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Characterization and chemical modification of the Na^+-dependent bile-acid transport system in brush-border membrane vesicles from rabbit ileum

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The Na^+-dependent uptake system for bile acids in the ileum from rabbit small intestine was characterized using brush-border membrane vesicles. The uptake of [^{3}H]taurocholate into vesicles prepared from the terminal ileum showed an overshoot uptake in the presence of an inwardly-directed Na^+-gradient ($[Na^+]_{out} > [Na^+]_{in}$), in contrast to vesicles prepared from the jejunum. The Na^+-dependent [^{3}H]taurocholate uptake was *cis*-inhibited by natural bile acid derivatives, whereas cholephilic organic compounds, such as phalloidin, bromosulphophthalein, bilirubin, indocyanine green or DIDS – all interfering with hepatic bile-acid uptake – did not show a significant inhibitory effect. Photoaffinity labeling of ileal membrane vesicles with 3,3-azo- and 7,7-azo-derivatives of taurocholate resulted in specific labeling of a membrane polypeptide with apparent molecular mass 90 kDa. Bile-acid derivatives inhibiting [^{3}H]taurocholate uptake by ileal vesicles also inhibited labeling of the 90 kDa polypeptide, whereas compounds with no inhibitory effect on ileal bile-acid transport failed to show a significant effect on the labeling of the 90 kDa polypeptide. The involvement of functional amino-acid side-chains in Na^+-dependent taurocholate uptake was investigated by chemical modification of ileal brush-border membrane vesicles with a variety of group-specific agents. It was found that (vicinal) thiol groups and amino groups are involved in active ileal bile-acid uptake, whereas carboxyl- and hydroxyl-containing amino acids, as well as tyrosine, histidine or arginine are not essential for Na^+-dependent bile-acid transport activity. The irreversible inhibition of [^{3}H]taurocholate transport by DTNB or NBD-chloride could be partially reversed by thiols like 2-mercaptoethanol or DTT. Furthermore, increasing concentrations of taurocholate during chemical modification with NBD-chloride were able to protect the ileal bile-acid transporter from inactivation. These findings suggest that a membrane polypeptide of apparent M_r 90000 is a component of the active Na^+-dependent bile-acid reabsorption system in the terminal ileum from rabbit small intestine. Vicinal thiol groups and amino groups of the transport system are involved in Na^+-dependent transport activity, whereas other functional amino acids are not essential for transport activity.

Introduction

Bile acids are acidic sterols synthesized from cholesterol in the liver. They undergo a biological recycling with the involvement of liver, terminal ileum, kidney and blood [1–5]. After synthesis in the liver, the bile acids are secreted into bile, emptied into the small intestine where they are absorbed and gain entry to the liver via the portal circulation. The uptake processes for bile acids in the ileum, the liver and the kidney are Na^+-dependent and a cotransport of Na^+ and bile acids is assumed [3–11]. The respective binding proteins for bile acids during enterohepatic circulation in blood [12], hepatocytes [13–20], cytosol of hepatocytes [20,21] and ileocytes [22], brush-border membranes of intestine [23–25] and kidney [26], as well as basolateral membranes of intestine [27] and kidney [28] have been identified by affinity labeling using (photo)reactive bile-acid derivatives [13,18,29–32].

The bile acids, reabsorbed in the ileum and recirculating to the liver, inhibit in a feedback mechanism the conversion of cholesterol to bile acids by modulating the activity of cholesterol-7α-hydroxylase [33]. Hence, the interruption of the enterohepatic circulation of bile acids is a very attractive principle to lower elevated

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serum cholesterol levels by stimulation of bile-acid synthesis [34]. The ileal Na^+-dependent bile-acid transport system shows a remarkable substrate specificity [4,5,23,35–41], recommending this transport system as a putative pharmaceutical target for the treatment of hypercholesterolemia by interruption of the enterohepatic circulation with specific inhibitors of bile-acid transport. Since the specificity of a transport system is caused by a specific interaction of amino-acid side-chains of the transport protein with definite epitopes of the respective ligand molecule, knowledge of these interactions essential for molecular recognition is of great importance for the development of transport inhibitors. Therefore, in the present study, we have characterized the Na^+-dependent bile-acid reabsorption system in rabbit ileum and have identified by chemical modification the amino-acid side-chains essential for the functionality of the Na^+-dependent bile-acid transporter.

Materials and Methods

Materials

Photoaffinity labeling was carried out with the sodium salts of (7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]-cholan-24-oyl)-2-aminoethanesulfonic acid (spec. act. 20.25 Ci/mmol) and (3,3-azo-7α,12α-dihydroxy-5β[7β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid (spec. act. 1.3 Ci/mmol) synthesized as described [29–31]. [^{3}H(G)]Taurocholic acid (spec. act. 2.1 Ci/mmol) and D-[U-^{14}C]glucose (spec. act. 258.5 mCi/mmol) were purchased from NEN Du Pont (NEN Division, Dreieich, Germany). Acrylamide, *N,N'*-methylenebisacrylamide, diethylpyrocarbonate (DEP), 5,5'dithiobis-(2-nitrobenzoic acid) (DTNB), fluorescein isothiocyanate (FITC), *N*-acetylimidazole (NAI), 7-chloro-4-nitrobenz-2-oxo-1,3-diazole (NBD-chloride), phenylarsine oxide (PAO), *p*-chloromercury-benzoate (PCMB), phenylglyoxal (PGO), phenylboronic acid, iodoacetamide, phenylmethylsulfonic acid (PMS), mercury-II-chloride, 2,4,6-trinitrobenzenesulfonic acid (TNBS), *N,N'*-dicyclohexylcarbodiimide (DCCD), bile acids and marker proteins for the determination of molecular weights were from Sigma (München, Germany). Serva Blue R-250 and all other materials for electrophoresis were from Serva (Heidelberg, Germany). Cellulose nitrate filters for transport measurements (25 mm diameter, 0.45 μm pore size ME 25) were from Schleicher& Schüll (Dassel, Germany) and scintillators Quickszint 501, 361 and Unisolve I from Zinsser Analytic (Frankfurt, Germany). The kits (Merckotest) for the determination of the activity of the marker enzymes aminopeptidase N, γ-glutamyltransferase and alkaline phosphatase were from Merck (Darmstadt, Germany). All other substances were obtained from the usual commercial sources and were of analytical grade.

Methods

Preparation of brush-border membrane vesicles. Brush-border membrane vesicles from the ileum and the jejunum of male white New Zealand rabbits (weighing 2.5–3 kg) were prepared by the Mg^{2+}-precipitation method [42] as described previously [24,43,44]. The entire small intestine was removed and divided into ten segments of equal length, numbered 1–10, proximal to distal. Segments 7–10 were used for the preparation of ileal brush-border membrane vesicles and segments 2–5 for the preparation of jejunal brush-border membrane vesicles. The brush border membranes were enriched (17.2 ± 4)-fold with regard to aminopeptidase N (EC 3.4.11.2), (16.6 ± 7.3)-fold for γ-glutamyltransferase (EC 2.3.2.2) and (14.4 ± 4)-fold for alkaline phosphatase (EC 3.1.3.1) Immediately after preparation, the vesicles were stored in liquid nitrogen without loss of transport and enzymatic activity for at least 4 weeks. The intactness of the vesicles was determined by measuring Na^+-dependent D-glucose uptake after 15 s of incubation; usually the overshoot uptake at 15 s was greater than 20-fold. The enzymatic activities of aminopeptidase N, γ-glutamyltransferase and alkaline phosphatase were determined with Merckotest N kits and protein was determined according to Bradford [45] using the Bio-Rad assay (Bio-Rad, München, Germany).

Transport measurements. Uptake of radiolabeled substrates by brush-border membrane vesicles was determined by the membrane filtration method [42] as described previously [24,43,44]. Typically, the transport reaction was initiated by adding 10 μl of the vesicle suspension (100 μg of protein) equilibrated with 10 mM Tris-Hepes buffer (pH 7.4), 300 mM mannitol to 90 μl of incubation medium containing the radioactively labeled substrate kept at 30°C. The composition of the incubation medium for measurements in the presence of a Na^+-gradient usually was 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 100 mM mannitol and in the absence of a Na^+-gradient 10 mM Tris-HCl (pH 7.4), 100 mM KCl, 100 mM mannitol. For measurement of taurocholate uptake, these media contained 50 μM (0.75 μCi) [^{3}H]taurocholate and for glucose uptake 19 μM (1 μCi) D-[U-^{14}C]glucose. At desired time-points, the transport reaction was terminated by the addition of 1 ml of ice-cold stop solution (10 mM Tris-Hepes (pH 7.4), 150 mM KCl). The entire content was pipetted onto the middle of a prewashed, prechilled filter kept under suction with the aid of a vacuum controller. The filter was rinsed immediately with 5 ml of ice-cold stop solution and then solubilized in scintillator Quickszint 361. The radioactivity remaining on the filter was counted with standard liquid scintillation techniques. After correction of medium radioactivity bound to the filter in the absence of membrane vesicles and eventual chemiluminescence, absolute solute uptake was calcu-

lated and expressed as nmol/mg protein. All experiments were performed in triplicate and uptake values are given as mean ± S.D.

Chemical modification of ileal brush-border membrane vesicles. Ileal brush-border membrane vesicles (50 μl, 10 mg/ml, 500 μg of protein) were incubated at 20°C for 1 h with 500 μl of 10 mM Tris-Hepes buffer (pH 7.4), 300 mM mannitol containing the respective amino-acid modifying agents in the concentration range of 10^{-6} M to 10^{-2} M as described [46,47]. For the preparation of these solutions some of the agents at first were dissolved in ethanol (TNBS, PITC, NBD-chloride, DCCD, phenylboronic acid) or DMSO (DTNB, PAO, FITC, PGO) and were then diluted with buffer; in each case, the concentration of the organic solvent was less than 5% and the uptake into control vesicles was measured in the presence of the same concentration of the respective organic solvent. Chemical modification with DEP was performed as described [46,47]. After 1 h of incubation the reaction was stopped by addition of 35 ml of ice-cold 10 mM Tris-Hepes buffer (pH 7.4), 300 mM mannitol and membrane vesicles were collected by centrifugation at 48000 × *g* for 30 min. After resuspension of the membranes in 35 ml of the abovementioned buffer the centrifugation was repeated. The vesicles were resuspended in buffer to a concentration of 10 mg/ml and used immediately for the transport measurements.

For the investigation of the reversibility of the inactivated bile-acid transport system by thiol-modifying agents, brush-border membrane vesicles (150 μl, 1.5 mg of protein) were suspended for 1 h at 20°C either in (a), 1 ml of 10 mM Tris-Hepes buffer (pH 7.4), 300 mM mannitol; (b), 1 ml of the above buffer containing 10^{-4} M NBD-chloride, or (c), 1 ml of above buffer containing 10^{-3} M DTNB. After dilution of these incubation mixtures with 35 ml of ice-cold buffer IV (10 mM Tris-Hepes (pH 7.4), 300 mM mannitol), vesicles were collected by centrifugation at 48000 × *g* for 30 min at 4°C and resuspended in 200 μl of buffer IV each. 40 μl (300 μg of protein) of these modified brush-border membrane vesicles were incubated for 1 h at 20°C in 1.5 ml either of buffer IV, 10 mM 2-mercaptoethanol in buffer IV, 10 mM DTT in buffer IV, 10 mM cysteine in buffer IV or 10 mM glutathione in buffer IV. After dilution with 35 ml of buffer IV, collection of the vesicles by centrifugation at 48000 × *g* for 30 min and resuspension in 40 μl of buffer IV, the uptake of [^{3}H]taurocholate was measured.

For the investigation of a possible protective effect of bile acids on the inactivation of the ileal bile-acid transporter by thiol-modifying agents, brush-border membrane vesicles (60 μl, 600 μg of protein) were incubated for 60 min at 20°C in 500 μl of buffer IV containing 0, 0.5, 1, 1.5, 2 or 2.5 mM taurocholate. After dilution with 35 ml of ice-cold buffer IV the membrane vesicles were collected by centrifugation at 48000 × *g* for 30 min and resuspended in 60 ml of buffer IV. One half of this suspension was added to 500 μl of buffer IV containing 1 mM NBD-chloride, 2% DMSO, the other half as control to 500 μl buffer IV, 2% DMSO. After incubation for 1 h at 20°C, addition of 35 ml of buffer IV and collection of the vesicles by centrifugation the 1 min uptake of [^{3}H]taurocholate was measured.

Photoaffinity labeling. Photoaffinity labeling with photoreactive bile acids was performed as described previously [12,14,25,29–31]. Typically, 25 μl of brush-border membrane vesicles (250 μg of protein equilibrated with buffer IV), were added in the dark to 175 μl of 10 mM Tris-Hepes buffer (pH 7.4), 100 mM NaCl, 100 mM mannitol containing the radiolabeled photoreactive 3,3-azo- or 7,7-azo-derivatives of taurocholic acid and the putative non-radioactively labeled inhibitors. After 5 min of preincubation, the suspensions were irradiated for 10 min at 350 nm in a Rayonet Photochemical Reactor RPR-100 (The Southern Ultraviolet Company, Hamden CT, USA) equipped with 16 RPR 3500 Å lamps. Afterwards, the suspensions were diluted with 1 ml of ice-cold buffer IV and centrifuged for 30 min at 48000 × *g*. The supernatant was carefully removed and the membranes were solubilized in 50 μl of 62.5 mM Tris-HCl buffer (pH 6.8), 2% SDS (w/v), 10% glycerol (v/v), 5% 2-mercaptoethanol (v/v), 0.001 Bromophenol blue (w/v) and submitted to SDS-PAGE.

Gel electrophoresis. SDS-PAGE was carried out in vertical slab gels (20 × 17 × 0.15 cm) using an electrophoresis System LE 2/4 (LKB Pharmacia Biotechnologie, Freiburg, Germany) as described [25,48]. After staining with Serva Blue R 250, the gels were scanned with a densitometer CD 50 (DESAGA, Heidelberg, Germany) and the individual lanes were cut into slices of 2 mm thickness. Each slice was solubilized with 250 μl of tissue solubilizer Biolute S overnight and after addition of 4 ml of scintillator Quickzint 501 the samples were counted for radioactivity.

Results

Na^+-dependent bile-acid uptake by brush-border membrane vesicles from rabbit ileum

To determine whether the ileum from rabbits possesses a Na^+-dependent bile-acid uptake system as the rat [3,49] and the guinea pig [4,5], the uptake of [^{3}H]taurocholate into membrane vesicles prepared from rabbit ileum and jejunum was measured. As shown in Fig. 1 (upper panel), [^{3}H]taurocholate showed a rapid uptake into ileal vesicles prepared in a Na^+-free medium and incubated in a Na^+-containing buffer. The uptake of taurocholate in the presence of an inwardly directed Na^+-gradient ($[Na^+]_{out}$ = 100 mM, $[Na^+]_{in}$ =

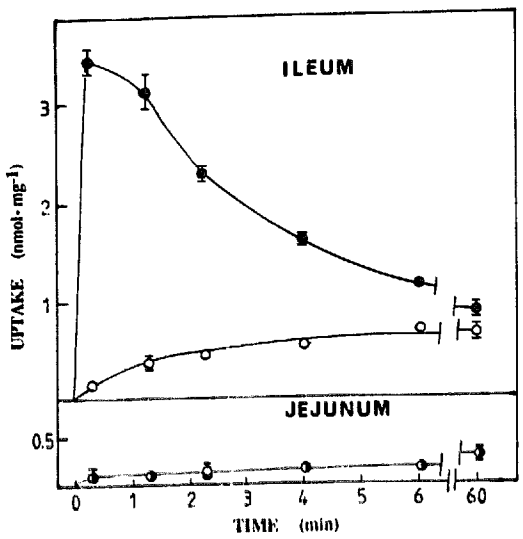

Fig. 1. Na^+-dependent uptake of [^{3}H]taurocholate into ileal and jejunal brush-border membrane vesicles from rabbit small intestine. Brush-border membrane vesicles (10 μl, 100 μg of protein) loaded with 10 mM Tris-Hepes buffer (pH 7.4), 300 mM mannitol were incubated at 30°C with 90 μl either of 10 mM Tris-Hepes buffer (pH 7.4), 100 mM mannitol, 50 μM (0.75 μCi) [^{3}H]taurocholate containing either 100 mM NaCl (●) or 100 mM KCl (○) and uptake was measured after the indicated incubation times, corrected for unspecific binding of [^{3}H]taurocholate to the filters.

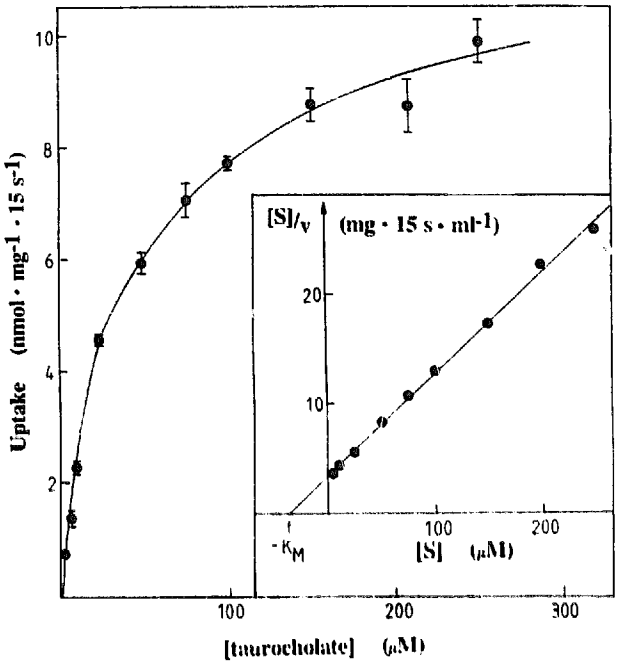

Fig. 2. Concentration-dependence of Na^+-dependent [^{3}H]taurocholate uptake into ileal brush-border membrane vesicles. Ileal brush-border membrane vesicles (10 μl, 50 μg of protein) loaded with 10 mM Tris-Hepes buffer (pH 7.4), 300 mM mannitol were incubated at 30°C with the indicated concentration of [^{3}H]taurocholate dissolved in 90 μl of 10 mM Tris-Hepes buffer, 100 mM NaCl, 100 mM mannitol. Uptake was measured for 15 s and uptake rates are expressed as nmol/mg protein per 15s, after correction for nonspecific filter binding. V_{max} and K_m-values were obtained from the [S]/v vs. [S] diagram.

0 mM) was greatly stimulated (Fig. 1, ●) compared with its uptake in the total absence of Na^+ ($[K^+]_{out} > [K^+]_{in}$) (Fig. 1, ○). The uptake occurred with a transient intravesicular accumulation with a maximum after 15 s of incubation (overshoot phenomenon), whereas the uptake in the absence of Na^+ did not exceed the equilibrium uptake at 60 min. The equilibrium uptake rates after 60 min of incubation were nearly identical, demonstrating that the presence of Na^+ had no significant effect on the binding of taurocholate to the brush-border membrane vesicles, as well as on the vesicle volume. In contrast, vesicles prepared from the jejunum of the same animals, did not show any Na^+-dependence of [^{3}H]taurocholate uptake and no overshoot phenomenon (Fig. 1, lower panel). Fig. 2 shows the concentration-dependence of [^{3}H]taurocholate uptake into ileal brush-border membrane vesicles in the presence of a Na^+-gradient. The uptake of taurocholate was a saturable process obeying Michaelis-Menten kinetics with a maximum uptake rate of 10.7 nmol/mg per 15 s and a Michaelis-Menten constant K_m of 36 μM.

To determine the substrate specificity of the Na^+-dependent bile-acid uptake system from rabbit ileum, the effect of non-radiolabeled compounds and bile-acid derivatives on the uptake of [^{3}H]taurocholate by ileal vesicles was determined. Fig. 3 shows that natural bile acids in the incubation medium led to a concentration-dependent inhibition of Na^+-dependent [^{3}H]taurocholate uptake, i.e., uptake under $[Na^+]_{out} > [Na^+]_{in}$

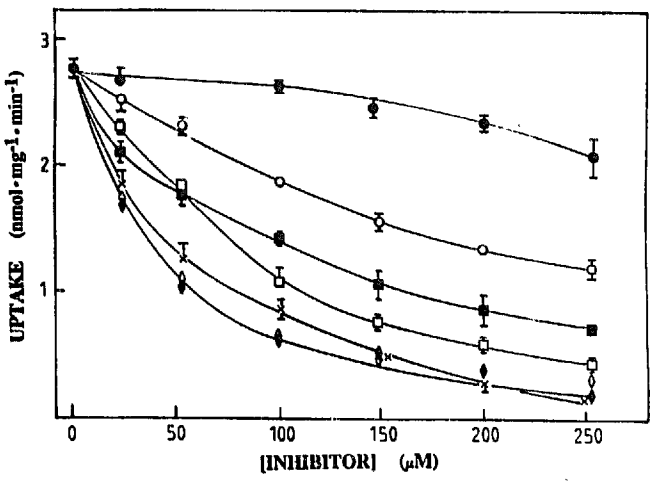

Fig. 3. Inhibition of Na^+-dependent [^{3}H]taurocholate uptake into ileal brush-border membrane vesicles by natural bile acids. Ileal brush-border membrane vesicles (10 μl, 100 μg of protein) loaded with 10 mM Tris-Hepes buffer (pH 7.4), 300 mM mannitol were incubated at 30°C with 30 μl of 10 mM Tris-Hepes buffer, 100 mM NaCl, 100 mM mannitol containing 50 μM (0.75 μCi) [^{3}H]taurocholate, either in the absence of inhibitors or in the presence of the indicated concentrations of the respective bile-acid derivatives. Uptake of [^{3}H]taurocholate was measured for 60 s and corrected for nonspecific filter binding. ●, lithocholate; ○, cholate; ■, ursodeoxycholate; □, taurolithocholate; ♦, chenodeoxycholate; ◇, taurochenodeoxycholate; ×, deoxycholate.

gradient minus uptake under $[K^+]_{out} > [K^+]_{in}$ gradient conditions. The order of inhibitory effect measured by a half-maximal inhibition (IC_{50}) on Na^+-dependent [^{3}H]taurocholate uptake was: chenodeoxycholate, taurochenodeoxycholate > deoxycholate > taurolithocholate > tauroursodeoxycholate > cholate ≫ lithocholate.

Cholephilic organic compounds like bromosulphophthalein, indocyanine green and bilirubin, the peptide phalloidin from the poisonous mushroom *Amanita phalloides* or the bifunctional anion transport inhibitor DIDS – compounds all interfering with hepatic bile-acid transport [17,19,20,50,51] – showed no significant inhibitory effect on the Na^+-dependent [^{3}H]taurocholate uptake as in the rat [35,52], suggesting different transport systems for bile acids in the ileum and the liver of rabbits (Fig. 4).

Photoaffinity labeling of ileal brush-border membrane vesicles

For the identification of bile-acid binding proteins in the brush-border membrane from rabbit ileocytes, photoaffinity labeling experiments using photoreactive derivatives of taurocholate carrying a carbene-generating diazirino-function at position 3 or 7 of the steroid nucleus were carried out. For these studies, brush-border membrane vesicles were incubated in media containing the tritium-labeled photoreactive bile-acid derivatives, subsequently exposed to UV light and after washing of the membrane vesicles the membrane proteins were separated by denaturing SDS-PAGE.

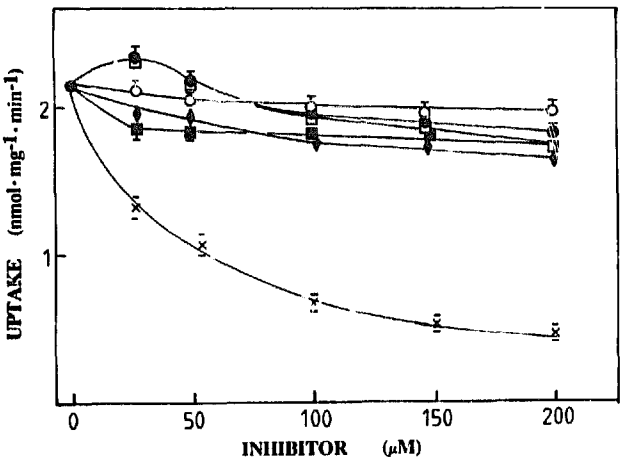

Fig. 4. Effect of cholephilic compounds on Na^+-dependent [^{3}H]taurocholate uptake by ileal brush-border membrane vesicles. Ileal brush-border membrane vesicles (10 μl, 100 μg of protein) loaded with 10 mM Tris-Hepes buffer (pH 7.4), 300 mM mannitol were incubated with 90 μl of 10 mM Tris-Hepes buffer (pH 7.4), 100 mM NaCl, 100 mM mannitol containing 50 μM (0.75 mCi) [^{3}H]taurocholate, either in the absence of inhibitors or in the presence of the indicated concentrations of taurochenodeoxycholic acid (×), DIDS (●), phalloidin (○), bromosulphophthalein (■), bilirubin (□) or indocyanine green (◆). Uptake of [^{3}H]taurocholate was measured for 60 s and corrected for unspecific binding to the filter membrane.

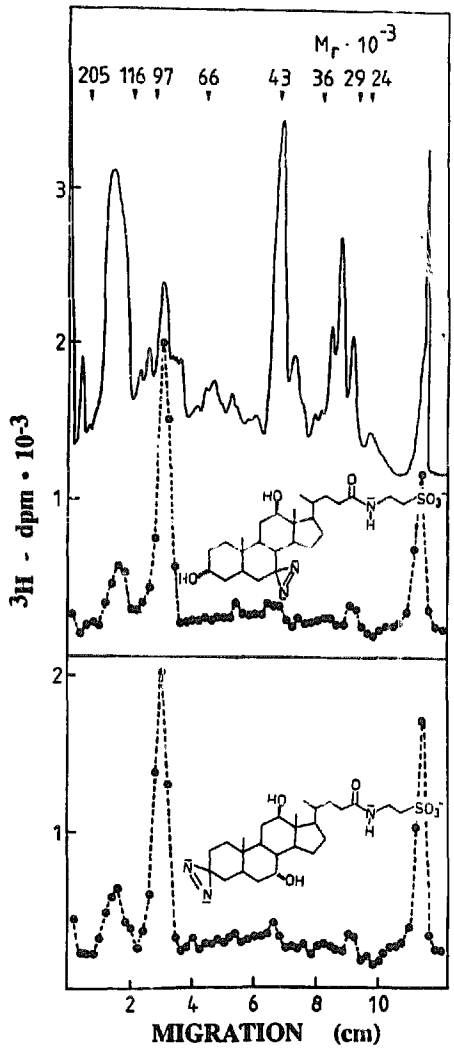

Fig. 5. Photoaffinity labeling of ileal brush-border membrane vesicles from rabbit small intestine with photolabile derivatives of taurocholate. Ileal brush-border membrane vesicles (25 μl, 250 μg of protein) loaded with 10 mM Tris-Hepes buffer (pH 7.4), 300 mM mannitol were mixed for 5 min in the dark with 175 μl of 10 mM Tris-Hepes buffer (pH 7.4), 100 mM NaCl, 100 mM mannitol containing either 0.49 μM (2 μCi) (7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid (top) or 6.6 μM (2 μCi) (3,3-azo-7α,12α-dihydroxy-5β[7β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid (bottom) and subsequently irradiated at 350 nm for 10 min. After washing of the vesicles, membrane proteins were separated by SDS-PAGE using 9% gels. The solid line shows the densitometer scanning of the polypeptides after staining, whereas the dotted lines indicate the distribution of radioactivity.

As shown in Fig. 5 (upper panel), photoaffinity labeling of membrane vesicles with the 7,7-azo derivative of taurocholic acid resulted in an incorporation of the radiolabel nearly exclusively into one polypeptide with an apparent molecular mass of 90 kDa and to a much lesser extent into a polypeptide with a molecular mass of 130 kDa. Irradiation in the simultaneous presence of nonlabeled taurocholate (250 μM) completely inhibited the labeling of the 90 kDa polypeptide, whereas labeling of the 130 kDa polypeptide as well as

TABLE I

Effect of bile acids and cholephilic compounds on photoaffinity labeling of the 90-kDa bile-acid binding protein in ileal brush-border membrane vesicles from rabbit small intestine

Ileal brush-border membrane vesicles (25 μl, 250 μg of protein) loaded with 10 mM Tris-Hepes buffer (pH 7.4), 300 mM mannitol) were mixed for 5 min in the dark with 175 μl of 10 mM Tris-Hepes buffer (pH 7.4), 100 mM NaCl, 100 mM mannitol containing 0.49 μM (2 μCi) (7,7azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oyl)-2-aminoethanesulfonic acid, either in the absence (control) or presence of 250 μM of the indicated substances. After irradiation at 350 nm for 10 min and subsequent SDS-PAGE, the amount of radioactivity incorporated into the 90-kDa bile-acid binding polypeptide was determined by liquid scintillation counting.

Compound	^{3}H incorporated into 90 kDa-protein (dpm)	% inhibition
Control	78358	0
+ taurocholate	27884	64.4
+ taurochenodeoxycholate	12611	83.9
+ taurolithocholate	8200	89.5
+ taurodeoxycholate	11772	84.9
+ cholate	30731	60.7
+ chenodeoxycholate	17338	77.9
+ lithocholate	67122	14.3
+ lithocholate-3-sulfate	56853	27.4
+ phalloidin	85611	0
+ DIDS	72951	6.9
+ bilirubin	57542	26.5
+ bromosulphophthalein	48609	37.9
+ indocyanine green	50349	35.7
+ L-carnosine	86647	0
+ glycine	71286	9.0

nonspecific background labeling remained unchanged, demonstrating the specificity of the 90 kDa protein for taurocholate (data not shown). Photoaffinity labeling of brush-border membrane vesicles with the 3,3-azo isomer resulted in an similar labeling pattern with prominent and specific labeling of the 90 kDa protein (Fig. 5, lower panel). In order to evaluate the binding specificity of the 90 kDa protein, competition photoaffinity labeling experiments in the presence of structurally different nonradiolabeled bile acids, as well as cholephilic compounds were performed. Table I shows that bile acids were able to inhibit the labeling of the 90 kDa protein by photoreactive taurocholate analogues whereas cholephilic compounds such as phalloidin, DIDS, bromosulphophthalein or bilirubin, as well as amino acids such as L-proline or substrates of the oligopeptide transporter such as L-carnosine or D-cephalexin [43,44] showed no or only a moderate effect on the extent of labeling.

Chemical modification of the Na^+-dependent bile-acid transport system in brush-border membrane vesicles from rabbit ileum

In order to identify the involvement of distinct amino acids of the Na^+-dependent intestinal bile-acid transporter in the uptake process of taurocholate, membrane vesicles were incubated for 1 h with different group-specific reagents dissolved in appropriate buffers in the concentration range of 10^{-6} to 10^{-2} M. After washing of the vesicles, the initial uptake of 50 μM [^{3}H]taurocholate was measured both in the presence and the absence of an inwardly directed Na^+-gradient ($[Na^+]_{out} > [Na^+]_{in}$). To discriminate between the modification of amino acids essential for active bile-acid transport and inhibition by nonspecific effects of the respective agents, we also measured in each modification experiments the effect on the Na^+-dependent D-glucose uptake system, because the amino acids which are essential for transport have been determined previously [47,53].

Since some of the modifying agents had to be dissolved in organic solvents, we at first investigated the effect of different concentrations of the solvents ethanol and DMSO on the Na^+-dependent uptake of both, [^{3}H]taurocholate and D-[^{14}C]glucose. It was found that ethanol up to a concentration of 5% (v/v) had no significant effect on taurocholate uptake, whereas D-glucose transport was slightly effected by these ethanol-concentrations in accordance with a previous study [54]. DMSO had no effect on both transport systems in concentrations up to 6 vol% (data not shown).

In a first series of experiments, we investigated the influence of several thiol-modifying reagents on Na^+-dependent taurocholate uptake. DTNB, NEM, iodoacetamide, $HgCl_2$, PCMB, as well as PMS led to a concentration-dependent inactivation of both, the Na^+-dependent taurocholate transporter as well as the uptake system for D-glucose (Fig. 6, Table II). The mercurials $HgCl_2$ and PCMB were strong inhibitors of both transporters with IC_{50}-values of $(3–6) \cdot 10^{-6}$ M, whereas a concentration of 3–5 mM DTNB was necessary for half-maximal inhibition. NEM was a strong inhibitor of D-glucose transport and a weak one for taurocholate uptake, whereas iodoacetamide had only slight inhibitory effects on both transporters. PAO, an inhibitor selective for vicinal thiol groups [55] led to a nearly complete inactivation of the bile-acid transporter at concentrations > 1 mM, indicating the involvement of vicinal thiol groups. On the other hand, thiol reducing agents like 2-mercaptoethanol or DTT neither inhibited bile-acid transport nor D-glucose uptake, excluding the involvement of disulfide bridges in [^{3}H]taurocholate transport.

The modification of amino groups with agents like TNBS or FITC also inactivated the bile-acid transport system. NBD-chloride, an agent modifying thiol- and amino-groups, resulted in an irreversible inhibition of the bile-acid transporter with an IC_{50} value of about $8 \cdot 10^{-4}$ M. The modification of carboxyl-groups of the acidic amino-acids glutamic or aspartic acid with DCCD and the reaction of hydroxyl-groups in the amino-acids

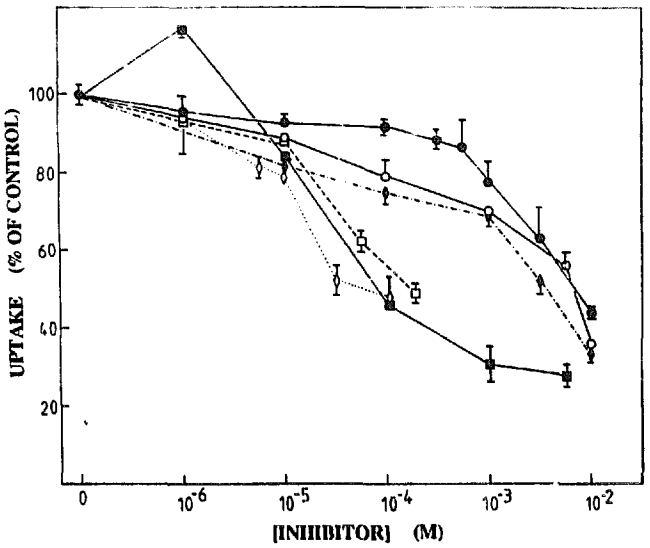

Fig. 6. Effect of thiol- and amino-group specific agents on Na^+-dependent [^{3}H]taurocholate uptake by ileal brush-border membrane vesicles. Ileal brush-border membrane vesicles (10 mg/ml) were treated with the indicated concentrations of amino-acid-modifying agents for 1 h at 20°C as described in Materials and Methods. After washing, the uptake of 50 μM (0.75 μCi) of [^{3}H]taurocholate was measured for 60 s in the presence of an inwardly-directed Na^+-gradient. Uptake is indicated as percentage of the respective control values after subtraction of filter background. ●, DTNB; ○, NEM; ■, PCMB; □, NBD-chloride; ◇, $HgCl_2$; ◆, FITC.

serine and threonine with PMSF or phenylboronic acid left the bile-acid transporter completely unchanged, like after modification of tyrosine residues with NAI or of histidyl residues with DEP. The results of these modification experiments are summarized in Table II.

The selective inhibition of the Na^+-dependent intestinal bile-acid transporter by thiol-modifying agents should be reversible by treatment with thiol agents. Therefore, ileal brush-border membrane vesicles, treated for 1 h with DTNB or NBD-chloride to inactivate the active bile-acid transport system, were subsequently exposed to various thiol-containing agents. After washing, the initial uptake of [^{3}H]taurocholate was measured and compared with the respective control vesicles, which have been exposed to the thiol-containing agents alone. Fig. 7 shows that the irreversible inhibition of [^{3}H]taurocholate uptake was nearly completely restored by treatment with 10 mM 2-mercaptoethanol. The protective effect of DTT and L-cysteine was significantly smaller, whereas glutathione was ineffective in reversing the inhibition by NBD-chloride. Presumably steric and electrostatic effects explain the high potency of the small and electrically neutral molecules 2-mercaptoethanol and DTT in contrast to the polar and bulky agents L-cysteine and glutathione.

TABLE II

Effect of amino-acid modifying agents on Na^+-dependent [^{3}H]taurocholate uptake and D-[U-^{14}C]glucose uptake in brush-border membrane vesicles rabbit ileum

Ileal brush-border membrane vesicles were treated with the respective amino-acid modifying agents for 1 h at 20°C in the concentration range of 10^{-6} to 10^{-2} in the appropriate buffers, as described in Materials and Methods. After washing with 10 mM Tris-Hepes buffer (pH 7.4), 300 mM mannitol the uptake of 50 μM [^{3}H]taurocholate and 19 μM D-[^{14}C]glucose was measured for 1 min at 30°C, both in the presence and the absence of an inwardly-directed Na^+-gradient. IC_{50} values were determined after substraction of the uptake in the absence of a Na^+-gradient from the uptake in the presence of an inwardly-directed Na^+-gradient.

Reagent	Selective for	Inhibition of Na^+-dependent taurocholate uptake (IC_{50}-value)		Inhibition of Na^+-dependent D-glucose uptake (IC_{50}-value)	
$HgCl_2$	SH	+++	($2\cdot10^{-5}$ M)	+++	($4.5\cdot10^{-5}$ M)
PCMB	SH	+++	($5\cdot10^{-5}$ M)	+++	($5\cdot10^{-5}$ M)
PMS	SH	++	($2.5\cdot10^{-4}$ M)	+++	($8\cdot10^{-5}$ M)
NEM	SH	++	($6\cdot10^{-4}$ M)	+++	($3\cdot10^{-5}$ M)
DTNB	SH	+	($5\cdot10^{-3}$ M)	+	($2.5\cdot10^{-3}$ M)
Iodoacetamide	SH	(+)	($>10^{-3}$ M)	(+)	($>10^{-3}$ M)
PAO	vicinal SH	++	($8\cdot10^{-4}$ M)	++	($2\cdot10^{-4}$ M)
DTT	disulfide	∅		∅	
2-Mercaptoethanol	disulfide	∅		∅	
NBD-chloride	SH, NH_2	+++	($7\cdot10^{-5}$ M)	+++	($6\cdot10^{-5}$ M)
TNBS	NH_2	(+)	($8\cdot10^{-3}$ M)	(+)	($>10^{-2}$ M)
FITC	NH_2	++	($6\cdot10^{-4}$ M)	+++	($8\cdot10^{-6}$ M)
PITC	NH_2	++	($6\cdot10^{-4}$ M)	+	($3\cdot10^{-3}$ M)
PMSF	OH	∅		∅	
DCCD	COOH	∅		∅	
Phenylboronic acid	Serine	∅		∅	
NAI	Tyrosine	∅		∅	($>10^{-2}$ M)
DEP	Histidine	∅		+++	($7\cdot10^{-5}$ M)
PGO	Arginine	∅	($>10^{-2}$ M)	∅	

In order to investigate whether the cysteine-residues – obviously essential for active bile acid transport – are located at or near the bile-acid binding site, substrate-protection experiments were carried out. Vesicles were incubated with increasing concentrations of nonlabeled taurocholate for 1 h to obtain equilibrium. After washing, the vesicles were either treated with NBD-chloride or incubated with buffer alone. After repeated washings, the initial uptake of [^{3}H]taurocholate into the membrane vesicles was measured. Fig. 8 demonstrates that increasing concentrations of taurocholate were able to protect the bile-acid transport system from inactivation by NBD-chloride. These findings are indicative for the presence of essential cysteine residues at the binding site for the bile-acid substrates.

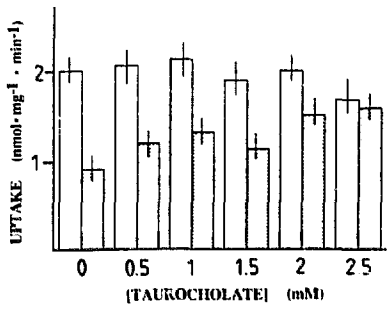

Fig. 8. Protective effect of taurocholate on the inactivation of the ileal bile-acid transport system by NBD-chloride. Ileal brush-border membrane vesicles (10 mg/ml) were incubated for 60 min at 20°C with the indicated concentrations of taurocholate. After washing, one half of the vesicles was incubated for 60 min with 1 mM NBD-chloride in buffer, whereas the other half was kept in buffer alone. After washing, the uptake of 50 μM (0.75 μCi) [^{3}H]taurocholate was measured for 60 s. Open bars, uptake into vesicles incubated with taurocholate alone. Hatched bars, uptake into vesicles treated with NBD-chloride in the presence of taurocholate.

Discussion

The active transport of polar and charged organic solutes across the brush-border membrane of small intestinal enterocytes is mediated by specific membrane carrier-proteins. Bile-acids are taken up in the ileum against a concentration gradient by a Na^+-dependent transport system which couples uphill bile-salt transport to Na^+-migration. The understanding of ileal bile-acid reabsorption on a molecular level is important for the design of potent transport inhibitors. Such an understanding of the transport mechanism and the structure/activity relationships of inhibitors involves the kinetic characterization of the transport system and the identification of the amino acids essential for molecular recognition, together with the identification and purification of the protein components, as well as reconstitution and sequencing of the transporter proteins.

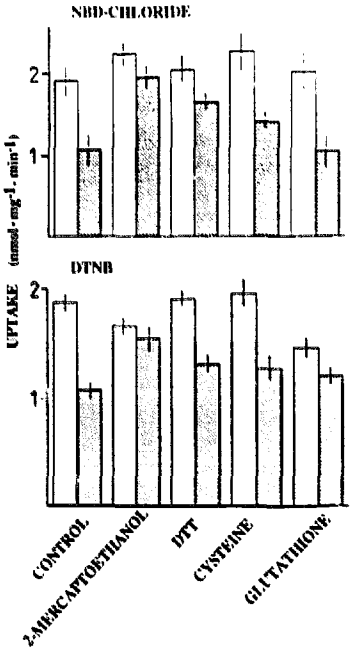

Fig. 7. Reversibility of the inhibiton of the ileal Na^+-dependent bile-acid transport system by NBD-chloride and DTNB with thiols. Ileal brush-border membrane vesicles (10 mg/ml) were incubated for 1 h at 20°C either with 10^{-4} M NBD-chloride (top) or 10^{-3} M DTNB (bottom). After washing the membrane vesicles were treated for 1 h at 20°C, either with buffer alone or with 10 mM solutions of 2-mercaptoethanol, DTT, L-cysteine or glutathione; for control, brush-border membrane vesicles were also incubated with the above-mentioned thiols without chemical modification by NBD-chloride or DTNB. After washing the uptake of 50 μM (0.75 μm) [^{3}H]taurocholate was measured for 60 s in the presence of an inwardly-directed Na^+-gradient. Open bars, uptake into vesicles treated with thiols alone. Hatched bars, uptake into vesicles first modified with DTNB or NBD-chloride and subsequently treated with thiols.

The Na^+-dependent bile-acid transport system in the ileum from rabbit small intestine was characterized using brush-border membrane vesicles. [^{3}H]Taurocholate accumulated against a concentration gradient in the presence of a Na^+-gradient in vesicles obtained from the ileum, whereas no active bile-acid transport was found in vesicles prepared from the jejunum. Photoaffinity labeling with 3- or 7-diazirino derivatives of taurocholic acid resulted in the specific labeling of one membrane polypeptide with an apparent molecular mass of 90 kDa. The specificity for binding to this 90 kDa polypeptide correlated very well with the specificity for transport: bile acids were able to inhibit both transport and photoaffinity labeling and the ranking of the inhibitory effect of the different bile-acids derivatives was also corresponding. Other cholephilic compounds which interfere with hepatic bile-acid uptake, such as phalloidin, bromosulphophthalein, indocyanine green or DIDS neither inhibited active ileal taurocholate transport nor prevented photoaffinity labeling of the 90-kDa protein significantly. These findings suggest that the 90-kDa protein is a component of the ileal Na^+-dependent bile-acid transport system in rabbit

ileum. The molecular mass of 90 kDa of the bile acid binding protein from rabbit ileum as well as the substrate specificity of this protein is similar to the putative ileal bile acid transporter in rats of 99 kDa [23–25,35] whose direct involvement in intestinal bile-acid reabsorption was shown recently with antibodies [56].

To obtain information about the molecular interaction of specific amino-acid residues in the transport protein with the substrate molecule, we have chemically modified the different amino-acid side-chains with a variety of group-specific reagents. Only the modification of thiol- and amino functions led to an irreversible inactivation of the Na^+-dependent [^{3}H]taurocholate uptake system. Thiol groups have also been found to be essential for Na^+-dependent bile-acid uptake by rat hepatocytes [57]. The involvement of thiol groups in active bile-acid reabsorption explains the susceptibility of this transport system in photoaffinity labeling experiments with short-wavelength ultraviolet light [24]. In contrast, transport systems without essential cysteine residues, such as the oligopeptide/β-lactam antibiotic transporter from rabbit small intestine are extremely resistent to photolytic damage [44,47]. The basic amino-acids histidine and arginine and the acidic amino-acids aspartic acid and glutamic acid do not play a role as the hydroxy-containing amino acids serine, threonine or tyrosine. The inhibition of Na^+-dependent taurocholate transport by PAO indicates the involvement of vicinal thiol groups. Agents which react with primary amino groups like PITC, FITC or TNBS also led to an inactivation of the bile-acid transporter, suggesting that lysine residues are also essential for transport activity. From these experimental findings, the structure/activity relationships elaborated by Lack [4] can be modified leading to the hypothesis shown in Fig. 9: (1) The positively-charged group in the transporter protein essential for coulombic interaction with the negatively-charged side-chain of the bile-acid molecule presumably is the ϵ-amino group of a lysine-residue. (2) It may be speculated that (vicinal) cysteine groups, essential for Na^+-dependent transport activity, represent the negatively-charged binding site in the transporter protein [4] presumably responsible for the binding of Na^+-ions.

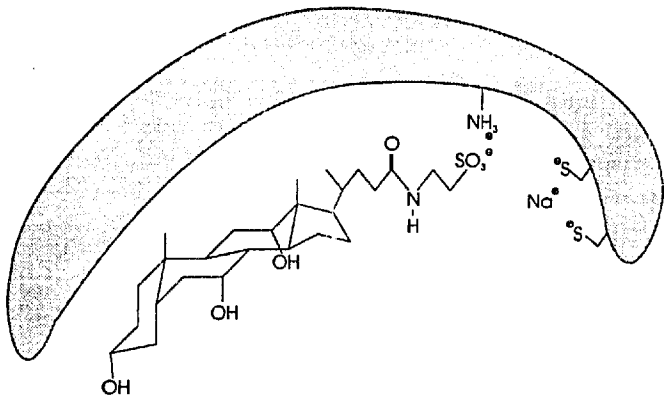

Fig. 9. Model for the interaction of bile acids with the ileal bile-acid transporter.

The susceptibility of the Na^+-dependent bile-acid transporter from rabbit ileum to chemical modification is very similar to that of the D-glucose uptake system [47,58,59] with essential thiol- and amino groups; in addition to the bile-acid transporter, histidine- and tyrosine residues are essential for Na^+-dependent D-glucose transport activity. The bile-acid and glucose transporters also differ in the apparent molecular masses with 72 kDa for the glucose transporter [60] and 90 kDa for the taurocholate transporter. Cloning of the intestinal bile-acid transporter is necessary to decide whether the Na^+-dependent bile acid and glucose transporters belong to a common family of transporters.

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