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Further characterization of 3′-isothiocyanatobenzamido[^{3}H]cholate binding to hepatocytes. Correlation with bile acid transport inhibition and protection by substrates and inhibitors

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Binding of 3′-isothiocyanatobenzamido[^{3}H]cholate ([^{3}H]IBCA) to hepatocytes correlates to its efficacy in inhibiting cholate uptake in isolated hepatocytes. The correlation is linear up to 20 μM [^{3}H]IBCA. Labeling of polypeptides is proportional to the degree of inhibition particularly for a protein of molecular weight 50 000. Transported substrates, competitive and non-competitive inhibitors of cholate transport protect against IBCA inhibition. Additionally binding of [^{3}H]IBCA to isolated plasma membranes is prevented by the same substrates and inhibitors of the cholate transport system. The prevention is achieved by taurocholate, iopodate, iodipamide, furosemide, BSP, cyclosporin A, and somatostatin analogs. Protection is correlated to the degree of transport inhibition and depends on the hydrophobicity of the compounds. Other inhibitors known to destroy the driving forces such as valinomycin do not protect membrane proteins against coupling with IBCA. Silybin, which preferentially alters membrane fluidity, has little effect on the labeling. The above results give further evidence that IBCA, when applied in concentrations below 20 μM, is a suitable label for the hepatocellular bile salt transporter.

Introduction

We have recently introduced IBCA (3′-isothiocyanatobenzamidocholate) [1] a derivative of cholate, as affinity label in bile acid transport studies. IBCA has been shown to bind to hepatocytes with a resultant inhibition of bile acid uptake. Binding to isolated plasma membranes is saturable and irreversible; polypeptides of molecular weight 37, 50, 54, 67 kDa are preferentially labeled [1].

Here we present evidence that shows IBCA is acting as a true affinity label [2] for the active Na^+-dependent, carrier-mediated cholate transport system in isolated hepatocytes [3–6].

We show that the inhibition of bile acid transport by IBCA occurs by a direct action of IBCA on the transport proteins and not by indirect effects. Using intact cells, substrates (e.g. cholate) or reversible inhibitors (e.g. iodipamide, iopodate [7], furosemide [8,9], BSP [10]) protect the bile acid transport system against irreversible inhibition by IBCA. Further, the binding to plasma membranes and the labeling of specific polypeptides by radiolabeled IBCA could be prevented by substrates of the bile acid transport system and also by inhibitors of the bile acid transport when these inhibitors acted at the level of the transport protein (e.g. DIDS [11]) but not by agents which inhibit bile

Abbreviations: IBCA, 3′-isothiocyanatobenzamidocholate; [^{3}H]IBCA, 3′-isothiocyanatobenzamido[^{3}H]cholate; BSP, sulfobromphthalein; [^{3}H]$_2$DIDS, 4,4′-diisothiocyanato-1,2-diphenylethane-2,2′-disulfonic acid.

acid transport by effecting driving forces (valinomycin [9]).

Silybin which modulates lipid fluidity [12–14] protects plasma membranes against IBCA binding, too.

Transport proteins are embedded in the lipid core of the membrane and their functions depend on the lipid composition and distribution [15].

Materials

IBCA and [^{3}H]IBCA (spec. act. 70–80 mCi/mmol) were synthesized as described previously. [^{14}C]Cholate (spec. act. 52 mCi/mmol) was purchased from Amersham Buchler, Braunschweig, F.R.G. SDS, iodoacetamide, ethanol, EDTA and chloroform were purchased from Merck Darmstadt, F.R.G. Benzamidine hydrochloride was purchased from Fluka AG, Switzerland, Lipoluma and Lumasolve from Baker Chemicals, Gross-Gerau, F.R.G. All other reagents were of analytical grade.

Methods

Isolation of hepatocytes. Hepatocytes were isolated according to Berry and Friend (1969) [16] with some modifications [17]. Viability was tested by Trypan blue exclusion. All experiments were performed within 2 h after cell isolation.

Evaluation of binding capacity of isolated hepatocytes. The binding capacity of isolated hepatocytes was evaluated by the addition of [^{3}H]IBCA at various concentrations (2, 10, 20, 100, 200 and 700 μM) to 1 ml of cell suspension ($2 \cdot 10^6$ hepatocytes/ml, 4 mg of cell protein). The reaction was terminated after 20 min by the addition of 500 μl 1 M Tris (pH 7.4) and the cells were washed three times with Tyrode buffer containing 1 mM phenylmethylsulfonyl fluoride and 5 mM EDTA. The cells were then resuspended in 20 mM Tris-HCl (pH 7.4) containing the same protease inhibitors mentioned above and lysed by sonication for 5 s. The homogenate was centrifuged for 10 min at $1000 \times g$ to remove nuclei and unbroken cells. Crude hepatocellular membranes were separated from cytosolic proteins by centrifugation at $100\,000 \times g$. In some experiments the lipid was extracted using chloroform/ethanol (1 : 1, v/v).

Studies on the protective effect of natural substrates, competitive and non-competitive inhibitors of the bile acid transport system on the inhibition of cholate transport by IBCA. Isolated rat liver cells ($2 \cdot 10^6$/ml) in Tyrode buffer were preincubated at 37°C for 1 min with competitive and non-competitive inhibitors and natural substrates of bile acid transport before adding 3.5 μM of IBCA. After 10 min the cells were washed extensively. The uptake of cholic acid was measured by adding a mixture of 1 μM [^{14}C]cholic acid (spec. act. 52 m Ci/mmol) and 6 μM of cholate to $2 \cdot 10^6$ hepatocytes/ml (final concentrations). Aliquots of 100 μl were withdrawn at timed intervals, and centrifuged through silicon oil according to Schwarz et al. [3].

Isolation of liver plasma membranes. Plasma membranes from rat liver were prepared according to Touster et al. (1970) [18] with some modifications [19].

Protection of isolated plasma membranes against binding of [^{3}H]IBCA by several substrates and inhibitors of the bile salt transporter. Isolated plasma membrane vesicles (2 mg/ml) in 40 mM phosphate buffer pH 7.4 containing 120 mM NaCl were incubated 1 min with the inhibitors at concentrations between 8- and 700-fold in molar excess of the label. [^{3}H]IBCA (12 μM) was added. After 20 min at 37°C the reaction was stopped with Tris-HCl (pH 7.4). The unbound label was removed by washing in 20 mM Tris-HCl containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM EDTA, 0.1 mM iodoacetamide).

SDS gel electrophoresis. Hepatocellular crude membranes and isolated rat liver membrane proteins were separated by SDS gel electrophoresis and the radioactive polypeptides were visualized by fluorography [20]. The radioactivity bound to the polypeptides was determined by slicing gel rods, followed by counting in Lipoluma/Lumasolve/water (10 : 1 : 0.2, v/v). Protein content was determined according to the method of Lowry et al. [21].

Results

Binding of [^{3}H]IBCA to crude hepatocellular membranes and to a 50 kDa polypeptide

Saturable binding of [^{3}H]IBCA to crude mem-

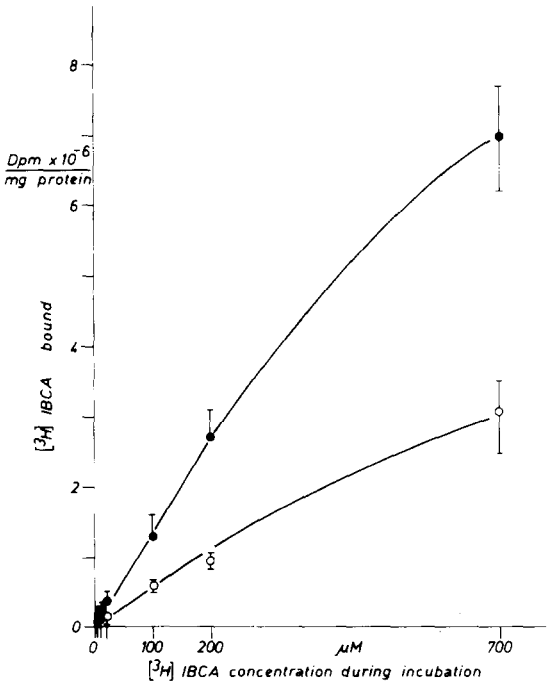

Fig. 1. Binding of [^{3}H]IBCA to crude membranes of isolated hepatocytes. Isolated hepatocytes ($2 \cdot 10^6$/ml) were incubated with 2, 10, 20, 100, 200 and 700 μM of [^{3}H]IBCA for 20 min at 37°C in O_2/CO_2 atmosphere. After the reaction was stopped with 1 M Tris (pH 7.4) the cells were washed extensively in Tyrode buffer. Thereafter cells were transferred to 20 mM Tris-HCl and lysed by sonication. Nuclei and unbroken cells were removed by $1000 \times g$ centrifugation. The radioactivity remaining in the crude membrane fraction after centrifugation at $100000 \times g$ was determined. ●——●, Crude membrane fraction; ○——○, post-lipid extraction. Values are means ± S.D. from four independent experiments.

branes before and after lipid extraction could be demonstrated up to an IBCA concentration of 700 μM (Fig. 1). Higher concentrations of IBCA solubilized the cells. Lipid extraction decreased the amount of bound label indicating that a large fraction of the hydrophobic label partitioned into the lipid bilayer or was bound by lipids or lipoproteins. Saturation kinetics was also observed in the radiolabeling of a 50 kDa polypeptide of the plasma membrane (Fig. 2), which is the most highly labeled polypeptide.

This polypeptide is discussed as a possible candidate for an important part of the transport system [1]. We therefore quantified the radioactivity bound to the corresponding band in SDS-polyacrylamide gels.

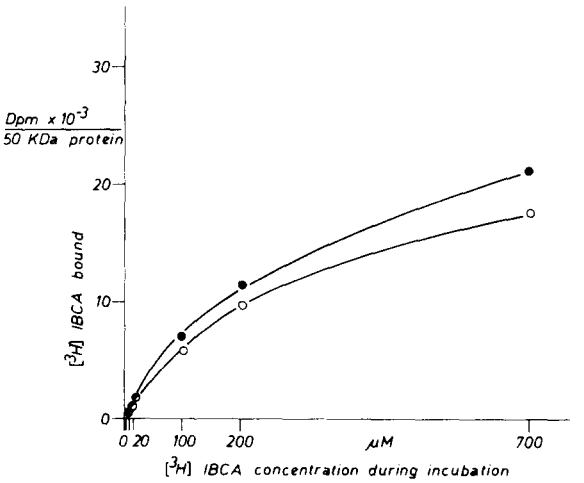

Fig. 2. Irreversible binding of [^{3}H]IBCA to the 50 kDa polypeptide. Isolated hepatocytes were incubated with 2, 10, 20, 100, 200 and 700 μM of [^{3}H]IBCA at 37°C in a shaking water bath in O_2/CO_2 atmosphere. After 20 min the reaction was stopped and the unbound label removed by washing. After disruption of the cells by sonication, $1000 \times g$ and $100000 \times g$ centrifugation, the proteins of the pellet were analyzed by SDS-polyacrylamide gel electrophoresis. The gel rods were sliced and the radioactivity in the 50 kDa region was counted in Lipoluma/Lumasolve/water (10:1:0.2, v/v) in a Packard Tri-Carb 2660 scintillation counter. The result of a typical experiment is shown ($n = 3$). ●——●, Radioactivity bound to the 50 kDa polypeptide; ○——○, post-lipid extraction.

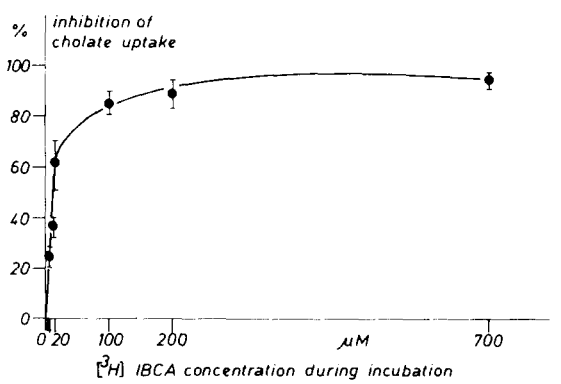

Fig. 3. Irreversible inhibition of cholic acid uptake by IBCA. Isolated hepatocytes ($2 \cdot 10^6$/ml) were incubated with 2, 10, 20, 100, 200 and 700 μM IBCA at 37°C. After 20 min the reaction was stopped and the cells were washed extensively. Thereafter uptake of [^{14}C]cholic acid was measured by adding a mixture of 1 μM [^{14}C]cholic acid and 6 μM cholate. The results are expressed as % inhibition of the initial uptake. Values are means ± S.D. from four different experiments.

Correlation of coupling of [^{3}H]IBCA to hepatocytes with its inhibitory action on cholate transport

The irreversible inhibition by IBCA of cholate transport into hepatocytes is concentration dependent and saturable.

50% inhibition of cholate transport (6 μM) was seen after preincubation of $2 \cdot 10^6$ hepatocytes/ml with 10 μM IBCA for 20 min (Fig. 3). At 50% inhibition of cholate uptake 1.3 nmol of [^{3}H]IBCA per mg of protein remained bound to crude membranes after washing. Lipid extraction reduced the fraction bound to 0.65 nmol/mg of protein. Correlation between uptake inhibition and binding was linear up to 20 μM [^{3}H]IBCA in the medium (Fig. 4). The same result was obtained by plotting irreversible inhibition of cholate uptake against [^{3}H]IBCA bound to the 50 kDa polypeptide (Fig. 5). At IBCA concentrations higher than 100 μM the relationship was no longer linear presumably because additional nonspecific sites were occupied by [^{3}H]IBCA.

Radiolabeling of membrane and cytosolic polypeptides

Binding of [^{3}H]IBCA to crude membranes and cytosolic proteins was examined by fluorographic visualization of the labeled polypeptides on SDS-polyacrylamide slab gels (Figs. 6A, 6B).

In crude membranes the lowest [^{3}H]IBCA concentration (2 μM) leads to labeling of a 50 kDa polypeptide. At higher concentration of [^{3}H]IBCA (10–20 μM) additional polypeptides of molecular weight of 67, 60, 54, 35 kDa were labeled. At 100–200 μM [^{3}H]IBCA nearly all polypeptides were substituted.

The labeled 50 kDa polypeptide is one of the major proteins of the liver plasma membrane. Lipid extraction did not alter the electrophoretic pattern of the membrane proteins (data not shown).

In the cytosolic fraction only one labeled band of 31–34 kDa could be detected at IBCA concentrations up to 100 μM. At higher concentrations of [^{3}H]IBCA additional polypeptides were identified (26, 42, 58 kDa; Fig. 6B).

Prevention of IBCA inhibition of cholate transport in the presence of various substrates and inhibitors of bile acid transport

If inhibition by IBCA results from a specific

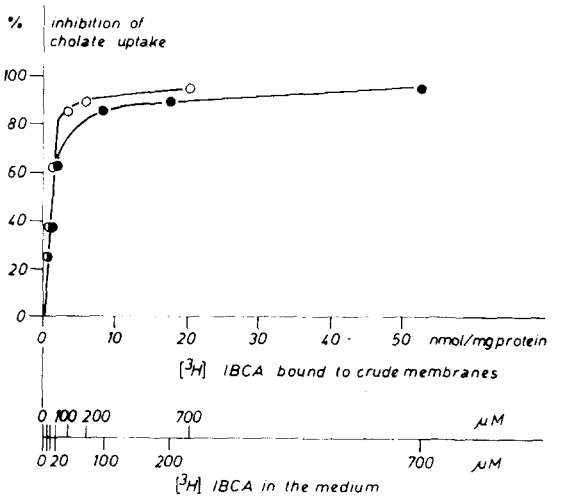

Fig. 4. Correlation between irreversible inhibition of cholic acid uptake by IBCA and irreversible binding of [^{3}H]IBCA to isolated hepatocytes. Isolated hepatocytes were preincubated with 2, 10, 20, 100, 200 and 700 μM of IBCA for 20 min at 37°C. After extensive washing the uptake of cholic acid was measured (for detail see Methods and legend to Fig. 3). With the same cell preparations binding of [^{3}H]IBCA (2–700 μM) to plasma membrane proteins was determined (for detail see legend to Fig. 1). ○——○, Crude membrane after lipid extraction; ●——●, crude membrane. Shown is the result of a typical experiment ($n = 3$). Ordinate: % inhibition of cholate transport. Abscissa: (a) ^{3}H bound nmol/mg protein; (b) μM [^{3}H]IBCA added.

reaction at a cholate binding site substrates of the transport system should protect the system against IBCA inhibiton. The same is to be expected for

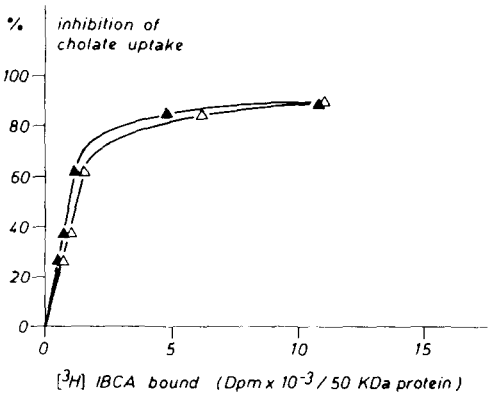

Fig. 5. Correlation between irreversible inhibition of cholic acid uptake by IBCA and irreversible binding of [^{3}H]IBCA to the 50 kDa subunit. △——△, after lipid extraction; ▲——▲, normal. Legend as in Fig. 2 and Fig. 3. Shown is the result of a typical experiment ($n = 3$).

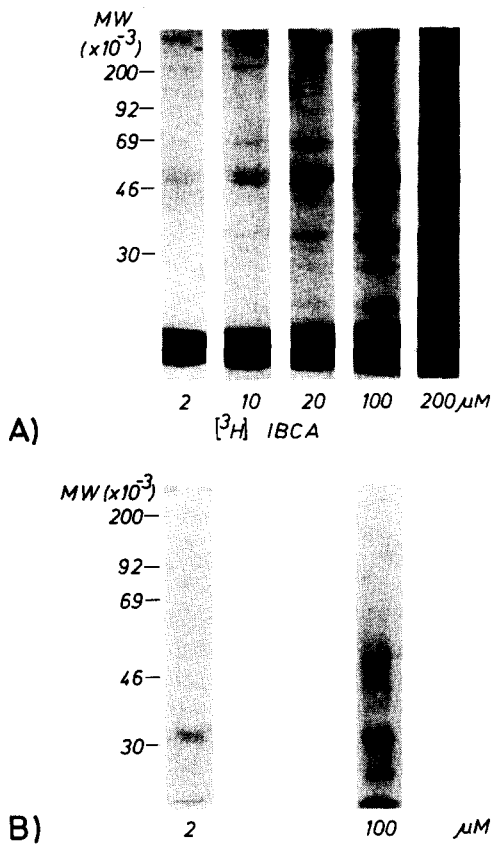

Fig. 6. Fluorographic visualization of labeled SDS subunits (A) in crude membranes, (B) in the cytosolic fraction. Isolated hepatocytes were labeled with [^{3}H]IBCA (2–200 μM) for 20 min at 37°C. After removal of unbound label the cells were lysed by sonication and the cytosol separated from the crude membranes by centrifugation at 1000 × g and, 100000 × g. 100 μg aliquots of membranes and cytosol were separated by SDS gel electrophoresis. Labeled proteins were detected by fluorography.

competitive and non-competitive inhibitors. On the other hand, inhibitors which do not interact with the transporter itself should be unable to decrease the inhibition (Table I).

Addition of cholate during preincubation with IBCA protected the bile acid transport system from irreversible inhibition by IBCA. The low cholate concentration was used because higher concentrations cannot be fully removed by washing.

Iopodate and iodipamide are competitive inhibitors of the cholate transport with IC_{50} of 20 μM and 50 μM, respectively [7]. Addition of either

TABLE I

INFLUENCE OF VARIOUS SUBSTRATES AND INHIBITORS OF THE BILE ACID TRANSPORT ON THE INHIBITION OF CHOLATE TRANSPORT BY IBCA

Isolated hepatocytes were preincubated with 3.5 μM IBCA alone or in the presence of 500 μM iopodate, iodipamide, furosemide, 5 μM BSP and 40 μM cholate. After 10 min the incubation medium was renewed and uptake of cholate was measured (for details see Methods). Values are presented as means ± S.D. (n = 4).

Incubation	% Inhibition of V_i	Cholate uptake, V_i (pmol/mg protein/min)
Control	0	264 ± 37
IBCA	50 ± 1.4	135 ± 15
IBCA + iopodate	25 ± 3.5	192 ± 42
IBCA + iodipamide	40 ± 12	162 ± 39
IBCA + BSP	35 ± 7	175 ± 49
IBCA + furosemide	30 ± 10	186 ± 23
IBCA + cholate	40 ± 5	158 ± 17

iopodate or iodipamide during preincubation with IBCA protected the cholate transport system from irreversible inhibition by IBCA; the protection achieved by iopodate was 1.6-times better then by iodipamide which agrees with their different IC_{50} values. The non-competitive inhibitors, furosemide [8,9] and BSP [10] were also effective protectors against IBCA inhibition. The low concentration of BSP was used because BSP at higher concentrations cannot be fully removed by washing, and causes inhibition of cholate transport.

It is evident that substrates and inhibitors, competitive as well as non-competitive ones, protect the bile acid transport system from IBCA inactivation, which suggests that the inhibition by IBCA results from a specific reaction at a cholate binding site.

Inhibition of binding of [^{3}H]IBCA to isolated plasma membranes by substrates or inhibitors of the bile acid transport system

Natural substrates, e.g. taurocholate protect plasma membranes against binding of [^{3}H]IBCA. Complete protection, however, could not be achieved, differential protection of only one polypeptide could also not be obtained. The radioactivity associated with the polypeptides is uniformally reduced (Table II).

The non-competitive inhibitor BSP is known to alter membrane charges and to solubilize membranes at concentrations above 200 μM [22]. Protection was achieved with 100 μM, an 8-fold excess with respect to the applied label. Cyclosporin A, one of the most potent inhibitors of cholate uptake (23, non-competitive) is also an effective protector against [^{3}H]IBCA labeling (Fig. 7). On the other hand cyclosporin is hydrophobic; its inhibitory effect on cholate transport is not fully reversible after prolonged preincubation [23].

The same is true for somatostatin analogs which are competitive inhibitors of cholate transport [24]. The more hydrophobic compounds stay longer in the membrane and compete more effectively with

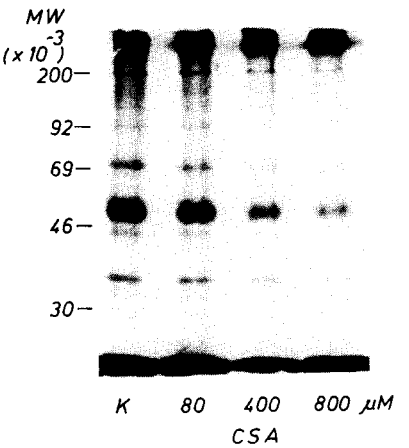

Fig. 7. Protection of plasma membranes by cyclosporin A against binding of [^{3}H]IBCA. Isolated plasma membranes were incubated with [^{3}H]IBCA (12 μM) alone (K) or with 80, 400, 800 μM cyclosporin A for 20 min. The reaction was stopped by Tris (pH 7.4) and the membranes washed by centrifugation. 100 μg of the membranes were separated by SDS gel electrophoresis and the radioactivity bound to the polypeptides was visualized by fluorography. Shown is the result of a typical experiment ($n = 3$).

TABLE II

PROTECTION OF ISOLATED PLASMA MEMBRANES AGAINST BINDING OF [^{3}H]IBCA BY SEVERAL TYPICAL SUBSTRATES AND INHIBITORS OF THE BILE SALT TRANSPORT SYSTEM

Isolated rat liver plasma membranes (Touster method) were incubated with 12 μM [^{3}H]IBCA and a 8–700-fold excess of protector. After 20 min at 37°C the reaction was stopped by addition of 1 M Tris (pH 7.4) and unbound label was removed by washing. 100 μg aliquots of the membrane proteins were separated by SDS gel electrophoresis. Quantification was done by slicing the SDS gel rods and counting the radioactivity in Lipoluma/Lumasolve/H_2O in a Packard Tricarb 2660 liquid scintillation counter. Labeled proteins in SDS-slab gels were visualized by fluorography. The inhibition of [^{3}H]IBCA binding to the 50 kDa protein region was determined. The experiment was performed three times with the same result.

Protector	x-fold excess	% Inhibition of binding (mean ± S.D.)
DIDS	70	70 ± 5
CSA	30	35 ± 16
	60	50 ± 7
BSP	8	20 ± 7
	70	50 ± 8
Somatostatin analogs	70	45 ± 5
Silybin	200	30 ± 6
Furosemide	250	20 ± 7
Taurocholate	370	30 ± 8
Iopodate	500	40 ± 8
Iodipamide	500	35 ± 6
Ouabain	700	0 –
Phalloidin	500	0 –
Valinomycin	100	0 –

IBCA at the transporter than furosemide (non-competitive) and iopodate or iodipamide (competitive) which are more hydrophilic.

Phalloidin, a cyclopeptide from *Amanita* phalloides, is taken up by liver cells via Na^+ dependent transport [9,25] which can be inhibited by all bile acids including IBCA, at very low concentrations [26]. Vice versa, phalloidin inhibits bile acid uptake only at very high concentrations [11]. Under our experimental conditions phalloidin did not prevent [^{3}H]IBCA binding.

Irreversible inhibitors like DIDS [11,19] which couples covalently to membrane proteins, have the most marked protective effect against binding of [^{3}H]IBCA.

Valinomycin decreases bile acid uptake by destroying driving forces [9]. As expected binding of [^{3}H]IBCA was not effected (data not shown). Ouabain, which also inhibits bile acid transport at high concentrations only [9,27], did not protect membranes against [^{3}H]IBCA binding. Silybin a flavonoid known to protect liver cells against phalloidin [12,13] penetrates into the lipid bilayer of liver cells [14] and is thought to influence the inward transport of cholate by an indirect mechanism. Interaction of silybin with membrane pro-

teins has not yet been demonstrated, our results show that silybin protects membranes against [^{3}H]IBCA (Table II).

Discussion

The following criteria have to be considered for a true affinity label of transport proteins: Is it a substrate or a competitive inhibitor of the transport? Do natural substrates or competitive inhibitors protect the protein against binding of the label? Are the binding sites saturable? Is there a stoichiometric incorporation of one reagent molecule per binding site? [2].

Saturability and stoichiometric inactivation, however, are not always applicable (e.g., to receptors) because of the high degree of nonspecific labeling that usually occurs at higher concentrations. In experiments with chemical affinity labels of transport proteins the reaction products depend on the coupling rates of the reactive group of the label as well as on the rates of change between different possible states of the transport system itself.

In our labeling experiments we used a cholate analog, IBCA, to identify the cholate carrier in rat liver. IBCA binds to saturable sites in the membrane of hepatocytes. Lipid extraction reduced the radioactivity associated with crude membranes by 50–60% indicating the hydrophobic character of the bile acid derivative.

Crude membranes were saturated by binding of 20 nmoles of [^{3}H]IBCA to one mg of protein (Fig. 4). This was achieved by incubation of $2 \cdot 10^6$ hepatocytes per ml with 700 μM of [^{3}H]IBCA. In spite of the high uptake capacity of liver cells for bile acids, this is a rather high value. Strange (1981) [28] calculated from binding data obtained by Accatino (1976) [29] (maximal binding capacity of 30 nmol/mg for binding of cholate at pH 6 to plasma membrane proteins) that all membrane proteins must be involved in transport to account for this value. There are additional binding sites besides those responsible for bile acid uptake. Most of the polypeptides of crude membranes were radiolabeled at saturating concentrations of [^{3}H]IBCA. On the other hand [^{3}H]IBCA below 20 μM modifies five polypeptides only. The binding to all of those polypeptides could be reduced by protecting substrates (Fig. 7) indicating a specific interaction.

The binding of [^{3}H]IBCA to crude membranes and to the 50 kDa polypeptide is stoichiometric up to 20 μM. This corresponds to a 60% inhibition of the transport. Additional sites at positions on the transporter not essential for bile acid transport are occupied at higher concentrations.

Polypeptides of identical molecular weight in SDS gel electrophoretograms may comprise several species with different functions and binding properties. In the present study we did not subdivide the different 50 kDa types. The presence of subtypes, however, is possible, as we have already shown [19] by two-dimensional electrophoresis using bromotaurodehydro[^{3}H]cholate and [^{3}H]$_2$-DIDS-labeled plasma membrane proteins: The 50 and 54 kDa polypeptides are composed of several polypeptides with different isoelectric points.

We were able to identify cytosolic proteins binding [^{3}H]IBCA. Intracellular bile acid binding has been proposed as an important mechanism for both hepatic transport of bile acids and protection from their physical effects [30]. The molecular weights of the [^{3}H]IBCA-labeled cytosolic proteins are in part in the similar range as previously detected binding proteins: Ligandin (22 + 22 kDa), glutathione S transferase B (22 + 25 kDa) [30], and a 33 kDa protein [31].

The most convincing evidence that IBCA is a true affinity label for bile acid transport proteins comes from protection studies with reversible inhibitors of cholate transport. They reduce the effect of IBCA on cholate transport.

Furthermore those substances protected against binding of [^{3}H]IBCA to isolated plasma membranes. Hydrophobic compounds (cyclosporin A, somatostatin analogs) possess a higher affinity to the transport system than hydrophilic ones (iopodate, iodipamide). Compounds influencing membrane fluidity (Silybin) interfered with the accessibility of the membrane protein. Other conditions known to alter membrane fluidity e.g. reduced temperature [32] also reduce the uptake of bile acids and phalloidin in hepatocytes.

Differential protection of only one single polypeptide, as Levy et al. observed with azo-7,7-dihydroxycholanoic acid [33], was not achieved. This could be due to different binding characteristics of

affinity and photoaffinity labels.

Even at very high concentrations phalloidin, ouabain, and valinomycin did not protect against [^{3}H]IBCA binding. This was expected for valinomycin and in part for ouabain (identity of the transport system for ouabain and cholate is discussed [27]) but not for phalloidin.

Using a photolabile phalloidin analog Wieland et al. [34] identified phalloidin binding proteins in the liver plasma membrane with the same molecular weight as those susceptible to IBCA. Furthermore, Kurz et al. [35] were able to induce phalloidin protection against binding of a photolabile derivative of cholate. Apparently unknown properties of our label prevent protection by phalloidin.

We feel the studies presented show that IBCA may be used as an affinity label for the bile acid transport system instead of photoaffinity labels. The use of IBCA has some technical advantages over photoaffinity labels e.g. no need for special equipment.

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