Substrate specificity of the rat liver \( \text{Na}^+ \)-bile salt cotransporter in \( \text{Xenopus laevis} \) oocytes and in \( \text{CHO} \) cells

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1Division of Clinical Pharmacology and Toxicology, Department of Medicine, University Hospital, CH-8091 Zurich; 2Drug Metabolism and Pharmacokinetics, Novartis Pharma, CH-4002 Basel, Switzerland; 3Division of Gastroenterology, Department of Medicine, University of California San Diego, La Jolla 92039-0813; and 4Ferring Research, La Jolla, California 92037

Schroeder, Alice, Uta Eckhardt, Bruno Stieger, Ronald Tynes, Claudio D. Schteingart, Alan F. Hofmann, Peter J. Meier, and Bruno Hagenbuch. Substrate specificity of the rat liver \( \text{Na}^+ \)-bile salt cotransporter in \( \text{Xenopus laevis} \) oocytes and in \( \text{CHO} \) cells. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G370–G375, 1998.—It has been proposed that the hepatocellular \( \text{Na}^+ \)-dependent bile salt uptake system exhibits a broad substrate specificity in intact hepatocytes. In contrast, recent expression studies in mammalian cell lines have suggested that the cloned rat liver \( \text{Na}^+ \)-taurocholate cotransporting polypeptide (Ntcp) may transport only taurocholate. To characterize its substrate specificity Ntcp was stably transfected into Chinese hamster ovary (CHO) cells. These cells exhibited saturable \( \text{Na}^+ \)-dependent uptake of \( [\text{H}] \)taurocholate (Michaelis constant \( K_m \) of \( \approx 34 \mu \text{M} \)) that was strongly inhibited by all major bile salts, estrone 3-sulfate, semutamidene, and cyclosporin A. Ntcp cRNA-injected \( \text{Xenopus laevis} \) oocytes and the transfected \( \text{CHO} \) cells exhibited saturable \( \text{Na}^+ \)-dependent uptake of \( [\text{H}] \)taurocholate (\( K_m \) of \( \approx 5 \mu \text{M} \)), \( [\text{H}] \)tauroursodeoxycholate (\( K_m \) of \( \approx 14 \mu \text{M} \)), and \( [\text{H}] \)glycocholate (\( K_m \) of \( \approx 27 \mu \text{M} \)). After induction of gene expression by sodium butyrate, \( \text{Na}^+ \)-dependent transport of \( [\text{H}] \)estrone 3-sulfate (\( K_m \) of \( \approx 27 \mu \text{M} \)) could also be detected in the transfected \( \text{CHO} \) cells. However, there was no detectable \( \text{Na}^+ \)-dependent uptake of \( [\text{H}] \)bumataneide or \( [\text{H}] \)glycocholate A. These results show that the cloned Ntcp can mediate \( \text{Na}^+ \)-dependent uptake of all physiological bile salts as well as of the steroid conjugate estrone 3-sulfate. Hence, Ntcp is a multispecific transporter with preference for bile salts and other anionic steroidal compounds.

UPTAKE OF ORGANIC ANIONS such as bile salts from blood into hepatocytes is mediated by \( \text{Na}^+ \)-dependent as well as \( \text{Na}^+ \)-independent transport systems (7). Previous kinetic uptake and inhibition studies in several experimental systems have indicated that the hepatocellular \( \text{Na}^+ \)-dependent bile salt uptake system exhibits a broad substrate specificity for a wide variety of amphipathic organic molecules, including conjugated bile salts, electronneutral steroids, cyclic oligopeptides, and a number of drugs, such as bumetanide and cyclosporin A (3, 14, 22). In contrast, recent expression studies in the liver-derived immortalized cell line HPCT-1E3 have suggested that the cloned rat liver \( \text{Na}^+ \)-taurocholate co-transporting polypeptide (Ntcp) may exclusively transport taurocholate and that other transport systems, such as epoxide hydrolase, account for \( \text{Na}^+ \)-dependent uptake of cholate and glycocholate into rat hepatocytes (15, 21). Because Ntcp is thought to represent a major bile salt transport system in mammalian liver (8) and because only taurocholate has been used as a transport substrate in previous Ntcp expression studies (15, 19), we have reexamined the substrate specificity of Ntcp in more detail in cRNA-injected \( \text{Xenopus laevis} \) oocytes and in transfected Chinese hamster ovary (CHO) cells. The results clearly demonstrate that Ntcp transports typical natural di- and trihydroxy-conjugated bile salts, as well as, albeit to a lesser degree, the steroid conjugate estrone 3-sulfate.

MATERIALS AND METHODS

Materials. \( [\text{H}] \)taurocholic acid (2.6 Ci/mmol), \( [\text{H}] \)cholic acid (13.2 Ci/mmol), \( [\text{H}] \)glycocholic acid (44.6 mCi/mmol), and \( [\text{H}] \)estrone 3-sulfate (49.0 Ci/mmol) were obtained from DuPont-New England Nuclear (Boston, MA). \( [\text{H}] \)glycocholate A (11.1 Ci/mmol) was purchased from Amersham International (Little Chalfont, Buckinghamshire, UK). \( [\text{H}] \)taurodeoxycholic acid (0.5 Ci/mmol) and \( [\text{H}] \)tauroursodeoxycholic acid (0.5 Ci/mmol) were conjugated as described previously (11). \( [\text{H}] \)bumataneide was kindly provided by E. Petzinger of Justus Liebig-Universität (Giessen, Germany). All cell culture media and reagents were obtained from Life Technologies (Paisley, UK). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

Expression of Ntcp in \( \text{Xenopus laevis} \) oocytes. \( \text{Xenopus laevis} \) oocytes were prepared as described previously (8). After an overnight incubation at 18°C, healthy oocytes were injected with 2.5 ng of Ntcp cDNA or water. After 3 days in culture, uptake of the indicated substrates was measured at 25°C in a medium containing 100 mM NaCl or choline chloride, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Tris(hydroxymethyl)aminomethane, pH 7.5, as described previously (6).

Stable transfection of \( \text{CHO} \) cells with Ntcp. Wild-type \( \text{CHO} \) cells (CHO-K1) were stably transfected with the coding region of the rat Ntcp (9 subcloned into vector pCMV vector-1 as previously described (18). The vector pCMV contains the strong cytomegalovirus promoter-enhancer unit excised from pcDNA1/NEO (Invitrogen) and used for user convenience into pBluescript (Stratagene, La Jolla, CA), which also contained a \( \text{Xenopus} \)-rabbit \( \text{5}’ \)-untranslated leader, the rabbit \( \beta \)-globin poly(A) signal, and a gastrin gene transcription stop signal. From the resulting transfected cell pool, single clones were isolated using cloning cylinders and tested for \( \text{Na}^+ \)-dependent transport of taurocholate. The best trans-
porting clone (CHO 9-6) was selected and used for all further experiments.

Cell culture. CHO cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 50 µg/ml l-proline, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.5 µg/ml Fungizone (amphotericin B) at 37°C with 5% CO2 and 95% humidity. Selective media contained an additional 400 µg/ml G418-sulfate (Geneticin).

Uptake studies in CHO cells. For the determination of Na\(^{+}\)-dependent uptake of potential substrates for Ntcp, the cells were grown to confluency on 35-mm dishes and rinsed three times with prewarmed (37°C) sodium or choline containing Earle's balanced saline solution (16) supplemented with 5.5 mM D-glucose. The cells were then incubated at 37°C in the presence of radiolabeled substrate. After the indicated time interval, the transport was stopped with 3 ml of ice-cold Na\(^{+}\)-containing Earle's solution. After two additional washing steps with the same stop solution, the cells were solubilized in 1 ml of 1% Triton X-100 and mixed with 5 ml of scintillation fluid (Ultima Gold; Canberra Packard International, Zurich, Switzerland). Radioactivity was determined in a Packard Tri-Carb 2200 CA liquid scintillation counter (Canberra Packard). For some experiments, expression of Ntcp was induced by incubating the cells for 24 h with culture medium supplemented with 5 mM butyrate as described previously (13).

RESULTS

To evaluate the proposed broad substrate specificity of Ntcp in a stably transfected cell line, we constructed an expression vector by inserting the coding region of Ntcp into pCMV vector-1 (18) and electroporated the resulting construct into CHO cells. Several G418-resistant cell clones were isolated using cloning cylinders and tested for Na\(^{+}\)-dependent taurocholate uptake. Clone CHO 9-6 exhibited the highest transport activity.

Time-dependent uptakes of taurocholate in wild-type and Ntcp-expressing CHO cells are compared in Fig. 1. Wild-type cells (Fig. 1A) showed no significant Na\(^{+}\)-dependent transport of taurocholate, reflecting the absence of Ntcp in these cells. In contrast, Ntcp-expressing CHO 9-6 cells (Fig. 1B) showed a strong intracellular accumulation of taurocholate in the presence, but not in the absence, of Na\(^{+}\). As shown in Fig. 2, the Na\(^{+}\)-dependent taurocholate uptake portion was strongly inhibited by cholate, taurodeoxycholate, taurochenodeoxycholate (TCDC), taoursodeoxycholate (TUDC), the sulfate-conjugated steroids estrone 3-sulfate and 17β-estradiol 3-sulfate, and the drugs bumetanide and cyclosporin A. Inhibition by the unconjugated steroids such as testosterone and progesterone was less pronounced.

To test whether the cis-inhibiting substrates are also transported by Ntcp, we performed uptake studies in the Xenopus laevis oocyte expression system. As summarized in Table 1, Na\(^{+}\)-dependent transport of all tested bile salts was stimulated between 10- and 100-fold in cRNA-injected oocytes. In contrast, Na\(^{+}\)-dependent uptake of radiolabeled estrone 3-sulfate was stimulated only twofold, and no Na\(^{+}\)-dependent uptake was observed for bumetanide and cyclosporin A. Similar re-
results were also obtained in stably transfected CHO 9-6 cells. As depicted in Fig. 3, both TCDC and TUDC accumulated in an Na\(^{+}\)-dependent manner in Ntcp-expressing CHO 9-6 cells, whereas no significant Na\(^{+}\)-dependent uptake was seen in wild-type CHO-K1 cells.

Because Na\(^{+}\)-dependent uptake of glycocholate was only minimally stimulated in uninduced CHO 9-6 cells (Fig. 4), we induced gene expression by 24 h preincubation with 5 mM sodium butyrate (13). As can be seen in Fig. 4, butyrate induction resulted in a 10-fold increase in the expressed Na\(^{+}\)-dependent glycocholate uptake rate, whereas no effect of butyrate was seen on Na\(^{+}\)-independent uptake. In contrast, wild-type CHO-K1 cells did not show any Na\(^{+}\)-dependent accumulation of glycocholate even after butyrate induction (Fig. 4).

Finally, we measured uptake of the cis-inhibiting substrate estrone 3-sulfate and determined the kinetics of substrate uptake in butyrate-induced Ntcp-expressing CHO 9-6 cells. In contrast, wild-type CHO-K1 cells did not show any Na\(^{+}\)-dependent transport signal. Furthermore, Na\(^{+}\)-dependent uptake of estrone 3-sulfate and various bile salts exhibited clear saturability with the highest affinity for TCDC, followed by TUDC > glycocholate = estrone 3-sulfate = taurocholate (Table 2).

### Table 1. Ntcp-mediated substrate transport in cRNA-injected Xenopus laevis oocytes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Uptake, fmol·oocyte(^{-1})·min(^{-1})</th>
<th>NaCl</th>
<th>Choline chloride</th>
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<tbody>
<tr>
<td>Cholate (1 µM)</td>
<td>6.1 ± 1.0</td>
<td>0.1 ± 0.01</td>
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<tr>
<td>Taurocholate (5 µM)</td>
<td>135.3 ± 60.0</td>
<td>1.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Glycocholate (50 µM)</td>
<td>728.4 ± 325.8</td>
<td>70.3 ± 15.1</td>
<td></td>
</tr>
<tr>
<td>Taurochenodeoxycholate (1 µM)</td>
<td>31.3 ± 13.2</td>
<td>2.6 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Tauroursodeoxycholate (1 µM)</td>
<td>56.8 ± 23.2</td>
<td>5.8 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Estrone 3-sulfate (1 µM)</td>
<td>3.6 ± 1.1</td>
<td>1.5 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Bumetanide (20 µM)</td>
<td>32.1 ± 6.0</td>
<td>29.5 ± 4.0</td>
<td></td>
</tr>
<tr>
<td>Cyclosporin A (10 µM)</td>
<td>1.7 ± 2.2</td>
<td>1.4 ± 1.3</td>
<td></td>
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</tbody>
</table>

Values represent means ± SD of 10–15 determinations. Xenopus laevis oocytes were injected with 2.5 ng of Na\(^{+}\)-taurocholate cotransporting polypeptide (Ntcp) cRNA. After 3 days in culture, uptakes of indicated substrates were measured at 25°C in NaCl or choline chloride buffers for 15 min.

As depicted in Fig. 3, both TCDC and TUDC accumulated in an Na\(^{+}\)-dependent manner in Ntcp-expressing CHO 9-6 cells, whereas no significant Na\(^{+}\)-dependent uptake was seen in wild-type CHO-K1 cells. Because Na\(^{+}\)-dependent uptake of glycocholate was only minimally stimulated in uninduced CHO 9-6 cells (Fig. 4), we induced gene expression by 24 h preincubation with 5 mM sodium butyrate (13). As can be seen in Fig. 4, butyrate induction resulted in a 10-fold increase in the expressed Na\(^{+}\)-dependent glycocholate uptake rate, whereas no effect of butyrate was seen on Na\(^{+}\)-independent uptake. In contrast, wild-type CHO-K1 cells did not show any Na\(^{+}\)-dependent accumulation of glycocholate even after butyrate induction (Fig. 4).

Finally, we measured uptake of the cis-inhibiting substrate estrone 3-sulfate and determined the kinetics of substrate uptake in butyrate-induced Ntcp-expressing CHO 9-6 cells. As in oocytes (Table 1) there was again a clear Na\(^{+}\)-dependent estrone 3-sulfate transport in the CHO 9-6 cells (Fig. 5), whereas wild-type CHO-K1 cells showed no Na\(^{+}\)-dependent transport signal. Furthermore, Na\(^{+}\)-dependent uptake of estrone 3-sulfate and various bile salts exhibited clear saturability with the highest affinity for TCDC, followed by TUDC > glycocholate = estrone 3-sulfate = taurocholate (Table 2). In contrast, several attempts failed to demonstrate Na\(^{+}\)-dependent uptake of bumetanide and cyclosporin A in butyrate-induced Ntcp-expressing CHO 9-6 cells. No difference in bumetanide (1 µM) uptake was found in the presence (1.42 ± 0.17 pmol·min\(^{-1}\)·mg protein\(^{-1}\)) and absence (1.30 ± 0.18 pmol·min\(^{-1}\)·mg protein\(^{-1}\)) of Na\(^{+}\) in transfected CHO cells. For cyclosporin A (1 µM) similar uptakes were found in transfected (175 ± 23 and 126 ± 9 pmol·min\(^{-1}\)·mg protein\(^{-1}\)) and in wild-type (172 ± 21 and 128 ± 9 pmol·min\(^{-1}\)·mg protein\(^{-1}\))
CHO cells. These results are similar to the data obtained in injected Xenopus laevis oocytes (Table 1). They prove the universal nature of Ntcp as a bile salt carrier and document its relative broad substrate specificity. However, the data also indicate that not all \( \text{cis} \)-inhibiting substrates are also transport substrates of Ntcp, thus emphasizing the need for direct transport studies rather than kinetic inhibition studies for the correct determination of the substrate specificity of a single carrier protein.

**DISCUSSION**

Previous kinetic inhibition studies with hepatocytes and isolated rat liver basolateral membrane vesicles have provided evidence for a rather broad substrate specificity of the hepatocellular Na\(^{+}\)-dependent bile salt uptake system (3, 14, 22). However, these transport studies in intact cells and isolated membrane vesicles could not distinguish between the presence of a single multispecific or several monospecific transport systems. To discriminate between these two possibilities, the involved transporting polypeptides must be isolated and transfected into a cell line that does not constitutively express the respective transport function. Although functional expression of Ntcp in Xenopus laevis oocytes (9) as well as in various mammalian cell lines (2, 15, 18, 19) has clearly established its function as an Na\(^{+}\)-dependent taurocholate uptake system, recent experiments in stably transfected HPCT-1E3 cells have suggested that Ntcp might not transport other bile salts such as glycocholate and cholate (15). In fact, additional Na\(^{+}\)-dependent bile salt transporting polypeptides, such as microsomal epoxide hydrolase (21), have been postulated to contribute to the obvious multispecificity of the Na\(^{+}\)-dependent bile salt uptake function of rat hepatocytes (20).

Contrary to these suggestions, the present study demonstrates that besides taurocholate, Ntcp also mediates transport of cholate, glycocholate, TCDC, and TUDC (Tables 1 and 2; Figs. 3 and 4). These results indicate that Ntcp can transport all \( \text{cis} \)-inhibiting bile salt derivatives (Fig. 2), although the extent of Ntcp-

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( K_m ) ( \mu \text{M} )</th>
<th>( V_{max} ) (pmoles·min(^{-1} )·mg protein(^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurocholate</td>
<td>34 ± 3</td>
<td>744 ± 28</td>
</tr>
<tr>
<td>Glycocholate</td>
<td>27 ± 6</td>
<td>241 ± 28</td>
</tr>
<tr>
<td>Taurochenodeoxycholate</td>
<td>5 ± 1</td>
<td>787 ± 36</td>
</tr>
<tr>
<td>Tauroursodeoxycholate</td>
<td>14 ± 3</td>
<td>584 ± 38</td>
</tr>
<tr>
<td>Estrone 3-sulfate</td>
<td>27 ± 15</td>
<td>451 ± 96</td>
</tr>
</tbody>
</table>

Initial 20 s uptake measurements were performed at 37°C in the presence and absence of Na\(^{+}\). Net Na\(^{+}\)-dependent uptake values (uptake in the presence of NaCl minus uptake in the presence of choline chloride) were fitted using nonlinear regression analysis. CHO, Chinese hamster ovary; \( K_m \), Michaelis constant; \( V_{max} \), maximum velocity.
mediated transport is different for various bile salts. Thus, based on the ratio of the maximum velocity (\(V_{\text{max}}\)) to the Michaelis constant, the best transport substrate of Ntcp was TCDDC followed by TUDC, taurocholate, estrone 3-sulfate, and glycocholate (Table 2). These data are consistent with the previously observed higher affinity of Na\(^+\)-dependent dihydroxy-conjugated bile salt uptake compared with trihydroxy-conjugated bile salt uptake in isolated hepatocytes (1, 20). Furthermore, the kinetic data presented in Table 2 demonstrate that maximal Na\(^+\)-dependent bile salt uptake in butyrate-induced Ntcp-expressing CHO 9-6 cells was as high as in short-term cultured hepatocytes (19), indicating that the adopted CHO cell system represents a suitable model for the correct delineation of the substrate specificity of Ntcp.

In addition to various physiological bile salts, the cis-inhibiting compound estrone 3-sulfate (Fig. 2) was also transported by Ntcp with an affinity similar to that of bile salts (Table 2) in butyrate-induced CHO 9-6 cells (Fig. 5), indicating that the substrate specificity of Ntcp indeed extends beyond bile salts as previously suggested in functional transport studies in isolated basolateral membrane vesicles (22). However, in comparison with bile salts, the Ntcp-mediated Na\(^+\)-dependent uptake portion of estrone 3-sulfate is small (Table 1). In transfected CHO 9-6 cells it could only be detected after butyrate-induced gene expression, indicating that the level of Ntcp expression is a critical factor for correct delineation of the true substrate specificity of Ntcp. In this regard, it is important to realize that even after butyrate induction, the transfected CHO 9-6 cells still expressed an approximately threefold lower \(V_{\text{max}}\) value for Na\(^+\)-dependent taurocholate uptake compared with freshly isolated hepatocytes (1). Hence, it cannot be definitely excluded that at higher expression levels Ntcp might also transport the cis-inhibiting substrates bumetanide and cyclosporin A (Fig. 2). However, this possibility appears unlikely, since bumetanide transport is encoded by a different rat liver mRNA species (10) and cyclosporin A exhibited noncompetitive inhibition of Na\(^+\)-dependent taurocholate uptake in isolated rat liver basolateral plasma membrane vesicles (22). The present study nevertheless demonstrates that the achieved expression level in butyrate-induced CHO 9-6 cells was sufficient to extend the substrate specificity of Ntcp to various physiological bile salts and to the estrogen conjugate estrone 3-sulfate. Because Ntcp expression is decreased in various forms of cholestatic liver disease (4, 5, 17), its downregulation may be associated with a continuous narrowing of the spectrum of transported substrates also in vivo. Our results indicate that even at low expression levels Ntcp would still maintain its transport preference for taurine-conjugated bile salts, whereas its less well-transported substrates could still be transported across the sinusoidal membrane of cholestatic hepatocytes by Na\(^+\)-independent systems, including members of the organic anion transporting poly peptide gene (oatp) family of transporters (12).

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