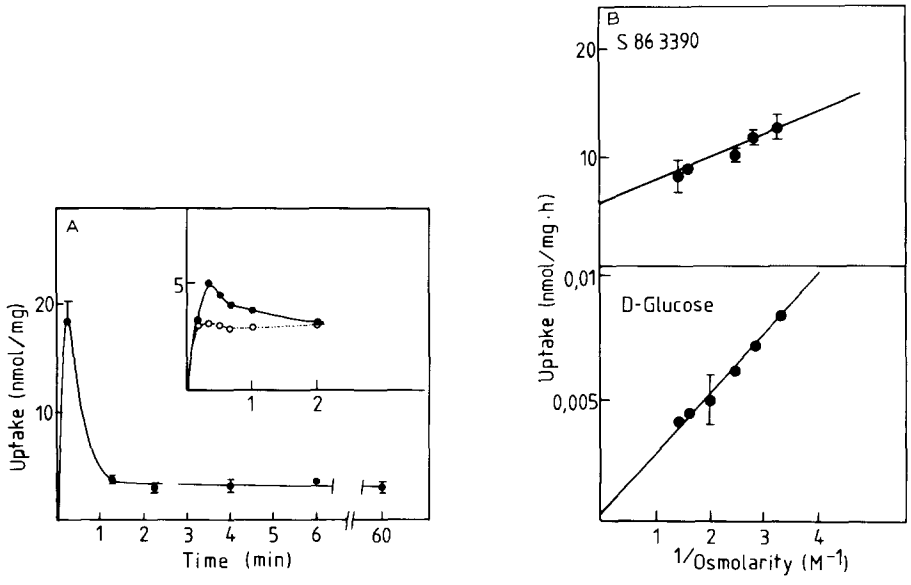


Fig. 5. Uptake of S 86 3390 into brush border membrane vesicles from rabbit small intestine. (A) Time-dependent uptake of S 86 3390 into membrane vesicles. Intestinal brush border membrane vesicles (100 μg, 10 μl) equilibrated with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol were mixed at 30 °C with 90 μl of 10 mM citrate-Tris buffer (pH 6.0)/140 mM KCl and uptake was measured. The inset shows the time-dependent uptake of S 86 3390 into brush border membrane vesicles in the presence (●) and in the absence (○) of an inwardly directed H^+-gradient. (B) Effect of medium osmolarity on the uptake of S 86 3390 (I) and D-[^{14}C]glucose (II) into membrane vesicles. Brush border membrane vesicles (100 μg, 10 μl) equilibrated with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol were incubated at 30 °C for 60 min with 90 μl 20 mM citrate-Tris buffer (pH 6.0) containing either 2 mM S 86 3390 (I) or 1 μCi (40 μM) of D-[U-^{14}C]glucose (II) and varying amounts of cellobiose to attain the desired medium osmolarity.

lated with the osmolarity of the medium. We therefore measured the equilibrium uptake of S 86 3390 into brush border membrane vesicles with different concentrations of cellobiose in the incubation medium. Fig. 5BI shows that the equilibrium uptake of S 86 3390 was inversely proportional to the medium osmolarity as for D-[^{14}C]glucose (Fig. 5BII) demonstrating a transport of S 86 3390 across the brush border membrane. In a next series of experiments the time-dependent uptake of S 86 3390 was measured in the presence of an inwardly-directed H^+-gradient ($pH_{out} = 6.0$, $pH_{in} = 7.4$). Fig. 5A shows that the uptake of S 86 3390 occurred with a transient accumulation compared to the equilibrium uptake value and was stimulated by an inwardly directed H^+-gradient. These studies clearly indicate that the peptide-derived renin inhibitors S 86 2033 and S 86 3390 share the intestinal uptake system for small peptides and orally active β-lactam antibiotics.

Interaction of the renin inhibitors S 86 2033 and S 86 3390 with the binding protein for small peptides and β-lactam antibiotics of M_r 127 000 in brush border membrane vesicles from rabbit small intestine

Consequently, further experiments were performed to determine whether the renin inhibitors S 86 2033 and S 86 3390 bind to the putative transport protein for peptides and β-lactam antibiotics in the brush border membrane of small intestinal enterocytes. Photoaffinity labeling studies of brush border membrane vesicles with photoreactive analogues of penicillins, cephalosporins and dipeptides have identified an integral membrane protein of molecular weight 127 000 as a component of the intestinal peptide transport system [19–22]. Competition labeling experiments and transport studies demonstrated a specificity for di- and tripeptides as well as for β-lactam antibiotics, whereas amino acids, hexoses or bile acids had no affinity [19–22]. In order to evaluate

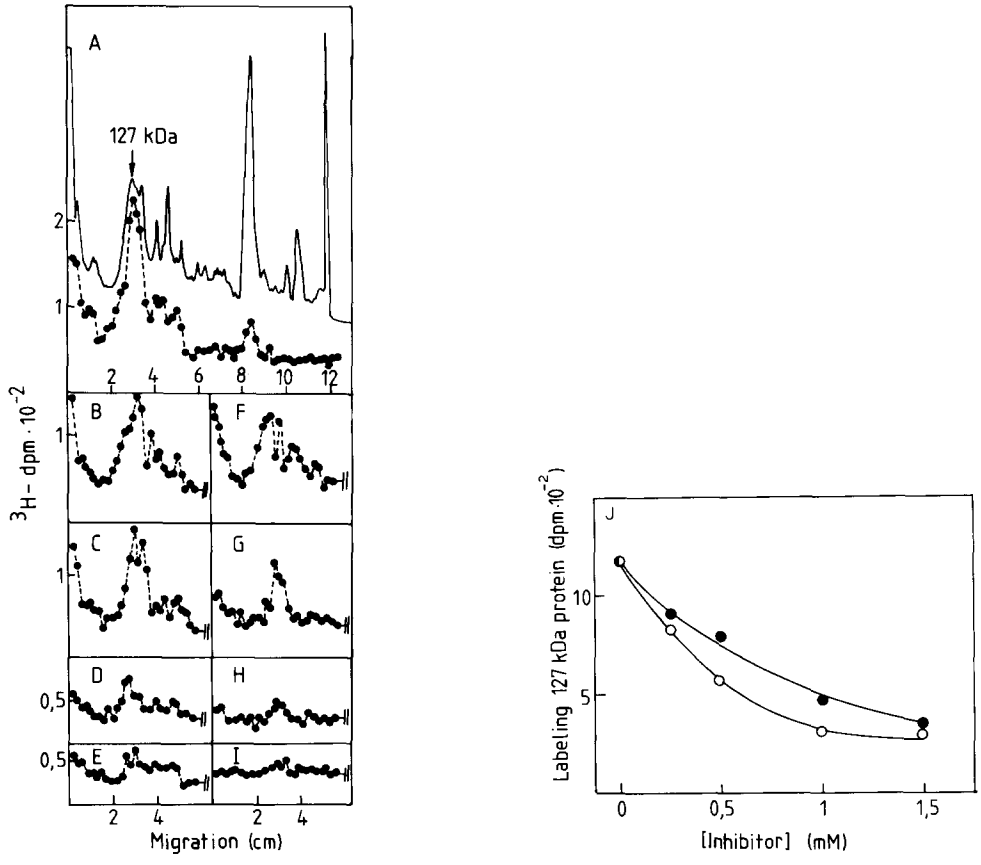


Fig. 6. Effect of the renin inhibitors S 86 2033 and S 86 3390 on photoaffinity labeling of brush border membrane vesicles with [^{3}H]benzylpenicillin. 200 μg (10 μl) of brush border membrane vesicles preloaded with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol were incubated in the dark for 5 min without (A) or with S 86 3390 (0.5 mM (B), 1 mM (C), 2 mM (D), 3 mM (E)) or S 86 2033 (0.5 mM (F), 1 mM (G), 2 mM (H), 3 mM (I)) in 100 μl of 10 mM citrate-Tris buffer (pH 6.0)/140 mM KCl/3% DMSO. After addition of 100 μl of 10 mM citrate-Tris buffer (pH 6.0)/140 mM KCl containing 3 μCi [^{3}H]benzylpenicillin (final concentration 0.88 μM) the vesicles were irradiated at 254 nm for 2 min. After washing the membrane proteins were precipitated and submitted to sodium dodecylsulfate polyacrylamide gel electrophoresis on 7.5% polyacrylamide gels. (A–I) Distribution of radioactivity after slicing of the gels into 2 mm pieces. The drawn line shows the distribution of Serva Blue R 250 stained polypeptides after densitometer scanning, whereas the dotted lines show the distribution of radioactivity. (J) Concentration-dependent inhibition of the labeling of the 127 kDa binding protein for oligopeptides and β-lactam antibiotics by the renin inhibitors S 86 2033 (○) and S 863390 (●). On the *y*-axis is given the radioactivity (in dpm) found in the 127 kDa band after sodium dodecylsulfate polyacrylamide gel electrophoresis.

a possible interaction of renin inhibitors with this putative peptide transport protein, we performed competition photoaffinity labeling experiments with S 86 2033 and S 86 3390 using [^{3}H]benzylpenicillin as photoprobe. If S 86 2033 and S 86 3390 bind to the 127 kDa binding protein like β-lactam antibiotics and oligopeptides, their presence during the photoaffinity labeling experiment should decrease the extent of incorporation of the radioactive photoprobe into the 127 kDa protein. Fig. 6 A–I shows that increasing concentrations of S 86 2033 and S 86 3390 led to a concentration-dependent decrease in the extent of labeling of the 127 kDa binding protein for oligopeptides and β-lactam antibiotics. In these competition labeling experiments S 86 2033 was more effective than S 86 3390; a half-maximal decrease in the extent of labeling of the 127 kDa protein was achieved with about 500 μM S 86 2033 whereas about 800 μM of S 86 3390 were necessary for a half maximal inhibition (Fig. 6J).

The present investigations demonstrate that the renin inhibitors S 86 2033 and S 86 3390 competitively bind to the putative transport protein for oligopeptides and β-lactam antibiotics of molecular weight 127000 in the brush border membrane of enterocytes from rabbit small intestine and also inhibit the peptide transport system. A direct involvement of this binding protein in the transport of α-amino-β-lactam antibiotics and dipeptides has been proven with antibodies against the purified protein [33–35]. Therefore, the specific interaction of the renin inhibitors S 86 2033 and S 86 3390 with the intestinal peptide transport system suggests that an orally active renin inhibitor is taken up into the enterocyte by this uptake system. It seems probable that orally active renin inhibitors must fulfill the structural requirements of a substrate for the intestinal peptide transport system to be transported across the intestinal brush border membrane.

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