Substrate specificity of the rat liver Na+-bile salt cotransporter in Xenopus laevis oocytes and in CHO cells

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MATERIALS AND METHODS

Materials. [3H]taurocholic acid (2.6 Ci/mmol), [3H]cholesterol acid (13.2 Ci/mmol), [14C]glycocholic acid (44.6 mCi/mmol), and [3H]estrone 3-sulfate (49.0 Ci/mmol) were obtained from Du Pont-New England Nuclear (Boston, MA). [3H]cyclosporin A (11.1 Ci/mmol) was purchased from Amersham International (Little Chalfont, Buckinghamshire, UK). [3H]taurocholate oxidase (0.5 Ci/mmol) and [3H]taurosododeoxycholate (0.5 Ci/mmol) were conjugated as described previously (11). [3H]betamethasone was kindly provided by E. Petzinger of J. usus 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 Xenopus laevis oocytes. 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 cRNA 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-hydroxyethylpiperezine-N′-2-ethanesulfonic acid (tris[hydroxymethyl]aminomethane, pH 7.5, as described previously (6).

Stable transfection of CHO cells with Ntcp. Wild-type CHO cells (CHO-K1) were stably transfected with the cDNA 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 pDNA1/NEO (Invitrogen) and used for user convenience into pBluescript (Stratagene, La Jolla, CA), which also contained a Xenopus-rabbit b-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 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 RADIOLABELLED 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 TAURUROCHOLATE UPTAKE. CLONE CHO 9-6 EXHIBITED THE HIGHEST TRANSPORT ACTIVITY.

TIME-DEPENDENT UPTAKES OF TAURUROCHOLATE 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 TAURUROCHOLATE, REFLECTING THE ABSENCE OF NTCP IN THESE CELLS. IN CONTRAST, NTCP-EXPRESSING CHO 9-6 CELLS (FIG. 1B) SHOWED A STRONG INTRACELLULAR ACCUMULATION OF TAURUROCHOLATE IN THE PRESENCE, BUT NOT IN THE ABSENCE, OF Na\(^+\). AS SHOWN IN FIG. 2, THE Na\(^+\)-DEPENDENT TAURUROCHOLATE UPTAKE PORTION WAS STRONGLY INHIBITED BY CHOLATE, TAURURODEOXYCHOLATE, TAURUCHENODEOXYCHOLATE (TCDC), TAUROURSODEOXYCHOLATE (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 RADIOLABELLED 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 an 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, and estrone 3-sulfate (10). In contrast, several attempts failed to demonstrate Na\(^{+}\)-dependent uptake of bumetanide and cyclosporin A in butyrate-induced Ntcp-expressing CHO 9-6 cells.

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>
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<tr>
<td>Cholate (1 µM)</td>
<td>6.1 ± 1.0</td>
</tr>
<tr>
<td>Taurocholate (5 µM)</td>
<td>135.3 ± 60.0</td>
</tr>
<tr>
<td>Glycocholate (50 µM)</td>
<td>728.4 ± 325.8</td>
</tr>
<tr>
<td>Taurochenodeoxycholate (1 µM)</td>
<td>31.3 ± 13.2</td>
</tr>
<tr>
<td>Tauroursodeoxycholate (1 µM)</td>
<td>56.8 ± 23.2</td>
</tr>
<tr>
<td>Estrone 3-sulfate (1 µM)</td>
<td>3.6 ± 1.1</td>
</tr>
<tr>
<td>Bumetanide (20 µM)</td>
<td>32.1 ± 6.0</td>
</tr>
<tr>
<td>Cyclosporin A (10 µM)</td>
<td>1.7 ± 2.2</td>
</tr>
</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.

Fig. 3. Time course of Na\(^{+}\)-dependent taurochenodeoxycholate (A, B) and tauroursodeoxycholate (C, D) uptake into CHO cells. Wild-type (CHO-K1) (A, C) or Ntcp-expressing (CHO 9-6) CHO cells (B, D) were incubated with 2.5 µM \(^{[3]}\)H]taurochenodeoxycholate (A, B) or 2.5 µM \(^{[3]}\)H]tauroursodeoxycholate (C, D) at 37°C for the indicated time periods in the presence (●) and absence (○) of an inwardly directed NaCl gradient. Data points represent means ± SD of triplicate determinations.
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 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 cis-inhibiting bile salt derivatives (Fig. 2), although the extent of Ntcp-

**Table 2. Kinetic parameters of Na\(^+\)-dependent substrate uptake in butyrate-induced Ntcp-expressing CHO 9-6 cells**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(K_m) (\mu)M</th>
<th>(V_{max}) pmoles (\cdot) min (^{-1}) (\cdot) 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>Tauursodeoxycholate</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>
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</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.

Fig. 4. \(Na^+\)-dependent uptake of glycocholate into CHO cells with and without butyrate induction. Before uptake measurements wild-type (CHO-K1) or Ntcp-expressing (CHO 9-6) CHO cells were incubated for 24 h without or with 5 mM sodium butyrate (butyrate). For uptake measurements cells were incubated with 2.2 \(\mu\)M \[^{14}\)C\]glycocholate at 37°C for 5 and 30 min in the presence (solid bars) and absence (open bars) of an inwardly directed NaCl gradient. Values represent means ± SD of triplicate determinations.

Fig. 5. Time course of \(Na^+\)-dependent estrone 3-sulfate uptake into CHO cells. Wild-type (CHO-K1; A) or Ntcp-expressing (CHO 9-6; B) CHO cells were incubated for 24 h in presence of 5 mM sodium butyrate. For uptake measurements cells were incubated at 37°C for the indicated time periods in the presence (●) and absence (○) of an inwardly directed NaCl gradient with 10 \(\mu\)M \[^{3}H\]estrone 3-sulfate. Data points represent means ± SD of triplicate determinations.
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 $\text{Ntcp}$ was TCDC followed by TUDC, taurocholate, estrone 3-sulfate, and glycocholate (Table 2). These data are consistent with the previously observed higher affinity of $\text{Na}^+$-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 $\text{Na}^+$-dependent bile salt uptake in butyrate-induced $\text{Ntcp}$-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 $\text{Ntcp}$.

In addition to various physiological bile salts, the cis-inhibiting compound estrone 3-sulfate (Fig. 2) was also transported by $\text{Ntcp}$ 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 $\text{Ntcp}$ 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 $\text{Ntcp}$-mediated $\text{Na}^+$-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 $\text{Ntcp}$ expression is a critical factor for correct delineation of the true substrate specificity of $\text{Ntcp}$. 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 $\text{Na}^+$-dependent taurocholate uptake compared with freshly isolated hepatocytes (1). Hence, it cannot be definitely excluded that at higher expression levels $\text{Ntcp}$ 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 $\text{Na}^+$-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 $\text{Ntcp}$ to various physiological bile salts and to the estrogen conjugate estrone 3-sulfate. Because $\text{Ntcp}$ 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 $\text{Ntcp}$ 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 $\text{Na}^+$-independent systems, including members of the organic anion transporting polypeptide gene (oatp) family of transporters (12).

This study was supported by Swiss National Science Foundation Grants 31–45536.95 and 31–45677.95 (to P. J. Meier and B. Hagenbuch). Work at the University of California San Diego was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-21506 as well as by a Grant-in-Aid from the Falk Foundation (Freiburg, Germany). B. Hagenbuch is a recipient of a Cloetta Foundation Fellowship.

A preliminary report of this study was presented at the annual meeting of the American Gastroenterological Association in New Orleans, in May 1994, and was published previously in abstract form (Gastroenterology 106: A979, 1994).

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Received 16 Jan 1994; accepted in final form 30 October 1997.

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