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Bile acid binding proteins in hepatocellular membranes of newborn and adult rats. Identification of transport proteins with azidobenzamidotauro[^{14}C]cholate ([^{14}C]ABATC)

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Neonatal hepatocytes are less active in uptake of bile acids than are mature hepatocytes. This phenomenon has been further investigated by transport studies with azidobenzamidotaurocholate (ABATC). Taurocholate, cholate and the photolabile ABATC were taken up by liver cells of adult rats by a sodium-dependent and by an additional sodium-independent mechanism. In the dark, ABATC inhibited the uptake of taurocholate and cholate. Taurocholate decreased the transport of ABATC in a competitive manner, both in the presence and absence of sodium. In neonatal hepatocytes the V_{max} for taurocholate and for ABATC was similar but was lower than in mature liver cells. In contrast, the K_m was similar for neonatal and mature hepatocytes. For identification of binding proteins in both kinds of cells ABATC was photolysed after preincubation with isolated hepatocytes. Under our experimental conditions (single ultraviolet flash) about 80% of the azido groups was converted to nitrene. The covalently binding nitrene derivative inhibited bile salt transport irreversibly. Photolabeling of intact hepatocytes or of isolated plasma membranes with ABATC resulted in radioindication of membrane proteins with 67, 60, 54, 50 and 43 kDa in mature plasma membranes but of proteins with masses of 67, 54, 43 and 37 kDa in neonatal basolateral membranes. The 50 kDa protein is largely lacking in membranes of 9-day-old rats. The process of photolabeling itself was sodium-independent when isolated cells were treated with ABATC. In contrast, the degree of labeling of intact hepatocytes was markedly reduced in the absence of sodium and chloride. 100-fold molar excess of taurocholate, benzamidotaurocholate (BATC), phalloidin or cyclosomatostatin protected isolated plasma membranes against coupling of ABATC. Photolabeling of hepatoma cells known to be deficient in bile salt transport did not result in radiomodification of membrane proteins.

Introduction

The hepatocellular uptake of bile acids is a carrier-mediated process [1–3] driven in part by an inwardly directed sodium gradient [4–5] which is maintained by the Na^+/K^+-ATPase localized at the basolateral membrane of hepatocytes [6]. Bile acid uptake is reduced in sodium-free and chloride-free medium [4,7]; in contrast, bile acid binding to isolated plasma membranes was shown to be sodium-independent [8,9]. Kinetic studies indicate that apart from bile acids, some other organic anions (DIDS in Refs. 10 and 11; iodipamide in Refs. 12–14) and also certain neutral cyclopeptides (antamanide, Ref. 15; somatostatins, Ref. 16; phalloidin, Ref. 17) are recognized as substrates of the sodium-dependent bile acid carrier. The above transport system was therefore termed 'multispecific transporter' [16]. It is immature in newborn rats. The uptake of bile acids in isolated hepatocytes of neonatal rats [18] is lower than that in adults. The uptake of phalloidin by liver cells increases during the maturation of the organ [19,20]. Protein components of the above system were identified by affinity [21–24] or by photoaffinity labeling [25,26]. Proteins with masses of 67, 54, 50 and 37 kDa were labeled in most of these studies. A main drawback of earlier photoaffinity labeling studies [25,26] was the long ultraviolet irradiation needed for photolysis of the labels (10 min). During this period, sensitive proteins may have been damaged. These problems were overcome by the introduction of a high-energy ultraviolet flash technique [27].

The aim of the study presented was the localization of the transport system responsible for bile acid and

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xenobiotic transport in liver parenchymal cells of adult and newborn rats by photoaffinity labeling using ultraviolet flashed light. The kinetics and inhibition properties of the photolabile taurocholate analog ABATC were compared with those of taurocholate and cholate. The dependence of photoaffinity labeling of isolated hepatocytes on sodium and chloride was investigated. The specificity of photoaffinity labeling with ABATC was demonstrated by protection studies and by labeling of AS 30 D ascites hepatoma cells, known to be bile acid transport-deficient.

Materials

[^{14}C]Cholic acid (sp. act. 1.85–2.2 GBq/mmol) and Amplify were purchased from Amersham Buchler, Braunschweig, F.R.G. Phenylmethylsulfonyl fluoride, acrylamide, *N,N'*-methylenbisacrylamide, Coomassie blue G, EDTA and SDS were from Serva Heidelberg, F.R.G. and [^{3}H]taurocholate (spec. act. 1.85–2.2 GBq/mmol) from New England Nuclear, Dreieich, F.R.G. Mercaptoethanol, iodacetamide and glycine were from Merck, Darmstadt, F.R.G.; leupeptin was from Sigma, Munich and benzamidine was from Fluka, Switzerland. All other chemicals were of at least analytical grade purity.

Methods

Isolation of liver parenchymal cells

Hepatocytes from adult male Wistar rats (250 g) were isolated according to the method of Berry and Friend [28] and from 9-day-old rats as described earlier [29], by perfusion of rat liver with 0.05% collagenase in a Ca^{2+}-free Krebs-Henseleit buffer. After equilibration in Tyrode buffer (137 mM NaCl/2.7 mM KCl/1 mM $MgCl_2$/12 mM $NaHCO_3$/5 mM glucose/0.42 mM NaH_2PO_4/1.8 mM $CaCl_2$) for 30 min, viability was tested by Trypan blue exclusion. Of the isolated cells 85–90% excluded the dye. Cells were used for experiments within 2 h after isolation.

Preparation of AS 30 D ascites hepatoma cells

AS 30 D 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 250 × g.

Uptake studies

Cholate, taurocholate and ABATC uptake by isolated hepatocytes was studied in the presence or absence of sodium in a Tyrode buffer (sodium-free Tyrode buffer: sodium was replaced by 137 mM LiCl/12 mM $LiCO_3$). The cellular uptake of the substrates was determined by a rapid centrifugation technique through a silicon oil layer [30]. Cells, $2 \cdot 10^6$/ml in Tyrode buffer, were incubated in the dark without (control) or with increasing concentrations of inhibitors (ABATC or taurocholate) 30 s before the addition of a mixture of 5.7–230 μM cholate plus 1 μM [^{14}C]cholate or 4.8–97 μM taurocholate and 1 μM [^{3}H]taurocholate or 3.6–73 μM and 1 μM [^{14}C]ABATC. During the period of linear uptake the initial rate was determined.

Isolation of liver plasma membranes from adult and newborn rats

Liver plasma membranes enriched in the basolateral fraction were isolated from newborn and adult rats according to the method of Touster [31]. All buffer solutions contained the following proteinase inhibitors: PMSF 0.5 mM/EDTA 5 mM/leupeptin 5 μg/ml/benzamidine 1 mM. The activity of Na^+/K^+-ATPase (a marker for basolateral plasma membranes) was enriched 22-fold, whereas Mg^{2+}-ATPase (a marker for canalicular membranes) was enriched 2-fold compared with the crude homogenate. Glucose-6-phosphatase was enriched 2-fold showing minor contaminations of the plasma membrane fraction with subcellular organelles.

Photoaffinity labeling

Isolated hepatocytes and isolated basolateral plasma membranes were photoaffinity labeled as described [27]. Briefly, ABATC was photolysed by a single ultraviolet flash. To determine the irreversible inhibition of taurocholate uptake by ABATC, isolated hepatocytes were incubated ($0.6 \cdot 10^6$ cells/ml Tyrode buffer) with ABATC (1–100 μM) for 5 min in the dark. After photolysis (one single ultraviolet flash) the unbound ABATC was removed by washing (three times in 5 ml of Tyrode buffer) and uptake of taurocholate was measured. For identification of binding proteins, isolated rat liver parenchymal cells or basolateral membranes from adult or newborn rats were incubated for 1–30 min in the dark at 37 °C with [^{14}C]ABATC before photolysis. After excessive washing in Tris-HCl buffer (plus 1 mM PMSF/1 mM benzamidine/5 mM EDTA/5 μg/ml leupeptin/0.1 mM iodacetamide), the proteins were solubilized with 1% SDS/1% dithiothreitol at 95 °C for 5 min and separated by SDS-polyacrylamide electrophoresis. The radiolabeled proteins were visualized by fluorography using Amplify as intensifying fluorochrome. Bound radioactivity was quantified by slicing SDS rod gels and by determining the protein-associated radioactivity by liquid scintillation counting. The protein content was determined according to the method of Lowry [32], using bovine serum albumin as standard.

To evaluate the sodium- or chloride-dependency of the photoaffinity labeling, isolated hepatocytes were incubated in sodium-free buffer of the following composition: 137 mM LiCl/2.7 mM KCl/1.05 mM $MgCl_2$/1.8 mM $CaCl_2$/12 mM $LiCO_3$/5.55 mM glucose/0.42 mM KH_2PO_4 (pH 7.4) or in sodium- and

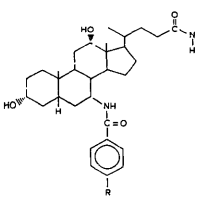

Fig. 1. Structure of the photolabile bile acid analog ABATC and its precursor BATC.

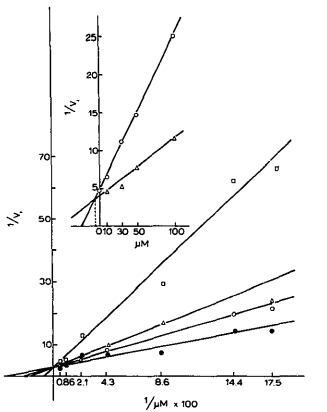

Fig. 2. Lineweaver-Burk plot of the sodium-dependent uptake of cholate in the presence of ABATC. The effect of ABATC (○) 30 µM; (△) 50 µM; (□) 100 µM on the uptake of cholate was analyzed in the presence or in the absence of sodium. V_i values were calculated within the period of linear uptake. The sodium dependency was evaluated by subtraction of V_i values for the uptake in lithium-Tyrode buffer from those in sodium-Tyrode buffer. The type of transport inhibition was determine dby plotting the data according to Lineweaver and Burk. $n = 3$. $K_m = 28$ µM; $V_{max} = 454.5$ pmol/mg per min. y scale: $1/V_i = (1/\text{pmol per mg per min}) \times 1000$. x scale: $(1/\mu M) \times 100$. The inset shows the Dixon plot of the inhibition by ABATC of the sodium-dependent cholate transport. The inhibition of 10, 30, 50 and 100 µM of ABATC on the sodium-dependent uptake of cholate ○, 11.5 µM; △, 23 µM was measured. Initial uptake rates were calculated and plotted according to Dixon. $n = 3$. $K_i = 5$ µM.

chloride-free buffer of the following composition: 140 mM K gluconate/1.05 mM magnesium gluconate/1.8 mM calcium gluconate/12 mM $KHCO_3$/5.55 mM glucose/0.42 mM KH_2PO_4.

Hepatocytes were incubated for 2 · 10 min in the above buffers followed by renewal of the buffer medium before incubation with [^{14}C]ABATC. Control cells were incubated in sodium Tyrode buffer.

Results

Comparison of kinetic properties of bile acid (taurocholate and cholate) and azidobenzamido-taurocholate uptake in isolated hepatocytes of adult and newborn rats

Hepatocytes from adult rats take up taurocholate, cholate and the photoreactive analog ABATC (Fig. 1) in a time- and concentration-dependent manner. Sodium-dependency was analyzed at 37° C in a lithium-Tyrode buffer. K_m and V_{max} values were calculated from Lineweaver-Burk plots [33] (Table I). Both the uptake of bile acids and the photoreactive derivative are mediated by sodium-dependent and -independent mechanisms in isolated hepatocytes of adult rats. In isolated liver cells of 9-day-old rats the total uptake, i.e., the uptake in the presence of sodium, was measured. Taurocholate and ABATC are taken up by those cells, but the V_{max} values are lower than in adult controls. In contrast, the K_m values are not different than those of adults. These results are in agreement with earlier studies of Suchy et al. [18] using taurocholate.

TABLE I

Kinetic parameters of the uptake of bile acids and ABATC in isolated hepatocytes of newborn and adult rats

Kinetic parameters were calculated from Lineweaver-Burk plots (n = 3). Standard deviations were calculated by linear regression analysis. Experiments with hepatocytes of newborn rats in sodium-Tyrode buffer; with hepatocytes of adult rats uptake was measured in sodium- as well as in lithium-Tyrode buffer.

	K_m (µM)	V_{max} (pmol/mg per min)
Hepatocytes from adult rats		
Taurocholate		
Na^+-dependent	25 ± 2	1929 ± 484
Na^+-independent	50 ± 13	550 ± 65
Cholate		
Na^+-dependent	27 ± 8	487 ± 58
Na^+-independent	91 ± 64	888 ± 96
ABATC		
Na^+-dependent	14 ± 2	562 ± 88
Na^+-independent	60 ± 12	350 ± 30
Hepatocytes from newborn rats		
Taurocholate	18 ± 7	515 ± 30
ABATC	16 ± 3	200 ± 25

Mutual inhibition of hepatocellular uptake of taurocholate, cholate and ABATC by isolated adult rat hepatocytes in the presence and absence of sodium

In the dark, ABATC reversibly inhibited the uptake of both taurocholate and cholate by isolated hepatocytes of adult rats in both the presence and in absence of sodium. The type of uptake inhibition was analyzed according to Lineweaver and Burk. In Figs. 2 and 3 the effect of ABATC on the sodium-dependent uptake of cholate and taurocholate is shown. The common intercept of the curves at the ordinate indicates a competitive type of transport inhibition (K_m of the control 28 μM; V_{max} 454.5 pmol/mg per min for cholate; and K_m 25 μM, V_{max} 1587 pmol/mg per min for taurocholate). ABATC is also a competitive inhibitor of the sodium-independent route of taurocholate uptake (Fig. 4): K_m 66.6 μM, V_{max} 625 pmol/mg per min.

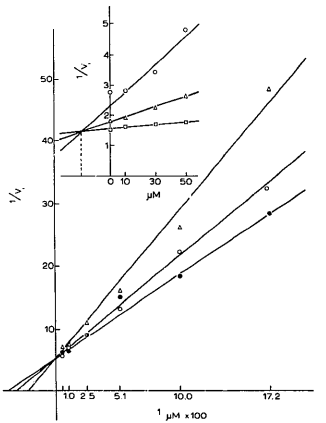

Fig. 3. Lineweaver-Burk diagram of the sodium-dependent uptake of taurocholate in the presence of ABATC. Isolated hepatocytes ($2 \cdot 10^6$ cells/ml sodium-Tyrode or lithium-Tyrode buffer) were preincubated for 30 s in the absence (●) or presence of 30 μM (○) or 50 μM (△) of ABATC before addition of a mixture of 1 μM [^{3}H]taurocholate plus 4.8–97 μM of taurocholate. Initial uptake rates were calculated for the sodium-dependent uptake and the data plotted according to Lineweaver and Burk. $n = 3$. $K_m = 25$ μM; $V_{max} = 1587$ pmol/mg per min. y scale: $1/V_i = (1/\text{pmol per mg per min}) \times 1000$. x scale: $(1/\mu M) \times 100$. The inset shows the Dixon plot of the inhibition by ABATC of the sodium-dependent taurocholate transport. The inhibitory effect of 10, 30 and 50 μM of ABATC on the sodium-dependent taurocholate uptake (○, 5.8 μM; △, 9.7 μM; □, 19.4 μM) was evaluated. Initial uptake rates were calculated and plotted according to Dixon. $n = 3$. $K_i = 20$ μM.

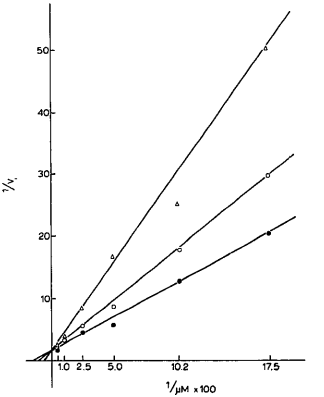

Fig. 4. Lineweaver-Burk diagram of the sodium-independent uptake of taurocholate in the presence of ABATC. The effect of ABATC (○) 30 μM; (△) 50 μM on taurocholate uptake was analyzed in the absence of sodium (replaced by lithium). Initial uptake rates were calculated and the data plotted according to Lineweaver and Burk. $n = 3$. $K_m = 66$ μM; $V_{max} = 625$ pmol/mg per min. y scale: $1/V_i = (1/\text{pmol per mg} \times \text{min}) \times 1000$. x scale: $(1/\mu M) \times 100$.

K_i values were determined according to Dixon [34] to be 5 μM for the sodium-dependent cholate uptake (inset to Fig. 2) and 20 μM (inset to Fig. 3) for the sodium-dependent taurocholate uptake. In a similar fashion, taurocholate blocks the sodium-dependent uptake of ABATC (Fig. 5) with a K_i of 25 μM (inset of Fig. 5).

Photoinactivation of taurocholate transport by ABATC

By flash photolysis of the azido group of ABATC a covalently binding nitrene is generated. This is evident by the lowered absorption maximum of the azido group at 267 nm (Fig. 6) after a single ultraviolet flash. Taurocholate uptake was irreversibly inhibited to 50% by 25 ± 3 μM of ABATC.

Photoaffinity labeling of isolated liver plasma membranes from adult and newborn rats

In isolated liver plasma membranes of adult rats [^{14}C]ABATC (10 μM) preferentially binds to plasma membrane proteins with masses of 67, 60, 54, 50 and 43 kDa (4% S.D.). In the experiment shown (Fig. 7) an additional 56 kDa protein was labeled, whereas a 37 kDa protein which was usually labeled with chemically reactive bile acid analogs (see Refs. 21–24) was not

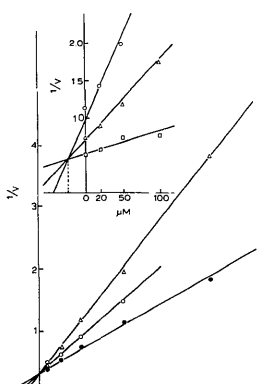

Fig. 5. Lineweaver-Burk plot of the sodium-dependent uptake of ABATC in the presence of taurocholate. The sodium-dependent uptake of ABATC (3.6–73 µM) was analyzed in the presence of 20 µM (○) and 50 µM (△) of taurocholate. V_i values were calculated. The type of transport inhibition was determined according to Lineweaver and Burk. $n = 3$; $K_m = 17.2$; $V_{max} = 625$ pmol/mg per min. y scale: $1/V_i = (1/\text{pmol per mg per min}) \times 100$. x scale: $(1/\mu M) \times 100$. The inset shows the Dixon plot of the inhibition by taurocholate of the transport of ABATC. The inhibition of the sodium-dependent uptake of ABATC (7.3 µM (○); 14.6 µM (△); 29.3 µM (□) by 20, 50 and 100 µM of taurocholate was measured. The K_i value was evaluated according to Dixon. $n = 3$; $K_i = 25$ µM.

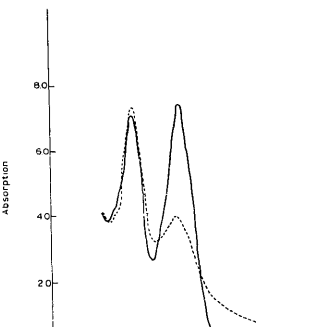

Fig. 6. Spectral changes of ABATC by treatment with a single ultraviolet flash. Absorption spectra of ABATC before (———) and after (– – – – –) one ultraviolet flash. The peak at 267 nm is reduced to 30% of the control after flash photolysis.

detected. In contrast, in newborn rat liver plasma membranes, proteins with masses of 67, 60, 54, 43 and 37 kDa were identified. The 50 kDa protein was evident only by shadowy label and was also lacking in the protein-stained electropherogram.

Photoaffinity labeling in sodium- and chloride-free buffer

The uptake of bile acids and the photolabile analog ABATC by isolated hepatocytes is sodium-dependent. In sodium-free buffer, e.g., lithium-Tyrode, cholate, taurocholate and ABATC, uptake was reduced. When isolated liver parenchymal cells were labeled with ABATC either in sodium- or lithium-Tyrode buffer, no difference of photoaffinity labeling was observed (Fig. 8a). Nevertheless, ABATC was shown to be a competitive inhibitor of taurocholate uptake in sodium-free buffer. In contrast, by excluding sodium plus chloride from the buffer (replacement by gluconate, see Methods) the labeling of hepatocellular plasma membrane proteins was significantly reduced (Fig. 8b).

Protection of photoaffinity labeling of isolated plasma membranes by an excess of taurocholate, benzamidotaurocholate, phalloidin or by a cyclic somatostatin analog (008)

Plasma membrane proteins (molecular masses 67, 60, 54, 50, 43 and 37 kDa) were protected against binding

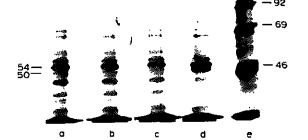

Fig. 7. Photoaffinity labeling of isolated plasma membranes from adult and newborn rats. Plasma membranes were isolated from adult and 9-day-old rats according to the method of Touster [31]. 0.6 mg protein per ml phosphate-buffered saline (pH 7.4) was incubated in the dark for 3 min with 2 µM of [^{14}C]ABATC before flash photolysis. Unbound label was removed by washing in Tris-HCl buffer in the presence of proteinase inhibitors (for details, see Methods). Proteins (100 µg) were separated on SDS polyacrylamide slab gels. Labeled proteins were visualized by fluorography. Lanes a–c present labeled proteins in three separate plasma membrane preparations from 9-day-old rats. d represents labeled proteins in isolated plasma membranes of adult rats. e represents standard proteins: 92 kDa phosphorylase; 69 kDa albumin; 46 kDa ovalbumin.

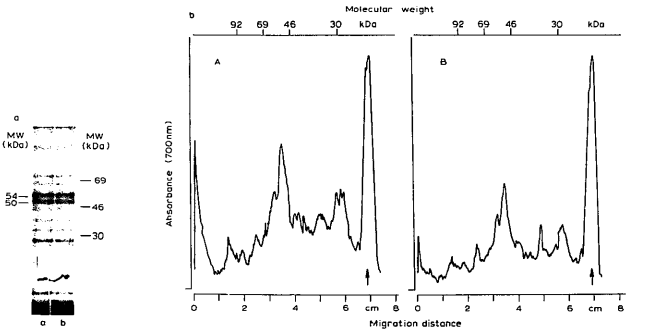

Fig. 8. (a) Photoaffinity labeling of isolated hepatocytes in sodium or lithium-Tyrode buffer. Isolated hepatocytes ($0.6 \cdot 10^6$ cells/ml sodium-Tyrode (a) or lithium-Tyrode (b) buffer) were incubated for 5 min with 20 μM of [^{14}C]ABATC before flash photolysis. Unbound label was removed by washing the cells in Tris-HCl buffer in the presence of proteinase inhibitors (for details, see Methods). Proteins (100 μg) were separated on 10% polyacrylamide slab gels. Visualization by fluorography. (b) Photoaffinity labeling of isolated hepatocytes in sodium and chloride-free buffer. Isolated hepatocytes were photolabeled in sodium-Tyrode (panel A) or gluconate buffer (panel B) (for details, see Methods). A scan of a fluorogram of an SDS slab gel is shown.

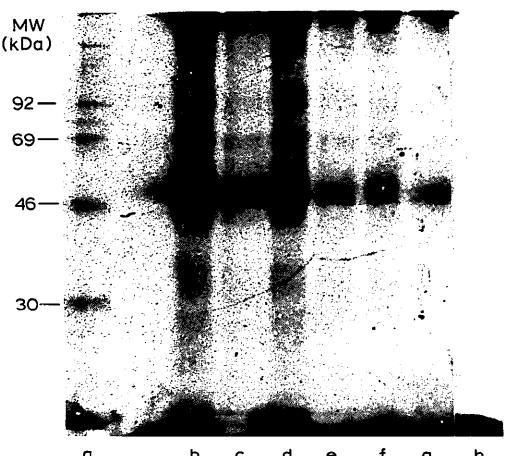

Fig. 9. Protection of plasma membrane proteins against photoaffinity labeling with ABATC. Plasma membranes (0.6 mg/ml phosphate-buffered saline) were preincubated for 3 min in the dark with 200 μM of taurocholate, cholate, BATC, 008 or phalloidin before the addition of 2 μM of [^{14}C]ABATC. The label was photolyzed by a single ultraviolet flash. After removal of unbound label, proteins were separated by SDS gel electrophoresis. A fluorogram of an SDS slab gel is shown. Lanes represent: (a) marker proteins; (b) control; (c) protection by ABATC; (d) protection by phalloidin; (e) protection by taurocholate; (f) protection by cholate; (g) protection by 008 and (h) photoaffinity labeling of AS 30 D ascites hepatoma cells.

of [^{14}C]ABATC by a 100-fold molar excess of the above-mentioned substrates. A complete or differential inhibition of binding could never be achieved. Phalloidin was less protective than taurocholate, a cyclic somatostatin analog (008) or BATC (Fig. 9). Control was 2460 ± 150 dpm in the 50–54 kDa region; in the presence of BATC: 1150 ± 65 dpm; phalloidin: 1980 ± 120 dpm; taurocholate: 950 ± 50; cholate 1000 ± 70 dpm; cyclic somatostatin analog: 940 ± 30 dpm; evaluated in sliced SDS rod gels.

Photoaffinity labeling of AS 30 D ascites hepatoma cells

As already shown in earlier studies [22,26] with certain other affinity markers, transformed hepatocytes are not specifically labeled by [^{14}C]ABATC (Fig. 9, lane h).

Discussion

The new photoaffinity label ABATC introduced in this paper was taken up by liver cells of adult rats by sodium-dependent and -independent mechanisms, as has been already shown for cholate and taurocholate [4–5]. In neonatal hepatocytes, the V_{max} value for the transport of the photosensitive analog was similar to that of taurocholate, but was lower than in mature liver cells as was also reported for taurocholate [18]. Bile acids (cholate and taurocholate) and the photosensitive taurocholate analog exert mutual competitive inhibition of the hepatocellular transport, in either the presence and absence of sodium. By flash photolysis of the azido group of ABATC, bile acid transport became irreversible. These studies show the suitability of ABATC to identify proteins of the bile acid transport system in the hepatocytes of adults as well as of newborn rats.

All substrates of the multispecific transporter so far tested are taken up less rapidly by neonatal hepatocytes than by mature liver cells [18–20,29]. The question was whether this property of basolateral membranes is due to a lower number of transport (or binding) proteins in neonatal cells, or to differences in the K_m, or to an immature energy supply of the transport system. The data shown above suggest that the 50 kDa protein always seen in bile salt transporting basolateral membranes [11,21–26] was labeled to a lesser extent with ABATC in neonatal hepatocytes. This means one of the hepatocellular membrane proteins which is undoubtably involved in bile salt transport is not fully expressed in liver cells of 9-day-old rats. AS 30 D ascites hepatoma cells, known to be completely deficient in uptake of bile acids, iodipamide and cyclosomatostatins [13,19], did not bind photolysed ABATC. Photolabeling with ABATC is therefore indicative of components of the multispecific transporter. We conclude that the 50 kDa component is expressed during the postnatal period (1–20 d). The K_m of the transport system is not different in neonatal and mature hepatocytes. This indicates that the low transport activity is more likely due to the number of available transport proteins than to qualitative differences in components of the system. A 37 kDa protein labeled with ABATC in neonatal hepatocytes appears to be lacking in plasma membranes of adult rats. At present, the interpretation of this result is difficult, since, using chemical reactive bile acid analogs (see Refs. 21–22), a 37 kDa protein could always be labeled in adult rat hepatocytes.

The binding of ABATC to the above proteins is specific as shown by protection studies, i.e., labeling in presence of other substrates of the transporter. Even phalloidin (in a 100-fold molar excess) protects proteins against binding of ABATC. In contrast, phalloidin did not inhibit binding of DIDS [11], oligobromotaurodehydrocholate [22] or isothiocyanatobenzamidocholate [23]. The identity of the binding sites for bile acids and phalloidin was demonstrated by Wieland et al. [35].

In many earlier studies, the uptake of bile acids and of foreign substrates by liver cells was found to be sodium-dependent [1–3]. One might expect that binding of photolabels derived from bile acids should also depend on the presence of sodium. Under our experimental conditions, no influence of sodium on the degree of labeling was seen. However, absence of both sodium and chloride reduced labeling with ABATC markedly. This indicates that binding of sodium to bile-acid-transporting proteins is not a prerequisite for binding of the substrate. On the other hand, it can not be excluded that in whole cells certain electrochemical conditions favor the entry of substrates into a transport channel. The uptake of various substrates of the multispecific transporter by hepatocytes depends on the membrane potential [17] and chloride participates notably in this process.

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