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HEPATOCELLULAR UPTAKE OF PEPTIDES—I

CARRIER-MEDIATED UPTAKE OF HYDROPHILIC LINEAR PEPTIDES WITH RENIN INHIBITORY ACTIVITY INTO ISOLATED RAT LIVER CELLS

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Abstract—The hepatic uptake of a hydrophilic, cationic linear peptide with renin inhibitory activity [5(4-amino-piperidyl-1-carbonyl)-L-2,6[^{3}H]phenyl-alanyl-β-alanyl-(4*S*-amino-3*S*-hydroxy-5-cyclohexyl)-pentan-carbonyl-L-isoleucyl-aminomethyl-4-amino-2-methyl-pyrimidine-citrat] (code number EMD 56133; EMD, E. Merck, Darmstadt) was investigated in isolated rat hepatocytes. EMD 56133 was taken up by isolated rat liver cells in a time-, concentration-, energy- and temperature-dependent manner. The uptake was a combination of diffusion and a carrier-mediated process. EMD 56133 was accumulated 4.5-fold in liver cells. Eighty-three per cent of the accumulated peptide was found in the cytosol, not bound to membrane proteins. Seventeen per cent was associated with membrane proteins after cell fractionation and centrifugation at 100,000 *g*. The permeability coefficient of the non-saturable uptake of EMD 56133 was $P = 1.973 \times 10^{-6}$ cm/sec. The kinetic constants for the carrier-mediated transport are $K_m = 92\ \mu M$ and $V_{max} = 128$ pmol/mg × min. Various substrate analogs inhibited the uptake of EMD 56133. AS-30D ascites hepatoma cells and Reuber hepatoma cells did not accumulate EMD 56133. The absence of oxygen or a decreased cellular ATP content blocked the hepatocellular uptake of the renin inhibitor. Temperatures above 20° increased the transport; the activation energy was determined to be $A_{app} = 41$ kJ/mol. The apparently active uptake of EMD 56133 was not sodium dependent. In contrast, the membrane potential might be a driving force for the transport of the positively charged EMD 56133.

Biologically active peptides, e.g. hormone modifications, superagonists and antagonists, are gaining importance in drug development. Unfortunately, peptides often have a short half-life *in vivo*. In the case of proteolytically stable peptides in the M_r range of 200–1200 this is the result of rapid biliary elimination.

The liver plays a major role in the clearance of endogenous and exogenous molecules from the circulation [1]. The rate at which the liver removes a given substance from the blood depends on the nature of the substance, the rate of delivery to the liver and the relative affinity of the substrate to carrier proteins in the liver cell membrane. Some hydrophobic molecules including certain hydrophobic linear peptides are eliminated from portal blood via transport systems that physiologically transport bile acids [2, 3]. We have shown that the short plasma half-life of linear hydrophobic renin-inhibiting peptides is due to their high affinity to a bile acid transport system, termed multispecific bile acid transporter (the physiological substrate is cholate), and to the sodium-dependent taurocholate carrier [2, 3]. In order to develop peptides with a longer duration of action, the structural requirements for affinity to bile acid carriers were determined. In these studies a correlation between the hydrophobic properties of peptides and the affinity to bile acid transport systems became evident [2, 3]. Therefore, hydrophilic peptides (e.g. EMD 56133; EMD,† E. Merck, Darmstadt) were synthesized with the aim of creating substances with a lower affinity to these transport systems.

The hydrophilic linear renin inhibitor EMD 56133 [5(4-amino-piperidyl-1-carbonyl)-L-2,6[^{3}H]phenyl-alanyl-β-alanyl(4*S*-amino-3*S*-hydroxy-5-cyclohexyl)-pentan-carbonyl-L-isoleucyl-aminomethyl-4-amino-2-methyl-pyrimidine-citrat], which is positively charged under physiological conditions (pK values: 5.9 and 8.9) was seen to have a lower affinity to bile acid transport systems. Studies *in vivo*, however, indicated that EMD 56133 was also eliminated via the biliary pathway. After 24 hr, 75% of the compound was eliminated via the liver of rats, 36% by this route in monkeys, unmetabolized (data provided by E. Merck). The aim of the present study was to characterize the special transport features of this hydrophilic, cationic peptide in isolated rat hepatocytes.

MATERIALS AND METHODS

Materials. The renin inhibitors EMD 56133, [^{3}H]-EMD 56133 (sp. act. 7.77 GBq/mmol), EMD 56162, EMD 55982, EMD 56617, EMD 56013, EMD 55843 and EMD 55981 were gifts from E. Merck (Darmstadt, F.R.G.). The chemical structure of

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† Abbreviations: EMD, E. Merck, Darmstadt; FAO, Reuber hepatoma cell line; CCCP, carbonylcyano-chlorophenylhydrazone.

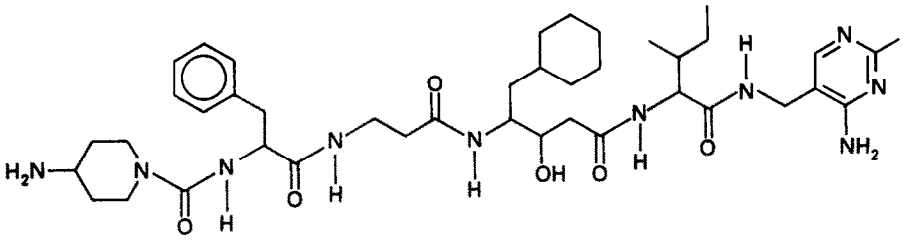

Fig. 1. Chemical structure of the hydrophilic renin inhibitor EMD 56133.

EMD 56133 is shown in Fig. 1. The Reuber hepatoma (FAO) cells were from Dr W. Föllmann (Gießen, F.R.G.). The cation lucigenin was a gift from Dr D. K. F. Meijer (Groningen, The Netherlands). All other chemicals were of the highest purity grade available.

Composition of the buffers. Na^+-Tyrode: 137 mM NaCl, 2.7 mM KCl, 1.05 mM $MgCl_2$, 1.8 mM $CaCl_2$, 12 mM $NaHCO_3$, 5.55 mM glucose and 0.42 mM NaH_2PO_4. Lithium-Tyrode buffer: 137 mM LiCl, 2.7 mM KCl, 1.05 mM $MgCl_2$, 1.8 mM $CaCl_2$, 12 mM Li_2CO_3, 5.55 mM glucose and 0.42 mM KH_2PO_4. Choline-Tyrode buffer: 137 mM choline chloride, 2.7 mM KCl, 1.05 mM $MgCl_2$, 1.8 mM $CaCl_2$, 12 mM Li_2CO_3, 5.55 mM glucose and 0.42 mM KH_2PO_4. Potassium-Tyrode buffer: 139.7 mM KCl, 1.05 mM $MgCl_2$, 1.8 mM $CaCl_2$, 12 mM K_2CO_3, 5.55 mM glucose and 0.42 mM KH_2PO_4. In the nitrate-, sulfate- and thiocyanate-Tyrode buffer the NaCl was replaced by either 137 mM $NaNO_3$, 137 mM Na_2SO_4, and 137 mM NaSCN, respectively. The pH of 7.4 at 37° was adjusted by appropriate acids. In the calcium-free-Tyrode buffer the $CaCl_2$ was replaced by 1.8 mM NaCl. All buffers were adjusted to pH 7.4 at 37°.

Isolation of rat hepatocytes. For the measurement of uptake into isolated rat hepatocytes, hepatocytes were prepared from male Wistar rats (body weight 180–240 g) by the method of Berry and Friend [4]. After 30 min of equilibration in Tyrode buffer, pH 7.4 at 37° under a carbogen atmosphere (95% O_2, 5% CO_2), the viability of the cells was tested by Trypan blue exclusion [5]. More than 80% of the cells excluded the dye.

Preparation of AS-30D ascites hepatoma cells. AS-30D ascites hepatoma cells were harvested 1 week after their transplantation into female Sprague–Dawley rats. The cells were washed in Tyrode buffer by centrifugation at 25 *g* until the blood was totally removed. More than 96% of the cells were viable as tested by Trypan blue exclusion. All experiments were performed within 2 hr of isolation of the hepatocytes or hepatoma cells.

Uptake studies. For all experiments, a cell suspension of 2×10^6 hepatocytes/mL Tyrode buffer (pH 7.4, 37°, carbogen atmosphere), or 1.4×10^7 hepatoma cells/mL buffer was used. Both cell suspensions contained a protein concentration of 4 mg/mL, determined with Bradford reagent [6].

Aliquots of 1 or 2 mL of the cell suspension were incubated with a mixture of 2.14 μM [^{3}H]EMD 56133 and different concentrations of unlabeled EMD 56133 dissolved in 50 μL phosphate-buffered saline, pH 7.4. At 15, 75, 135, 195 and 255 sec, and at later times, depending on the experiment, uptake was measured. Aliquots (100 μL) of the cell suspension were withdrawn and put into Microtest® tubes. The uptake of the substances was stopped by silicone oil centrifugation [7] using a Beckman Microfuge B. The amount of substance taken up into the cells was determined by radioactivity measurements in the sediment fraction with a fluid scintillation counter.

The initial velocity of the uptake was estimated from the gradient of the uptake vs time graphs; the IC_{50} value was estimated from the graph of the percentage inhibition versus log of the concentration. In the kinetic studies, the initial uptake rates and the concentration of the substrate were plotted according to Lineweaver and Burk [8], Woolf [9] and Hofstee [10] and Cornish-Bowden [11]; the K_i was determined according to Dixon [12].

Uptake in the absence of oxygen. The measurement of the uptake of the peptide was done after incubating the hepatocytes for 30 min under a nitrogen atmosphere (95% N_2/5% CO_2) in a diaphragm-sealed vessel. Aliquots of the cell suspension were withdrawn with an airtight, sealed syringe to avoid O_2 contamination. To prove that a possible reduction of the uptake is not caused by the death of the cells they were reincubated in oxygen for 30 min and the uptake of EMD 56133 was measured again.

Uptake measurements in sodium-/chloride-free Tyrode buffer. The hepatocytes were diluted to a concentration of 2×10^6 cells/mL Tyrode buffer and washed twice in Na^+- or Cl^--free Tyrode buffer for 2–3 sec in an Eppendorf centrifuge. The pellets were dissolved in Na^+- or Cl^--free Tyrode buffer. After 10 min equilibration time the peptide was added.

Temperature dependence of the substrate uptake. The hepatocytes were incubated at selected temperatures (7°, 17°, 22°, 27°, 32° or 37°) for 10 min before the peptide was added.

Determination of the distribution of EMD 56133 between the cytosol and plasma membrane fraction of hepatocytes. Liver cell suspension (1.5 mL; 2×10^6 cells/mL buffer) was incubated for 5 min with 77.14 μM EMD 56133/[^{3}H]EMD 56133. Of this mixture, 1 mL was washed twice in an Eppendorf tube at 30 *g*. The cell suspension was then frozen in liquid nitrogen to disrupt the cells. After thawing and shaking of the cell fragment suspension at 37°, 100 μL were withdrawn in order to determine the total activity of [^{3}H]EMD 56133. Samples of 500 μL of the suspension were centrifuged for 20 min at

100,000 *g*. The activity of 100 μL of the supernatant fraction and of 100 μL of the sediment fraction, after resuspension with 500 μL buffer, was measured. The activity of the supernatant and of the sediment fraction was plotted as percentage of the total activity of the suspension.

Determination of the ATP content of rat hepatocytes after incubation with metabolic inhibitors. Two milliliters of a hepatocyte suspension of 10×10^6 cells/mL buffer were incubated in a 10 mL Erlenmeyer tube under a carbogen atmosphere at 37°. At 30 sec and again at 10 min after the addition of 10 μL of the metabolic inhibitor carbonylcyanochlorophenylhydrazone (CCCP) (12 μg), antimycin A (30 μg) or oligomycin (30 μg), 1.5 mL of the cell suspension was withdrawn and added to 1.5 mL ice-cold 12% trichloroacetic acid. After 5 min the samples were centrifuged at 3000 rpm to obtain a clear supernatant. Aliquots of 0.5 mL supernatant were used in the test.

To determine the ATP content, a test-kit of Sigma Diagnostics was used. The ATP content of the liver cells (0.75 ± 0.06 μmol/mL) was not detectable after 10 min incubation with metabolic inhibitors. After 30 sec incubation with CCCP, antimycine A or oligomycine the ATP of the hepatocytes was almost completely metabolized (0.0056 ± 0.0004, 0.008 ± 0.0007 or 0.014 ± 0.001 μmol/mL, respectively).

Statistical methods. All experiments were performed with three different cell preparations; the average was used for further calculations. The plots were calculated by linear regression analysis, the IC_{50} values by logarithmic regression analysis.

Statistical significance ($\alpha = 0.01$) was assessed with the program SPSS/PC+. The experiment was considered significant when $P \leq 0.01$.

RESULTS

Kinetic data of the uptake of EMD 56133

Uptake of the hydrophilic linear peptide EMD 56133 by hepatocytes was time and concentration dependent (Fig. 2). Uptake of EMD 56133 shows a curvilinear dependence of initial transport velocity (V_i) on the peptide concentration (Fig. 3). The mixed-type kinetics can be interpreted as a superimposition of a saturable and a non-saturable part of the uptake. This conclusion is supported by the non-linearity in a Woolf–Hofstee plot (Fig. 4a).

The saturable part of the uptake was obtained after subtracting the contribution of the unsaturable component at 6°, which is equivalent to diffusion, from the uptake rates at 37° (Fig. 3). From the slope of the diffusion curve a permeability coefficient of $P = 1.973 \times 10^{-6}$ cm/sec was determined. The kinetic constants for the saturable uptake obtained from a double reciprocal Lineweaver–Burk plot were $K_m = 92$ μM and $V_{max} = 128$ pmol/mg × min (Fig. 2 inset). As is evident from the Woolf–Hofstee plot (Fig. 4b) only one saturable transport system is responsible for the uptake of EMD 56133 (K_m = 130 μM, $V_{max} = 170$ pmol/mg × min).

The intracellular enrichment was 4.5 times the extracellular concentration at its highest level

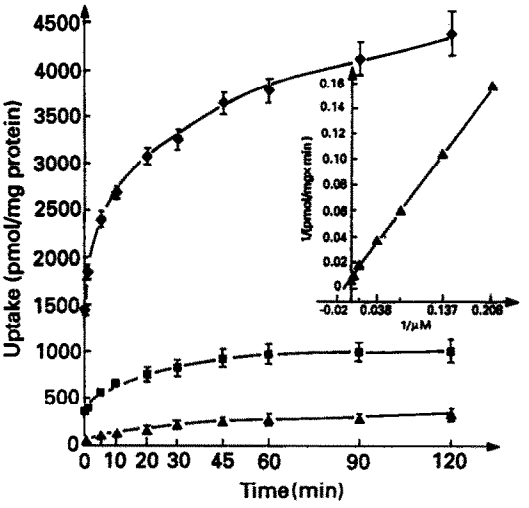

Fig. 2. Time and concentration dependence of the uptake of EMD 56133 in isolated hepatocytes. Aliquots of 2 mL of liver cell suspension (2×10^6 cells/mL) were incubated at 37° with 13 (▲), 130 (■), or 630 (◆) μM EMD 56133/[^{3}H]EMD 56133. Uptake was measured at the times indicated as described in Materials and Methods. N = 3; means ± SD. Inset: Lineweaver–Burk plot of the saturable uptake of EMD 56133. The data of the carrier-mediated uptake of Fig. 3 were plotted in a double-reciprocal Lineweaver–Burk diagram. The kinetic constants were calculated by linear regression analysis. $K_m = 92$ μM; $V_{max} = 128$ pmol/mg × min; $r = 0.9995$; N = 3.

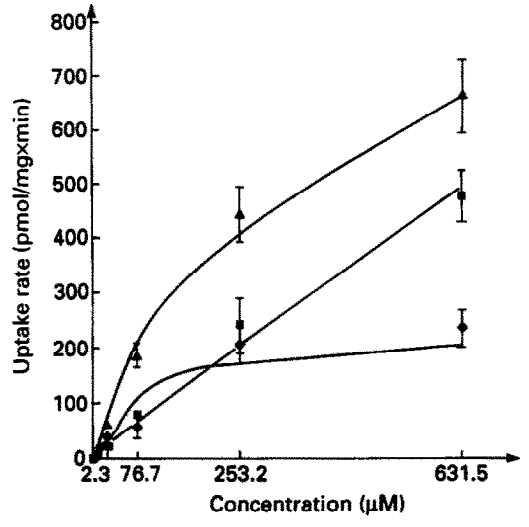

Fig. 3. Kinetics of the uptake of EMD 56133 at 37° and 6° and determination of the saturable part of the transport. Aliquots of 1 mL of the hepatocyte suspension were incubated with 2.14 μM [^{3}H]EMD 56133 and different concentrations of unlabeled EMD 56133. The uptake was measured at 37° (▲) and 6° (■). Initial uptake was calculated between 15 and 255 sec. The saturable uptake (◆) was determined by subtracting the 6° curve from the 37° curve. The permeability coefficient of $P = 1.973 \times 10^{-6}$ cm/sec was determined from the slope of the diffusion curve (6°) by linear regression analysis. r = 0.9948; N = 3 means ± SD.

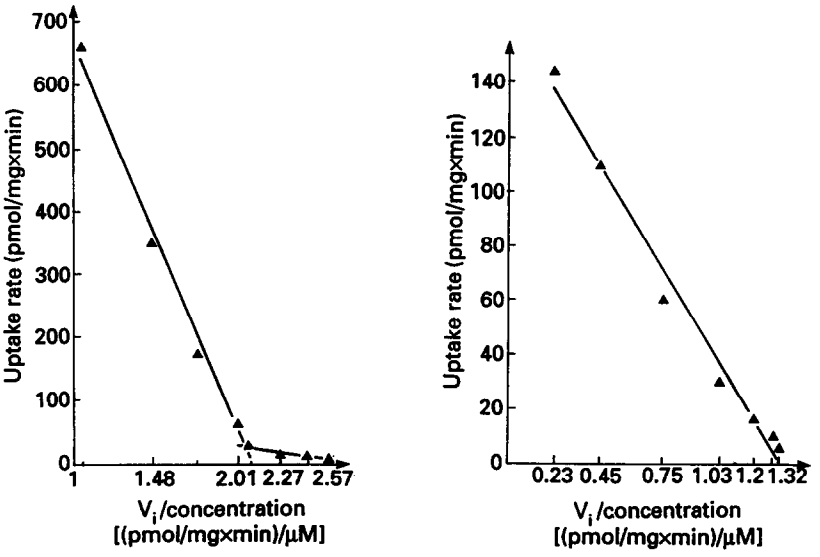

Fig. 4. (a) Woolf–Hofstee plot of the initial uptake rates of the total uptake of EMD 56133 in isolated rat hepatocytes. $r = 0.995$, for V_i/concn = 1.05–2.08; $r = 0.96$, for V_i/concn = 2.08–2.57. N = 3; SD < 21.4%. (b) Woolf–Hofstee plot of the saturable uptake of EMD 56133 in hepatocytes. $r = 0.991$; N = 3.

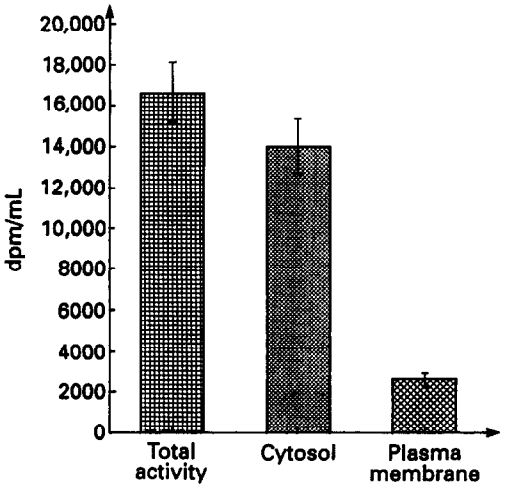

Fig. 5. Distribution of [^{3}H]EMD 56133 between the cytosol and plasma membrane fraction of rat liver cells. Aliquots of 2×10^6 cells/mL were incubated for 5 min in 77.14 μM EMD 56133/[^{3}H]EMD 56133. One milliliter of this mixture was treated as described in Materials and Methods. The activity of the supernatant and of the sediment fraction was plotted. N = 3; means ± SD.

(concentration of EMD 56133 1–3 μM). Eighty-three per cent of the accumulated peptide was found in the cytosol, not bound to membrane proteins. Seventeen per cent was associated with membrane proteins (Fig. 5).

Peptide uptake in Ca^{2+}-free buffer was not different than that under control conditions (data not shown), suggesting that the hepatocytes do not take up the renin inhibitor by receptor-mediated endocytosis. In addition, lucigenin, that undergoes absorptive endocytosis [13, 14] in liver cells, did not interfere with the uptake of EMD 56133.

Cell-specific uptake of EMD 56133

To test whether transport of the renin inhibitor is liver cell specific the uptake was measured in hepatocytes, AS-30D ascites hepatoma cells [15] and FAO cells [16]. Neither hepatoma cell line accumulated as much peptide as the liver cells (Fig. 6). The initial uptake as well as the uptake at equilibrium of EMD 56133 into hepatocytes was three times higher than into AS-30D ascites hepatoma cells and 15 times higher than into FAO cells.

Influence of substrate analogs

The uptake of EMD 56133 was decreased by the substrate analogs EMD 55981, EMD 55982, EMD 55843, EMD 56013, EMD 56162 and EMD 56617 in a concentration-dependent manner (Table 1). EMD 55981, a hydrophobic compound, non-competitively inhibited the uptake of EMD 56133 (Table 2). In contrast, EMD 55982, a hydrophilic compound, caused a competitive inhibition (Table 2).

Energy dependence of EMD 56133 *uptake*

Uptake of EMD 56133 *in the absence of oxygen.* Incubation of liver cells for 30 min with 95% N_2/5% CO_2 resulted in 33% inhibition of EMD 56133 uptake. Transport could be restored by as much as 85% by regenerating the cells for 30 min in carbogene (Fig. 7).

Preincubation of the hepatocytes with metabolic

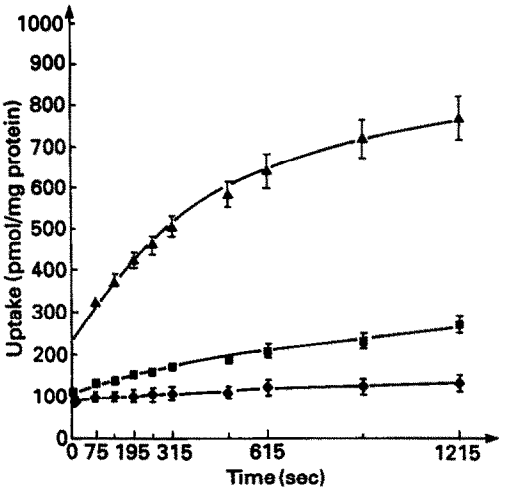

Fig. 6. Total uptake of EMD 56133 into AS-30D ascites hepatoma cells and into FAO cells. The uptake of 75 μM EMD 56133/[^{3}H]EMD 56133 was measured either in 2 mL of a hepatocyte suspension (2×10^6 cells/mL) (▲) or in a suspension of 1.4×10^7 AS-30D ascites hepatoma cells (■) or FAO cells (◆), per milliliter Tyrode buffer. All cell suspensions correspond to a protein concentration of 4 mg/mL medium. Initial uptake was calculated between 15 and 255 sec. N = 3; means ± SD.

Table 1. Influence of substrate analogs on the uptake of EMD 56133

Substrate analog (M_r)	Rank of hydrophilicity	IC_{50} (μM)
EMD 55843 (779,990)	2	63
EMD 55981 (778,020)	6	345
EMD 55982 (822,030)	1	0.65
EMD 56013 (835,070)	3	2.22
EMD 56162 (821,080)	5	216
EMD 55617 (794,020)	4	1.29

The hepatocytes were preincubated with three concentrations (10, 100 and 500 μg) of the substrate analogs EMD 55843, EMD 55981, EMD 55982, EMD 56013, EMD 56162 and EMD 56617. After 30 sec 75 μM EMD 56133/[^{3}H]EMD 56133 were added. Initial velocity was calculated from the linear part of the uptake curve. The IC_{50} values correspond to the concentrations of the inhibitors required for 50% inhibition of the initial uptake of 75 μM EMD 56133. They were estimated from a semilogarithmic plot by logarithmic regression analysis. Hydrophilicity was measured by a chromatographic method as described in [2].

LC_{50} values are means: N = 3.

Table 2. Kinetics of inhibition of the total uptake of EMD 56133 by the substrate analogs EMD 55981 and EMD 55982

Inhibitor	K_i (μM)	Type of inhibition
EMD 55981	400	Non-competitive
EMD 55982	50	Competitive

The initial uptake of increasing concentrations of EMD 56133/[^{3}H]EMD 56133 (6.3–630 μM) was measured 30 sec after the addition of four increasing concentrations of EMD 55981 and EMD 55982. The initial uptake rates and the concentrations of the substrates were plotted according to Lineweaver–Burk [8], Woolf [9], Hofstee [10] and Cornish-Bowden [11]; the K_i was determined according to Dixon [12].

N = 3.

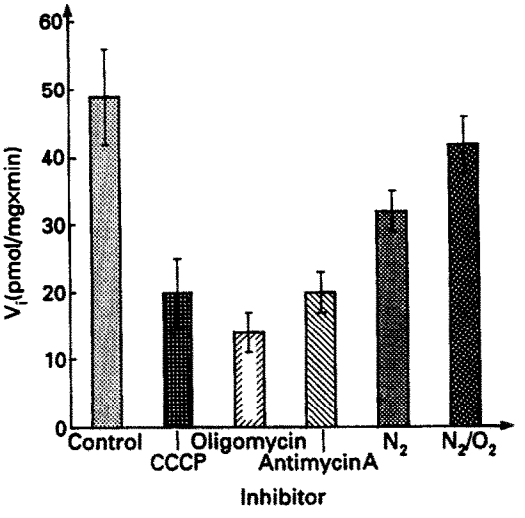

Fig. 7. Total uptake of EMD 56133 in the presence of metabolic inhibitors and under anaerobic conditions. Aliquots of 2 mL liver cell suspension were incubated for 10 min with 10 μg/mL antimycin A, 4 μg/mL CCCP or 10 μg/mL oligomycin prior to the addition of 75 μM EMD 56133/[^{3}H]EMD 56133. N = 3; means ± SD; $P < 0.0001$. Measurement under anaerobic conditions was performed after incubating 2 mL of the hepatocytes for 30 min under an oxygen (95% O_2/5% CO_2) or a nitrogen (95% N_2/5% CO_2) atmosphere. Three probes were first incubated under a nitrogen and then an oxygen atmosphere in diaphragm-sealed vessels. N = 3; means ± SD; P = 0.0074. Initial uptake was calculated between 15 and 255 sec.

inhibitors. The three metabolic inhibitors CCCP, antimycin A and oligomycin, which have different targets in the respiratory chain of the liver cell [17, 18], significantly inhibited the transport of the peptide (Fig. 7). The cellular ATP content was rapidly decreased after 10 min preincubation with the inhibitors (see Materials and Methods).

Temperature dependence of the uptake of EMD 56133. The initial velocity of the peptide

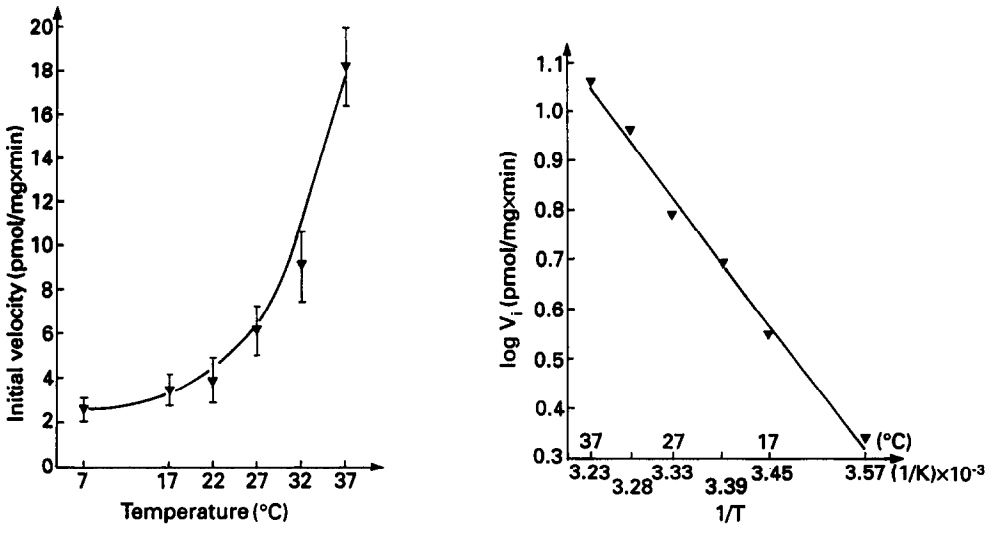

Fig. 8. Temperature dependence of the uptake of EMD 56133. (a) The uptake of 75 μM EMD 56133/[^{3}H]EMD 56133 into isolated hepatocytes was measured after preincubation for 10 min at 7°, 17°, 22°, 27°, 32° and 37°. Initial velocity was determined from the linear part of the uptake. N = 3; means ± SD. (b) The results of (a) were plotted in an Arrhenius diagram. An apparent activation energy (A_{app}) of 41 kJ/mol was calculated from the equation: $A_{app} = 2.303 \times R \times \delta \log V_i \times (\delta 1/T)^{-1}$.

uptake increased in an exponential manner with increasing temperature (Fig. 8a). The Q_{10} values at temperatures higher than 17° are greater than 2.0. The apparent activation energy of the transport could be calculated ($A_{app} = 41$ kJ/mol) from an Arrhenius diagram (Fig. 8b).

Driving forces of the uptake of EMD 56133

Uptake in sodium-free buffer. No significant effect on the uptake could be seen by replacing Na^+ in the Tyrode buffer by lithium, choline or potassium (Fig. 9).

Dependence of the uptake on the membrane potential. The initial uptake of EMD 56133 was measured either in Cl^--Tyrode buffer or in nitrate-, sulfate- or thiocyanate-Tyrode buffer. The permeation of hydrophilic sulfate through the membrane is slower than that of chloride due to the greater hydration coat of SO_4^{2-}. The inner side of the membrane is therefore more positive and the entry of negatively charged substrates is facilitated by this anion. The lipophilic anions SCN^- and NO_3^-, which have a smaller solvation coat and permeate faster through the cell membrane, induce a diffusion potential which is more negative inside than the Cl^- distribution potential. The entry of a positively charged substrate is facilitated by these anions [19]. The transient negative diffusion potential accelerated the uptake of the positively charged EMD 56133. The hydrophilic sulfate ion, on the other hand, decreased the uptake of EMD 56133 (Fig. 9).

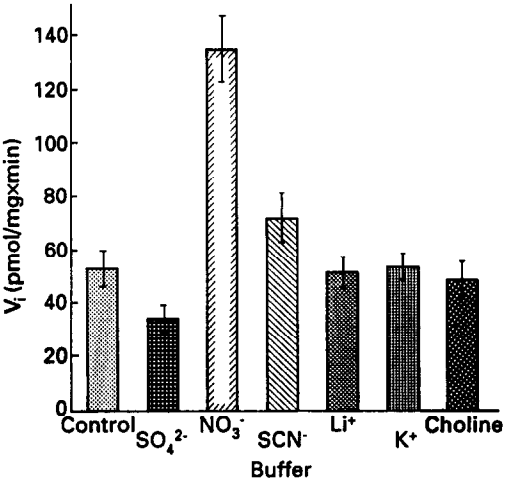

Fig. 9. Lack of dependence on sodium and influence of membrane potential on the uptake of EMD 56133 at 37°. Two milliliters of hepatocytes were preincubated in either NaCl-Tyrode (control), Li^+-Tyrode, choline-Tyrode, K^+-Tyrode, NO_3^--Tyrode, SO_4^{2-}-Tyrode or SCN^--Tyrode for 10 min before the uptake of 75 μM EMD 56133/[^{3}H]-EMD 56133 was measured. Initial velocity was calculated as described in Fig. 8a. P = 0.0516–0.1143 for cation exchange experiments; P < 0.0001 for anion exchange experiments. N = 3; means ± SD.

DISCUSSION

The hepatocellular uptake of the hydrophilic cationic linear renin inhibitor EMD 56133 (pK values 5.9 and 8.9) occurs by two mechanisms. One saturable component ($K_m = 92$ μM, $V_{max} = 128$ pmol/mg × min) can be detected, whereas the second component of the uptake system shows no saturation within the concentration range used. As

would be expected for a hydrophilic compound taken up by a carrier-mediated mechanism, the permeability coefficient of 1.973×10^{-6} cm/sec is much lower than those of hydrophobic compounds such as cyclosporins [20] or aflatoxins [21] (1.37×10^{-4} or 2.4×10^{-4} cm/sec) and hydrophobic linear renin inhibitors (8.123×10^{-6} cm/sec) [2, 3]. The temperature dependency, saturability and competitive inhibition by hydrophilic substrate analogs suggest involvement of a carrier-mediated process in the hepatic uptake of EMD 56133. The observed Q_{10} values greater than 2 and the activation energy of 41 kJ/mol support this conclusion. Hydrophilic EMD 56133 analogs such as EMD 55982 competitively inhibit the uptake of EMD 56133. Hydrophobic analogs such as EMD 55981 however are non-competitive inhibitors of the EMD 56133 uptake. Thus, it appears that hydrophobic and hydrophilic peptides are transported into liver cells via different transport systems.

The importance of the physicochemical properties of the peptides as major determinants of hepatic extraction has already been shown [2, 3, 22]. Hydrophobic peptides are more rapidly extracted by the liver [22] than are hydrophilic peptides [2]. Hydrophobic peptides such as the linear renin inhibitor EMD 51921 have a high affinity to their hepatocellular transport system ($K_m = 2\ \mu M$). The affinity of the hydrophilic EMD 56133 to its transporter is 50-fold lower ($K_m = 92\ \mu M$). The V_{max} values however are in the same range (EMD 56133 128 pmol/mg × min, EMD 51921 160 pmol/mg × min).

Transport systems that physiologically transport the bile acid cholate were claimed to be responsible for the rapid hepatocellular extraction of hydrophobic peptides such as the renin inhibitor EMD 51921 and cyclosomatostatins. The affinity of hydrophilic peptides to bile acid transporters is 200-fold lower than their hydrophobic counterparts. Transport inhibition is non-competitive [23]. In contrast, hydrophobic peptides and bile acids are mutually competitive transport inhibitors.

A further difference in the transport characteristics of hydrophobic linear renin inhibitors is the intracellular accumulation of the peptides. EMD 56133 is accumulated 4.5-fold intracellularly, whereas the maximal enrichment of the hydrophobic compound EMD 51921 is 244-fold [3]. This suggests that the hydrophilic peptide does not bind to proteins in the endoplasmic reticulum. Indeed, 83% of the accumulated peptide is found in the cytosol. The remaining 17% is associated with membrane proteins after cell fractionation and centrifugation at 100,000 *g*. *In vivo* studies and TLC of the cytosol indicated that this peptide is not metabolized by the liver. The native compound is found in liver cells and bile (data not shown). Although the intracellular accumulation of the cationic EMD 56133 is only 4.5 fold, which may be due to the negative inside membrane potential of the hepatocyte (−30 to −40 mV), the uptake is energy dependent [1].

An energy requirement for transport of EMD 56133 appears likely since various metabolic inhibitors reduced the uptake velocity. Both inhibitors of the oxidative phosphorylation (oligomycin) and substrates that interfere with electron transport (antimycine A) decrease the cellular ATP content and the uptake of EMD 56133, indicating that the uptake is at least partly dependent upon metabolic energy. The same can be concluded from the uptake studies in the presence of N_2. The results cannot be explained by loss of cell viability, because cellular appearance and Trypan blue exclusion were not affected during the experiments with metabolic inhibitors. Additionally, reoxygenation of cells previously incubated under an N_2 atmosphere restored the uptake to 80% of the control.

To identify the potential driving forces for EMD 56133 uptake into rat liver cells the influence of extra- to intracellular sodium gradients and the effect of changing the membrane potential were studied. Replacing Na^+ by Li^+, K^+ or choline had only insignificant effects on the uptake of EMD 56133 indicating that the hepatocellular uptake of this peptide is sodium independent. This has also been shown for most of the peptides studied, such as hydrophobic linear renin inhibitors [2, 3], cyclosomatostatins [24] and cholecystokinin octapeptide [22]. The only exception is the uptake of phalloidin [25], a heptapeptide isolated from the mushroom *Amanita phalloides*, which is driven by an inwardly directed sodium gradient.

Replacement of Cl^- by NO_3^- and SCN^- stimulated the uptake of EMD 56133. Substitution of Cl^- by NO_3^- results in a rapid hyperpolarization of the cell (from −30 to −59 mV) [26]; this leads to a higher uptake of the positively charged peptide. Depolarization of the cells, which occurs by replacement of Cl^- by SO_4^{2-}, decreased the uptake of EMD 56133 [19]. These results suggest that EMD 56133 is transported into liver cells as a positively charged compound. As was found for linear hydrophobic renin inhibitors (EMD 51921) the membrane potential might be a driving force for the uptake of EMD 56133.

Uptake of EMD 56133 seems to be liver cell specific, as 30D ascites hepatoma cells take up 3-fold lower and FAO cells 15-fold lower concentrations than liver cells. During malign transformation carrier proteins might be lost or altered.

We conclude, therefore, that EMD 56133 is accumulated into rat liver cells by a liver cell-specific energy dependent carrier-mediated process, which is not sodium dependent, but is dependent on the membrane potential. It is not yet clear what kind of physiological transport system is responsible for the elimination of hydrophilic linear peptides. Therefore, additional studies to elucidate the endogenous transport system have been undertaken [27]. The results indicate that hydrophilic peptides, in contrast to their hydrophobic counterparts are not substrates or hepatocellular bile acid transporters.

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