Contribution of Sodium Taurocholate Co-Transporting Polypeptide to the Uptake of Its Possible Substrates Into Rat Hepatocytes

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ABSTRACT

As one of the Na\(^+\)-dependent transporters responsible for the hepatic uptake of ligands, sodium taurocholate (TC) co-transporting polypeptide (NTCP) has been cloned from rat liver and its substrate specificity has been clarified by examining the inhibition of TC uptake mediated by NTCP. The contribution of NTCP to the Na\(^+\)-dependent uptake of ligands into rat hepatocytes, however, still needs to be clarified. To determine the contribution of NTCP, we examined the uptake of ligands into primary cultured hepatocytes (cultured for 4 h) and into COS-7 cells, transiently expressing NTCP, and normalized the uptake of ligands with TC as a reference compound. Western Blot analysis indicated that NTCP was glycosylated much less extensively in the transfected COS-7 cells, although the expression level was comparable for the cultured hepatocytes and transfectant. Kinetic parameters for the Na\(^+\)-dependent uptake of TC were similar for the cultured hepatocytes and NTCP-transfected COS-7 cells (\(K_m = 17.7\) vs. 17.4 \(\mu\)M; \(V_{\text{max}} = 1.63\) vs. 1.45 nmol/min/mg protein). Glycocholic acid and cholic acid were taken up by NTCP-transfected COS-7 cells. The contribution of NTCP to the Na\(^+\)-dependent uptake of glycocholic acid and cholic acid into rat hepatocytes was approximately 80%, whereas that of cholic acid was 40%. In addition, the analysis indicated that the contribution of NTCP to the Na\(^+\)-dependent uptake of several ligands (ouabain, ibuprofen, glutathione-conjugate of bromosulphophthalein, glucuronide- and sulfate-conjugates of 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole) was negligible. Thus, this is a convenient method to determine the contribution of NTCP to the uptake of ligands into hepatocytes. It is also suggested that multiple transport mechanisms are responsible for the Na\(^+\)-dependent uptake of organic anions into hepatocytes.

In addition to renal excretion, hepatic elimination is one of the main pathways involved in the detoxification of xenobiotics. Hepatic uptake is the initial process for the elimination of xenobiotics mediated by metabolism and/or biliary excretion. The mechanism for the hepatic uptake of ligands has been studied by kinetic analysis of the experimental data obtained in vivo, in situ with perfused liver, in vitro in isolated and/or cultured hepatocytes and isolated sinusoidal membrane vesicles (Suchy, 1993; Elferink et al., 1995; Yamazaki et al., 1996). For the hepatic uptake of bile acids, it has been established that approximately 80 and 40% of TC and CA uptake, respectively, are mediated by a Na\(^+\)-dependent mechanism (Yamazaki et al., 1993). In addition, based on the kinetic studies, it has been suggested that some ligands are transported across the sinusoidal membrane via mechanisms shared with bile acids. Zimmerli et al. (1989) found that the Na\(^+\)-dependent uptake of TC into isolated sinusoidal membrane vesicles was competitively inhibited by bile acids (such as CA, taurochenodeoxycholate and chenodeoxycholate), steroids (such as progesterone and 17-\(\beta\)-estradiol-3-sulfate), bumetanide, furosemide, verapamil and phal-loidin, and suggested a broad substrate specificity for the Na\(^+\)-dependent bile acid transporter. With isolated hepatocytes, cumulative evidence has been obtained to support the hypothesis that several compounds can act as possible substrates of Na\(^+\)-dependent bile acid transporter (Frimmer and Ziegler, 1988). In addition, recently, Terasaki et al. (1995) and Nakamura et al. (1996) found that octreotide (a soma-

ABBREVIATIONS: NTCP, sodium taurocholate co-transporting polypeptide; OATP, organic anion transporting polypeptide; TC, taurocholate, taurocholic acid; GGA, glycocholate, glycocholic acid; CA, cholate, cholic acid; BSP, bromosulphophthalein; BSP-SG, glutathione-conjugate of bromosulphophthalein; E3040, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole; SD, Sprague-Dawley; \(K_m\), Michaelis constant; \(V_{\text{max}}\), maximum transport velocity; CL\(_{\text{uptake}}\), uptake clearance; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; SSC, saline sodium citrate; SDS, sodium dodecyl sulfate; mEH, microsomal epoxide hydrolase; TBS-T, Tris-buffered saline containing 0.05% Tween 20.
tostatin analog) and a cyclic peptide (BQ-123, an endothelin antagonist) are taken up by isolated hepatocytes in a Na+-dependent manner and demonstrated that the uptake of the two ligands is competitively inhibited by TC. In the same manner, Blitzer et al. (1982) and Petzinger et al. (1989) provided kinetic evidence to support the hypothesis that butyrate and TC share a common transport mechanism. Cumulative evidence suggests that the Na+-independent uptake mechanism of many organic anions is shared by CA (Petzinger, 1994; Elferink et al., 1995; Meier, 1995; Yamazaki et al., 1996).

To get more detailed information on the mechanism for hepatic uptake, the cDNA species for NTCP and OATP1 were isolated from rat liver based on expression cloning with Xenopus laevis oocytes (Hagenbuch et al., 1991; Jacquesmin et al., 1994). Moreover, the human homologs of these transporters (NTCP and OATP) have been cloned (Hagenbuch and Meier, 1994; Kullak-Ublick et al., 1995). The sinusoidal localization of these transporters was confirmed with antibodies (Ananthanarayanan et al., 1994; Stieger et al., 1994), and in the transport properties were characterized with oocytes injected with cRNA and mammalian cells transfected with cDNA (Meier, 1995; Hagenbuch and Meier, 1996). Functional analysis of NTCP, has shown that NTCP-mediated TC uptake is inhibited by several bile acid derivatives (such as taurochenodeoxycholate, chenodeoxycholate, tauroursodeoxycholate, ursodeoxycholate and CA), butyrate and BSP (Hagenbuch et al., 1991; Hagenbuch and Meier, 1994; Boyer et al., 1994). However, much less information is available on whether the previously described inhibitors of TC uptake are transported via NTCP. Platte et al. (1996) reported that the transport of CA and GCA into an immortalized liver-derived cell line was not stimulated by transfection of NTCP, although the two bile acid derivatives were effective inhibitors of NTCP-mediated TC uptake in this transfected cell line.

The purpose of the present study is to examine whether several organic anions which are uptake by hepatocytes via a Na+-dependent mechanism can be a substrate for NTCP. In addition, we propose a convenient method to examine the contribution of NTCP to the Na+-dependent uptake of ligands, because multiple systems may be responsible for their hepatic uptake. For this purpose, we examined the uptake of ligands into primary cultured hepatocytes and into COS-7 cells transiently expressing NTCP and normalized the uptake of ligands with TC as a reference compound.

Materials and Methods

Materials. COS-7 cells were purchased from American Type Culture Collection (Rockville, MD). [3H]TC (128.4 GBq/mmol), [3H]ICA (906.5 GBq/mmol) and [3H]ouabain (758.5 GBq/mmol) were purchased from New England Nuclear (Boston, MA). [14C]GCA (2.11 GBq/mmol) and [3H]ibuprofen (18.5 GBq/mmol) were purchased from Amersham International (Buckinghamshire, England). The glucuronide- and sulfate-conjugates of [3H]ES040 (1.85 GBq/mmol), prepared according to the method described previously (Hibi et al., 1994), were kindly donated by Eizai Co., Ltd. (Tokyo, Japan). The [3H]BSP-SG was synthesized according to the method described by Saxena and Henderson (1995) with BSP (Aldrich, Milwaukee, WI) and [3H]glutathione (1739 GBq/mmol, New England Nuclear). All other chemicals were commercially available and of reagent grade.

Transient expression of NTCP cDNA in COS-7 Cells. Full-length cDNA for NTCP was cloned initially by screening the rat liver cDNA library with the reported sequence according to the method described previously in detail (Ito et al., 1997). NTCP cDNA rescued into the pBluescript II S/K (−) vector (TOYOBO, Osaka, Japan) was excised with XhoI and XbaI (Takara, Tokyo, Japan) to perform the subcloning into the XhoI site in the pCAGGS vector (Niwa et al., 1991) after converting to blunt ends.

For transfection, COS-7 cells were cultured in 150-mm dishes in DMEM supplemented with 5% fetal bovine serum. At 30% confluence, cells were exposed to serum-free DMEM containing plasmid (1 μg/ml) and Lipofectamine (1 μg/ml; BRL, Gaithersburg, MD). At 8 h after transfection, the plasmid-Lipofectamine solution was removed, and the medium consisting of DMEM supplemented with 5% fetal bovine serum was allowed to culture overnight. Then, the transfected cells were treated with trypsin to seed approximately 1.6 × 106 cells onto 22-mm dishes and cultured overnight. An uptake study was performed at 48 h after transfection.

Primary cultured rat hepatocytes. Rat hepatocytes were isolated from male SD rats (200 250 g, Nihon Ikagaku Dobutsu Shizai Kenkyusyo, Tokyo, Japan) after perfusion of the liver with collagenase. Cell viability was checked routinely by the trypan blue (0.4% w/v) exclusion test. After preparation, freshly isolated cells were suspended in Williams’ medium E. Approximately 5 × 105 cells were placed on collagen-coated 22-mm dishes and cultured for 4 h.

Northern Blot analysis. Northern Blot analysis was performed as described previously (Ito et al., 1997). Poly(A)+ RNA (0.5 or 2 μg), prepared from COS-7 cells 48 h after transfection and SD rat liver, were separated on 0.8% agarose gel containing 3.7% formaldehyde and transferred to a nylon membrane before fixation by baking for 2 h at 80°C. Blots were prehybridized in medium containing 4 × SSC, 5 × Denhardt’s solution, 0.2% SDS, 0.1 mg/ml sonicated salmon sperm DNA and 50% formamide at 42°C for 2 h. We used 0.9 kbp NTCP cDNA (nucleic acid, 260–1186 bp) as a hybridization probe and hybridization was performed overnight in the same buffer containing 106 cpm/ml 32P-labeled cDNA prepared by the random primed labeling method. As a control, 32P-labeled cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Clontech Laboratories, Inc., Palo Alto, CA) was used. The hybridized membrane was washed in 2 × SSC and 0.1% SDS at 55°C for 20 min and then in 0.1 × SSC and 0.1% SDS at 55°C for 20 min. After the membrane was exposed for 1 h to the imaging plate at room temperature, it was analyzed by Bio-Image Analyzer (Bas 2000; Fuji Film, Tokyo, Japan).

Western Blot analysis. For the Western Blot analysis crude membrane fraction was prepared from COS-7 cells 48 h after transfection, rat hepatocytes cultured for 4 h and SD rat liver according to the method of Gant et al. (1991). Cells were homogenized in five volumes 0.1 M Tris-HCl buffer (pH 7.4) containing 1 μl/μg leupeptin and pepstatin A and 50 μg/ml phenylmethylsulfonyl fluoride with 20 strokes of a Dounce homogenizer. The supernatant, after centrifugation (1500 × g for 10 min) of homogenate, was centrifuged again (100,000 × g for 30 min). The precipitate was suspended in Tris-HCl buffer and centrifuged again (100,000 × g for 30 min). The crude membrane fraction was resuspended in the 0.1 M Tris-HCl buffer containing the proteinase inhibitors with 5 strokes of a Dounce homogenizer and stored at −80°C before being used for Western Blot analysis. All procedures were performed at 0 to 4°C. The membrane protein concentrations were determined according to the method of Lowry et al. (1951). Fifty micromgrams crude membrane was dissolved in 10 μl of 2 × 0.25 M Tris-HCl buffer containing 2% SDS, 30% glycerol and 0.01% bromphenol blue (pH 6.8) and was loaded on a 7.5% SDS-polyacrylamide gel electrophoresis plate with a 4.4% stacking gel. Molecular weight was assessed with a prestained protein marker (NEB, Beverly, MA). Proteins were transferred electrophoretically to a nitrocellulose membrane (Millipore, Bedford, MA) with a blotter (Bio-Rad Laboratories, Richmond, CA) at 15V for 1 h. The membrane was blocked with TBS-T and 5% BSA for 1 h at room temperature. After washing with TBS-T (5 × 5 min), the membrane was incubated with anti-rat NTCP serum (dilution...
1:5000), which was kindly donated by Dr. PJ Meier (Stieger et al., 1994), in TBS-T containing 5% BSA overnight at 4°C and then washed with TBS-T (3×5 min). The membrane was allowed to bind to 125I-labeled sheep anti-rabbit Ig in TBS-T containing 5% BSA for 1 hr room temperature and then washed with TBS-T (3×5 min). After the membrane was exposed overnight to the imaging plate at room temperature, it was analyzed by Bio-Image Analyzer (Bas 2000; Fuji Film, Tokyo, Japan).

**Uptake study**

Uptake was initiated by adding the radiolabeled ligands to the medium after the culture dishes had been washed three times and preincubated with Krebs-Henseleit buffer or choline buffer at 37°C for 5 min. The Krebs-Henseleit buffer consisted of 142 mM NaCl, 23.8 mM NaHCO3, 4.83 mM KCl, 0.96 mM KH2PO4, 1.20 mM MgSO4, 12.5 mM HEPES, 5.0 mM glucose and 1.53 mM CaCl2 adjusted to pH 7.3. The composition of the choline buffer was the same as the Krebs-Henseleit buffer except that the NaCl and NaHCO3 were replaced with isotonic choline chloride and choline bicarbonate, respectively. The final concentration of [3H]TC, [3H]CA, [3H]BSP-SG, [3H]ouabain and [3H]ibuprofen was 1 μM; that of [14C]E3040 glucuronide and [14C]E3040 sulfate was 2 μM whereas that of [14C]GCA was 10 μM. At designated times, the reaction was terminated by adding ice-cold Krebs-Henseleit buffer. Just before the designated times, 50 μl medium was transferred to scintillation vials. Then cells were washed three times with 2 ml ice-cold Krebs-Henseleit buffer and solubilized in 500 μl aliquots of cell lysate were used to determine protein concentrations by the method of Lowry et al. (1951) with BSA as a standard. The ligand uptake is given as the volume of distribution, determined as the amount of ligands associated with the cells (pmol/mg protein) divided by the medium concentration (μM). 

Student’s t test was used to evaluate significant differences in the uptake of ligands into rat hepatocytes in the presence and absence of Na+, and that into COS-7 cells transfected with pCAGGS alone and pCAGGS containing NTCP.

**Determination of kinetic parameters.** The TC uptake for 2 min was used because the initial rates of TC uptake appeared linear during this period. The kinetic parameters for TC uptake were estimated from the following equation:

\[ V_0 = \frac{(V_{max} \times S)}{(K_m + S)} \]  

where \( V_0 \) is the initial uptake rate of TC (nmol/min/mg protein), \( S \) is the TC concentration in the medium (μM), \( K_m \) is the Michaelis constant (μM), and \( V_{max} \) is the maximum uptake rate (nmol/min/mg protein). The uptake data were fitted to this equation by a nonlinear least-squares method with a MULTI program (Yamaoka et al., 1981) to obtain estimates of the kinetic parameters. The input data were weighted as the reciprocals of the squares of the observed values.

**Estimation of the contribution of NTCP to the Na+-dependent uptake of ligands into rat hepatocytes.** The Na+-dependent uptake was calculated by subtracting the Na+-independent uptake (measured in choline buffer) from the total uptake (measured in Krebs-Henseleit buffer). NTCP-mediated uptake was calculated by subtracting the uptake into COS-7 cells transfected with pCAGGS (measured in Krebs-Henseleit buffer) from that into COS-7 cells transfected with pCAGGS containing NTCP (measured in Krebs-Henseleit buffer). The initial uptake velocity for the Na+-dependent and NTCP-mediated uptake of ligands was calculated with linear regression applied to the initial two or three data points. The clearance of uptake and NTCP-mediated uptake of ligands (CLuptake in μl/min/mg protein) was assumed to be the initial velocity for the uptake in pmol/min/mg protein divided by the substrate concentration in the medium (μM). For the determination of CLuptake under linear conditions, the uptake of ligands at tracer concentrations was examined.

\[ R_{hap} = \frac{CL_{uptake of ligands into hepatocytes}}{CL_{uptake of TC into hepatocytes}} \]  

\[ R_{COR} = \frac{CL_{uptake of ligands into COS-7 cells}}{CL_{uptake of TC into COS-7 cells}} \] 

The contribution of NTCP to Na+-dependent uptake of ligands by rat hepatocytes was estimated from the following equation:

\[ \text{Contribution} (%) = \frac{R_{COR}}{R_{hap}} \times 100 \]

**Results**

**Expression of NTCP in COS-7 cells.** The expression of transfected NTCP in COS-7 cells was examined by Northern and Western Blot analyses. As shown in figure 1, the NTCP transcript was found at approximately 2.4 kb in transfected COS-7 cells (lanes c and d), the length of which was longer than that in liver (approximately 2.1 kb; lane a). Western Blot analysis (fig. 1) indicated, however, that the molecular weight of the NTCP product in COS-7 cells (lane h) was approximately 33 kDa, which was significantly lower than that in the cultured hepatocytes (51 kDa; lane g). Although the amount of the transcript of NTCP was 60- to 70-fold higher in NTCP-transfected COS-7 cells than SD rat liver, the amount of NTCP expressed on the membrane was similar for hepatocytes and transfectant (fig. 1). No expression of NTCP was observed in COS-7 cells transfected with pCAGGS vector (lane e).

**Quantification of ligand transport.** The uptake of TC, GCA and CA by cultured hepatocytes and NTCP-transfected COS-7 cells exhibited Na+-dependence, whereas the extent of uptake of these bile acid derivatives by vector-transfected COS-7 cells was minimal (fig. 2). Kinetic analysis of the Na+-dependent uptake of TC by cultured hepatocytes gave a \( K_m \) of 17.7 ± 2.8 μM and a \( V_{max} \) of 1.63 ± 0.15 nmol/min/mg protein (fig. 3). In the same manner, the \( K_m \) and \( V_{max} \) of NTCP-mediated TC uptake was 17.4 ± 3.3 μM and 1.45 ± 0.16 nmol/min/mg protein, respectively (fig. 3).

The Na+-dependent uptake of TC, GCA and CA by cultured hepatocytes was compared with that mediated by NTCP in COS-7 cells (fig. 4). The CLuptake for the Na+-dependent uptake of TC, GCA and CA by the cultured hepatocytes was 61, 26 and 18 μl/min/mg protein, respectively (table 1). In the same manner, the NTCP-mediated CLuptake of TC, GCA and CA was calculated to be 81, 28 and 9 μl/min/mg protein, respectively (table 1). The expression of NTCP to the Na+-dependent uptake of GCA and CA by cultured hepatocytes was 81 and 39%, respectively (table 1). Although the uptake of BSP-SG, E3040 glucuronide and sulfate, ibuprofen and ouabain by primary cultured rat hepatocytes was mediated predominantly by an Na+-independent
mechanism, part of the uptake exhibited Na\(^{+}\)-dependence (fig. 5). In contrast, transfection of NTCP did not affect the uptake of these ligands by COS-7 cells (fig. 5). Accordingly, the contribution of NTCP to the uptake of these ligands into hepatocytes was minimal (table 1).

Discussion

In the present study, we compared the ligand transport between primary cultured rat hepatocytes and NTCP-transfected COS-7 cells. Because the expression of transporters and their function have been reported to decrease in hepatocytes cultured for more than 6 h (Liang et al., 1993; Ishigami et al., 1995), the culture period was restricted to 4 h or less in the present study (Torchia et al., 1996). TC, GCA, CA and E3040 sulfate partially were taken up by primary cultured hepatocytes in a Na\(^{+}\)-dependent manner (figs. 2 and 5), which is consistent with the previously reported transport characteristics of these ligands by freshly isolated and/or cultured hepatocytes (Anwer and Hegner, 1978; Van Dyke et al., 1982; Takenaka et al., 1997). Although the uptake of BSP-SG, E3040 glucuronide, ibuprofen and ouabain by cultured hepatocytes was mediated predominantly by a Na\(^{+}\)-independent mechanism (fig. 5), part of the uptake exhibited Na\(^{+}\)-dependence (fig. 5). These data do not agree with previous reports in which no significant Na\(^{+}\)-dependent uptake of ouabain and E3040 glucuronide into freshly isolated hepatocytes was observed (Eaton and Klaassen, 1978; Takenaka et al., 1997). We have no satisfactory explanation for the difference between the present results and previous reports. The discrepancy may be accounted for by differences in the experimental conditions in that cultured (for 4 h) and freshly isolated hepatocytes were used in the present and previous studies, respectively. It is plausible that unidentified Na\(^{+}\)-dependent transporter(s) for these ligands may have been up-regulated during the 4 h incubation and/or digested with the enzymes used in the preparation of the freshly isolated hepatocytes.

The expression of NTCP cDNA was studied in transfected COS-7 cells (fig. 1). In our preliminary experiments, we examined the expression of the transfected cDNA product into COS-7 cells with β-galactosidase gene inserted into pCAGGS vector. The analysis indicated that the expression of the enzyme was highest at 48 h after transfection. Examination by microscopy indicated that the enzyme was expressed in approximately 70% of COS-7 cells. To optimize the sensitivity of the experiments, we performed the transport studies at 48 h after transfection of NTCP cDNA. It was assumed that the transport properties of NTCP molecules per se do not change as a function of the time after transfection. Northern Blot analysis indicated that the length of the transcript (approximately 2.4 kb) is longer than that observed in SD rat liver (approximately 2.1 kb), which agrees with the report by Boyer et al. (1994). Western Blot analysis indicated that the molecular mass of NTCP expressed in COS-7 cells (approximately 33 kDa) was much smaller than in cultured hepatocytes (approximately 51 kDa). This lower molecular mass of NTCP in the transfected COS-7 cells may be accounted for by a much lower degree of glycosylation of this transporter; previous Western Blot analysis indicated that the molecular mass of NTCP in isolated rat basolateral membrane (51 kDa) is shifted to 33.5 kDa by N-glycosidase F treatment (Stieger et al., 1994). In addition, Hagenbuch et al. (1991) incubated cRNA-injected Xenopus laevis oocytes with \(^{35}\)S)methionine and analyzed the membrane to find a molecular mass of 41 kDa for NTCP in oocytes. Treatment of the oocyte membrane with N-glycosidase F yielded the molecular mass of 35 kDa (Hagenbuch et al., 1991). They also reported the production of 33 kDa protein in an in vitro translation study with wheat germ extract and reticulocyte lysate systems (Hagenbuch et al., 1991). Collectively, the results of the present study suggest that the glycosylation of NTCP in transfected COS-7 cells is minimal. As shown in fig. 1, we found that, although the mRNA levels in COS-7 cells are 60- to 70-fold higher than in the liver, the expression of NTCP was similar for the two cell lines. The minimal glycosylation of NTCP in COS-7 cells may be related to the lower expression of this transporter on the plasma membrane, because it is well established that glycosylation of a protein is closely related to the stability of the protein as well as the intracel-

![Northern blot analysis](image1)

![Western blot analysis](image2)

**Fig. 1.** Expression of NTCP in transfected COS-7 cells was examined by Northern (lanes a–d) and Western (lanes e–h) Blot analyses. Poly (A)\(^{+}\) RNA from SD rat liver, NTCP- and vector-transfected COS-7 cells were used in Northern Blot analysis. The membrane hybridized with \(^{32}\)P-labeled NTCP cDNA fragment (nucleic acid, 260–1186 bp) and rehybridized with \(^{32}\)P-labeled glyceraldehyde-3-phosphate dehydrogenase cDNA was exposed for 1 h at room temperature with an intensifying screen. Lanes a, b and d were loaded with 2 µg poly (A)\(^{+}\) RNA from rat liver, vector- and NTCP-transfected COS-7 cells, respectively. Lane c was loaded with 0.5 µg poly (A)\(^{+}\) RNA from NTCP-transfected COS-7 cells. The crude membrane from SD rat liver, primary cultured rat hepatocytes, NTCP- and vector-transfected COS-7 cells were used in Western Blot analysis. The membrane incubated with anti-rat NTCP serum was exposed overnight at room temperature with an intensifying screen. Lanes e, f, g and h were loaded with 50 µg crude membrane from vector-transfected COS-7 cells, liver, primary cultured hepatocytes and NTCP-transfected COS-7 cells.

**Table 1.** Expression of NTCP in transfected COS-7 cells and primary cultured rat hepatocytes.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>COS-7 Cells</th>
<th>Primary Cultured Hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>Zn(2.2)</td>
<td>Zn(0.8)</td>
</tr>
<tr>
<td>GCA</td>
<td>Zn(2.2)</td>
<td>Zn(0.8)</td>
</tr>
<tr>
<td>CA</td>
<td>Zn(2.2)</td>
<td>Zn(0.8)</td>
</tr>
<tr>
<td>E3040</td>
<td>Zn(2.2)</td>
<td>Zn(0.8)</td>
</tr>
<tr>
<td>BSP-SG</td>
<td>Zn(2.2)</td>
<td>Zn(0.8)</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>Zn(2.2)</td>
<td>Zn(0.8)</td>
</tr>
<tr>
<td>Ouabain</td>
<td>Zn(2.2)</td>
<td>Zn(0.8)</td>
</tr>
</tbody>
</table>

Zn indicates a significant decrease.
lular sorting of synthesized proteins. Because the culture membrane fractionized in the Western Blot analysis (fig. 1) also contains the membrane of intracellular organelles, it is possible that the transfected NTCP product also is located intracellularly. Although Stieger et al. (1994) indicated that NTCP is expressed on the plasma membrane in NTCP-transfected CHO cells, no information is presently available on the intracellular localization of NTCP in COS cells.

Our kinetic analysis indicated that the $K_m$ value for TC was similar in cultured hepatocytes and NTCP-transfected COS-7 cells (17.7 vs. 17.4 μM). These values are in good agreement with previous reports in which the $K_m$ of NTCP for TC was examined in cRNA injected oocytes (25 μM) and cDNA-transfected COS-7 cells (29 μM), and in isolated and/or primary cultured hepatocytes (20~30 μM) (Boyer et al., 1994). Furthermore, we found that the $V_{max}$ for TC was similar in hepatocytes and NTCP-transfected COS-7 cells (1.63 vs. 1.45 nmol/min/mg protein). Because Western Blot analysis indicated that the expression of NTCP was similar in the two cell lines (fig. 1), the results suggest that the glycosylation of NTCP may affect neither the affinity nor the velocity of transport. In addition, the transfected COS-7 cells
may be used for quantitatively predicting NTCP activity in hepatocytes after correction of its expression by Western Blot analysis.

With NTCP-transfected cells, we showed that GCA and CA are substrates for NTCP. Although CA has been hypothesized to be a substrate for NTCP, based on the finding that CA inhibits the NTCP-mediated transport of TC, Platte et al. (1996) failed to demonstrate NTCP-mediated uptake of CA in

**TABLE 1**

Contribution of NTCP to Na⁺-dependent uptake of ligands by rat hepatocytes

<table>
<thead>
<tr>
<th>Compound</th>
<th>CLutake (Hepatocyte) µl/min/mg protein</th>
<th>R_hep</th>
<th>CLutake (COS-7) µl/min/mg protein</th>
<th>R_COS</th>
<th>Contribution of NTCP %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurocholate</td>
<td>60.8</td>
<td>1.00</td>
<td>80.9</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td>Glycocholate</td>
<td>26.4</td>
<td>0.434</td>
<td>28.4</td>
<td>0.351</td>
<td>81</td>
</tr>
<tr>
<td>Cholate</td>
<td>18.0</td>
<td>0.296</td>
<td>9.30</td>
<td>0.115</td>
<td>39</td>
</tr>
<tr>
<td>BSP-SG</td>
<td>22.0</td>
<td>0.362</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>E3040 sulfate</td>
<td>18.9</td>
<td>0.311</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>E3040 glucuronide</td>
<td>3.79</td>
<td>0.0623</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>14.3</td>
<td>0.235</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ouabain</td>
<td>1.16</td>
<td>0.0191</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data shown in figures 2 and 5 were used to determine the clearance for the Na⁺-dependent uptake by hepatocytes and that for NTCP-mediated uptake by transfected COS-7 cells. Based on these clearance values, R_hep and R_COS values were determined to calculate the contribution of NTCP.
HPCT cells. In addition, transfection of NTCP did not stimulate uptake of GCA in this transfected cell line (Platte et al., 1996). The discrepancy between the observation in the present study and that by Platte et al. (1996) may be accounted for, at least in part, by the difference in the expression level of NTCP: the $V_{m}$ of TC into NTCP-transfected HPCT cells was approximately 0.6 µl/min/mg protein (Platte et al., 1996), which is much smaller than observed in the present study (81 µl/min/mg protein) (figs. 2 and 3). It is possible that the lower expression of NTCP hindered detection of the NTCP-mediated transport of GCA and CA because of their uptake via passive diffusion (Platte et al., 1996). Our results are supported further by the finding by Schroeder et al. (1998), of the NTCP-mediated transport of CA and GCA in cDNA-injected and cDNA-transfected CHO cells (Schroeder et al., 1988). The present kinetic analysis indicated that Na$^{+}$-dependent hepatic uptake of GCA is accounted for predominantly by NTCP (table 1). In contrast, NTCP contributed approximately 40% to the Na$^{+}$-dependent CA uptake (table 1), which suggests that another transporter(s), such as mEH (von Dippe et al., 1996), may be involved in the Na$^{+}$-dependent uptake of CA.

We also examined the uptake of ouabain and nonbile acid organic anions in NTCP-transfected COS-7 cells. Although BSP-SG, E3040 sulfate and glucuronide, ibuprofen and ouabain were taken up by hepatocytes, at least in part, in an Na$^{+}$-dependent manner, transfection of NTCP did not stimulate the uptake of these ligands into COS-7 cells (fig. 5), which suggests the presence of multiple transport systems for organic anions. These results are consistent with previous work of Blitzer et al. (1982) and Petzinger et al. (1989), who showed that bumetanide is taken up by isolated rat hepatocyte in an Na$^{+}$-dependent manner and reported mutual inhibition between bumetanide and TC. However, with oocytes, Na$^{+}$-dependent transport of TC and bumetanide was coded by different mRNA fractions in rat liver (Honscha et al., 1993). In addition, injection of NTCP cRNA into oocytes did not stimulate the Na$^{+}$-dependent uptake of bumetanide (Petzinger et al., 1996). Some transporter(s), other than NTCP, may be responsible for the Na$^{+}$-dependent hepatic uptake of organic anions.

In the present study, we also proposed a method to determine the contribution of NTCP to the hepatic uptake of ligands. This method is valid if the Na$^{+}$-dependent uptake of TC by hepatocytes is mediated predominantly by NTCP. This assumption has been justified by the previous finding by Hagenbuch et al. (1996); they used antisense oligonucleotide against NTCP to inhibit the expression of this particular transporter in oocytes injected with total rat liver mRNA. Simultaneous injection of an antisense oligonucleotide almost completely (approximately 95%) abolished the Na$^{+}$-dependent uptake of TC, which suggests that the Na$^{+}$-dependent hepatic uptake of TC is mediated predominantly by NTCP. Although mEH has been identified as the Na$^{+}$-dependent transporter for TC in hepatocytes (von Dippe et al., 1996), the contribution of mEH to hepatic uptake of TC might be less marked than that of NTCP.

We must interpret the data cautiously, however, because the determination of the magnitude of the contribution may not be appropriate if we assume a synergistic or allosteric interaction of the transporter with unidentified membrane protein(s). In addition, it is also possible that the post-translational modification of NTCP may affect the substrate specificity and affinity of NTCP, although the $K_{m}$ value for TC was very similar for hepatocytes and NTCP-transfected COS-7 cells (fig. 3). These two types of problems are associated with any experiments designed to examine the transport properties of cloned cDNA product. A complete answer to these questions may be obtained by comprehensively examining the transport properties of cDNA-injected oocytes and following cDNA-transfection of many kinds of mammalian cell lines. Irrespective of those kinds of limitations, the methodology described in this manuscript may also be used to determine the contribution of other transporters. Indeed, we recently determined the contribution of OATP1 to the hepatic uptake of several ligands with estradiol-17β-d-glucuronide as a reference compound (Kouzuki et al., submitted).

In conclusion, we have a convenient method to determine the contribution of NTCP to the Na$^{+}$-dependent uptake of ligands by hepatocytes with NTCP-transfected COS-7 cells. Although the contribution of NTCP can be estimated by simultaneous injection of antisense oligonucleotide against NTCP with total rat liver mRNA, the method described in the present study may be more useful because we often have difficulty in observing a significant uptake of test compounds into oocytes injected with total liver mRNA. The present analysis shows that Na$^{+}$-dependent uptake of GCA is mediated predominantly by NTCP, whereas transporter(s) other than NTCP may be responsible for the Na$^{+}$-dependent uptake of CA. In addition, Na$^{+}$-dependent uptake of ligands examined in the present study was not mediated by NTCP, which suggests the presence of multiplicity for the Na$^{+}$-dependent transport mechanism across the sinusoidal membrane.

References


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