Taurocholate Transport by Rat Liver Canalicular Membrane Vesicles

Evidence for the Presence of An Na⁺-independent Transport System

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bstract. To elucidate the mechanism of vectorial translocation of bile acids in the liver, taurocholate transport was studied in isolated liver canalicular membrane vesicles by a rapid filtration method. The membrane vesicles revealed temperature-dependent, Na⁺-independent transport of taurocholate into an osmotically reactive intravesicular space. In the absence of sodium, taurocholate uptake followed saturation kinetics (apparent $K_{\rm m}$ for taurocholate = 43 μ M and V_{max} = 0.22 nmol/mg protein \times 20 s at 37°C) and was inhibited by cholate and probenecid. Transstimulation by unlabeled taurocholate was also demonstrated. When the electrical potential difference across the membranes was altered by anion replacement, a more positive intravesicular potential stimulated, and a more negative potential inhibited, transport of taurocholate by the vesicles. Valinomycin-induced K⁺diffusion potential (vesicle inside-positive) enhanced the rate of taurocholate uptake that was not altered by imposed pH gradients.

These results indicate that rat liver canalicular plasma membrane contains a sodium-independent taurocholate transport system that translocates the bile acid as an anion across the membrane. In intact hepatocytes, the electrical potential difference across the canalicular membrane probably provides the driving force for taurocholate se-

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© The American Society for Clinical Investigation, Inc. 0021-9738/84/03/0659/05 \$1.00 Volume 73, March 1984, 659-663 cretion. The contribution of nonionic diffusion to taurocholate secretion appears to be minimal.

Introduction

Vectorial transport of organic anion, such as bile acids and bilirubin, is an important liver function. Hepatic secretion of bile acids involves uptake at the sinusoidal plasma membranes, intracellular interaction with cytoplasmic proteins, and translocation across the canalicular membranes. To elucidate the molecular mechanism of hepatic vectorial transport, events in the plasma membrane must be differentiated from intracellular processes. Previous studies (1, 2) reported isolation of highly purified rat liver sinusoidal and canalicular membrane vesicles. The presence of an Na⁺-taurocholate cotransport system was demonstrated in sinusoidal membrane vesicles (1, 3, 4); however, the mechanism of bile acid transport across canalicular membranes is not defined.

The present work reports the mechanism of taurocholate transport by isolated rat liver canalicular membrane vesicles. The results provide evidence for a potential-sensitive Na^+ -independent taurocholate transport system. Part of these results were reported in a preliminary form (5).

Methods

Materials. ³H-labeled taurocholic acid and D-glucose were obtained from New England Nuclear, Boston, MA. Ouabain, disodium adenosinetriphosphate, *p*-nitrophenylphosphate, NADH, γ -glutamyl-*p*-nitroanilide, cholic acid, taurocholic acid, and probenecid were purchased from Sigma Chemical Co., St. Louis, MO. All reagents were of analytical grade.

Rat liver canalicular membrane vesicles. Canalicular plasma membrane vesicles were prepared from livers of male Sprague-Dawley rats by differential centrifugation, nitrogen cavitation, and calcium precipitation as described previously (2). The membranes were enriched at least 55-fold in aminopeptidase M and γ -glutamyltransferase as compared with crude homogenate; no significant enrichment of intracellular organelles was observed. Na⁺,K⁺-ATPase, a marker of sinusoidal plasma membranes (6), was enriched 2.5-fold. By electron microscopy, membrane samples consisted exclusively of vesicles with no detectable contaminating organelles or junctional complexes. Isolated vesicles were suspended in 10 mM Hepes-Tris buffer, pH 7.4, which contained 0.25 M sucrose and 0.2 mM CaCl₂, and stored in small portions (\sim 3 mg of membrane protein/ml) at -70° C. Storage of membranes at -70° C for 3 mo did not alter transport properties for taurocholate. Assays for marker enzymes and membrane proteins were performed as described (1).

Transport. Transport of ³H-labeled taurocholate was measured by a rapid filtration technique (7). Unless otherwise indicated, the assay mixtures contained, in a final volume of 0.15 ml, 10 mM Hepes-Tris buffer, pH 7.4, 0.25 M sucrose, 0.2 mM CaCl₂, 10 mM MgCl₂, 0.1 M KNO_3 , ~0.15 mg vesicles in protein, and varying concentrations of ³H-labeled taurocholate. Other conditions are indicated in the individual experiments. Frozen stock vesicles were thawed quickly at 40°C and stored in ice until used. Transport was started by adding 20-µl membrane vesicles to 130 μ l of incubation mixtures that were preincubated for 10 min at 37°C. At timed intervals, 20-µl aliquots were removed and diluted in 1 ml of ice-cold stop solution. The stop solution contained 10 mM Hepes-Tris buffer, pH 7.4, 0.25 M sucrose, 0.2 mM CaCl₂, 10 mM MgCl₂, 0.1 M NaCl, and varying concentrations of unlabeled taurocholate. The diluted samples were immediately filtered through a Millipore filter (Millipore/Continental Water Systems, Bedford, MA) (HA, 0.45 μ m) followed by washing with 3 ml of ice-cold stop solution. Radioactivity on the filters was measured in a liquid scintillation counter using NEN-Formula 963 as scintillant. Values were corrected for radioactivity on the filters in the absence of membrane vesicles. Variations in transport activity with different vesicle preparations were small and all experiments were repeated at least three times with similar results.

Results

Taurocholate transport and binding to the canalicular membranes. The time course for uptake of taurocholate by rat liver



Figure 1. Temperature-dependent uptake of taurocholate by rat liver canalicular membrane vesicles. Taurocholate uptake was measured in a transport medium containing, in a total volume of 0.15 ml, 0.25 M sucrose, 0.2 mM CaCl₂, 10 mM MgCl₂, 10 mM Hepes-Tris buffer, pH 7.4, 50 μ M ³H-labeled taurocholate, and 0.1 M NaNO₃ (\circ , \Box), or KNO₃ (\bullet , \bullet)

at 37°C (\odot , •) or at 0°C (\Box , •). Reaction was started by adding membrane vesicles. At timed intervals, 20-µl aliquots were withdrawn and diluted into 1 ml ice-cold stop solution that contained 0.25 M sucrose, 0.2 mM CaCl₂, 10 mM MgCl₂, 10 mM Hepes-Tris buffer, pH 7.4, 0.1 M NaCl, and 1 mM unlabeled taurocholate. The diluted samples were immediately filtered through 0.45-µm millipore filters, and washed with 3 ml of ice-cold stop solution. Radioactivity associated with vesicles on the filters was determined. Details are described in the text. Each point represents mean value of triplicate experiments (deviation < 13%).



Figure 2. Membrane binding and transport of radioactive taurocholate. After equilibrium uptake $(37^{\circ}C \text{ for } 30 \text{ min})$ of $50 \ \mu\text{M}$ ³Hlabeled taurocholate (\bigcirc, \square) or 2 mM ³H-labeled D-glucose (\bullet, \bullet) , membrane-associated radioactivity was determined with the intact (\bigcirc, \bullet) and heat-denatured vesicles (\square, \bullet) . Heat denaturation of the vesicles was performed at 100°C for 3

min. The stop solution contained varying concentrations of unlabeled taurocholate. Other conditions were the same as in Fig. 1. Each point represents mean value of triplicate determinations (deviation <8%).

canalicular membrane vesicles is shown in Fig. 1. Taurocholate uptake was enhanced at 37°C, and was higher in a sodium gradient than in a potassium gradient (inside-outside). After incubation for 20-60 min, uptake reached equilibrium. The initial rate of uptake at 0°C was slow either in an Na⁺ or K⁺ gradient and equilibrium was not reached after 60 min of incubation. This temperature dependence provides evidence that the early period of uptake represents transmembrane transport of taurocholate rather than binding to the outer surface of the vesicles. Further proof for this assumption was provided when the effect of increasing the amount of taurocholate in the washing solution on the uptake was investigated (Fig. 2). Even at the highest concentration of taurocholate used, only 15% of uptake observed after 30 min could be replaced under conditions where vesicles' integrity, as shown by D-glucose content, was undisturbed; 15% is almost identical to the amount of taurocholate taken up at 0°C and probably represents binding to the outside of the vesicular membranes.

Fig. 2 also shows experiments in which vesicles were opened up by heat inactivation; ³H-labeled taurocholate binding to the membrane fraction was easily reduced by adding unlabeled taurocholate in the washing solution, suggesting that the canalicular membrane vesicles have binding sites for taurocholate, the majority of which are inside the vesicles. The presence of these binding sites was also evident when osmotic dependence of taurocholate and D-glucose uptake were compared (Fig. 3). After 30 min of incubation, increase in the osmotic gradient across the membrane from 200 to 500 mosM raffinose decreased Dglucose uptake by 50%, whereas taurocholate uptake changed only by 20%, indicating that only a small portion of taurocholate taken up by the vesicles was free in solution. After correcting binding capacity for the intravesicular space of 0.95 μ l, which was obtained from the control vesicles for D-glucose, the vesicular content of taurocholate was 1.85 nmol higher than that expected from simple equilibration of the 50- μ M taurocholate concentration. If the estimate of 15% for binding to the outside is correct at 50 μ m, ~1.6 nmol of taurocholate can, therefore, be bound per milligram protein. Vesicular uptake can be considered to predominantly represent translocation of taurocholate across the membrane followed by intravesicular binding. Further ev-



Figure 3. Effect of medium osmolarity on the uptake of taurocholate and D-glucose. The incubation medium contained, in a final volume of 0.15 ml, 0.25 M sucrose, 0.2 mM CaCl₂, 10 mM Hepes-Tris buffer, pH 7.4, 10 mM NaCl, varying concentrations of raffinose (0– 0.5 M), and 50 μ M [³H]taurocholate (B) or 2 mM ³H-D-glucose (A). After 30 min of incubation at 37°C, the amount of vesicle-associated radioactivity was determined as in Fig. 1 with the stop solution containing 0.3 M sodium chloride. Raffinose concentration in the incubation medium is plotted on the abscissa. The amount of taurocholate and D-glucose taken up by the vesicles in the absence of raffinose was 1.0 and 1.9 nmol/mg protein, respectively. Each point represents mean value of triplicate determinations.

idence for this assumption was obtained when transstimulation of taurocholate uptake was investigated (Table I). Both at 50 and 100 μ M, initial taurocholate uptake was markedly stimulated by preloading of vesicles. Transstimulation provides strong evidence for a taurocholate transport system in the canalicular membranes.

Table I. Transstimulation of Taurocholate Uptake in Canalicular Membrane Vesicles

| Taurocholate concentration | Uptake of ³ H-labeled taurocholate | | |
|----------------------------|---|------|--------|
| | 20 s | 60 s | 30 min |
| μM | | | |
| 50 | 1 235% | 151% | 143% |
| | 2 171% | 186% | 123% |
| | X 203% | 169% | 136% |
| 100 | 1 179% | 140% | 113% |
| | 2 151% | 123% | 100% |
| | X 163% | 135% | 107% |

Preloading was achieved by incubation of the vesicles for 1 h at 25°C in vesicle buffer containing 50 or 100 μ M taurocholate (final concentration). Control membrane vesicles were incubated concurrently without taurocholate. Uptake was measured at 50 or 100 μ M taurocholate in the presence of a 100-mM KNO₃ gradient so that, in the case of preloaded vesicles, no chemical gradient for cholate existed across the membrane. Individual and mean values, from two experiments that were performed in duplicate, are given in percent of the respective control.

Dose-dependent transport of taurocholate. Fig. 4 shows the dose dependence of the initial rate of Na⁺-independent taurocholate transport by canalicular membrane vesicles. Uptake followed saturation kinetics with respect to taurocholate concentration. Double-reciprocal plots of uptake vs. taurocholate concentration gave a straight line from which apparent K_m of 43 μ M and V_{max} of 0.22 nmol/mg protein \times 20 s at 37°C were derived. No saturation was observed for taurocholate uptake in the range of 12.5–200 μ M after incubation for 30 min. These results indicate that intravascular binding sites are not responsible for the saturation kinetics observed after 20 s of incubation, and suggest that the saturation phenomenon is caused by presence of a sodium-independent taurocholate transport system in the membrane.

Specificity of the transport system. As shown in Fig. 5, cholic acid (0.5 mM) inhibited taurocholate transport by 30% in the first minute of incubation; after long-term incubation, cholate decreased taurocholate uptake by 13%. Probenecid (5 mM), a well-known inhibitor for the weak organic acid transport system, also inhibited taurocholate uptake by $\sim 60\%$ in the early period of incubation; at equilibrium, 22% inhibition was observed. The greater inhibition during the initial uptake phase indicates that membrane translocation of taurocholate is inhibited by cholate and probenecid. Reduced inhibition after long-term incubation probably reflects competition between inhibitors and taurocholate for intravesicular binding sites. The nature of the inhibition remains to be elucidated.

Temperature dependency, transstimulation, saturability, and inhibition by substrate analogues are consistent with the presence of a sodium-independent taurocholate transport system in canalicular membrane vesicles. To determine the role of nonionic diffusion in taurocholate uptake, experiments were performed in which pH gradients were established across the vesicular membrane. As indicated in Table II, a 10-fold proton gradient, either directed from outside the vesicle to the inside or vice versa, did not affect taurocholate uptake.

Effect of membrane potential on taurocholate transport. The potential difference across biological membranes represents an important driving force for electrogenic solute transport systems. To test the membrane potential dependency of tauro-



Figure 4. Dose-dependency of taurocholate transport. Incubation medium contained, in a final volume of 0.15 ml, varying concentration of [³H]taurocholate, 0.25 M sucrose, 0.2 mM CaCl₂, 10 mM MgCl₂, 10 mM Hepes-Tris buffer, pH 7.4, and 0.1 M NaNO₃. The reaction was started by

adding vesicles. After 20 s of incubation at 37° C, radioactivity taken up by the vesicles was determined as in Fig. 1. (B) Double reciprocal plots of A.



Figure 5. Effect of organic anions on taurocholate transport. Incubation medium contained, in a final volume of 0.15 ml, 0.25 M sucrose, 0.2 mM CaCl₂, 10 mM MgCl₂, 10 mM Hepes-Tris buffer, pH 7.4, 0.1 M KNO₃, and 50 μ M radioactive taurocholate. Taurocholate transport was measured at 37°C in the absence (\odot) or presence of 0.5 mM cholic acid (\bullet) and 5 mM probenecid (\Box). Other conditions were as in Fig. 1.

cholate translocation, the effect of artificially imposed membrane potential difference on the transport activity was studied using the anion replacement method (Fig. 6). In the presence of a potassium gradient (K⁺ inside, K⁺ outside), replacement of Cl⁻ by a more permeant anion, NO₃⁻, decreased the initial rate of taurocholate uptake. When a less permeant anion, SO₄⁻⁻, was used instead of Cl⁻, the rate of uptake increased, indicating transport of taurocholate as an anion without simultaneous symport of a cation or antiport of an anion.

Further evidence for this assumption was obtained from experiments applying a valinomycin-induced K⁺-diffusion potential. As shown in Fig. 7, treatment of vesicles with valinomycin increased the rate of taurocholate by the vesicles. The intravesicular space of the membranes remained unchanged $(1 \ \mu l/$ mg protein) after valinomycin treatment as measured with radioactive D-glucose (data not shown). These results reveal a specific effect of valinomycin-induced K⁺-diffusion potential (vesicle inside-positive) rather than nonspecific increase in membrane permeability for small molecular weight components due to membrane perturbation by the ionophore. Thus, taurocholate can be translocated across the canalicular membranes as an anion by a sodium-independent transport system.

 Table II. Effect of pH Gradients on Taurocholate Uptake by

 Canalicular Membrane Vesicles

| Incubation time | pH₀/pHi | | | | |
|-----------------|---------|---------|---------|---------|--|
| | 6.9/6.9 | 7.9/6.9 | 6.9/7.9 | 7.9/7.9 | |
| 20 s | 0.182 | 0.176 | 0.160 | 0.170 | |
| 1 min | 0.279 | 0.260 | 0.280 | 0.258 | |
| 30 min | 0.568 | 0.507 | 0.582 | 0.506 | |

The final membrane preparation was suspended in vesicle buffer of the pH indicated by pH_i, sedimented for 30 min at 20,000 g, and resuspended in the buffer used for their initial suspension. Uptake experiments were performed in the presence of a 100-mM KNO₃ gradient and 50 μ M taurocholate at the pH indicated by pH₀. Results are the mean values derived from two experiments performed in duplicate. They are expressed as cpm $\times 10^{-3}$ /mg protein.



Figure 6. Effect of anion replacement on taurocholate transport. Taurocholate uptake was determined in the transport medium containing, in a final volume of 0.15 ml, 50 μ M ³H-labeled taurocholate, 0.25 M sucrose, 0.2 mM CaCl₂, 10 mM MgCl₂, and 10 mM Hepes-Tris buffer, pH 7.4; 0.1 M KCl (\bullet) in the medium was replaced by 0.1 M KNO₃ (\circ), or by 50 mM K₂SO₄ plus 50 mM mannitol (\Box). Uptake was mea-

sured at 37°C. Other conditions were the same as in Fig. 1. Each points represents mean value of triplicate determinations.

Discussion

This paper reports taurocholate transport by a plasma membrane fraction that is predominantly derived from canalicular membranes of hepatocytes. Since taurocholate is amphipathic, a problem in studying its transport in isolated membranes is that differentiating binding to membrane surface from transmembrane transport can be difficult. Evidence for translocation was obtained from transstimulation, temperature-dependence, saturation, and inhibition experiments. The first criterion is the strongest argument for the presence of a transport system. If only binding of taurocholate occurred, the binding sites would have been saturated by preloading, and decreased uptake of labeled taurocholate would be expected; the opposite results were obtained. The strong temperature dependence of uptake also supports our conclusion (8); however, in a few instances, binding to membranes decreases when the incubation temperature is lowered (1, 3, 9, 10). Saturability and competition can be expected for both transport and binding; however, in our experiments, binding was not saturated in the concentration range tested, and thus can be assumed to contribute insignificantly to the observed saturation of the uptake process. Thus, transport rather than binding represents the saturable component.

The apparent affinity of this transport system is of the same order of magnitude as that of the recently described sodium-



Figure 7. Effect of valinomycin on the uptake. Membrane vesicles were preincubated for 10 min at 25°C in the presence (•) or absence (0) of valinomycin (25 μ g/ mg protein). Uptake was measured in the transport solution containing, in a final volume of 0.15 ml, 50 μ M ³H-labeled taurocholate, 0.25 M sucrose, 0.2 mM CaCl₂, 10 mM MgCl₂, 10 mM Hepes-Tris buffer, pH 7.4, and 0.1 M KCl. Other conditions were the same as in Fig. 1. taurocholate cotransport system in the sinusoidal membranes (1). However, in contrast to the latter transport system, transstimulation and saturation were observed in the canalicular membrane transport system, thus demonstrating an important difference in the main routes of taurocholate transport across the two membranes. Another difference between the two systems is lower sensitivity of the canalicular membrane translocase to probenecid when compared with that of the sinusoidal membrane transport system. With 0.2 mM probenecid, the initial rate of taurocholate uptake was inhibited by 72% in sinusoidal membrane vesicles; 5 mM of probenecid was required to inhibit taurocholate transport in canalicular membrane vesicles by 60%. Interestingly, a similar difference in the inhibitory effect of probenecid is observed with the renal transtubular transport system for weak organic acids (11). The transport system for p-aminohippuric acid in renal basolateral plasma membranes is more sensitive to probenecid than that in tubular lumenal membranes. The reason for such difference in the inhibitory action of probenecid in the two topographically different plasma membrane domains is not known.

A third difference between the transport properties of the sinusoidal and canalicular membranes is their response to changes in the electrical potential difference across the vesicle membrane. In the sinusoidal membrane, intravesicular negativity stimulates sodium-taurocholate cotransport, while sodium-independent transport in the canalicular membrane is inhibited. In addition, intravesicular positivity inhibits transport in the sinusoidal membranes but stimulates transport in canalicular membranes. It is apparent that different transport systems operate in the two membranes. Taurocholate uptake by canalicular membrane vesicles is slightly higher in an Na⁺ gradient than in a K⁺ gradient (Fig. 1). Although the membrane fractions were highly enriched with marker enzymes of canalicular membranes (at least 55-fold), Na⁺, K⁺-ATPase, a marker of sinusoidal membranes, was also enriched 2.5-fold as compared with crude homogenate (2). The small amount of sodium-dependent uptake of taurocholate by canalicular membranes may reflect contamination by sinusoidal membranes. This possibility should be studied further in canalicular membrane vesicles that are completely free from contaminating sinusoidal membranes.

The present and previous studies (1) demonstrate the mechanism of vectorial transport of taurocholate in the liver. Uptake across the sinusoidal membrane occurs predominantly by a sodium-dependent transport system that is driven by the transmembrane sodium gradient and the cellular electrical potential difference (inside-negative). At the canalicular membrane, taurocholate leaves the cell by a sodium-independent transport system; the driving force for cellular exit is the electrical potential difference that favors efflux of taurocholate anion from hepatocytes (2, 4).

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