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Binding proteins for linear renin-inhibiting peptides in basolateral plasma membranes of rat liver

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A linear hydrophobic peptide, (Code no. EMD 55068), a synthetic renin-antagonist, competitively inhibits the uptake of taurocholate and of another linear peptide (EMD 51921) but not of oleic acid, serine or thiamin hydrochloride into isolated rat liver cells. EMD 55068 was attached to a gel matrix at a position that is not involved in the protein ligand interaction. The gel matrix used did not interact nonspecifically with solubilized proteins from rat liver. The quantity of bound ligand was determined to be 3.6 mg/ml of gel matrix. In the fraction of EDTA extracted hydrophilic membrane-associated proteins, no binding proteins were detected. Affinity chromatography of integral plasma membrane proteins resulted in four protein bands with molecular masses of 46, 49, 53 and 56 kDa in SDS-PAGE. In contrast, solubilized plasma membrane proteins from AS-30D ascites hepatoma cells, which are unable to transport bile acids and linear peptides, did not bind specifically to the affinity matrix.

Introduction

Linear renin-inhibiting peptides have a short survival in vivo. This behaviour is not due to proteolytic degradation, which is often assumed to be the limiting effect [1,2]. Various linear hydrophobic peptides stable against proteolysis are rapidly eliminated in the bile without previous biotransformation [3]. It was shown that a liver-specific clearance is responsible for first-pass elimination [4]. The hepatocellular uptake of such compounds is carrier-mediated, with a K_m of 2 μM and a V_{max} of 160 pmol/mg per min [5]. Uptake depends on energy supply but not on a sodium-gradient. During carrier-mediated uptake into liver cells, the linear hydrophobic renin-inhibiting peptides tested interfere with the uptake of bile acids as well as of BSP but not with that of amino acids, cationic compounds, fatty acids or hexoses [6]. A mutually competitive inhibition of uptake of bile acids and of linear hydrophobic peptides has been demonstrated, whereas the uptake of BSP was non-competitively influenced [7]. Our data suggest that the linear hydrophobic peptides use transport systems common to those of bile acids. A series of further anionic compounds and certain cyclopeptides were also shown to be foreign substrates of bile acid carriers [8]. The system, which physiologically transports cholate, was previously termed 'the multispecific bile acid transporter' [9]. Furthermore, the sodium-dependent taurocholate carrier is also capable of transporting foreign substrates [10]. The linear hydrophobic renin-inhibitors used in this study have affinity to transport proteins of both systems. Comparison of the kinetics of transport of bile acids and of linear hydrophobic renin-inhibitors provides evidence that linear hydrophobic peptides have a higher affinity to the carrier proteins than do bile acids (K_m for bile acids, 20–60 μM; for hydrophobic peptides, 2 μM). In contrast, V_{max} values are always higher for bile acids. The main difference between transport of bile acids and the above hydrophobic peptides is the sodium dependency, which could not be demonstrated for linear peptides. On the other hand, AS-30D ascites hepatoma cells, known to be unable to transport bile acids and cyclopeptides [11,12], are also unable to transport linear hydrophobic renin-inhibitors.

Unfortunately, at present no chemically reactive or photoreactive analogues of linear hydrophobic renin-inhibitors are available. Binding proteins for linear hydrophobic peptides have not yet been identified. For other substrates of bile acid transporters, binding proteins with molecular masses of 48–50, 52–54, 67 and 37

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kDa were detected in isolated rat liver basolateral plasma membrane vesicles (blPmv) using (photo) affinity labels derived from bile acids and also from cyclopeptides [13–18]. Since the affinity of linear hydrophobic peptides to the transport system was 10–30-fold higher than that of bile acids, it might be possible to identify binding proteins by affinity chromatography using a linear peptide as ligand.

The aim of the present study, therefore, was to prepare an affinity matrix which specifically interacts with binding proteins for linear peptides in isolated rat liver basolateral plasma membrane vesicles (blPmv). Some of the results have already been described in a preliminary report (19).

Materials and Methods

Materials

Renin inhibitors EMD 55068 (M_r 780.0), 55450 (M_r 914.2), 51921 (M_r 753) and [^{3}H]51921 (spec. act. 2.9 GBq/mmol) were gifts from Merck, Darmstadt, FRG. The chemical structures of 55068 and 55450 are shown in Fig. 1.

[^{14}C]Cholic acid sodium salt (spec. act. 2.07 GBq/mmol), [^{14}C]oleic acid (spec. act. 2.1 GBq/mmol), [^{3}H]taurocholic acid sodium salt (spec. act. 222 GBq/mmol) and [^{3}H]serine (spec. act. 32.5 GBq/mmol) were purchased from Du Pont-De Nemours, Dreieich, FRG. [^{14}C]Thiamin hydrochloride was a gift from Dr. Hahn, Giessen, FRG.

Affi-Gel 10 and 15 were purchased from Bio-Rad, Munich, FRG, CNBR-activated Sepharose 4B and Epoxy-activated Sepharose 6B from Pharmacia, Freiburg, FRG. Nonidet P-40, Triton X-100 and X-114, octyl glycoside, CHAPS and sulfobetaines were purchased from Serva, Heidelberg, FRG. All other chemicals were of analytical grade purity or better.

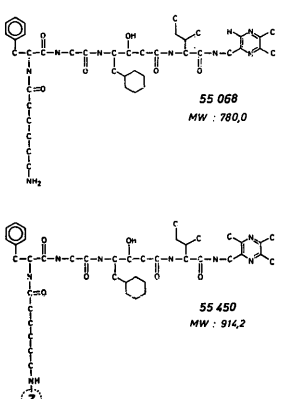

Fig. 1. Structure of EMD 55068 and EMD 55450.

Methods

Isolation of rat hepatocytes

Rat liver parenchymal cells were isolated from male Wistar rats by the perfusion technique of Berry and Friend [20]. Only preparations with less than 15% non-viable cells, as determined by Trypan blue exclusion, were used. The uptake studies were performed within 2 h after the isolation of the hepatocytes.

Uptake into isolated hepatocytes

Hepatocytes were diluted to $2 \cdot 10^6$ cells/ml Tyrode buffer, corresponding to 4 mg/ml of cell protein, determined by the Biuret method. Aliquots of 1 ml were incubated for 30 s with three different concentrations of the renin-inhibitors. A constant concentration of [^{14}C]cholate or [^{3}H]taurocholate mixed with various concentrations of unlabeled substrate was then added. The initial uptake rates of the substrates were plotted according to Lineweaver and Burk or Cornish-Bowden [21]. K_i was determined according to Dixon [22]. The uptake of oleic acid, serine and thiamin hydrochloride was measured as described [6].

Preparation of sinusoidal plasma membranes (blPmv) from rat liver

Membranes were prepared from livers of male Wistar rats according to the method of Blitzer and Donovan [23] with the following modifications: After homogenization of the livers with a loose Dounce homogenizer and a motor driven teflon pestle the homogenate was diluted 10:1 (w/v) with 10 mM Tris-HCl (pH 7.6), containing 250 mM sucrose and 1 mM phenylmethylsulfonyl fluoride (PMSF) and was centrifuged at 2500 $\times g$ for 15 min in a GSA-rotor (Sorvall). The continuous Percoll gradient was performed by centrifugation in a SS 34-rotor (Sorvall) at 24000 $\times g$ for 35 min. Finally, membranes were suspended in 10 mM Hepes-KOH (pH 7.5), containing 300 mM sucrose and 1 mM PMSF, with a protein concentration of 4 mg/ml, and were stored at −70°C.

Preparation of plasma membranes from AS-30D ascites hepatoma cells

One week after intraperitoneal inoculation of AS-30D ascites hepatoma cells into female Sprague-Daw-

ley rats, ascites liquid was taken from the decapitated and blood-drained rats. After washing the cell suspension bloodless three times with PBS buffer (pH 7.4), containing 1 mM PMSF and centrifugation at $20 \times g$ for 5 min, the tumor cells were disrupted in a cell disrupter bomb (NECo). The preparation of the membranes followed according to the method of Touster et al. [24]. Membranes were stored at −70°C.

Analysis of enzyme activities

The plasma membrane fractions were characterized by determination of the marker enzymes. Na^+/K^+-ATPase was determined according to the method of Scharschmidt [25]. Glucose-6-phosphatase was determined according to Harper [26] and 5'-nucleotidase according to Michell and Hawthorne [27]. Release of anorganic phosphate from both enzymes was measured according to the method of Chen, modified by Ames [28].

Taurocholate and EMD 51921 uptake into isolated blPmv

The uptake of taurocholate into blPmv was measured as described previously [23]. For determination of the uptake of EMD 51921, 3 μM of the linear peptide was incubated with 60–80 μg of blPmv in 125 μl incubation buffer (pH 7.5), which contained 100 mM sodium chloride, 100 mM sucrose and 10 mM Hepes-KOH.

Separation of hydrophilic membrane associated proteins from integral hydrophobic proteins

Plasma membranes were dialysed overnight at 4°C against 10 mM EDTA (pH 8.0). Thereafter, hydrophilic membrane proteins were separated from integral proteins by centrifugation at $100000 \times g$ for 30 min. Alternatively, hydrophilic and hydrophobic proteins were separated by Triton X-114 phase separation as described below.

Photoaffinity labeling of isolated blPmv with ^{14}C-Azidobenzamidotaurocholate (ABATC)

Isolated blPmv in PBS buffer were labeled as described [18].

Solubilization of the plasma membranes

Triton X-114. Triton X-114 was precondensed according to the method of Bordier [29] and was then added to the plasma membranes at a final concentration of 1%. After solubilization at 4°C for 60 min and centrifugation for 60 min at $100000 \times g$ supernatant was incubated at 37°C for 5 min and centrifuged for 3 min in an Eppendorf centrifuge. The supernatant, corresponding to the aqueous phase containing the hydrophilic proteins of the solubilized membranes, was stored at −70°C for column procedure. The pellet, containing the hydrophobic proteins of the membranes, was diluted in PBS buffer (pH 7.4), 1 mM PMSF and stored at −70°C for column procedure.

Zwittergent 3–12. The sinusoidal plasma membranes were extracted with EDTA and were then stirred with 1% Zwittergent 3–12 for 120 min at 4°C, followed by centrifugation at $100000 \times g$ for 60 min. The supernatant was stored at −70°C for affinity chromatography.

Nonidet P-40. Membranes were dialyzed against 10 mM EDTA (pH 8.0), at 4°C overnight to dissolve associated hydrophilic membrane proteins. After centrifugation at $100000 \times g$ for 30 min, the pellet was resuspended in PBS buffer (pH 7.4), containing 1 mM PMSF and 2% Nonidet P-40. The sample was stirred for 90 min at 4°C, centrifuged at $100000 \times g$ for 60 min, and the supernatant was used for affinity-chromatography.

CHAPS. Plasma membranes, after EDTA extraction, were solubilized with 2% CHAPS for 1 h at 4°C and insoluble proteins were then separated from the soluble fraction by centrifugation at $100000 \times g$ for 1 h.

Octyl glycoside. Plasma membranes after EDTA extraction were solubilized with 2% octyl glycoside for 1 h at 4°C.

Column preparation

Control columns with the following gel matrices were prepared as described by the manufacturer, without addition of the ligand: CNBR-activated Sepharose, Epoxy-activated Sepharose and Affi-Gel 10 and 15.

Preparation of 55068-affinity columns

After washing the gel bed (Affi-Gel 10) several times with methanol, 17 μmol EMD 55068 per ml of gel, dissolved in methanol, were added and coupled by gentle aggitation at 37°C or at room temperature overnight. Subsequently, 100 μl 1 M ethanolamine (pH 8.0) per ml gel were added and stirred for 60 min at room temperature to block free binding sites. The gel was then washed with PBS buffer (pH 7.4), containing 1 mM PMSF, to remove methanol and uncoupled ligand, degassed for 30 min and packed in a column. The column was equilibrated with starting buffer (PBS buffer (pH 7.4), containing 1 mM PMSF and 0.05% Nonidet P-40 or Triton X-100 or X-114 or 0.3% CHAPS or Zwittergent 3–12 or 0.5% octyl glycoside) and stored at 4°C. The coupling results were determined by HPLC, which was kindly performed by Dr. Raddatz, E. Merck, Darmstadt, FRG.

Affinity chromatography

Affinity chromatography was done at 4°C. The solubilized plasma membranes were applied to the column and allowed to penetrate. After incubation for 60 min,

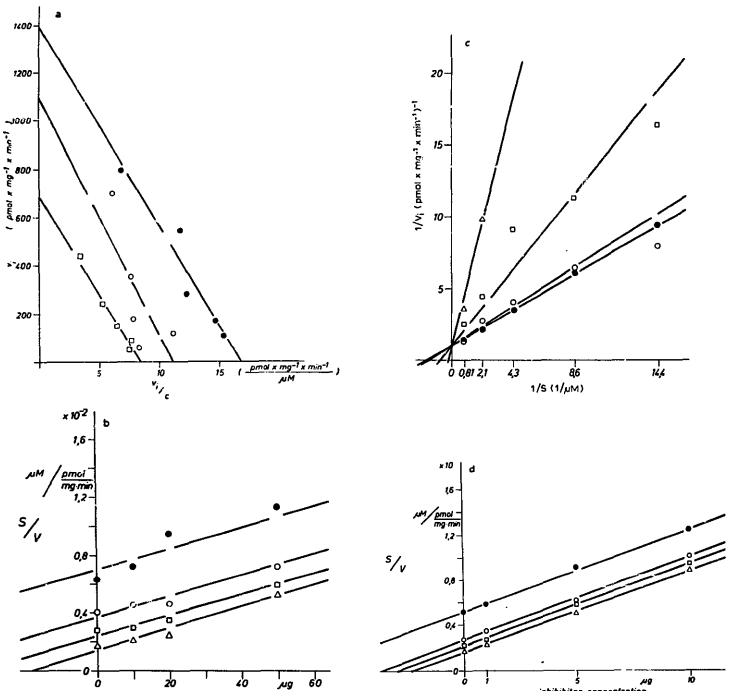

Fig. 2. Isolated rat liver cells ($2 \cdot 10^6$ cells/ml Tyrode buffer, pH 7.4) were incubated with increasing concentrations of EMD 55068 or EMD 55450 30 s prior to the addition of a mixture of radiolabeled and unlabeled cholate or taurocholate (6.9–115.2 µM cholate or 5.8–93 µM taurocholate). Initial uptake rates were determined and plotted according to Lineweaver and Burk, Eadie-Hofstee and Cornish-Bowden (21). (a) Eadie-Hofstee plot on the inhibition of cholate uptake by EMD 55068. ●——●, Control. EMD 55068: ○——○, 10 µg; □——□, 20 µg. (b) Cornish-Bowden plot on the inhibition of taurocholic acid uptake by EMD 55068. ●——●, 95.2 µM; ○——○, 38 µM; □——□, 19 µM; △——△ 5.7 µM. (c) Lineweaver-Burk plot on the inhibition of cholate uptake by 55450. ●——●, Control; ○——○, 1 µM; □——□, 10 µM; △——△, 20 µM. (d) Cornish-Bowden plot on the inhibition of taurocholic acid uptake by EMD 55450. ●——●, 95.2 µM; ○——○, 38 µM; □——□, 19 µM; △——△, 5.7 µM.

the column was washed with starting buffer (flow rate 0.1 ml/min) to remove unadsorbed material. A KCl-gradient from 0 to 1 M (flow rate 0.15 ml/min) followed to exclude unspecific binding. To further increase the specificity of the column, additional washing steps were performed with 30% ethanol, piperonyl butoxyde from 0 to 500 µM, PBS and KSCN gradient from 0 to 1 M.

Bound proteins were eluted with a taurocholate gradient from 0 to 20 mM (flow rate 0.15 ml/min). Peak fractions were pooled and concentrated with a Centricon™ microconcentrator, exclusion limit 30

kDa. The enriched proteins were examined by SDS-PAGE.

Results

Interaction of EMD 55068 or EMD 55450 with the transport of cholate, taurocholate, EMD 51921, oleic acid, serine and thiamin hydrochloride into isolated rat hepatocytes

To determine the suitability of EMD 55068 as a possible ligand for binding proteins for bile acids and linear peptides, EMD 55068 was used as inhibitor of the uptake of the above substrates by liver cells. The first step in transport is binding of the substrates to membrane proteins.

Since the free NH_2 group in EMD 55068 is used for coupling to the gel matrix, another compound EMD 55450 in which the NH_2 group was blocked by a Z-group (benzyloxycarbonyl) was also tested. This compound resembles EMD 55068 bound to the gel matrix. If the free NH_2 group is needed for binding to the binding proteins EMD 55068 would not be a suitable compound.

EMD 55068 turned out to be a competitive inhibitor of the uptake of EMD 51921 and taurocholate, whereas the uptake of cholate was non-competitively blocked (Fig. 2). EMD 55450 with the Z-group, however, competitively inhibited the uptake of both bile acids and EMD 51921 (Fig. 2). The affinity of EMD 55450 to the transport systems was 20-fold higher than that of EMD 55068 (Table I). In addition, preincubation of liver cells with EMD 55450 for 30 s or 10 min resulted in an inhibition of bile acid transport which was partly irreversible, since removal of EMD 55450 by washing the cells leads only to a 30% restoration of bile acid transport (Table II).

In contrast, the uptake of oleic acid, serine and thiamin hydrochloride was not affected by the linear peptides tested (Table II). This points to the fact that these peptides should bind to protein components of peptide and bile acid binding systems but not to those for monovalent organic cations, amino acids or long chain fatty acids.

TABLE I

Inhibition of the hepatocellular uptake of cholate, taurocholate and EMD 51921

Isolated rat hepatocytes ($2 \cdot 10^6$ cells/ml) were incubated with three or four different concentrations of EMD 55068 or EMD 55450 30 s prior to the addition of a mixture of radioactively labeled and unlabeled cholate, taurocholate or EMD 51921 as described in the Method section. The type of transport inhibition and the K_i values were determined according to Cornish-Bowden and Dixon [21,22].

Inhibitor	Substrate	Type of inhibition	K_i (μM)
EMD 55068	cholate	non-competitive	12.2
	taurocholate	competitive	26.0
	EMD 51291	competitive	22.3
EMD 55450	cholate	competitive	1.1
	taurocholate	competitive	0.8
	EMD 51921	competitive	2.2

TABLE II

Reversibility of the inhibition of the transport of taurocholate by EMD 55450

Isolated rat liver cells ($2 \cdot 10^6$ cells/ml) were preincubated for 30 s or 10 min with increasing concentrations of EMD 55450. Thereafter, cells were washed two times in fresh Tyrode buffer and uptake measurements were started by addition of 1.2 μM [^{14}C]cholate and 5.8 μM cholate. Initial uptake rates were determined.

Inhibitor	Preincubation time	% uptake of the control
EMD 55450	30 s	28 ± 1
	10 min	29 ± 3

Membrane preparation

The quality of the membrane preparation was tested by measuring marker enzyme enrichments. Na^+/K^+-ATPase, a marker for blPm, was enriched 22–26-fold, Mg^{2+}-ATPase and 5'-nucleotidase, both markers for canalicular membranes 3.2–3.5- and 3.2–3.4-fold, respectively. In all preparations an enrichment of the microsomal marker enzyme Glucose-6-phosphatase of 1.5–1.8-fold could be determined, pointing to contamination of the blPmv preparation with intracellular membranes.

Uptake of taurocholate and EMD 51921 into isolated blPmv

To test the suitability of the plasma membrane preparation for the identification of binding proteins for bile acids and linear peptides, the uptake of taurocholate and EMD 51921 into blPmv was measured. As

TABLE III

Influence of EMD 55068 and EMD 55450 on the hepatocellular uptake of oleic acid, serine and thiamin hydrochloride

The hepatocellular uptake of the above substrates was measured in the presence of the linear peptides as described in the Method section. Initial uptake rates are shown.

Substrate	Inhibitor	V_i (pmol/mg per min)	
		control	plus 100 μM peptide
Thiamin hydrochloride	EMD 55068	39.3 ± 1	37.7 ± 2
	EMD 55450	40.3 ± 2	39 ± 2
Oleic acid	EMD 55068	418.3 ± 32	469 ± 42
	EMD 55450	420 ± 18	430 ± 22
Serine	EMD 55086	215 ± 23	220 ± 25
	EMD 55450	212 ± 20	218 ± 19

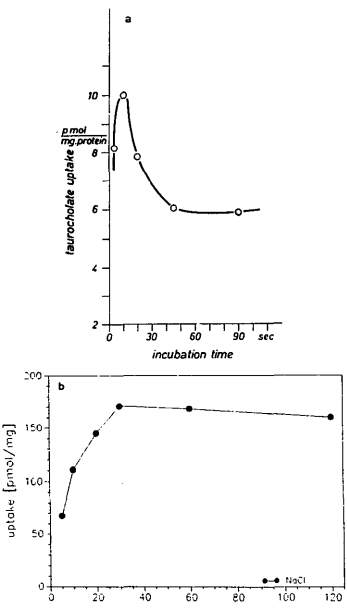

Fig. 3. Basolateral membrane vesicles (50–80 μg) were incubated with 1 μM [^{3}H]taurocholate or 3 μM [^{3}H]EMD 51921. At the times indicated the uptake of the substrates was measured by a rapid filtration technique [23]. In the case of EMD 51921 a hydrophilic membrane was used (Durapore hydrophil from Millipore). (a) Uptake of taurocholate into isolated blPmv. (b) Uptake of EMD 51921 into isolated blPmv.

can be seen in Fig. 3a, the vesicle preparation showed overshooting taurocholate uptake. In contrast, the uptake of EMD 51921 was not stimulated above equilibrium values, even in the presence of an inwardly directed sodium gradient (Fig. 3b). This agrees with studies in isolated rat liver cells [5].

Separation of membrane-associated and integral membrane proteins

The plasma membrane bilayer carries integral membrane spanning and associated peripheral proteins. Binding proteins in both fractions needed to be investigated. Therefore, the blPmv were dialysed against 10 mM EDTA, pH 8.0 to separate associated proteins from integral proteins. In addition, EDTA serves as a proteinase inhibitor. Of the initial plasma membrane proteins 27–30% could be extracted by EDTA. The integral part of the membrane, accounting for 70–73% of the protein content, had to be solubilized with detergents for further processing. As shown in earlier studies using chemically reactive bile acid analogs, labeled proteins with molecular masses of 67 and 37 kDa could be extracted with EDTA [14–16]. Alternatively, hydrophilic proteins were separated from hydrophobic proteins by Triton X-114 phase separation. In this case, 26% of the proteins were found in the hydrophilic fraction.

Solubilization of isolated EDTA extracted blPmv

EDTA extracted blPmv were solubilized with different detergents. Since it is known that detergents with a hydrophilic-lipophilic balance (HLB) of 12–14.5 are best for extracting and solubilizing integral membrane proteins without denaturation, we investigated the solubilizing effects of the following detergents: Triton X-100 (HLB 13.3), Triton X-114 (HLB 12.4), Nonidet P-40 (HLB 13.1) and octyl glycoside. The zwittergents CHAPS and Zwittergents 3–12 were used since these detergents have a high CMC (< 1 mM) and therefore their removal will be easy.

To follow the efficiency of the individual detergent in solubilizing proteins which might be involved in binding of peptides and bile acids, [^{14}C]ABATC-labeled blPmv were solubilized with the detergents at a protein to detergent ratio of 1 : 10.

TABLE IV

Solubilization of blPmv

Isolated [^{14}C]ABATC-labeled blPmv from rat liver (2 mg/ml) in PBS buffer were solubilized with different detergents as described in the Method section. The amount of solubilized protein was determined by the method of Lowry [30]. The radioactivity in the soluble fraction was determined in a Packard Tricarb 2660 scintillation counter.

Detergent	% Solubilization yield	
	protein	radioactivity
CHAPS	76 ± 14	85 ± 7
Zwittergent 3-12	89 ± 1.5	77 ± 5
Triton X-100	80 ± 5	80 ± 10
Triton X-114	62 ± 2.8	68 ± 13
Nonidet P-40	65 ± 14	83 ± 8
Octyl glycoside	70 ± 12	85 ± 7

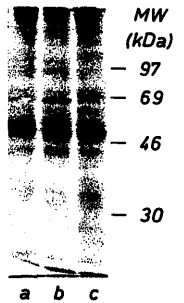

Fig. 4. Fluorographic visualization of [^{14}C]ABATC-labeled proteins in the fraction of solubilized proteins. Photoaffinity labeled rat liver membrane proteins were solubilized with the detergents indicated. The proteins in the supernatant after 100000× g centrifugation were separated on 10% SDS-PAGE. The labeled proteins are visualized by fluorography. a, Nonidet P-40; b, Zwittergent 3–12; c, in this case proteins were first solubilized with octyl glycoside and then photolabeled with [^{14}C]ABATC.

As shown in Table IV all detergents used succeeded in solubilizing the integral membrane proteins. The yield of solubilized proteins varied between 62 and 89%. The amount of radioactivity in the soluble fraction varied between 70 and 80%. As visualized in a fluorogram, labeled proteins with molecular masses of 48–50, 52–34, 43 and 67 kDa could be found in the soluble fraction after detergent solubilization. We have shown in earlier studies [15,16] that labeling of these membrane proteins is also possible after Nonidet P-40 and Triton X-100 solubilization. The same is true for octyl glycoside, CHAPS and Zwittergent 3–12 (Fig. 4).

Testing of suitable gel matrices

The following gel matrices for affinity chromatography were tested in order to find a gel matrix with low unspecific interactions with the solubilized proteins: CNBR-activated Sepharose, Epoxy-activated Sepharose, Affi-Gel 10 and 15.

The columns without bound ligand were used for chromatography of solubilized proteins. In the case of Sepharose matrices and Affi-Gel 15, unspecific bound proteins could be detected in a KSCN eluate. In contrast, using Affi-Gel 10 no unspecific bound proteins were found. Therefore, Affi-Gel 10 was used as gel matrix for affinity chromatography.

Coupling of EMD 55068 to Affi-Gel 10

EMD 55068 was coupled to Affi-Gel 10 under anhydrous conditions. After solubilization of EMD 55068 in methanol, 17 μmol of ligand were added per ml of gel matrix. The coupling reaction was performed at room temperature or at 37°C overnight. Unreacted groups were blocked by addition of ethanolamine. Determination of the amount of bound ligand per ml gel was carried out by indirect evaluation. With HPLC, the amount of the ligand recovered in the liquid phase and the pooled washings after finishing coupling was determined to be 3.6 mg/ml at 37°C and 2.16 mg/ml at room temperature. Therefore, all further coupling was performed at 37°C.

Affinity chromatography of EDTA extracted detergent solubilized bPmv proteins on EMD 55068 affinity columns

To exclude any unspecific reactions of the solubilized proteins with the gel matrix, especially with the spacer arm, proteins were first chromatographed on Affi-Gel 10 columns without ligand. The flow-through

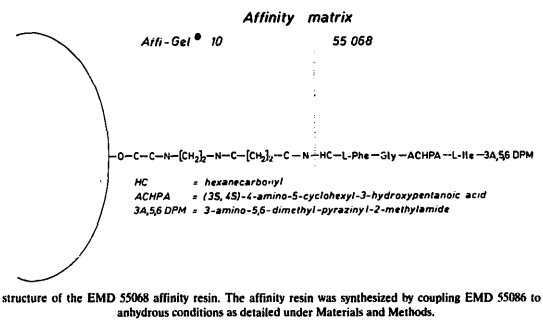

Fig. 5. Chemical structure of the EMD 55068 affinity resin. The affinity resin was synthesized by coupling EMD 55086 to Affi-Gel 10 under anhydrous conditions as detailed under Materials and Methods.

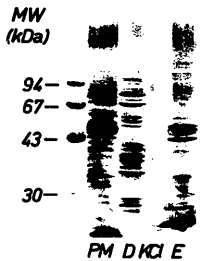

Fig. 6. Affinity chromatography of Nonidet P-40 solubilized integral rat liver blPm proteins. Shown is a silver-stained SDS gel (10%). Membrane associated proteins were removed by EDTA extraction. The residual integral membrane proteins were then solubilized with Nonidet P-40 as described in Methods. PM, Plasma membrane proteins solubilized with Nonidet P-40; D, flow through fraction; KCl, proteins eluted with a KCl gradient; E, proteins bound to the affinity-column and eluted with taurocholate.

fraction was forwarded to the EMD 55068 affinity column. For efficient adsorption the column and the sample were equilibrated for 1 h before starting the washing procedure. The column was washed with starting buffer. Thereafter several elution steps were performed as described in the methods section. Specific bound proteins were desorbed using a competitive inhibitor of the uptake of hydrophobic linear peptides,

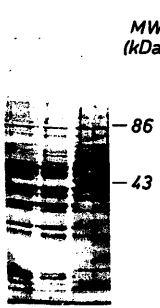

Fig. 7. Affinity chromatography of Zwittergent 3-12 solubilized integral rat liver blPm proteins. Shown is a silver stained SDS-PAGE (10%, 10 μg protein per lane). a, Plasma membranes solubilized with Zwittergent 3-12; b, flow through fraction; c, proteins bound to the affinity-column and eluted with taurocholate.

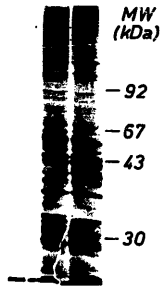

Fig. 8. Affinity chromatography of Nonidet P-40 solubilized rat AS-30D ascites hepatoma cell membrane proteins, shown on SDS-PAGE (10%), silver stained. Hepatoma cell membrane proteins were separated in membrane associated and integral proteins by EDTA extraction. The integral proteins were then solubilized with Nonidet P-40. a, Proteins bound to the affinity-column; b, flow through fraction; c, solubilized plasma membrane proteins from AS-30D ascites hepatoma cells.

taurocholate. The use of the ligand EMD 55068 itself was not possible because of solubility problems.

Using a 0–20 mM taurocholate gradient a fraction of proteins could be eluted. As shown in the SDS-PAGE four proteins with molecular masses of 46 ± 2, 49 ± 2, 53 ± 1 and 56 ± 3 kDa were enriched in this fraction (Fig. 5–7). This protein pattern could be found after chromatography of Nonidet P-40, Triton X-100, CHAPS, octyl glycoside as well as Zwittergent 3–12 solubilized proteins.

In contrast, using EDTA extracted Nonidet P-40 solubilized plasma membranes from AS-30D ascites hepatoma cells, which are unable to transport bile acids and linear hydrophobic peptides, no proteins in this molecular weight range could be eluted (Fig. 8).

Affinity chromatography of hydrophilic proteins

Chromatography of membrane associated hydrophilic proteins extracted with EDTA on EMD 55068 affinity columns did not result in the detection of binding proteins. In contrast, in the Triton X-114 phase separated hydrophilic protein fraction binding proteins with molecular masses of 46, 49, 53 and 56 kDa were found (Fig. 9).

Discussion

From kinetic studies it became evident that several linear hydrophobic renin-inhibitors are cleared from

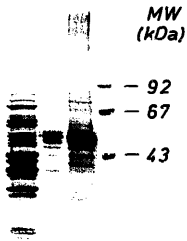

Fig. 9. Affinity chromatography of hydrophobic and hydrophilic rat liver plasma membrane proteins. Shown is a silver-stained SDS-PAGE (10%), 10 μg protein per lane. BlPm proteins were solubilized with Triton X-114. Hydrophilic proteins were separated from hydrophobic ones by phase separation as described in Methods. a, Flow through fraction of hydrophilic proteins; b, hydrophilic proteins bound to the affinity-column and eluted with taurocholate; c, hydrophobic proteins bound to the affinity-column and eluted with taurocholate.

the circulation via an energy-dependent carrier-mediated uptake mechanism, which is related to bile acid transporters. The linear peptides tested here inhibited the uptake of taurocholate in a competitive manner; the inhibition of the uptake of cholate, however, was often non-competitive. A further difference in the kinetics is the sodium-dependence which was not found in the case of linear hydrophobic peptides. Therefore, to further evaluate our working hypothesis of common transport systems for bile acids and peptides, we tried to identify binding proteins for these peptides in isolated blPmv from rat liver. Since no photoreactive analogs were available, we questioned if the affinity of the hydrophobic peptides to the blPmv proteins is high enough to identify binding proteins by affinity-chromatography.

For this purpose, two linear hydrophobic renin-inhibitors (EMD 55068 and EMD 55450) were synthesized and the affinity of those compounds to the transport systems was tested. Although our main interest is in binding proteins we investigated the effect of the peptides on the transport of bile acids and peptides. We believe this is legitimate, since binding is the first event in the transport of substrates across a membrane. When binding is influenced, transport is also altered. On the other hand, the binding proteins identified, may also be involved in transport of the substrates. Whereas the peptides had no affinity to the uptake systems for amino acids, long chain fatty acids and monovalent organic cations, the affinity to the bile acid and linear peptide transporters was high (in the range of 10^{-6} M). As was shown in earlier studies [6], transport inhibition by EMD 55068 in the case of taurocholate was competitive. EMD 55068, the peptide with a free NH_2 group, however, non-competitively inhibited the uptake of cholate. Blocking the NH_2 group with a hydrophobic residue (Z-group: benzyloxycarbonyl) in EMD 55450, making the whole compound more lipophilic, increased the affinity to the transport system and changed the type of cholate transport inhibition to competitive. This peptide, EMD 55450 with the Z-group, resembles 55068 bound to the affinity matrix. The free amino group, therefore, is not needed for binding of the peptide to the proteins. In addition, 30% of the inhibition of bile acid transport by EMD 55450 was irreversible. This phenomenon might be due to binding of the compound to proteins, but also to the lipid phase of the membrane. It is difficult to remove the lipid-bound fraction of EMD 55450 even by careful washing of the cells. The lipid-bound fraction may change the fluidity of the membrane and alter transport functions [31]. A tight protein binding can complicate the elution of the proteins bound to the affinity matrix.

The membrane preparation used is able to transport taurocholate and EMD 51921. The blPmv fraction, however, is contaminated with intracellular membranes, especially from the ER fraction. To lower contaminants from ER proteins in the fraction of bound proteins the column was washed with an inhibitor of cytochrome *P*-450 isoenzymes, piperonyl butoxyde, after affinity adsorption. Electrophoresis of the concentrated eluted fraction gave negative results. The same was found for a KCl eluate, which should release any proteins bound by nonspecific ionic interactions. Only in the taurocholate eluate protein bands could be detected in SDS-PAGE. The minimal concentration needed for elution of proteins was 2 mM which is below the critical micellar concentration of the compound. For total removal of all proteins bound to the affinity-gel the concentration of taurocholate had to be increased to 10–20 mM. The question therefore remains whether this is a specific elution or a detergent effect. Nevertheless, a specific enrichment in four proteins is seen.

Since binding proteins might belong to cell associated hydrophilic proteins as well as to the integral part of the membrane we separated both fractions. With EDTA, hydrophilic proteins bound to the membrane via calcium are extracted. With Triton X-114 hydrophobic and hydrophilic proteins are separated. Although, 30% of the membrane proteins could be extracted from the integral part of the membrane by both methods and although the pattern of the proteins in SDS-PAGE was comparable, the affinity chromatographic results were different. In the EDTA-extracted

protein fraction no binding proteins could be identified. In contrast, after Triton X-114 phase separation hydrophilic proteins with molecular masses of 46, 49, 53, 56 kDa were bound to the affinity-gel and eluted with a taurocholate gradient. Interestingly, proteins in the same molecular weight range bind to the affinity column after chromatography of detergent solubilized integral membrane proteins. At present it is not fully understood why these proteins are found in both fractions. On the other hand, for albumin, a soluble protein, binding sites for Triton X-100 were described [32].

The specificity of the proteins bound to the affinity matrix becomes evident by comparison of the protein pattern after chromatography of membrane proteins from AS-30D ascites hepatoma cells and blPmv. In hepatoma cell membranes, which do not transport linear peptides, proteins in the molecular mass range of 25–35 kDa are bound, whereas no proteins could be detected in the range of 45–60 kDa. The proteins in the range of 46–56 kDa, however, are labeled with photoreactive bile acid analogs pointing to the fact that they may be components of the transport system.

With the 55068-affinity matrix, binding proteins for linear peptides can be identified in solubilized blPmv irrespective of the detergent used. Further studies are needed for isolation and functional reconstitution of these proteins.

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