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Hepatocellular transport of cyclosomatostatins: evidence for a carrier system related to the multispecific bile acid transporter

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The uptake of the cyclopeptide c(Phe-Thr-Lys-Trp-Phe-D-Pro) (008), an analog of somatostatin with retro sequence, was studied in isolated hepatocytes. 008 is taken up by hepatocytes in a concentration-, time-, energy- and temperature-dependent manner. Since 008 is hydrophobic, it binds rapidly to liver cells. This is evident by the positive intercept at the *y*-axis in the uptake curves. At higher concentrations, a minor part of the transport occurs by diffusion at a rate of $8.307 \cdot 10^{-6}$ cm/s. This part of diffusion is measured at 4°C and can be subtracted from the uptake at 37°C resulting in the carrier mediated part of uptake which is saturable. Kinetic parameters for the saturable part of uptake are K_m 1.5 μM and V_{max} 40.0 pmol/mg per min. The transport is decreased in the absence of oxygen and in the presence of metabolic inhibitors. Uptake is accelerated at temperatures above 20°C. The activation energy was determined to be 30.77 kJ/mol. The membrane potential and not a sodium gradient is the main driving force for 008 transport. Cholate (a typical substrate of the multispecific bile acid transporter) and taurocholate are mutual competitive inhibitors of 008 uptake. Phalloidin, antamanide and iodipamide, typical foreign substrates of the transporter, interfere with the uptake of 008. AS 30D ascites hepatoma cells, known to be unable to transport bile acids, phalloidin and iodipamide, are also unfit to transport 008. Interestingly, sulfobromophthalein (BSP) but not rifampicin, both foreign substrates of the bilirubin carrier, inhibits the transport of 008 in a competitive manner.

1. Introduction

Efforts to develop drugs with peptidal structure, e.g., analogs of somatostatin have been hindered by rapid biodegradation, short duration of biological response or low oral activity. By manipulations of the peptide conformation and structure, e.g., by cyclization (cyclosomatostatins), analogs with stability to proteolytic enzymes could be obtained [1]. However, the in vivo activity of such compounds is low because of rapid biliary excretion. Pharmacokinetic studies in the rat have shown that somatostatin [2,3] and especially cyclic analogs are rapidly taken up by the liver and subsequently excreted as intact peptides in the bile [4].

Based on the results of preliminary experiments, we presumed that the transport of cyclosomatostatins into liver cells might be carrier mediated. Recent studies on isolated hepatocytes have shown that cyclosomatostatins are powerful competitive inhibitors of the hepatocellular bile acid uptake system [5]. This transport system, (termed multispecific bile acid transporter, MT, [6]) translocates, besides its physiological substrates, certain foreign compounds like cyclopeptides (phalloidin and antamanide). The assumption of cyclosomatostatin uptake via the multispecific bile acid transporter was further supported by photoaffinity labeling studies. Using photoreactive bile acid or cyclopeptide analogs, proteins with identical molecular weight were identified as binding and/or transporting proteins [7].

The aim of the present study was to characterize the uptake of cyclosomatostatins into isolated rat hepatocytes. A tritiated cyclic somatostatin analog (code number 008) with retro sequence, showing high affinity to the bile acid transporter, was used. Results indicate carrier-mediated uptake related to the multispecific bile acid carrier. Some of the results have been reported in preliminary form [8].

Abbreviations: 008, cyclopeptide c(Phe-Thr-Lys-Trp-Phe-D-Pro); BSP, sulfobromophthalein; MT, multispecific bile acid transporter.

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Materials and Methods

Materials

Cyclic somatostatin analogs were synthesized as described earlier [9]. Specific activity was 11 GBq/mmol. The radiochemical purity of the compound was tested by thin-layer chromatography. [^{14}C]Cholate (specific activity 1.85–2.2 GBq/mmol) was purchased from Amersham Buchler, Braunschweig, F.R.G., [^{3}H]taurocholate (specific activity 74–185 GBq/mmol) from New England Nuclear, Dreieich, F.R.G. All chemicals used were of at least analytical grade purity.

Methods

Isolation of rat hepatocytes and AS 30D ascites hepatoma cells. Rat liver cells were isolated from male Wistar rats weighed 230–260 g according to the method of Berry and Friend [10]. The liver was flushed in situ with 37 °C Ca^{2+} free Krebs-Henseleit buffer to remove blood. Thereafter, the liver was perfused for 15 min at 37 °C with 0.05% collagenase in Ca^{2+} free Krebs-Henseleit buffer. After dissociation of the cells, hepatocytes were separated from cell debris and nonparenchymal cells by centrifugation at $40 \times g$ for 3 min and resuspended in Tyrode buffer. Prior to each experiment cells were equilibrated for 30 min in Tyrode buffer (pH 7.4) at 37 °C in carbogene atmosphere (95% O_2/5% CO_2). Cell viability was tested by Trypan blue exclusion. From 5–15% of the cells were damaged. Experiments were performed within 2 h after the isolation. AS 30D ascites hepatoma cells were isolated from Sprague-Dawley rats 1 week after intraperitoneal injection of hepatoma cells. Hepatoma cells were washed two times in Tyrode buffer by centrifugation at $25 \times g$.

Uptake studies. Uptake of the radiolabeled compound into isolated rat liver cells was measured using 1 or 2 ml of liver cell suspension containing $2 \cdot 10^6$ cells/ml Tyrode buffer, corresponding to 3.3 mg of cell protein. Protein content was determined by a Biuret method. After addition of the tritiated compound, 100 µl aliquots of the cell suspension were withdrawn at 15, 45, 75, 105 and 135 s, 5 and 10 min. Other conditions are indicated in the legends of the figures. The extracellular medium was separated from hepatocytes by the silicon oil centrifugation technique [11]. Silicon oil layer density (1.0325 g/l) was obtained by mixing silicon oil AR 20 with AR 200 at a ratio of 1 : 3. The extracellular fluid contamination in the cell pellet, as determined with [^{3}H]inulin was less than 0.05% of the added radioactivity. Cell associated radioactivity was measured in a Packard 2660 liquid scintillation counter, after cutting the centrifugation tubes. The initial rates of uptake were determined during the first minute of uptake using linear regression analysis. The binding component of 008 (positive segment at the y-axis at time zero) was not further investigated in this study.

The part of diffusion was determined by uptake studies performed at 4 °C. Carrier-mediated uptake was calculated by subtracting the uptake values determined at 4 °C from those determined at 37 °C. Sodium dependence of uptake was measured in the absence of sodium using lithium-, choline- and potassium-containing Tyrode buffer (sodium-Tyrode buffer: 137 mM NaCl, 2.7 mM KCl, 1.05 mM $MgCl_2$, 1.8 mM $CaCl_2$, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4 and 5.6 mM glucose; lithium-Tyrode buffer: 137 mM LiCl, 12 mM $LiCO_3$ and 0.4 mM KH_2PO_4 instead of the corresponding salts; choline-Tyrode buffer: 137 mM choline chloride, 12 mM $LiCO_3$ and 0.4 mM KH_2PO_4; potassium-Tyrode buffer: sodium was replaced by corresponding potassium salts). The liver cell suspension was pelleted and washed twice with the appropriate buffer. After a preincubation of 3 min uptake studies were started.

Membrane potential dependence of uptake was studied by replacing chloride with the appropriate concentration of SCN^-, NO_3^- or SO_4^{2-} in the Tyrode buffer. Liver cells were processed as described above.

Energy dependence of uptake was studied in a N_2/CO_2 atmosphere. Cells were preincubated for 30 min in N_2/CO_2 before starting the uptake measurements.

Temperature dependence of uptake was measured at 4, 11, 18, 25, 30 and 37 °C. The initial rate of uptake ($\log V_i$) was plotted versus temperature ($1/T$) in the Arrhenius diagram. From the slope of the graph the apparent activation energy was calculated according to the equation $A_{app} = 2.303\ R \times \log V_i \times (1/T)^{-1}$, where R is the gas constant.

Statistics

The experiments were performed four or five times. Experimental values are given by ±S.D. Mean values were used for further calculations. The lines in the Lineweaver-Burk and Woolf-Hofstee plots were calculated by a linear regression analysis. Significance of the data was proven by one or two-factorial variance analysis with the program BMDP 2V on a Cyber 860 computer.

Results

Time and concentration dependence of 008 uptake

Isolated hepatocytes incorporate 008 in a time- and concentration-dependent manner (Fig. 1). Time-dependent saturation of uptake is reached after 2–4 min depending on the concentration of 008. The zero-time values of the uptake curves (positive segment at the y-axis) are indicative for a rapid binding of 008 to the plasma membranes. This binding component depending on the concentration of 008 is further investigated in a forthcoming paper.

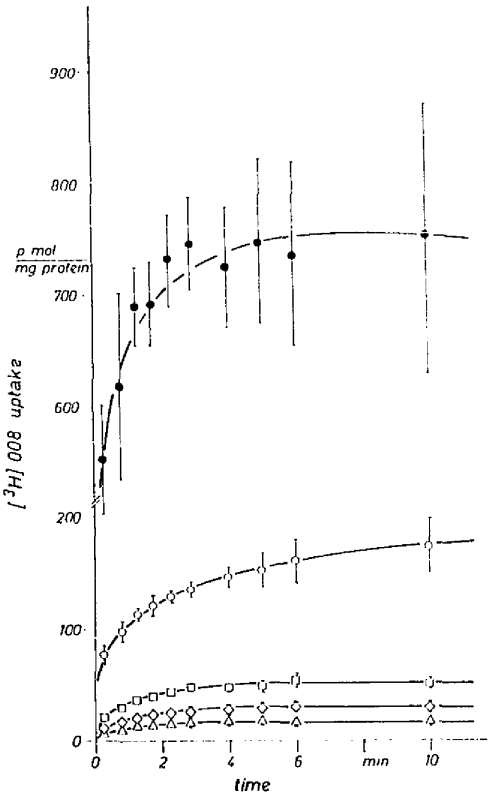

Fig. 1. Concentration and time dependence of 008 uptake. Isolated hepatocytes ($2 \cdot 10^6$/ml Tyrode buffer (pH 7.5) = 3.3 ± 0.2 mg of protein/ml) were incubated at 37°C with 0.1, 0.2, 0.4, 2.3 and 22.6 μM of 008. At 15, 45, 75, 135, 195 s and 4, 5, 6 and 10 min 100 μl aliquots were withdrawn. Cells were separated from the medium by centrifugation through silicon oil. The cell-associated radioactivity was measured in Lipoluma/Lumasolve/water (1:10:0.2) in a Packard 2660 liquid scintillation counter. $n = 5$ ±S.D.; 22.6 μM, ●——●; 2.3 μM, ○——○; 0.4 μM, □□; 0.2 μM, ◇——◇; 0.1 μM, △△.

The uptake of 008 into isolated rat liver cells follows mixed-type kinetics in the concentration range tested. Higher concentrations of 008 (> 20 μM) were no longer soluble in 1% DMSO. Increase of the concentration of DMSO damaged the cells.

The plot V_i versus substrate concentration is curvilinear (Fig. 2A). This points to the fact that at higher concentrations diffusion plays a role in total uptake of 008. The part played by diffusion can be extrapolated [12] or can be measured at 4°C and can be subtracted from total uptake (K_m 3.51 μM; V_{max} 93 pmol/mg per min). The latter method was employed in this study to determine the carrier-mediated uptake. The resulting curve represents the carrier-mediated transport (Fig. 2A). The diffusion of 008 is evident at a rate of $8.307 \cdot 10^{-6}$ cm/s.

Plotting the data for total uptake of 008 according to Woolf-Hofstee [13] resulted in a curvilinear function, demonstrating again two types of transport systems, e.g., diffusion and carrier-mediated uptake. In contrast, the V_0 versus V_0/s plot of the carrier mediated uptake is a straight line (Fig. 2B, inset) indicating one type of saturable transport. K_m and V_{max} values determined according to Woolf-Hofstee differ slightly from those determined according to Lineweaver and Burk [14] (legend to Fig. 2B).

008 is accumulated at equilibrium 16.6-fold over the extracellular concentration in liver cells at an initial extracellular concentration of 22.6 μM and 73-fold at an initial extracellular concentration of 110 nM, assuming an intracellular volume of 3.35 μl/10^6 cells [15].

Energy dependence of 008 uptake

The uptake of 008 (540 nM) is energy dependent. This becomes evident by the inhibition of transport in the absence of oxygen (Fig. 3). The initial uptake rate in the absence of oxygen was reduced to 33% of that of the control (V_i in the presence of oxygen 11.39 ± 3.1 pmol/mg per min; V_i in the absence of oxygen 3.76 ± 1.5; highly significant $P = 0.3213$ E-08 by two-factorial variance analysis). Reoxygenation led to 80% restoration of the transport ($V_i = 9.59 \pm 1.38$).

Metabolic inhibitors (dinitrophenol, oligomycin, antimycin A and CCCP) blocked the transport of 008 (Fig. 4). Only 10–20% of 008 uptake were resistant to these blockers.

Temperature dependence of 008 uptake

The uptake of 008 (540 nM) was measured at different temperatures (4–37°C). Uptake is temperature dependent showing accelerated uptake above 25°C. The Q_{10} value obtained for a temperature range of 25–35°C is 2.25. The apparent activation energy calculated from an Arrhenius diagram is 30.77 kJ/mol for 0.54 μM of 008 (Fig. 5A, B).

Sodium dependence of 008 uptake

The uptake of 008 (540 nM) is not clearly sodium dependent. In the absence of sodium but in the presence of lithium or choline the initial uptake rate is reduced to 90 or 80% of the control. These differences are not significant (Table I). Substitution of sodium by potassium reduced the uptake significantly (42% inhibition). Potassium, however, provokes additional effects on the membrane potential.

Preincubation of isolated hepatocytes for 10 min with 1 mM ouabain which totally blocks the sodium-potassium ATPase [16] has no effect on the uptake of 008 (data not shown).

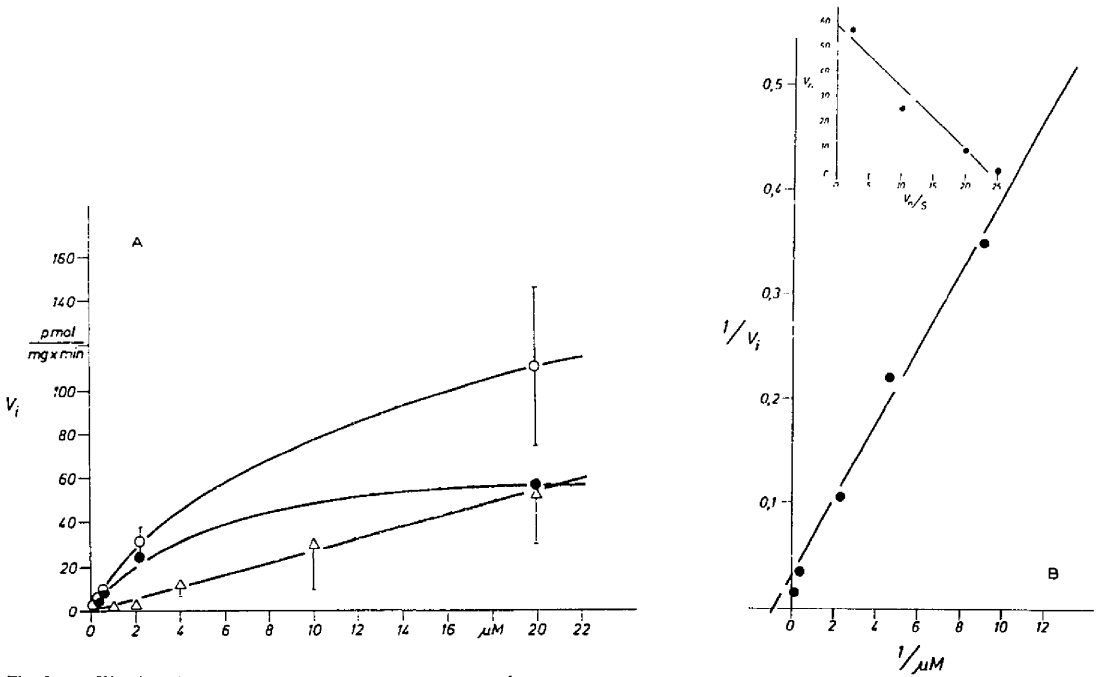

Fig. 2. (A) Kinetics of 008 uptake. Isolated rat liver cells ($2 \cdot 10^6$/ml Tyrode buffer (pH 7.5) were incubated at 37°C or at 4°C in carbogene atmosphere (95% O_2/5% CO_2) with increasing concentrations of 008 (22.6, 2.35, 0.44, 0.22, 0.11 µM at 37°C and 20, 10, 4, 2, 1 µM at 4°C). The initial rate of uptake was calculated during the period of linear uptake by linear regression analysis. V_i (pmol/mg per min) was plotted versus the concentration of 008, ○———○, representing total uptake of 008 at 37°C; △———△, the part of diffusion at 4°C; ●———●, the carrier-mediated uptake. (B) Lineweaver-Burk plot of the uptake of 008. The data for carrier mediated uptake of 008 were plotted according to Lineweaver and Burk. K_m and V_{max} values were calculated by linear regression analysis. $n = 5$, $K_m = 1.5$ µM, $V_{max} = 40$ pmol/mg per min, $r = 0.991$. Inset: the data for carrier mediated uptake were plotted according to Woolf-Hofstee. K_m and V_{max} values were calculated by linear regression analysis. $K_m = 2.4$ µM, $V_{max} = 58.5$ pmol/mg per min, $r = 0.97$.

Effects of alterations in the membrane potential on the uptake of 008

In the presence of a potassium chloride gradient which leads to a depolarization of the membrane potential [17] initial rates of 008 uptake were reduced. This is indicative of a membrane potential dependence of 008 uptake. Therefore, uptake was studied in the presence of lipophilic anions SCN^- and NO_3^- inducing a diffusion potential which is more negative inside as compared with the chloride distribution potential. In addition, the more hydrophilic less permeant anion SO_4^{2-} inducing a more positive inside of the cell is substituted for chloride.

In the presence of a sodium gradient, replacement of chloride by SCN^- and SO_4^{2-} inhibits significantly the 008 uptake. Substitution of chloride by NO_3^- had no effect on 008 uptake (Fig. 6).

Effects of the ionophores nigericin, monensin, gramicidin and valinomycin and absence of an ion gradient on the uptake of 008

The sodium-selective ionophores nigericin, inducing sodium–potassium exchange in living cells [18] and monensin which permits sodium–proton exchange [19], dissipate the sodium gradient without alterations to the membrane potential [20]. Monensin (10 µg/ml) and nigericin (1 µg/ml) inhibited the initial uptake of 008 to 80 or 64% of the control. In the case of monensin this inhibition was not significant (Table II).

Gramicidin is a channel-creating ionophore that permits the passage of sodium, potassium, lithium, protons and chloride thereby disturbing the membrane potential [21]. In the concentration tested (10 µg/ml) gramicidin had a small, but not significant effect on the uptake of 008.

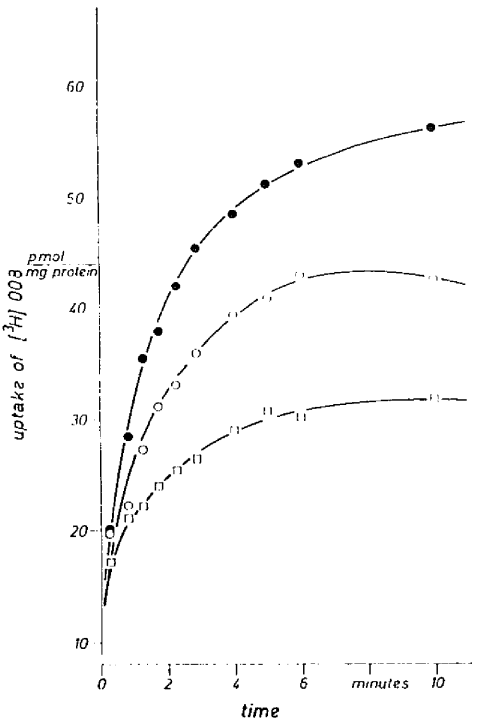

Fig. 3. Uptake of 008 (540 nM) in the presence and absence of oxygen and after reoxygenation. $2 \cdot 10^6$ cells/ml Tyrode buffer were incubated for 30 min either in 95% O_2/5% CO_2 (●———●) or 95% N_2/5% CO_2 (□———□) before starting uptake measurements. Part of the cells were first exposed to 95% N_2/5% CO_2 and were then reoxygenated (○○). At the times indicated 100 µl aliquots were withdrawn and centrifuged through silicon oil as described in Materials and Methods. Shown is the result of a typical experiment. $n = 4$.

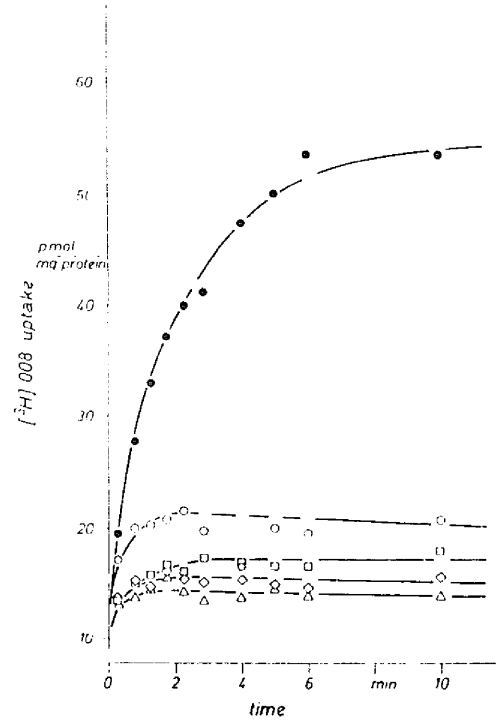

Fig. 4. Uptake of 008 in the presence of metabolic inhibitors. Isolated hepatocytes were preincubated for 10 min with 10 µg/ml of antimycin A or oligomycin, 4 µg/ml CCCP or 100 µM dinitrophenol. Uptake measurements were started by adding 540 nM of 008. $n = 5$. ●, control; ○, dinitrophenol; □, CCCP; ◇, antimycin; △, oligomycin.

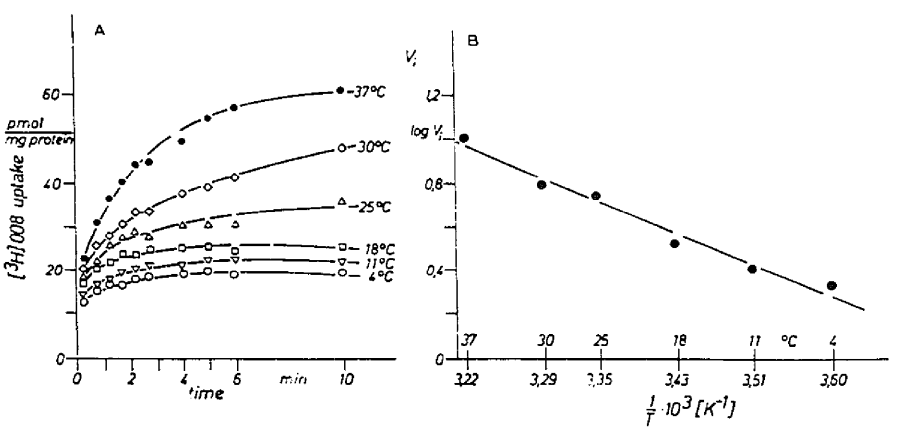

Fig. 5. (A) Temperature dependence of 008 uptake. Isolated hepatocytes ($2 \cdot 10^6$/ml Tyrode buffer (pH 7.5) were preincubated for 10 min at 4, 11, 18, 25, 30, and 37 °C before starting uptake measurements by adding 540 nm of 008. At the times indicated 100 µl aliquots were withdrawn. The cell associated radioactivity was determined. $n = 6$. Arrhenius-diagram. The initial uptake rates of 008 at different temperatures were calculated by linear regression analysis. The logarithm of V_i was plotted versus $1/T$ according to Arrhenius. An apparent activation energy of 31 kJ/mol was determined.

TABLE I

Sodium dependence of 008 (540 nM) uptake

Isolated liver cells were washed and incubated in lithium-, sodium-, choline- or potassium-Tyrode buffer (the composition of the buffers is given in Materials and Methods) 3 min before starting 008 uptake measurements.

Buffer system	V_i	% Inhibition	Significance (P)
Na^+-Tyrode	11.82 ± 3.0	–	
Li^+-Tyrode	10.33 ± 1.68	13	0.379
Choline-Tyrode	9.6 ± 2.3	20	0.133
K^+-Tyrode	6.91 ± 0.62	42	0.0423

TABLE II

Inhibitory effects of ionophores on the uptake of 008 (540 nM)

Isolated hepatocytes were preincubated for 10 min with 10 μg/ml monensin, 1 μg/ml nigericin, 10 μg/ml gramicidin and 0.1 μg/ml valinomycin. The inhibition of the initial uptake rates was determined. The significance of the inhibition was determined by one factorial variance analysis.

	% Inhibition of the control	Significance (P)
Monensin	21.2	0.578
Nigericin	44	0.0481
Gramicidin	14.3	0.733
Valinomycin	no effect	

Valinomycin, inducing an efflux of potassium out of the cells thereby disturbing the membrane potential [22], had no effect on 008 transport.

Preincubation of liver cells in the absence of an ion gradient (incubation in sucrose medium) led to a 73% inhibition of the transport (Fig. 7). Therefore, 25% of 008 uptake is not coupled with ion gradients.

Specificity of 008 uptake

Cholate and taurocholate were used as inhibitors of 008 uptake. Both bile acids blocked 008 uptake in a competitive manner (Fig. 8A, B). In earlier studies, it was shown that 008 competitively inhibits the uptake of cholate and taurocholate [5]. The affinity of 008 to the bile acid transporting protein is higher compared with that of bile acids (Table III). Other foreign substrates of the MT, e.g., phalloidin, antamanide, iodipamide and ouabain (for review see Ref. 6) interfere with 008 uptake, showing different affinities (Table IV).

Interestingly, BSP, a putative substrate of the bilirubin carrier, also inhibits 008 transport in a competitive manner (Fig. 9A). Unfortunately, mutual competitive transport inhibition studies with radioactive labeled BSP were not possible, since the isotope labelled substance is no longer available commercially. Rifampicin, a further

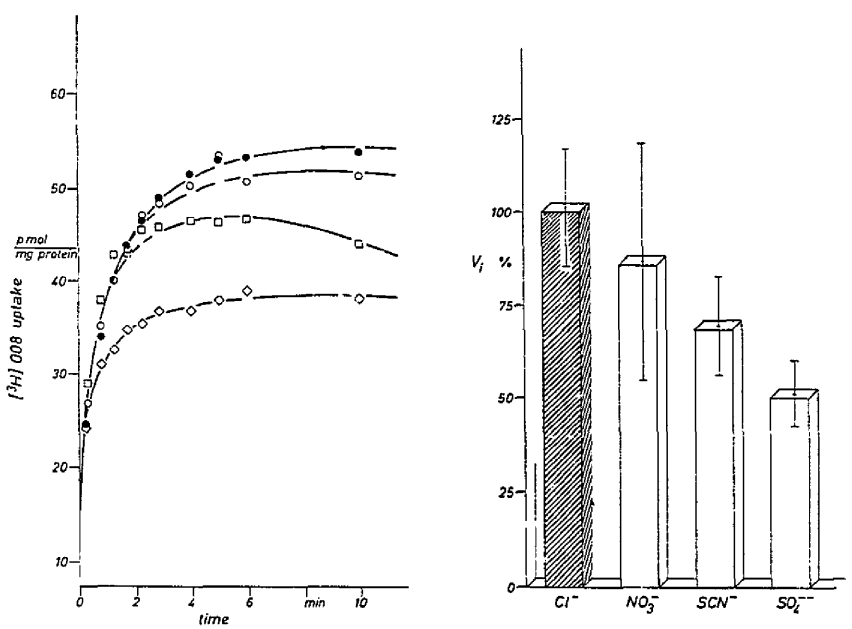

Fig. 6. Effect of chloride substitution on the uptake of 008 (540 nM). Isolated hepatocytes were incubated in SCN^-, NO_3^- or SO_4^{2-} Tyrode buffer (for details see Materials and Methods) for 10 min at 37 °C before starting uptake measurements. In the left graph uptake curves are shown, in the right graph the inhibition of the initial uptake in the different buffers is shown ($X \pm$ S.D.). $n = 5$. ●, Cl^-; ○, NO_3^-; □ SCN^-; ◇, SO_4^{2-}.

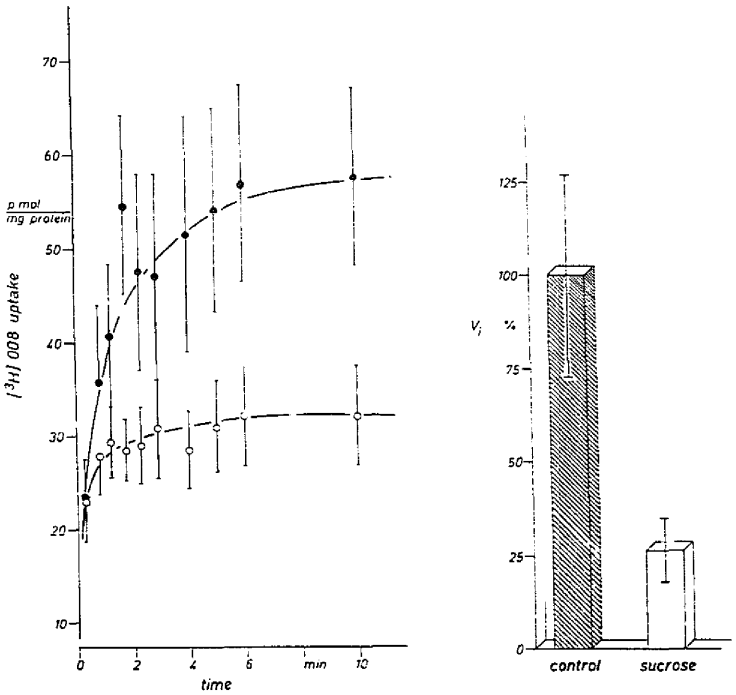

Fig. 7. Uptake of 008 (540 nM) in the absence of an ion gradient. Isolated rat liver cells were incubated either in Tyrode buffer (control) or in a unbuffered sucrose medium. The osmolarity of the medium was 290–300 mosmol. $n = 4$. $X \pm$S.D. ●, Tyrode buffer; ○, sucrose medium.

foreign substrate of the bilirubin carrier, inhibits 008 uptake in a mixed competitive/non-competitive manner (Fig. 9b). In accordance with the results with cholate, phalloidin and iodipamide uptake [6], AS 30D ascites hepatoma cells did not incorporate 008. Hepatoma cells bind small amounts of 008 without exhibiting time-dependent uptake (Fig. 10).

Discussion

Earlier studies have shown that somatostatin and especially cyclic analogs are rapidly eliminated via the biliary pathway [2–4]. In the rat, 30% of injected cyclic peptides are found in the liver 1 min after i.v. injection. The hepatocellular uptake mechanism for those cyclosomatostatins, however, has not been characterized as yet.

Previous studies [5] in this laboratory demonstrated that certain cyclosomatostatins with retro sequence, which were synthesized as cytoprotective agents, posess high affinities to a bile acid carrier in the liver termed multispecific bile acid carrier [6]. The clinical usefulness of such cytoprotective cyclosomatostatins is questiona-

TABLE III

K_i values of the mutual competitive transport inhibition of bile acids and 008

The kinetics of the transport inhibition of 008 or bile acids by 008 or bile acids was determined and the K_i values were calculated according to Dixon.

Substrate	Inhibitor	K_i value (μM)
008	cholate	7 ± 0.5
008	taurocholate	13 ± 1.4
Taurocholate	008	2 ± 0.3
Cholate	008	3 ± 0.6

TABLE IV

IC_{50} values of the inhibition of 008 uptake (540 nM) by certain substrates of the multispecific bile acid transporter

Isolated hepatocytes were incubated for 30 s with increasing concentrations of the inhibitors before addition of [^{3}H]008. The concentration inhibiting 50% of the uptake of 008 was determined.

	IC_{50} (μM)
Cholate	120 ± 12
Taurocholate	35 ± 3
Phalloidin	400 ± 23
Iodipamide	7.5 ± 3
Antamanide	2.1 ± 0.7
Ouabain	1000

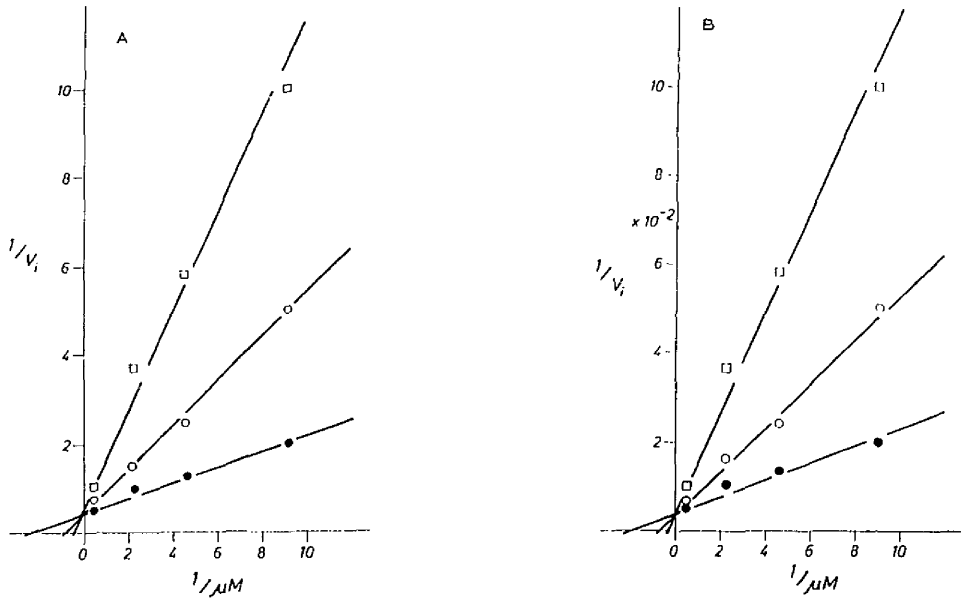

Fig. 8. (A) Lineweaver-Burk plot on the inhibition of 008 uptake by cholate. Isolated rat liver cells were incubated for 30 s without (control) or with 10 or 50 μg of cholate before addition of increasing concentrations (2.35; 0.44; 0.22; 0.11 μM) of [^{3}H]008. Initial uptake rates were determined and plotted according to Lineweaver and Burk, $n = 3$. control, ●; 10 μg, ○; 50 μg, □ of cholate. (B) Lineweaver-Burk plot on the inhibition of 008 uptake by taurocholate. Rat hepatocytes ($2 \cdot 10^6$/ml Tyrode buffer) were preincubated for 30 s without (control) or with 20 or 50 μg of taurocholate before addition of [^{3}H]008. Initial uptake rates were determined and plotted according to Lineweaver and Burk, $n = 3$. control, ●; 20 μg, ○; 50 μg □ of taurocholate.

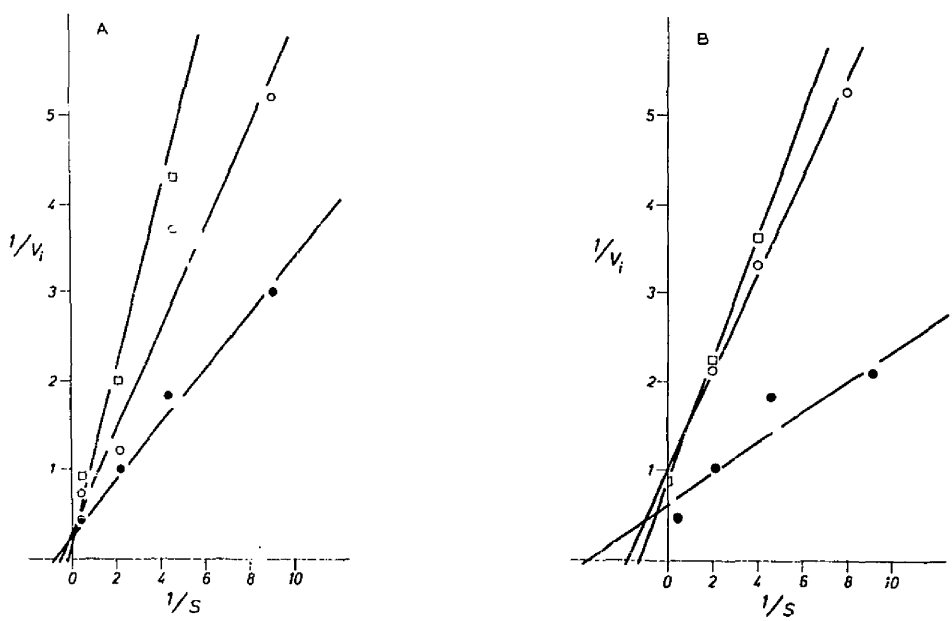

Fig. 9. (A) Lineweaver-Burk plot of the inhibition of 008 uptake by BSP. The BSP concentrations used were 5 and 10 μg/ml. Other conditions are as in Fig. 8A. control, ●; 5 μg, ○; 10 μg, □ of BSP. (B) Lineweaver-Burk plot on the inhibition of 008 uptake by rifampicin. The rifampicin concentrations used were 5 and 10 μg/ml. control, ●; 5 μg, ○; 10 μg, □ of rifampicin.

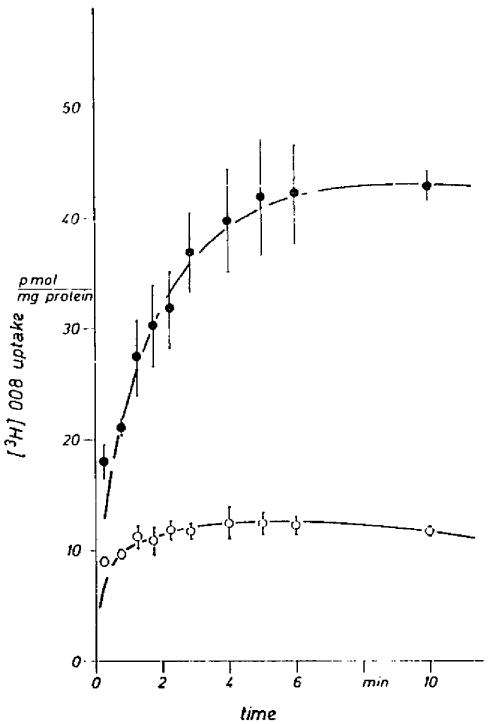

Fig. 10. Specificity of 008 uptake: uptake in AS 30D ascites hepatoma cells. The uptake of 540 nM of 008 was measured either in $2 \cdot 10^6$ hepatocytes/ml Tyrode buffer or in $14 \cdot 10^6$ hepatoma cells/ml Tyrode buffer corresponding to 3.3 mg of protein/ml buffer. $n = 3$; $X \pm$ S.D. control, ●; AS 30D ascites cells, ○.

ble, since they cause cholestasis due to a competitive inhibition of the uptake of bile acids in liver cells.

We hypothesized that those cyclosomatostatins are taken up into hepatocytes by a carrier-mediated process related to the MT. The results of the studies presented indicate that the uptake mechanism for cyclosomatostatins and bile acids are closely related; however, with some differences of the driving forces. Liver cells accumulate 008 at 37°C 16–77-fold over the extracellular concentration. Since the cyclopeptide is not metabolized by liver cells (Kessler, personal communication) metabolic trapping of radioactivity derived from 008 in the cells can be excluded. Carrier-mediated clearly saturable uptake as well as diffusion take part in the accumulation at 37°C. Diffusion occurs at a rate of $8.307 \cdot 10^{-6}$ cm/s. The kinetic parameters K_m of 1.5 μM and V_{max} of 40.0 for the saturable part of the uptake show that cyclopeptides have a high affinity for the transport system. The affinity is higher compared to that of other cyclopeptides, e.g., phalloidin and antamanide ($K_m = 20$–36 μM) and especially higher than that of bile acids ($K_m = 20$–60 μM). The V_{max} values of 008, however, are in the range of that of phalloidin and antamanide but lower compared to that of bile acids.

As earlier shown for bile acids, the uptake of 008 is energy and temperature dependent. Lowering the ATP content of the cells either by metabolic inhibitors or by anoxia, blocked 80% of 008 uptake. Therefore, 20% of total uptake is due to diffusion independent of the cellular energy sources and still existing at 4°C. Increase of the incubation temperature leads to a stimulation of total uptake. Q_{10} values higher than 2 and an apparent activation energy of 31 kJ/mol are further arguments for a carrier-mediated process.

Cholate and taurocholate competitively inhibit 008 uptake. The potency of inhibition ($K_i = 7$–13 μM) is lower compared to that of 008 inhibition of the total uptake of bile acids ($K_i = 2$–3 μM). Other foreign compounds of the MT also interfere with 008 uptake. Antamanide, also a cyclopeptide, had the highest affinity. The existence of a mutual competitive transport inhibition for bile acids and cyclopeptides is the main evidence for a common transport system of those chemically different compounds. A further evidence for this hypothesis comes from the fact that AS 30 D ascites hepatoma cells, which are bile acid transport deficient, do not transport 008.

Interestingly, BSP, a foreign substrate of the bilirubin carrier, also inhibits 008 uptake in a competitive manner. At present mutual transport inhibition studies could not be performed because BSP is no longer available in radioactive form.

Looking at the driving forces of 008 and bile acids, several differences can be detected. The uptake of 008 is not clearly sodium dependent (20% inhibition of uptake in choline buffer). Dissipation of the sodium gradient either by substitution of sodium by lithium or choline or by blocking the $(Na^+ + K^+)$-ATPase by ouabain has no significant effect on 008 uptake. For taurocholate the sodium gradient drives over 80% of the transport. In the case of cholate a sodium-independent saturable transport is described [25–27]. This sodium-independent transport system is also supposed to drive the uptake of other organic anions like BSP [27,28]. In the study presented, BSP turned out to competitively block 008 uptake. In addition, photoaffinity labeling studies with analogs of bile acids, 008 and BSP (Ref. 7, for review see Ref. 6) resulted in identification of proteins with identical molecular weights. It seems that the sodium-independent transport systems of 008, bile acids and other organic anions (BSP) is somehow related. It has to be mentioned, however, that a photoreactive 008 analog [7] competitively inhibits the sodium-dependent as well as the sodium-independent part of taurocholate uptake.

Uptake of 008 must be coupled to ion gradients, since in sucrose medium 80% of the uptake is blocked.

Therefore, we suggested that the membrane potential is a driving force for 008. This was also shown for cholate. Actually, alterations of the membrane potential, e.g., uptake in the presence of potassium chloride or substitution of chloride by SCN^- or SO_4^{2-} or preincubation with ionophores inhibits 008 uptake. At present, we are not able to predict whether 008 is taken up as a positive or negative charged component, since uptake is inhibited an inside more-negative as well as by an inside more-positive membrane potential. Therefore, studies with plasma membrane vesicles are needed to further characterize the driving forces for the uptake of 008 and the relationship of 008 uptake to the uptake of other organic anions like BSP and bile acids.

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