ATP-dependent bile-salt transport in canalicular rat liver plasma-membrane vesicles

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The present study identifies and characterizes a novel ATP-dependent bile-salt transport system in isolated canalicular rat liver plasma-membrane (cLPM) vesicles. ATP (1–5 mM) stimulated taurocholate uptake into cLPM vesicles between 6- and 8-fold above equilibrium uptake values (overshoot) and above values for incubations in the absence of ATP. The ATP-dependent portion of taurocholate uptake was 2-fold higher in the presence of equilibrated KNO_3 as compared with potassium gluconate, indicating that the stimulatory effect of ATP was not due to the generation of an intravesicular positive membrane potential. Saturation kinetics revealed a very high affinity ($K_m \sim 2.1 \mu\text{M}$) of the system for taurocholate. The system could only minimally be stimulated by nucleotides other than ATP. Furthermore, it was preferentially inhibited by conjugated univalent bile salts. Further strong inhibitory effects were observed with valinomycin, oligomycin, 4,4'-di-isothiocyano-2,2'-stilbene disulphonate, sulphobromophthalein, leukotriene C_4 and *N*-ethylmaleimide, whereas nigericin, vanadate, GSH, GSSG and daunomycin exerted only weak inhibitory effects or none at all. These results indicate the presence of a high-affinity primary ATP-dependent bile-salt transport system in cLPM vesicles. This transport system might be regulated *in vivo* by the number of carriers present at the perspective transport site(s), which, in addition to the canalicular membrane, might also include pericanalicular membrane vesicles.

INTRODUCTION

In order to maintain ongoing enterohepatic circulation and bile formation, liver parenchymal cells must continuously transport large amounts of bile salts from sinusoidal blood plasma into bile canaliculi [1]. This vectorial transcellular bile-salt secretory process occurs against unfavourable cell-to-plasma ($\sim 10:1$) and bile to cell ($10\text{--}100:1$) concentration gradients, thus requiring active ('uphill') bile-salt transport processes at both the basolateral (sinusoidal and lateral) and apical (canalicular) surface domains of hepatocytes [2,3]. Although the former is well known to be mediated predominantly by a secondary active Na^+ /bile-salt co-transport system that has recently been cloned and sequenced [4], canalicular secretion of monoanionic conjugated bile salts (e.g. taurocholate) has been suggested to be driven, at least in part, by the intracellular negative membrane potential of approx. -35 mV [2,3,5,6]. This concept of electrogenic canalicular bile-salt secretion has been supported by the observations that (a) inside-positive or -negative K^+ diffusion potentials increase taurocholate uptake into [7] or taurocholate efflux from [8,9] isolated canalicular rat liver plasma-membrane (cLPM) vesicles respectively, (b) agonists that increase the membrane potential enhance bile-salt efflux from isolated hepatocytes [10,11], and (c) membrane hyperpolarization in isolated hepatocyte couplets also increases taurocholate-dependent canalicular secretion [12]. However, membrane-potential-driven canalicular secretion of monomeric bile salts alone could only account for a 3–4-fold intracanalicular concentration of monoanionic bile salts, whereas the physiological cell-to-lumen bile-salt concentration gradients amounts to $1:10\text{--}100$ [1,2]. Hence, additional driving forces might be involved in the establishment of the large physiological concentration gradients of amidated bile salts (e.g. taurocholate, glycocholate) within the bile-canalicular lumen. In fact, recent findings in isolated cLPM vesicles indicate that ATP-dependent primary active bile-salt transport

might also occur at the canalicular membrane *in vivo* [13,14]. The present study confirms and extends these previous observations. The data indicate that ATP-driven canalicular transport of taurocholate and other univalent conjugated bile salts occurs largely independently of the membrane potential and, because of an exceptionally high bile-salt affinity of the transport system involved, might be regulated *in vivo* by the number of carriers present at the bile-canalicular front of hepatocytes.

Part of this work has been presented at the Annual Meeting of the American Association for the Study of Liver Diseases, Chicago, IL, November 1991, and has appeared in abstract form [14a].

EXPERIMENTAL

Materials

[6- ^3H]Taurocholic acid (2.1–6.6 Ci/mmol) and KS^{14}CN (50–60 mCi/mmol) were obtained from Du Pont–New England Nuclear (Boston, MA, U.S.A.) and Amersham International (Amersham, Bucks., U.K.) respectively. ATP (sodium and Tris salts; vanadium contents $< 1 \text{ p.p.m.}$) were purchased from Boehringer (Mannheim, Germany) and Sigma Chemical Co. (St. Louis, MO, U.S.A.). Creatine kinase and phosphocreatine were from Boehringer, valinomycin and nigericin from Sigma, and the potential-sensitive fluorescent dye 3,3'-diethylthiadicarbocyanine iodide [$\text{DiS-C}_2(5)$] from Eastman–Kodak (Rochester, NY, U.S.A.). All other chemicals used were of reagent grade and were obtained from BDH (Poole, Dorset, U.K.), Boehringer, Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany) or Sigma. Nitrocellulose filters ($0.45 \mu\text{m}$ pore size) were ordered from Sartorius (Göttingen, Germany).

Animals

Male Sprague–Dawley rats (SUT–SDT; Süddeutsches Tierzuchtinstitut, Tuttlingen, Germany) weighing 200–250 g were

Abbreviations used: bLPM, basolateral rat liver plasma membranes; cLPM, canalicular rat liver plasma membranes; DIDS, 4,4'-di-isothiocyano-2,2'-stilbenedisulphonate; $\text{DiS-C}_2(5)$, 3,3'-diethylthiadicarbocyanine iodide; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole.

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Fig. 1. Effect of ATP on taurocholate uptake in rat liver cLPM and bLPM vesicles

The vesicles were resuspended in 250 mM-sucrose/10 mM-Tris/HCl, adjusted to pH 7.4. The incubation medium consisted of 50 mM-sucrose, 100 mM-NaCl or -KCl, 10 mM-MgCl₂, 10 mM-phosphocreatine, 100 µg of creatine kinase (Boehringer)/ml and 10 mM-Tris/HCl, pH 7.4. ATP (sodium salt; Boehringer) was added in a final concentration of 1 mM as indicated. Taurocholate (1 µM) uptake was determined at 37 °C. Data represent means ± S.D. of triplicate determinations in one representative out of three membrane preparations. Symbols: ●, +ATP, ○, no ATP ([NaCl] outside > inside); ▲, +ATP, △, no ATP ([KCl] outside > inside).

used throughout this study. The animals had free access to water, were fed *ad libitum* (690 diet; Nafag, Gossau, Switzerland), and housed in a constant alternating 12 h-light (06:30–18:30 h)/12 h-dark cycle. Fed animals were regularly killed by decapitation between 07:30 and 08:30 h.

Isolation of canalicular (cLPM) and basolateral (bLPM) liver plasma-membrane subfractions

Highly purified cLPM and bLPM vesicles were isolated from rat liver as described previously [15]. The membrane subfractions were resuspended in appropriate buffer media, the exact compositions of which are given in the corresponding Figure legends. The membrane suspensions were stored frozen in liquid nitrogen (protein concentration > 5 mg/ml) for up to 6 months without loss of transport functions. Immediately before use, the samples were quick-thawed by immersion into a 37 °C water bath.

Determination of ATP-dependent taurocholate uptake in cLPM and bLPM vesicles

[³H]Taurocholate uptake in the absence and presence of extravesicular ATP was determined by a rapid filtration assay as described previously [8,9]. ATP-dependent uptake was determined with (Fig. 1) and without (Figs. 3–5, 7; Tables 1 and 2) an ATP-regenerating system. The vesicle protein concentrations were routinely adjusted to 50–60 µg/100 µl. Where indicated, the vesicles were pretreated with valinomycin (10 µg/mg of protein dissolved in dimethyl sulphoxide; Fig. 3) or with nigericin (10 µM; Fig. 5) for 5 min at 37 °C. The exact compositions of the vesicle-preloading buffers (= intravesicular buffers) as well as of the various transport media (= extravesicular buffers) are detailed in the corresponding Figure legends. After the indicated time periods, tracer taurocholate uptakes were terminated by addition of 2 ml of ice-cold stop solution, consisting of 50 mM-sucrose, 100 mM-KCl and 10 mM-Tris/HCl pH 7.4. After two additional washing steps, the vesicle-associated radioactivity retained on the filters was determined by liquid-scintillation counting as described [16].

Fluorescence measurements of membrane potential changes in cLPM vesicles

The potential-sensitive fluorescent dye DiS-C₂(5) was used to

monitor valinomycin- and ATP-induced potential changes in the presence of inwardly directed K⁺ gradients or K⁺-equilibrated conditions respectively (Fig. 6). The measurements were essentially performed as previously described [17–19]. Samples (20 µl) of cLPM vesicles (250 µg of protein) were added to 2.02 ml of incubation media, the exact compositions of which are detailed in the legend of Fig. 6. The concentration of DiS-C₂(5) in all incubation media was 3 µM. Fluorescence changes were recorded in an RF 510 spectrofluorimeter (Shimadzu, Tokyo, Japan) equipped with a red-sensitive photomultiplier R 446U. The excitation wavelength was set to 622 nm (slit width 5 nm) and the emission was recorded at 669 nm (slit 10 nm).

Other methods

Protein concentrations were determined by a modification of the Lowry procedure [20].

RESULTS

As illustrated in Fig. 1, incubation of isolated cLPM vesicles at 37 °C in the presence of ATP (1 mM) and an ATP-regenerating system stimulated taurocholate (1 µM) uptake by 6–8-fold above equilibrium values at 60 min ('overshoot') or above those for incubations in the absence of ATP. This ATP-dependent intravesicular accumulation of taurocholate was maintained over a time period of 10–20 min, indicating effective regeneration of ATP continuously hydrolysed by the canalicular 'ecto-ATPase' [21]. Furthermore, replacement of NaCl by KCl did not change the ATP-stimulatory effect on taurocholate uptake, indicating an Na⁺-independent transport process. Quantitatively the ATP-stimulated portion of taurocholate uptake was surprisingly large if one considers that only some 20–30 % of cLPM vesicles as isolated in this study are oriented in an inside-out configuration [8], thus permitting direct access of the exogenously added ATP to the cytoplasmatically oriented ATP-binding site(s) of the canalicular transporter. In simultaneously isolated bLPM vesicles, ATP stimulated taurocholate uptake only to the extent of approx. 30 % of the maximal uptake values in cLPM vesicles (Fig. 1), a finding that is compatible with the routine degree of contamination of the bLPM subfraction with canalicular membranes [15,22]. Hence, ATP-stimulated taurocholate transport

Fig. 2. Hypothetical mechanisms for ATP-driven taurocholate uptake into inside-out-oriented (extravesicular surface = cytoplasmic side in intact hepatocytes) cLPM vesicles

For further explanations see the text. Key: X^+ , H^+ or Mg^{2+} ; TCA^- , taurocholate anion.

appears to be selectively localized to the bile-canalicular domain of rat hepatocytes.

Hypothetically, ATP could stimulate taurocholate uptake either indirectly through generation of an intravesicular positive potential (e.g. by a H^+ -ATPase), followed by taurocholate anion influx across the previously characterized electrogenic pathway

Fig. 4. ATP-dependent taurocholate uptake in the presence of equilibrated permeant (nitrate) and impermeant (gluconate) anions in cLPM vesicles

The vesicles were resuspended in 50 mM-sucrose/100 mM- KNO_3 or -potassium gluconate/10 mM-Hepes/Tris, adjusted to pH 7.4. Incubations were carried out in the same media supplemented with 10 mM- $Mg(NO_3)_2$ or -magnesium gluconate respectively. Where indicated, ATP (Tris salt) was added at a final concentration of 5 mM. Taurocholate (1 μM) uptake was determined at 37 °C. Data represent means $\pm$ S.D. of triplicate determinations in two separate membrane preparations. Symbols: $\bullet$, KNO_3 , +ATP; $\circ$, KNO_3 , no ATP; $\blacksquare$, potassium gluconate, +ATP; $\square$, potassium gluconate, no ATP.

[7–9], or directly via an ATP-dependent primary active bile-salt pump that could principally function in an anionic, electroneutral or cationic mode (for illustration see Fig. 2). In order to differentiate between these possibilities, we first investigated the effects of membrane-potential changes on ATP-stimulated taurocholate uptake in cLPM vesicles. These as well as all further experiments were performed only with 5 mM-ATP, in order to exclude any possible confounding effects from inhibition or stimulation of the ATP-regenerating system [23]. Although

Fig. 3. Effect of valinomycin on ATP-driven taurocholate uptake in cLPM vesicles

The vesicles were resuspended in 50 mM-sucrose/100 mM-potassium gluconate (left) or -sodium gluconate (right)/10 mM-Hepes/Tris, pH 7.4. They were treated with valinomycin (10 μg /mg of protein) as described in the Experimental section. Control membranes were treated with corresponding amounts of DMSO. Incubation media were of similar compositions to the vesicle resuspension buffers, except for the additional presence of 10 mM-magnesium gluconate. ATP (Tris salt, Sigma) was added at a final concentration of 5 mM. No ATP-regenerating system was included. Taurocholate (1 μM) uptake was determined at 37 °C. Data represent means $\pm$ S.D. of triplicate determinations in two separate membrane preparations. Symbols: $\blacksquare$, +ATP, +valinomycin; $\bullet$, +ATP, no valinomycin; $\square$, no ATP, +valinomycin; $\circ$, no ATP, no valinomycin.

Fig. 5. Role of ΔpH and membrane potential on ATP-stimulated taurocholate uptake in cLPM vesicles

(a) Effect of nigericin. Incubation conditions were identical with those described in Fig. 3, except that a portion of vesicles was also pretreated (5 min, 37 °C) with 10 μM -nigericin (pH-clamp). (b) Effect of ATP on thiocyanate (SCN⁻) uptake. KS¹⁴CN (20 μM) uptake was determined in separate portions of the same vesicle preparations and under conditions identical with those for taurocholate uptake in panel (a) (except no nigericin). All data represent means $\pm$ S.D. of triplicate determinations in two separate membrane preparations. Symbols: $\bullet$, KNO₃, +ATP; $\circ$, KNO₃, no ATP; $\blacktriangle$, KNO₃, +ATP, +nigericin; $\blacksquare$, gluconate, +ATP; $\square$, gluconate, no ATP.

slightly decreased, the omission of the ATP-regenerating system did not preclude the detection of ATP-dependent transient intravesicular accumulation of taurocholate within cLPM vesicles (Figs. 3–5). Unfortunately, however, the K⁺ ionophore valinomycin inhibited ATP-dependent taurocholate uptake to similar degrees in the presence of K⁺- as well as Na⁺-equilibrated conditions (Fig. 3), thus precluding the use of valinomycin-induced K⁺ diffusion potentials for the evaluation of the exact role of the membrane potential in ATP-driven cLPM uptake of taurocholate. This direct inhibitory effect of valinomycin on ATP-dependent taurocholate uptake was also observed in sucrose medium and after replacement of gluconate by chloride (results not shown).

A valinomycin-independent way of studying the electrogenicity of membrane transport processes is the comparison of solute transport rates in the presence of membrane-permeant and -impermeant anions such as, for example, nitrate (or thiocyanate) and gluconate, respectively. As demonstrated in Fig. 4, ATP-driven taurocholate uptake into cLPM vesicles was considerably higher under KNO₃-equilibrated conditions as compared with equilibrated potassium gluconate. In contrast, no effect of the anion substitution was observed on ATP-independent taurocholate uptake. These results are not compatible with an intravesicular positive potential created by a cation-pumping ATPase mediating secondary electrogenic taurocholate anion uptake,

Fig. 6. Valinomycin- and ATP-induced potential changes in KNO₃- and potassium gluconate-preloaded cLPM vesicles

The vesicles were resuspended in 50 mM-sucrose/10 mM-Hepes/Tris, pH 7.4, supplemented with either 100 mM-KNO₃ (a–d) or 100 mM-potassium gluconate (e–h). Fluorescence changes were determined with the potential-sensitive dye DiS-C₂(5) (3 μM) as described in the Experimental section. A decrease in fluorescence indicates uptake of the positively charged dye into the vesicles (inside negative potential), whereas an increase in fluorescence reflects release of dye from the vesicles. The incubation media consisted of 50 mM-sucrose/10 mM-Hepes/Tris, pH 7.4, and were supplemented with the following additions (final concns.): (a), 98 mM-NaNO₃; (b)–(d), 100 mM-KNO₃; (e), 98 mM-sodium gluconate (Naglug); (f)–(h), 100 mM-potassium gluconate (Kgluc). After recording the baseline, valinomycin (40 $\mu\text{g}/\text{mg}$ of protein), ATP (Tris salt; 4.46 mM) or ATP and taurocholate (TCA; 2.23 μM) were added as indicated. The data shown are from one representative out of three experiments.

since, in the case of such an 'indirect coupling' (see Fig. 2), the reverse results would have been expected, i.e. higher ATP-dependent taurocholate uptake in the presence of the impermeant (gluconate) as compared with the more permeant (nitrate) anion. Thus the data indicate the occurrence of direct ATP-coupled primary active taurocholate anion transport (see Fig. 2), with the transfer of a net negative charge into the cLPM vesicles being counterbalanced by the efflux of a nitrate anion.

Direct rather than indirect coupling between ATP hydrolysis and taurocholate transport can also be inferred from the data presented in Figs. 5 and 6. Fig. 5 first demonstrates that pH-clamping of the vesicles with nigericin in the presence of equilibrated KNO₃ had no effect on ATP-dependent taurocholate uptake, arguing against the involvement of intravesicular acidification by a H⁺-ATPase in the ATP-stimulated taurocholate uptake process. Furthermore, and most importantly, although ATP induced a transient intravesicular accumulation of the membrane-permeant anion SCN⁻ in gluconate-equilibrated vesicles (Fig. 5b), no effect of ATP on SCN⁻ uptake was found in KNO₃-equilibrated vesicles (Fig. 5b). These data indicate that

Fig. 7. Kinetics of ATP-dependent taurocholate uptake in cLPM vesicles

The vesicles were resuspended in 50 mM-sucrose/100 mM-KNO₃/10 mM-Hepes/Tris, pH 7.4. Incubations were carried out in the same medium supplemented with 10 mM-Mg(NO₃)₂. Uptakes (20 s values) of increasing concentrations of taurocholate (1–60 μM) were determined at 37 °C in the presence (●) or absence (○) of 5 mM-ATP (Tris salt). Data represent means ± S.D. of triplicate determinations in three separate membrane preparations. $K_m = 2.1 \mu\text{M}$; $V_{\text{max}} = 724 \text{ pmol/min per mg}$. The broken line indicates the ATP-specific portion of taurocholate uptake.

Table 1. Effects of various nucleotide derivatives on taurocholate uptake in cLPM vesicles

Values are means ± S.D. of six determinations in two separate cLPM preparations. Abbreviations: p[NH]ppA, adenosine 5'-[βγ-imido]-triphosphate; ATP[S], adenosine 5'-[γ-thio]triphosphate.

Nucleotide (5 mM)	Taurocholate uptake	
	(pmol/20 s per mg of protein)	(%)
None	24.4 ± 0.5	(12%)
ATP	196.8 ± 9.8	(100%)
UTP	49.2 ± 3.2	(25%)
CTP	30.3 ± 0.3	(15%)
GTP	53.1 ± 0.8	(27%)
ADP	29.1 ± 2.0	(15%)
AMP	27.8 ± 2.1	(14%)
p[NH]ppA	30.2 ± 1.9	(15%)
ATP[S]	30.2 ± 1.1	(15%)
Adenosine	27.2 ± 1.2	(14%)

ATP could not induce the generation of an inside-positive potential in cLPM vesicles under the conditions of maximal ATP-dependent taurocholate uptake (Figs. 4 and 5a). This assumption was further verified with the potential-sensitive fluorescent dye DiS-C₂(5) [24]. Since this dye is positively charged, its increased uptake into inside-negative membrane vesicles can be detected as a decrease in fluorescence [17–19], whereas the release of dye from inside positively charged membrane vesicles or from vesicle surface binding sites will lead to an increase in fluorescence. As previously shown [18,19], the dye's sensitivity to inside-negative potentials is greater than to inside-positive potentials. Especially, a high negative membrane surface charge density, which has to be assumed to be present in most (i.e. the right-side-out oriented portion) of the cLPM vesicles [25], markedly decreases the sensitivity of the dye to inside-positive membrane potentials, most probably because of too extensive surface binding of the dye [18]. However,

Table 2. Effects of various organic compounds on ATP-dependent taurocholate uptake in cLPM vesicles

Data represent the means ± S.D. of six determinations in two separate membrane preparations.

	Taurocholate uptake (% of control)
Bile salt derivatives	
Cholate (20 μM)	77 ± 13
Taurocholate (20 μM)	39 ± 2
Chenodeoxycholate (20 μM)	46 ± 6
Taurochenodeoxycholate (20 μM)	20 ± 4
Deoxycholate (20 μM)	74 ± 8
Taurodeoxycholate (20 μM)	38 ± 3
Ursodeoxycholate (20 μM)	85 ± 6
Tauroursodeoxycholate (20 μM)	55 ± 4
Dehydrocholate (50 μM)	92 ± 9
Taurodehydrocholate (50 μM)	95 ± 7
Other compounds	
Taurine (1 mM)	111 ± 11
Glycine (1 mM)	104 ± 9
GSH (1 mM)	97 ± 6
GSSG (1 mM)	97 ± 7
Sulphobromophthalein (10 μM)	50 ± 8
Leukotriene C ₄ (10 μM)	45 ± 4
Daunomycin (100 μM)	84 ± 13
DIDS (200 μM)	50 ± 5
Ouabain (1 mM)	75 ± 4
Amiloride (1 mM)	93 ± 4
Vanadate (100 μM)	79 ± 3
NBD-Cl (20 μM)	76 ± 4
N-Ethylmaleimide (20 μM)	27 ± 2
Oligomycin (10 μg/ml)	31 ± 2
NaN ₃ (1 mM)	95 ± 10

membrane-potential-induced fluorescence changes are transient and reversible in nature, and therefore can be differentiated from the virtually instantaneous and irreversible deflections induced by mere displacement of membrane-bound dye. On the basis of these considerations, the data presented in Fig. 6 permit the following conclusions. First, valinomycin and outwardly directed K⁺ gradients induced a greater and longer-lasting transient intravesicular negativity in the presence of the relatively impermeant anion gluconate (Fig. 6e) as compared with nitrate (Fig. 6a), demonstrating that membrane-potential changes can be adequately detected with DiS-C₂(5) in cLPM vesicles. Second, under K⁺-equilibrated conditions, addition of ATP, of ATP and taurocholate, and of valinomycin all induced a rapid and irreversible increase in fluorescence, probably resulting from displacement of the dye from cLPM surface binding sites. And third, ATP induced a transient, albeit small, intravesicular positivity under potassium gluconate- (Fig. 6, traces f–h), but not under KNO₃- (Fig. 6, traces b–d) equilibrated conditions, thus corroborating the results obtained with thiocyanate in Fig. 5. Furthermore, since ATP-dependent taurocholate uptake was highest in the presence of equilibrated KNO₃ (Figs. 4 and 5), these data further support the conclusion of a direct rather than indirect coupling between canalicular ATP-hydrolysis and taurocholate transport (see Fig. 2).

Finally, we investigated the kinetics and substrate specificity of the canalicular ATP-dependent bile-salt uptake system. As demonstrated in Fig. 7, the ATP-coupled portion of uptake demonstrated a surprisingly high affinity for taurocholate ($K_m \sim 2.1 \mu\text{M}$). Taurocholate uptake rates also were dependent on the concentrations of ATP and Mg²⁺ in the transport medium (results not shown). Thereby, half-maximal taurocholate (2 μM)

uptake rates were obtained in the presence of 500 μM -ATP and 1 mM- Mg^{2+} . Stimulation of taurocholate uptake was relatively specific for ATP and was not observed to any comparable degrees with other nucleotides, non-hydrolysable ATP derivatives or ATP-degradation products (Table 1). Most interestingly, the ATP-dependent portion of taurocholate uptake was strongly inhibited by conjugated, but less so by unconjugated, tri- and di-hydroxylated bile salts (Table 2), suggesting that amidation with taurine (and possibly also with glycine) is a requirement for optimal fit of bile salts to this transporter. Further strong inhibitory effects ($> 50\%$) on ATP-driven taurocholate uptake into cLPM vesicles were observed with sulphobromophthalein, leukotriene C_4 , 4,4'-di-isothiocyano-2,2'-stilbenedisulphonate (DIDS), *N*-ethylmaleimide and oligomycin, whereas other compounds tested, such as the amino acids taurine and glycine, GSH and GSSG, daunomycin, ouabain, amiloride, vanadate, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) and NaN_3 exerted no or only small inhibitory effects.

DISCUSSION

The present study confirms and extends recent observations suggesting the occurrence of ATP-dependent bile-salt (taurocholate) transport in isolated cLPM vesicles [13,14]. Most importantly, the data indicate the following. (a) ATP-dependent cLPM uptake of taurocholate is not secondary to an intravesicular positive potential generated by an ATP-driven cation pump such as, for example, a vacuolar H^+ -ATPase [26], but rather represents direct coupling between ATP hydrolysis and bile salt uptake (Figs. 4–6). (b) The ATP-driven transient maximal accumulation of taurocholate within the cLPM vesicles was considerably higher (Figs. 1, 3–5) than the previously demonstrated effects of valinomycin-induced transmembrane K^+ -diffusion potentials [7–9] and appears to be mediated by a high-affinity transport system (Fig. 7) that could well account for the generation of the high physiological bile-to-cell concentration gradient of univalent conjugated (amidated) bile salts. (c) The ATP-dependent bile-salt transport system is different from other canalicular ATP-driven organic anion [27] and cation [28] efflux pumps, as suggested by its distinct substrate specificity and sensitivity to various transport inhibitors.

Efficient ATP-dependent intravesicular accumulation of organic cationic, neutral and anionic compounds is well known to occur secondary to the generation of an intravesicular positive membrane potential by vacuolar types of H^+ -ATPases [26] in a variety of extrahepatic cells and organelles, including the chromaffin granules of the chromaffin cells of the adrenal medulla [29] and brain synaptic [30,31] as well as other secretory vesicles [32]. Furthermore, the vacuolar H^+ -ATPase is also widely distributed in intracellular membrane-bound compartments of hepatocytes such as endosomes [26], lysosomes [33], trans-Golgi membranes [34] and multivesicular bodies [35]. Contamination of the cLPM subfraction with some of these H^+ -ATPase-containing organelles [15,22] probably explains the finding of an ATP-induced small intravesicular positive potential (Figs. 5b and 6), although a direct contribution of a so-far unidentified canalicular-membrane-associated H^+ -ATPase cannot be definitely excluded. However, no positive intravesicular potential could be detected under KNO_3 -equilibrated conditions (Figs. 5b and 6), where the ATP-stimulated portion of taurocholate uptake was maximal. Since KNO_3 has also been shown to inhibit the vacuolar H^+ -ATPase in rat liver Golgi membranes [34], the findings strongly indicate the presence of an ATP-dependent bile-salt pump at the bile-canalicular front of rat hepatocytes.

Quantitatively ATP stimulated the transmembrane transport

of taurocholate to an approx. 10-fold greater extent (Fig. 1) than the valinomycin-induced K^+ -diffusion potentials previously characterized in similarly isolated cLPM vesicles [8,9]. Although part of these differences might be explained by the different incubation temperatures (25 °C in the former versus 37 °C in the present study), the ATP-dependent system also exhibited a surprisingly high affinity for taurocholate ($K_m \sim 2.1 \mu\text{M}$; Fig. 7), considering that total intracellular physiological bile-salt concentrations are estimated to range between 100 and 300 μM [2]. Although binding to cytosolic proteins and to various subcellular organelles might decrease the free cytosolic bile acid concentrations by over 90% [36], the dissociation constants for binding of bile acids by the three major classes of cytoplasmic binding proteins have been reported to range between 20 and 500 μM in rat liver [37]. Thus the possibility cannot be excluded that the ATP-dependent bile-salt transporter might operate *in vivo* under substrate-saturation conditions, which would mean that the overall ATP-dependent bile-salt transport capacity in intact liver is primarily dependent on the number of carriers per canalicular surface area. In this regard it is interesting to estimate the transport capacity *in vivo* from the V_{max} value of approx. 0.72 nmol/min per mg of cLPM protein (see Fig. 7). Considering the sidedness of cLPM vesicles ($\sim 25\%$ transport-competent inside-out vesicles [8]) and the average recovery and content of cLPM protein per g of liver (0.12 mg = 5% [15]), the estimated ATP-driven maximum taurocholate secretion by normal rat liver would amount to approx. 6.9 nmol/min per g of liver ($0.72 \times 4 \times 0.12 \times 20$). This value is clearly far below the reported bile-salt secretion maxima of 170–350 nmol/min per g of liver [1,2,38–40] and also is approx. 10-fold lower than the bile-salt secretion rates of normal rat liver operating under non-saturating conditions [1,38,40]. Thus it appears unlikely that canalicular bile-salt secretion *in vivo* is exclusively mediated by the ATP-dependent transport system, although such a possibility can never be definitely excluded on the basis of transport kinetics *in vitro* alone. However, the additional operation of a high-capacity electrogenic canalicular bile-salt secretory pathway has been well established not only in isolated cLPM vesicles [6–9], where conductive pathways could be artificially created during the membrane isolation procedure [41], but also in intact hepatocytes [10,11], isolated hepatocyte couplets [12] and more recently also in the isolated perfused rat liver [42]. It remains unknown at present which of the two bile-salt secretion systems (electrogenic or ATP-dependent) provides a closer link to the so far largely unknown molecular mechanisms involved in the coupling between canalicular bile salt and biliary lipid secretion [43]. Since the ATP-dependent system is probably saturated at low bile-salt transport rates, whereas biliary lipid secretion is continuously increasing up to the total secretory bile-salt maximum, one might speculate that the electrogenic system is more closely associated with the recruitment of biliary lipids. On the other hand, an increased number of ATP-dependent carriers in the canalicular membrane would also increase the total canalicular bile-salt output, and might therefore also stimulate biliary lipid secretion [43]. The latter assumption, however, would require a mechanism of rapid incorporation of ATP-dependent bile-salt carriers into the canalicular membrane from a pre-existing carrier pool located in intracellular vesicles situated in close proximity to the bile canaliculi ('pericanalicular vesicles'). Regulation of membrane transport processes by such vesicle incorporation/retrieval mechanisms has indeed been shown to occur for different transport systems in other cells [44]. In addition, canalicular exocytosis of pericanalicular vesicles appears to be involved in the regulation of intracellular pH, cell volume and bile secretion in rat liver [45]. The possibility that these pericanalicular vesicles also exhibit ATP-dependent bile-salt transport cannot be excluded at present, since pericanalicular

vesicles might well contaminate the cLPM subfraction as isolated in the present study [15,22].

Further investigations are also required to elucidate the relationship(s) between the ATP-dependent system and the previously characterized electrogenic canalicular bile-salt secretion pathway. The latter has been shown to be mediated by a 100 kDa membrane glycoprotein that has been isolated, partially purified and reconstituted in proteoliposomes [16,46,47]. It is unknown at present whether this 100 kDa protein could also mediate ATP-dependent taurocholate transport, or whether the two bile-salt transport systems function entirely independently from each other. Alternatively, the 100 kDa protein could be part of a multimeric transport complex [48], with the membrane potential and the ATP-dependent bile-salt translocation sites being mediated by distinct protein subunits. These questions can only be answered by separate isolation and functional reconstitution of the proteins involved in the two transport functions.

Finally, the cLPM-associated ATP-dependent taurocholate transport system exhibited a unique *cis*-inhibitory pattern towards a variety of organic compounds tested (Table 2). The finding that univalent taurine-conjugated bile salts showed greater *cis*-inhibitory effects than did their unconjugated analogues further supports the view that intracellular amidation and canalicular secretion of bile salts are highly regulated and probably closely related events. No significant *cis*-inhibition was found with daunomycin and GSSG, indicating that the ATP-dependent bile-salt transport system is different from the multi-drug-resistance gene product or Gp170 [28] and also from the recently characterized multi-organic-anion transporter (M.O.A.T. [27,49–52]). The latter conclusion, however, requires further kinetic analysis, since the two M.O.A.T. substrates sulphobromophthalein [27] and leukotriene C₄ [50] demonstrated > 50 % inhibitory effects (Table 2), and since, in contrast with previous findings [49], GSSG has been very recently claimed to be not a substrate for the M.O.A.T. system [52]. However, clear differences in the *cis*-inhibition pattern between the M.O.A.T. system and the ATP-dependent bile-salt transporter have been found by others [14], further indicating that the two systems represent distinct transport entities, although they might exhibit overlapping substrate specificities. In contrast with the present study (Table 2), the study by Nishida *et al.* [14] found no inhibitory effects of the thiol-reacting agent *N*-ethylmaleimide, but a dose-dependent inhibition by vanadate (50 % inhibition at ~ 20 μ M), indicating that an ATPase activity might be involved. The reason why vanadate, the classical inhibitor of ATPases with a phosphorylated intermediate [26], exerted only minimal inhibitory effects in our study, is unknown, but a similar low sensitivity to vanadate has recently also been reported for the ATP-dependent glutathione *S*-conjugate carrier, which is identical with the M.O.A.T. system [53]. Finally, it should be realized that the strong inhibitory effects of oligomycin (Table 2), a typical inhibitor of the mitochondrial F₀F₁-ATPase [26], was not due to mitochondrial contamination of the cLPM subfraction, since NaN₃ exerted no inhibitory effects (Table 2) and isolated mitochondria and submitochondrial particles did not exhibit ATP-dependent taurocholate uptake (results not shown).

In conclusion, the present study provides further strong evidence for the presence of a primary active ATP-dependent bile-salt transport system at the bile canalicular front of rat hepatocytes. Its primary localization most probably is the canalicular membrane, but it might also be present in pericanalicular vesicles destined to fuse with the canalicular membrane, thus providing a possible link to the mechanisms involved in canalicular exocytosis and biliary lipid secretion. The exact physiological role of this apparently high-affinity/low-capacity bile-salt pump and its relationship to the membrane-potential-

sensitive canalicular bile-salt transporter remain to be established. The same is also true with respect to its potential involvement in the pathophysiology of inherited, as well as endogenously and exogenously (toxic) induced, cholestatic liver diseases.

Note added in proof (received 17 February 1992)

After submission of this manuscript, two further studies have appeared that also demonstrate the occurrence of ATP-dependent bile salt transport in the rat liver canalicular plasma membrane [54,55]. Most importantly, the study by Müller *et al.* [54] indicates that the canalicular ATP-dependent bile salt transport system is mediated by a 110 kDa glycoprotein, which has been immunoprecipitated and apparently reconstituted into liposomes.

This study was supported by Swiss National Science Foundation Grants 31-26334.89 and 32-29878.90. We thank Professor Heini Murer and his collaborators for giving us access to their spectrofluorimeter.

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Received 18 September 1991/28 November 1991; accepted 10 December 1991