Properties of the canalicular bile acid transport system in rat liver

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4,4-Di-isothiocyanostilbene-2,2'-disulphonic acid inhibition of taurocholate efflux from canalicular vesicles was used to demonstrate that potential driven and 'carrier'-mediated canalicular excretion of taurocholate occur via a common, rather than two separate, pathways. This electrogenic canalicular bile acid 'carrier' preferentially transports trihydroxylated and conjugated dihydroxylated bile acids, but not the unphysiological oxo bile acids, and possibly extends its substrate specificity to other amphipathic molecules such as sulphobromophthalein.

INTRODUCTION

In order to maintain efficient enterohepatic circulation, the liver must continuously excrete bile acids into the minute channels of bile canaliculi that are formed by the canalicular plasma-membrane domains of two adjacent hepatocytes (Boyer, 1980; Blitzer & Boyer, 1982). This canalicular excretion represents the rate-limiting step in overall transport of bile acids from blood into bile (Reichen & Paumgartner, 1976). Previous studies with cLPM vesicles indicated that excretion of taurocholate, the major conjugated bile acid in the rat, is governed by a saturable transport system localized at the bile canalicular membrane (Inoue *et al.*, 1984a; Meier *et al.*, 1984b). Furthermore, the same studies provided evidence that canalicular efflux of taurocholate from hepatocytes can be driven, at least in part, by the physiological intracellular negative electric potential of approx. -30 to -40 mV. However, it remained unclear from these studies whether this potential-driven pathway for the canalicular excretion of taurocholate was identical with, or separate from, the 'carrier'-mediated process. In order to address this question we compared the effects of the anion-transport inhibitor DIDS on *trans*-stimulated as well as potential-driven taurocholate efflux from cLPM vesicles. Furthermore, the substrate specificity of the canalicular bile acid carrier was investigated by *cis*-inhibition and *trans*-stimulation experiments. The results provide definite evidence for the identity of the electrogenic and 'carrier'-mediated canalicular excretory process for taurocholate. This canalicular bile acid transport system preferentially transports primary bile acids, with conjugation being a prerequisite for efficient transport for chenodeoxycholate, but less so for cholate. In addition, BSP appears to be a co-substrate for the canalicular bile acid 'carrier'. Thus, as observed for organic-anion transporters that facilitate sinusoidal uptake into liver (Wieland *et al.*, 1984; Ziegler *et al.*, 1984; Hugentobler & Meier, 1986), the canalicular bile acid transport system may also share substrate specificity with other amphipathic molecules.

EXPERIMENTAL PROCEDURES

Materials

[6- 3 H]Taurocholic acid (sp. radioactivity 6.7 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). DIDS was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade and obtained from BDH (Poole, Dorset, U.K.), Calbiochem (Lucerne, Switzerland), Fluka AG (Buchs, Switzerland) or Sigma Chemical Co.

Animals

Male Sprague-Dawley rats (SUT; SDT Sdddeutsches Tierzuchtinstitut, Tuttlingen, Germany) weighing 200–250 g were 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-temperature constant-humidity environment with alternating 12 h light (06:30–18:30 h) and dark cycles. Fed animals were regularly killed by decapitation between 07:30 and 08:30 h.

Isolation of cLPM vesicles

cLPM vesicles were isolated as previously described (Meier *et al.*, 1984a). The membranes were resuspended in a solution comprising (mM): potassium gluconate, 100; sucrose, 100; calcium gluconate, 0.2; magnesium gluconate, 5; Hepes/Tris, 20; pH 7.5. This buffer system will hereafter be called 'standard membrane suspension' (SMS, pH 7.5) buffer. Portions of membranes were stored frozen in liquid N₂ for up to 2 weeks without loss of transport functions. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Determination of taurocholate efflux from cLPM vesicles

Frozen membrane suspensions were quickly thawed in a 37 °C water bath and diluted to the desired protein concentration (4 mg·ml⁻¹). After the addition of 10 µM-

Abbreviations used: DIDS, 4,4-di-isothiocyanostilbene-2,2'-disulphonic acid; cLPM, canalicular rat liver plasma-membrane; BSP, sulphobromophthalein; SMS, standard membrane suspension.

[^3H]taurocholate (final concentration), the membranes were vesiculated by 20 passages through a 25-gauge needle. Preloading of the vesicles with labelled taurocholate was completed by additional preincubation at 25 °C for 20 min. Subsequently all vesicle suspensions were treated with the potassium ionophore valinomycin ($10\text{ }\mu\text{g}\cdot\text{mg}$ of protein $^{-1}$). Tracer taurocholate efflux was determined at 25 °C either under voltage-clamped conditions ($[\text{K}^+]_{\text{in}} = [\text{K}^+]_{\text{out}}$) or in the presence of an intravesicular negative potassium diffusion potential ($[\text{K}^+]_{\text{in}} > [\text{K}^+]_{\text{out}}$). For this purpose 20 μl of [^3H]taurocholate-preloaded vesicles (40–80 μg of protein) were incubated in 180 μl of either SMS, pH 7.5 ($[\text{K}^+]_{\text{in}} = [\text{K}^+]_{\text{out}} = 100\text{ mM}$) or a solution comprising (mM): tetramethylammonium gluconate, 100; sucrose, 100; calcium gluconate, 0.2; magnesium gluconate, 5; Hepes/Tris, 20; pH 7.5 ($[\text{K}^+]_{\text{in}} = 100$; $[\text{K}^+]_{\text{out}} = 10$). After incubation for the indicated time intervals, taurocholate efflux was terminated by addition of 3 ml of ice-cold SMS, pH 7.5. Membrane-vesicle-associated ligand was separated from free ligand by rapid filtration through a $0.45\text{ }\mu\text{m}$ -pore-size nitrocellulose filter (Sartorius, Göttingen, Germany), that had been presoaked in cold deionized water and additionally rinsed with 1 mM-taurocholate in order to reduce unspecific filter binding. After two washes with 3 ml of cold SMS, pH 7.5, the filters were dissolved in 5 ml of liquid-scintillation cocktail (Filter-Count; Packard Instruments, Zurich, Switzerland) and the radioactivity counted in either a Tri-Carb 460 CD (Packard Instruments) or a Beckman LS 7000 liquid-scintillation counter. All experimental data were obtained from triplicate analyses of at least two separate membrane preparations.

RESULTS AND DISCUSSION

Since cLPM vesicles are predominantly oriented right-side-out (Meier *et al.*, 1984b), efflux rather than uptake represents the physiological direction of taurocholate transport in intact liver. Therefore efflux experiments were exclusively performed in the present study. The data in Fig. 1 extend our previous findings (Meier *et al.*, 1984b) and demonstrate that, under voltage-clamped conditions ($[\text{K}^+]_{\text{in}} = [\text{K}^+]_{\text{out}}$; valinomycin), taurocholate efflux from cLPM vesicles can be stimulated by the simultaneous presence of 50 μM -taurocholate or -glycocholate on the outside of the vesicles. This phenomenon of *trans*-stimulation or countertransport is characteristic of a 'carrier'-mediated process. As further demonstrated in Fig. 1, both basal as well as *trans*-stimulated taurocholate efflux could be inhibited to the same level when 1 mM-DIDS was present in the extravesicular media. Since DIDS was added in these experiments after preloading of the vesicles with [^3H]taurocholate, these results suggest that DIDS may exert its *trans*-inhibitory effects by blocking the involved 'carrier' from the outside of the vesicles. Similar effects of DIDS were also observed for taurocholate efflux when an intravesicular negative K^+ diffusion potential was imposed. As demonstrated in Fig. 2, diffusion-potential-driven taurocholate efflux could be completely blocked by the addition of DIDS on the outside of the vesicles. These results strongly suggest that *trans*-stimulated and potential-driven taurocholate efflux from cLPM vesicles is mediated by a common DIDS-inhibitable transport system rather than by two separate pathways. Thus the

Fig. 1. Effect of DIDS on taurocholate (TC) efflux from cLPM vesicles

Vesicles were preloaded with 10 μM -[^3H]taurocholate and voltage-clamped with valinomycin in the presence of equilibrated K^+ concentrations ($[\text{K}^+]_{\text{in}} = [\text{K}^+]_{\text{out}}$; 100 mM) as described in the Experimental procedures section. Taurocholate efflux was determined in the presence of either no additions ($\bullet$) or 50 μM -taurocholate ($\blacksquare$), or 50 μM -glycocholate (GC) ($\blacktriangle$) on the outside of the vesicles. Treatment of the vesicles with 1 mM-DIDS inhibited basal ($\circ$) and *trans*-stimulated ($\square$, $\triangle$) taurocholate efflux to the same level.

Symbol	$[\text{TC}]_{\text{in}}$ (μM)	$[\text{GC}]_{\text{out}}$ (μM)	$[\text{DIDS}]_{\text{out}}$ (μM)	$[\text{TC}]_{\text{out}}$ (μM)
$\triangle$	10	50	1000	—
$\square$	10	—	1000	50
$\circ$	10	—	1000	—
$\bullet$	10	—	—	—
$\blacksquare$	10	—	—	50
$\blacktriangle$	10	50	—	—

previously documented canalicular membrane bile acid 'carrier' (Inoue *et al.*, 1984a; Meier *et al.*, 1984b) can be 'driven', at least in part, by the physiological intracellular negative electrical membrane potential. Furthermore, the strong inhibitory action of DIDS on transcanalicular transport of taurocholate suggests that affinity labelling with DIDS might be a useful approach to identify the protein(s) involved in canalicular excretion of bile acids, as has previously been demonstrated for the sinusoidal bile acid uptake transporters (Ziegler *et al.*, 1984).

In order to delineate the substrate specificity of the canalicular bile acid 'carrier', *trans*-stimulation as well as *cis*-inhibition experiments were performed in the presence of various bile acids on the outside or inside of the vesicles respectively. For clarity the structural differences between the various bile acid substrates used are summarized in Fig. 3. *trans*-Stimulation and *cis*-

Fig. 2. Effect of DIDS on electrogenic efflux of taurocholate (TC) from cLPM vesicles

[^3H]Taurocholate ($10\ \mu\text{M}$)-preloaded vesicles were incubated either under voltage-clamped conditions (valinomycin; $[\text{K}^+]_{\text{in}} = [\text{K}^+]_{\text{out}}$, 100 mM) (B, D) or in the presence of an intravesicular negative K^+ diffusion potential (valinomycin; $[\text{K}^+]_{\text{in}} = 100\ \text{mM}/[\text{K}^+]_{\text{out}} = 10\ \text{mM}$) (A, C). Under both conditions treatment of the vesicles with 1 mM-DIDS inhibited taurocholate efflux to the same levels (C, D). The inset demonstrates the DIDS-sensitive portions of basal ($\bullet$) and potential-driven ($\circ$) taurocholate efflux. Results are means $\pm$ S.D. of nine determinations on three separate membrane preparations.

Symbol	$[\text{K}^+]_{\text{in}}$ (mM)	$[\text{K}^+]_{\text{out}}$ (mM)	DIDS
$\bullet$	100	100	—
$\circ$	100	100	+
$\blacksquare$	100	10	—
$\square$	100	10	+

inhibition of taurocholate efflux from cLPM vesicles were consistently observed for cholates, taurocholate and taurochenodeoxycholate (Table 1). In contrast, although taurodeoxycholate still exhibited *cis*-inhibition without having any *trans*-stimulatory effects, the unconjugated dihydroxylated bile acids chenodeoxycholate and deoxycholate inhibited taurocholate efflux from canalicular membrane vesicles when placed on either side of the membranes (*trans*- and *cis*-inhibition). No effects on taurocholate efflux were observed with the non-physiological bile acid taurodehydrocholate, in which the hydroxy groups on the 3,7 and 12 positions of the steroid moiety are replaced by oxo groups.

Table 1. *trans*-Stimulation and *cis*-inhibition of taurocholate (TC) efflux from cLPM vesicles by various bile acids

cLPM vesicles were resuspended in SMS, pH 7.5, preloaded with $10\ \mu\text{M}$ - $[\text{^3H}]$ taurocholate and treated with valinomycin. (a) Vesicles were incubated in SMS, pH 7.5 ($[\text{K}^+]_{\text{in}} = [\text{K}^+]_{\text{out}}$) containing either none (controls) or a $50\ \mu\text{M}$ concentration of the indicated bile acids. (b) In addition to $[\text{^3H}]$ taurocholate the vesicles were also preloaded with a $50\ \mu\text{M}$ concentration of the indicated bile acids. Incubations were performed in a solution comprising (mM): tetramethylammonium gluconate, 100; sucrose, 100; calcium gluconate, 0.2; magnesium gluconate, 5; Hepes/Tris, 20; pH 7.5 ($[\text{K}^+]_{\text{in}} = 100\ \text{mM}$; $[\text{K}^+]_{\text{out}} = 10\ \text{mM}$).

Results are means $\pm$ S.D. for nine determinations on three different membrane preparations. Statistical analysis was done by analysis of variance with two-way layout. The levels of significant differences from controls were: * $P < 0.001$; ** $P < 0.05$.

	Efflux of TC (pmol/mg of protein)		
	15 s	30 s	180 min
(a) <i>trans</i> -Stimulation			
Controls	17 $\pm$ 6	29 $\pm$ 7	123 $\pm$ 20
Cholate	28 $\pm$ 5*	52 $\pm$ 13*	112 $\pm$ 11
Taurocholate	32 $\pm$ 8*	42 $\pm$ 5*	103 $\pm$ 14
Chenodeoxycholate	9 $\pm$ 7**	18 $\pm$ 6*	100 $\pm$ 16
Taurochenodeoxycholate	30 $\pm$ 10**	42 $\pm$ 8*	133 $\pm$ 19
Deoxycholate	13 $\pm$ 4	13 $\pm$ 4*	99 $\pm$ 21
Taurodeoxycholate	22 $\pm$ 9	32 $\pm$ 11	122 $\pm$ 9
Taurodehydrocholate	18 $\pm$ 5	31 $\pm$ 8	115 $\pm$ 10
(b) <i>cis</i> -Inhibition			
Controls	38 $\pm$ 7	57 $\pm$ 9	—
Cholate	22 $\pm$ 4*	38 $\pm$ 9*	—
Taurocholate	20 $\pm$ 9*	28 $\pm$ 10*	—
Chenodeoxycholate	35 $\pm$ 13	40 $\pm$ 14*	—
Taurochenodeoxycholate	10 $\pm$ 7*	25 $\pm$ 12*	—
Deoxycholate	31 $\pm$ 20	40 $\pm$ 9*	—
Taurodeoxycholate	21 $\pm$ 13**	33 $\pm$ 9*	—
Taurodehydrocholate	36 $\pm$ 8	52 $\pm$ 12	—

These results (Table 1) permit several additional conclusions about the characteristics of the canalicular bile acid 'carrier'. First, the canalicular bile acid transport system appears to have the following order of substrate preference: cholate = taurocholate = taurochenodeoxycholate > taurodeoxycholate > chenodeoxycholate, deoxycholate. Since conjugation of the side chain of bile acids provides a higher negative charge, preference of the 'carrier' for conjugated bile acids would favour electrogenic driving forces for their canalicular excretion. Indeed, the inhibitory effects of the two unconjugated dihydroxy bile acids suggest that the transport of these less-polar bile acids is not effectively mediated by this potential-driven 'carrier'. Evidence for the importance of a negative side-chain charge for biliary excretion of bile acid derivatives has also been shown in the isolated perfused rat liver as well as in the intact animal (Vessey *et al.*, 1983; Anwer *et al.*, 1985). Secondly, the *cis*- and *trans*-inhibitory effects of the two unconjugated dihydroxy bile acids chenodeoxycholate and deoxycholate on taurocholate efflux from cLPM

Fig. 3. Chemical structure of the various bile acids discussed in Table 1

Cholate and chenodeoxycholate are synthesized in liver cells (primary bile acids), whereas deoxycholate is formed in the intestine by bacterial 7 α -dehydroxylation (secondary bile acid). Taurodehydrocholate is a synthetic non-micelle-forming bile-acid analogue. In the rat most of the bile acids are conjugated with taurine. This conjugation decreases the pK_a from 5 (cholate) or 6 (chenodeoxycholate, deoxycholate) to approx. 2 for all three physiological bile acids. Thus, at physiological pH, side-chain conjugation increases the negatively charged bile-acid species by factors of 10^3 and 10^4 respectively. Despite these high proportions of ionized molecules, the term 'bile acids' rather than 'bile salts' is generally used as a generic term for the entire group of substances (Hofmann, 1984).

Table 2. *trans*-Stimulation and *cis*-inhibition of taurocholate (TC) efflux from cLPM vesicles by various none-bile-acid substrates

cLPM vesicles were resuspended in SMS, pH 7.5, preloaded with 10 μ M-[3 H]taurocholate and treated with valinomycin. (a) Vesicles were incubated in SMS, pH 7.5 ($[K^+]_{in} = [K^+]_{out}$), containing either none (controls) or the indicated concentrations of the various substrates. (b) In addition to [3 H] taurocholate, the vesicles were also preloaded with the various substrates. Incubations were performed in the presence of an in-to-out $[K^+]$ gradient ($[K^+]_{in} = 100$ mM; $[K^+]_{out} = 10$ mM). Results are means $\pm$ S.D. for nine determinations on three different membrane preparations. Statistical analysis was done by analysis of variance with a two-way layout. The levels of significant differences from controls were: * $P < 0.05$; ** $P < 0.001$.

Non-bile-acid substrate	Efflux of TC (pmol/mg of protein)		
	15 s	30 s	180 min
(a) <i>trans</i> -Stimulation			
Controls	21 $\pm$ 5	31 $\pm$ 16	116 $\pm$ 11
BSP (1 mM)	28 $\pm$ 6	48 $\pm$ 5*	130 $\pm$ 19
GSH (5 mM)	17 $\pm$ 7	34 $\pm$ 6	121 $\pm$ 10
GSSG (1 mM)	18 $\pm$ 6	37 $\pm$ 10	115 $\pm$ 21
Glutamate (5 mM)	23 $\pm$ 5	33 $\pm$ 7	111 $\pm$ 11
K ₂ SO ₄ (10 mM)	20 $\pm$ 4	44 $\pm$ 9	109 $\pm$ 13
(b) <i>cis</i> -Inhibition			
Controls	33 $\pm$ 6	40 $\pm$ 7	
BSP (0.1 mM)	9 $\pm$ 3**	16 $\pm$ 2**	
GSH (5 mM)	23 $\pm$ 7	37 $\pm$ 5	
GSSG (1 mM)	30 $\pm$ 15	44 $\pm$ 8	
Glutamate (5 mM)	26 $\pm$ 6	34 $\pm$ 12	
K ₂ SO ₄ (5 mM)	31 $\pm$ 8	37 $\pm$ 12	

vesicles are most probably caused by high-affinity binding to the 'carrier' protein(s) rather than by unspecific effects on the membranes, since equilibrium values at 180 min were similar for all bile acid derivatives studied (Table 1). Thus unconjugated-dihydroxy-bile-acid binding and inhibition of the canalicular 'carrier' might partly explain the higher cholestatic potency of dihydroxylated compared with trihydroxylated bile acids (Drew & Priestley, 1979). Finally, alterations of the oxidation state of the steroid ring by replacement of the hydroxy groups by oxo groups no longer allows interaction with the bile acid carrier, since taurodehydrocholate neither *cis*-inhibited nor *trans*-stimulated taurocholate efflux (Table 1). Therefore taurodehydrocholate must be excreted into bile canaliculi by a separate, as yet unidentified, canalicular transport system. Preliminary studies suggest that the hepatic uptake of taurodehydrocholate is also facilitated by organic-anion transporters on the sinusoidal membrane that are different from the bile acid 'carriers' (Hardison *et al.*, 1984). In summary, although these studies will have to be complemented by separate delineations of the transport characteristics for each of the various bile acid substrates, the findings in Table 1 are consistent with the concept that the number of hydroxy groups, a negatively charged side chain and the structure of the steroid backbone are important determinants for efficient transport of bile acids by the canalicular taurocholate transport system. These conditions are remarkably similar to the structural requirements of the ileal and sinusoidal taurocholate uptake systems (Wilson, 1981; Hardison *et al.*, 1984). They raise the possibility that there may be certain common structural characteristics of bile acid 'carriers' that are involved in the transmembrane movement of bile acids throughout the enterohepatic circulation.

Finally, the substrate specificity of the canalicular bile acid 'carrier' was further assessed by *trans*-stimulation and *cis*-inhibition experiments with other organic and inorganic anions known to be excreted and concentrated in bile. As demonstrated in Table 2, in the concentrations used, GSH and GSSG, glutamate and sulphate had no effects on taurocholate efflux from cLPM vesicles, confirming that these cholephilic anions are transported into bile by separate transport systems. These transport systems have recently been identified and partially characterized in canalicular membrane vesicles (Inoue *et al.*, 1983; Akerboom *et al.*, 1984; Ballatori *et al.*, 1986; Meier *et al.*, 1986). However, the exogenous organic anion BSP *cis*-inhibited, and at 30 s also significantly *trans*-stimulated taurocholate efflux from cLPM vesicles (Table 2). Thus unconjugated BSP might be a co-substrate for the canalicular bile acid transport system. Whether this is also true for the glutathione conjugate of BSP, which has been reported not to be transported by the GSSG-transport system (Inoue *et al.*, 1984b), remains to be investigated. Considerable further studies will be required to determine the extent to which the canalicular bile acid transport carrier shows specificity towards other amphipathic molecules or their metabolites.

Conclusions

The present study demonstrates that electrogenic and 'carrier'-dependent canalicular excretion of taurocholate are mediated by a common transport system rather than by two separate pathways. This electrogenic canalicular taurocholate 'carrier' preferentially transports tri-hydroxylated and conjugated dihydroxylated bile acids. Thus introduction of a negative side-chain charge by conjugation appears to be an essential prerequisite for potential-driven canalicular excretion of primary and secondary bile acids. In addition, as observed in the ileal and hepatic sinusoidal taurocholate uptake systems, the number of hydroxy groups as well as the structure of the steroid moiety are important determinants of substrate specificity for the canalicular membrane taurocholate 'carrier'. Hence, despite differences in driving forces (Na^+ -dependent taurocholate uptake at the ileal brush-border and sinusoidal liver plasma membrane; Na^+ -independent electrogenic bile acid excretion at the canalicular pole of hepatocytes), all bile acid membrane transport systems within the enterohepatic circulation may share some common structural characteristics. More definite characterization of the similarities, as well

as differences, between the various bile acid transport systems awaits further studies, including isolation and reconstitution of the protein 'carrier' involved.

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