ELSEVIER

Biochimica et Biophysica Acta 1511 (2001) 7–16

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Characterization of bile acid transport mediated by multidrug resistance associated protein 2 and bile salt export pump

Hidetaka Akita [a], Hiroshi Suzuki [a,*], Kousei Ito [a], Setsuo Kinoshita [a], Norihito Sato [a], Hajime Takikawa [b], Yuichi Sugiyama [a]

[a] *Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[b] *Department of Medicine, Teikyo University School of Medicine, Kaga, Itabashi-ku, Tokyo 173-0003, Japan*

Received 20 March 2000; received in revised form 13 October 2000; accepted 25 October 2000

Abstract

Biliary excretion of certain bile acids is mediated by multidrug resistance associated protein 2 (Mrp2) and the bile salt export pump (Bsep). In the present study, the transport properties of several bile acids were characterized in canalicular membrane vesicles (CMVs) isolated from Sprague–Dawley (SD) rats and Eisai hyperbilirubinemic rats (EHBR) whose Mrp2 function is hereditarily defective and in membrane vesicles isolated from Sf9 cells infected with recombinant baculovirus containing cDNAs encoding Mrp2 and Bsep. ATP-dependent uptake of [^{3}H]taurochenodeoxycholate sulfate (TCDC-S) (K_m = 8.8 μM) and [^{3}H]taurolithocholate sulfate (TLC-S) (K_m = 1.5 μM) was observed in CMVs from SD rats, but not from EHBR. In addition, ATP-dependent uptake of [^{3}H]TLC-S (K_m = 3.9 μM) and [^{3}H]taurocholate (TC) (K_m = 7.5 μM) was also observed in Mrp2- and Bsep-expressing Sf9 membrane vesicles, respectively. TCDC-S and TLC-S inhibited the ATP-dependent TC uptake into CMVs from SD rats with IC_{50} values of 4.6 μM and 1.2 μM, respectively. In contrast, the corresponding values for Sf9 cells expressing Bsep were 59 and 62 μM, respectively, which were similar to those determined in CMVs from EHBR (68 and 33 μM, respectively). By co-expressing Mrp2 with Bsep in Sf9 cells, IC_{50} values for membrane vesicles from these cells shifted to values comparable with those in CMVs from SD rats (4.6 and 1.2 μM). Moreover, in membrane vesicles where both Mrp2 and Bsep are co-expressed, preincubation with the sulfated bile acids potentiated their inhibitory effect on Bsep-mediated TC transport. These results can be accounted for by assuming that the sulfated bile acids *trans*-inhibit the Bsep-mediated transport of TC.

Keywords: Multidrug resistance-associated protein; Bile salt export pump; Bile acid; Canalicular membrane vesicle

1. Introduction

As far as bile formation is concerned, continuous vectorial secretion of bile salts from blood to bile via hepatocytes is an important factor [1]. It has been established that transporters located on the sinusoidal (basal) and canalicular (apical) membrane are involved in transcellular transport of bile acids [2–4]. Uptake of bile salts into hepatocytes is mediated by both Na^+-dependent and -independent transporters. Recently, rat and human Na^+-taurocholate co-transporting polypeptide (Ntcp) and organic anion transporting polypeptides (oatp) have been cloned [5,6].

* Corresponding author. Fax: +81-3-5800-6949; E-mail: seizai.suzuki@nifty.ne.jp

0005-2736/01/$ – see front matter
PII: S0005-2736(00)00355-2

In addition, it has been shown that the biliary excretion of organic anions is mediated by multidrug resistance associated protein 2 (Mrp2) [7,8]. The substrate specificity of Mrp2 has been investigated by comparing transport across the bile canalicular membrane in normal rats and Mrp2-deficient rats. These mutant rats include Transport-Deficient (TR^-), Groningen Yellow (GY), and Eisai hyperbilirubinemic rats (EHBR), which are a good model for studying the pathogenesis of Dubin–Johnson syndrome in humans [7,8]. It has been shown that the substrates for Mrp2 include glutathione- and glucuronide-conjugates and non-conjugated organic anions [7,8].

The transport of monovalent bile acids (such as taurocholate; TC) across the bile canalicular membrane, however, is mediated by another transporter referred to as the bile salt export pump (Bsep). Using membrane vesicles isolated from Sf9 cells infected with recombinant baculovirus containing cDNA for the sister of P-glycoprotein, which was cloned as a homologue of MDR1 P-glycoprotein, it has been shown that this protein exhibits Bsep activity [9]. It was also demonstrated that the mutation in the Bsep gene results in PFIC 2 in human subjects [10].

In contrast to the previously described monovalent bile acids, it has been suggested that glucuronide- or sulfate-conjugates of bile acids are transported via Mrp2 and this hypothesis is based on the following in vivo findings. Kuipers and his collaborators found that the biliary excretion of cholate 3-*O*-glucuronide, taurochenodeoxycholate-3-sulfate (TCDC-S) and taurolithocholate-3-sulfate (TLC-S) was impaired in GY rats [11,12]. This indicates that the transport of these bile acid conjugates is mediated by Mrp2. This conclusion was further confirmed by Takikawa et al. [13] by in vivo experiments. In the present study, we have performed in vitro experiments to characterize the transport properties of sulfated bile acids (TCDC-S and TLC-S) along with their interaction with Bsep, since it has been demonstrated that the sulfated bile acids affect the disposition of TC in vivo [14,15]. In our experiments, we used CMVs from SD rats and EHBR and membrane vesicles from Sf9 cells infected with the recombinant baculovirus containing cDNAs for Mrp2 and Bsep.

2. Materials and methods

2.1. Materials

[^{3}H]TC (2–5 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA). [^{3}H]TLC-S and [^{3}H]TCDC-S were synthesized from lithocholate-3-sulfate and chenodeoxycholate-3-sulfate, respectively, using [2-^{3}H]taurine (30.3 Ci/mmol) (NEN Life Science Products) as described previously [16]. Chenodeoxycholate-3-sulfate was synthesized as described previously [17]. Unlabeled TC, TLC, TCDC, TLC-S, 17β-estradiol-17β-D-glucuronide ($E_2$17βG), ATP, ADP, AMP, GTP, CTP, UTP, creatine phosphate and creatine phosphokinase were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were commercially available and of reagent grade.

2.2. Preparation of CMVs

CMVs were prepared from male SD rats and EHBR as described previously [18] and kept as a suspension in 50 mM Tris buffer (pH 7.4) containing 250 mM sucrose. The membrane vesicles were then frozen in liquid N_2 and stored at −80°C until use. Protein concentrations were determined as reported previously [18], using an assay kit (Bio Rad Laboratories, Richmond, CA) with bovine serum albumin as a standard.

2.3. cDNA cloning of rat Bsep

Rat Bsep cDNA was cloned based on a sequence described by Gerloff et al. [9]. A cDNA probe was prepared by a reverse transcription–polymerase chain reaction (RT–PCR) on rat liver poly(A)$^+$ RNA using the forward (5′-dGAGCTGCTAGAAAGAAAAG-G-3′) and reverse primers (5′-dACCAGAATGTAG-TGCCATTC-3′). Reverse transcription was performed using oligo(dT) primer at 30°C for 10 min, 42°C for 30 min and 99°C for 5 min. Then, PCR was carried out at 94°C for 30 s, 37°C for 30 s, and 72°C for 1 min for 40 cycles using Taq polymerase (Takara Shuzo Co. Kyoto, Japan). RT–PCR resulted in the amplification of a partial sequence of rat Bsep (246 bp: 1924–2270 bp) [9]. This fragment was used as a probe to screen the full length cDNA from the

cDNA library prepared from size-fractionated poly-(A)$^+$ RNA (4.0–6.0 kbp) using the ZAP ExpressTM cDNA Synthesis and ZAP ExpressTM cDNA Gigapack III Gold Cloning kit (Stratagene, La Jolla, CA).

2.4. Preparation of membrane vesicles from Sf9 cells expressing rat Bsep and Mrp2

The Bac-to-Bac system (Life Technologies, Gaithersburg, MD) was used to establish the recombinant baculovirus. Full length of Bsep and Mrp2 [19] cDNAs were inserted into a donor plasmid (Life Technologies) downstream of the polyhedrin promoter. Sf9 cells were infected with the virus at 27°C for 72 h. To establish co-expressed cells for Bsep and Mrp2, Sf9 cells were infected with the 1-to-1 mixed virus sup for these clones. To prepare membrane vesicles, Sf9 cells were scraped from culture dishes, centrifuged at $1500 \times g$, and suspended in hypotonic buffer consisting of 1 mM Tris–HCl (pH 7.0) and gently stirred for 1.5 h. Then, the solution was centrifuged at $10\,000 \times g$, and diluted with isotonic buffer consisting of 10 mM Tris–HCl (pH 7.4) and 250 mM sucrose. Disrupted cells were homogenized with a glass/glass homogenizer, and mixed with 38% sucrose solution (Tris–Hepes, pH 7.4). After centrifugation at $280\,000 \times g$ for 45 min, the turbid layer was recovered and further centrifuged at $100\,000 \times g$. The pellet was diluted with the transport buffer (10 mM Tris, 250 mM sucrose and 10 mM $MgCl_2 \cdot 6H_2O$, pH 7.4), and passed through a 25-gauge needle. These membrane vesicle preparations were rapidly frozen in liquid N_2 and kept at −80°C until use.

2.5. Uptake experiments

The uptake of [^{3}H]TC, [^{3}H]TCDC-S and [^{3}H]TLC-S was examined as reported previously [18]. The transport medium contained the isotopically labeled ligand, 5 mM ATP or 5 mM AMP, and an ATP-regenerating system (10 mM creatine phosphate and 100 μg/ml creatine phosphokinase). A 16-μl aliquot of transport medium was mixed rapidly with 4 μl vesicle suspension (10 μg protein). In the inhibition study, TCDC-S and TLC-S were dissolved in DMSO and diluted to a final concentration (0.1% DMSO) with transport buffer. In the control study, the same concentration of DMSO was added to the transport medium.

In the present study, we also examined the inhibitory effect of sulfated bile acids on Bsep-mediated transport of [^{3}H]TC, by preloading the membrane vesicles with TLC-S and TCDC-S. In the preloading experiments, 10 μg of membrane vesicles was incubated in a 5-μl aliquot of transport buffer containing 5 mM ATP or 5 mM AMP, ATP-generating system and 1 μM TCDC-S or 1 μM TLC-S for 0 min, 1 min and 3 min, and then a 45-μl aliquot of transport medium containing 1 μM [^{3}H]TC, 5 mM ATP or 5 mM AMP, and an ATP-regenerating system was added rapidly to examine the uptake of [^{3}H]TC for 2 min.

The transport reaction was stopped by the addition of 1 ml ice-cold buffer containing 250 mM sucrose, 0.1 M NaCl and 10 mM Tris–HCl (pH 7.4). The stopped reaction mixture was filtered through a 0.45-μm HA filter (Millipore Corp., Bedford, MA), and then washed twice with 5 ml stop solution. The radioactivity retained on the filter and in the reaction mixture was measured in a liquid scintillation counter (LS 6000SE, Beckman Instruments, Fullerton, CA) following the addition of scintillation cocktail (Clear-sol I, Nacalai Tesque, Tokyo, Japan). Ligand uptake was normalized in terms of the amount of membrane protein.

The ATP-dependent uptake was determined by subtracting the uptake in the absence of ATP from that in the presence of ATP. The kinetic parameters were estimated as described previously [18].

2.6. Western blot analysis

Antiserum for rat Bsep was raised in rabbits against an oligopeptide (the carboxyl terminal of rat Bsep; AYYKLVITGAPIS) coupled with keyhole limpet hemocyanin via *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. Antiserum for rat Mrp2 (CP-2) was supplied by Dr J. Nakayama in Kumamoto University, Kumamoto, Japan, and this was raised against the upstream region of carboxy-terminal NBD (amino acid residues 1272–1285). For the Western blotting, membrane proteins were solubilized in a sample buffer consisting of 2% sodium dodecyl sulfate (SDS), 30% glycerol and 0.01% bro-

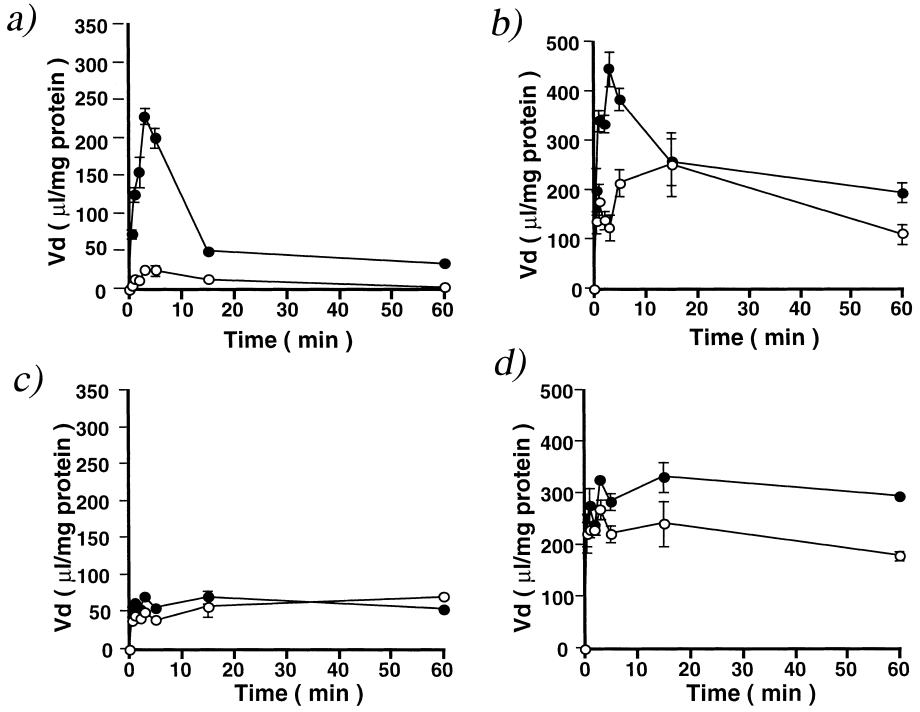

Fig. 1. Time-dependent uptake of [^{3}H]TCDC-S and [^{3}H]TLC-S by CMVs. CMVs prepared from SD rats (a,b) and EHBR (c,d) were incubated at 37°C with (●) or without (○) ATP and ATP-regenerating system in the medium. The concentration of [^{3}H]TCDC-S (a,c) and [^{3}H]TLC-S (b,d) was 1 μM. Each point and vertical bar represent the mean ± S.E. of three different experiments.

mophenol blue (pH 6.8). The suspension was subjected to SDS–polyacrylamide gel electrophoresis (PAGE), electrotransferred to a polyvinylidene difluoride membrane (Millipore) and blotted as described [20]. Blots were incubated with the antiserum (diluted 1:1000) and visualized with [^{125}I] anti-rabbit antibody (Amersham Corp., Buckinghamshire, UK), followed by exposure to Fuji imaging plates (Fuji Photo Film Co., Kanagawa, Japan) for 3 h at room temperature, and analyzed with an imaging analyzer (BSA 2000, Fuji Photo Film Co.).

3. Results

3.1. Uptake of [^{3}H]TCDC-S and [^{3}H]TLC-S by CMVs

The time profile for the uptake of [^{3}H]TCDC-S (1 μM) and [^{3}H]TLC-S (1 μM) by CMVs from SD rats and EHBR was examined (Fig. 1). In CMVs from SD rats, but not from EHBR, uptake of [^{3}H]TCDC-S and [^{3}H]TLC-S was stimulated by the presence of ATP, and increased linearly up to 2 min. Kinetic analysis revealed that the ATP-dependent uptake of [^{3}H]TCDC-S and [^{3}H]TLC-S into CMVs from SD rats could be described by one saturable component with $K_m = 8.8 \pm 1.3$ μM and $V_{max} = 659 \pm 58$ pmol/min per mg protein, and $K_m = 1.5 \pm 0.3$ μM and $V_{max} = 112 \pm 15$ pmol/min per mg protein, respectively (Fig. 2).

3.2. Uptake of [^{3}H]TC and [^{3}H]TLC-S by membrane vesicles from Sf9 cells expressing Bsep and Mrp2

cDNA product for Bsep and Mrp2 was expressed in Sf9 cells with the baculovirus expression system. In order to confirm the expression of these transporters, Western blotting was performed in CMVs from SD rats and membrane vesicles from Sf9 cells expressing Bsep and Mrp2 (Fig. 3). In the case of anti-serum against rat Bsep, approximately 170 and 160 kDa proteins were detected in CMVs and in membrane vesicles from Bsep-expressing Sf9 cells, respectively (Fig. 3b). In the same manner, approx-

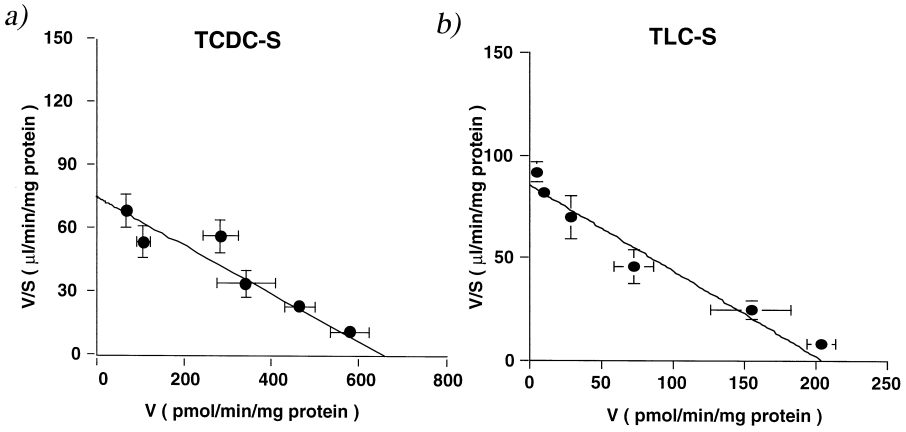

Fig. 2. Eadie–Hofstee plot for the uptake of [^{3}H]TCDC-S and [^{3}H]TLC-S by CMVs prepared from SD rats. CMVs prepared from SD rats were incubated at 37°C for 2 min, with or without ATP and ATP-regenerating system in the medium containing different concentration of TCDC-S (a) and TLC-S (b). The ATP-dependent uptake was obtained by subtracting the value in the absence of ATP from that in its presence. Each point and bar represent the mean ± S.E. of three different experiments.

imately 190 and 180 kDa proteins were detected by antiserum for Mrp2 in CMVs and membrane vesicles from Mrp2-expressing Sf9 cells, respectively (Fig. 3a).

The time profile for the uptake of [^{3}H]TC by membrane vesicles prepared from rat Bsep-expressing Sf9 cells is shown in Fig. 4a. The Bsep-mediated uptake of [^{3}H]TC (1 μM) was stimulated by the presence of ATP and the uptake was linear up to 2 min. In contrast, no ATP-dependent uptake of [^{3}H]TLC-S was observed in Bsep-expressing membrane vesicles (data not shown). Kinetic analysis of the concentration-dependent uptake of [^{3}H]TC gave a K_m of 7.5 ± 1.7 μM and a V_{max} of 295 ± 38 pmol/min per mg protein (Fig. 4c).

ATP-dependent uptake of [^{3}H]TLC-S by Mrp2 was also confirmed by membrane vesicles from Mrp2-expressing Sf9 cells. [^{3}H]TLC-S was taken up linearly by these membrane vesicles in an ATP-dependent manner up to 2 min (Fig. 4b). Kinetic analysis revealed that the K_m and V_{max} values for the ATP-dependent uptake of [^{3}H]TLC-S via Mrp2 were 3.9 ± 0.4 μM and 1486 ± 101 pmol/min per mg protein (Fig. 4d). In contrast, no ATP-dependent uptake of [^{3}H]TC was observed in Mrp2 expressing membrane vesicles (data not shown).

To determine the nucleotide dependence, transport of [^{3}H]TLC-S was measured in the presence of several kinds of nucleotide phosphates. As shown in Fig. 5a, replacement of ATP by ADP or AMP reduced the uptake of [^{3}H]TLC-S, suggesting that ATP hydrolysis is necessary for uptake of [^{3}H]TLC-S. Of

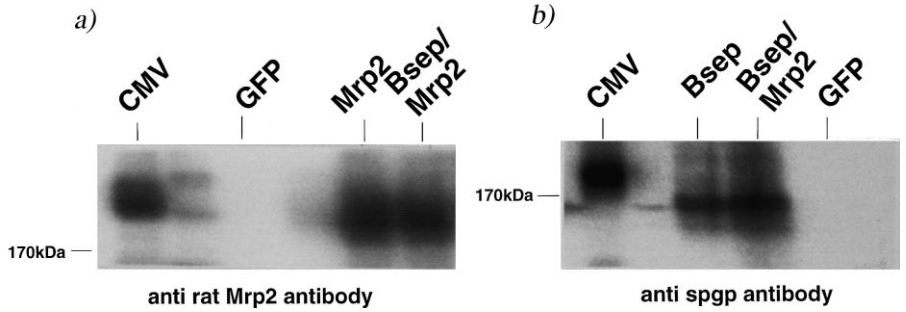

Fig. 3. Western blotting for CMVs and membrane vesicles prepared from Mrp2 and Bsep-expressing Sf9 cells. (a) Plasma membrane vesicles (30 μg) were size fractionated by 10% SDS–electrophoresis and electrophoretically transferred to a polyvinylidene difluoride membrane for immunoblotting with antiserum against MRP2. (b) Plasma membrane vesicles (100 μg) were applied for immunoblotting with antiserum against Bsep.

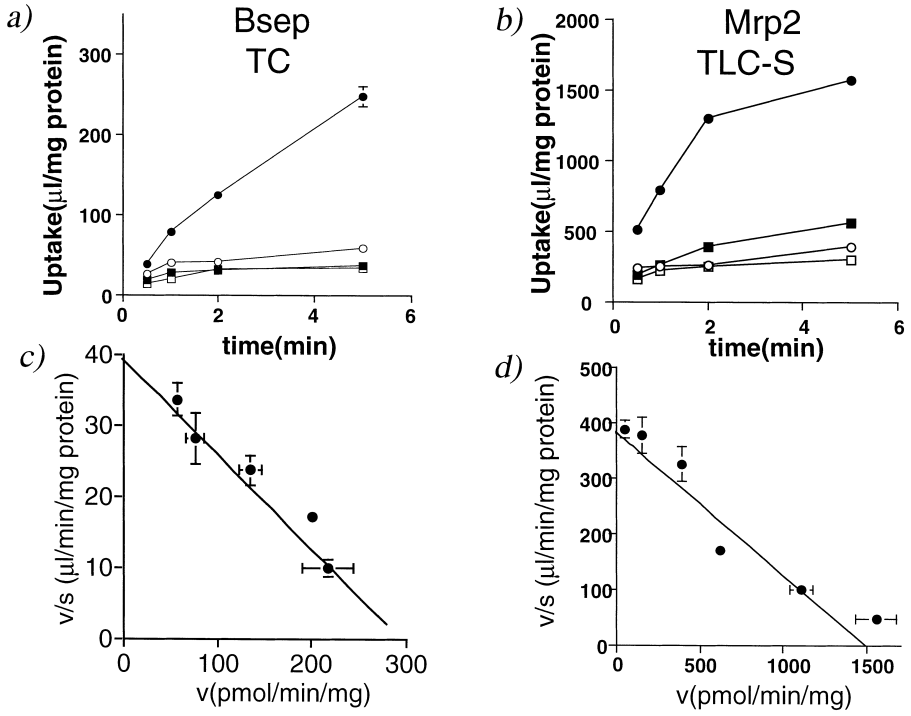

Fig. 4. Uptake of [^{3}H]TC and [^{3}H]TLC-S by membrane vesicles from Sf9 cells expressing Bsep and MRP2. Membrane vesicles isolated from Bsep (a) or Mrp2 (b) expressing Sf9 cells were incubated at 37°C for the indicated period, with (●) or without (○) ATP and ATP-regenerating system in the medium containing 1 μM [^{3}H]TC and [^{3}H]TLC-S, respectively. For comparison, the uptake into the control membrane vesicles in the presence (■) and absence (□) is indicated in panels a and b. The ATP-dependent uptake of ligands was obtained by subtracting the value in the absence of ATP from that in its presence. The saturable Bsep-mediated TC transport (c) and Mrp2-mediated TLC-S transport (d) are shown as Eadie–Hofstee plots. Each point and bar represent the mean ± S.E. of three different experiments.

the four nucleotide triphosphates tested, ATP was the most potent in stimulating the uptake of [^{3}H]TLC-S (Fig. 5a). Moreover, in order to confirm that the isotope count associated with the vesicles reflects transport into membrane vesicles, rather than binding to the membrane surface, the uptake of [^{3}H]TLC-S was measured in the presence of several different concentrations of sucrose in the transport medium (Fig. 5b). The uptake was osmotically sensitive, suggesting that the major part of the ATP-dependent uptake of [^{3}H]TLC-S is due to uptake, and not to adsorption to the membrane vesicle surface.

3.3. *Trans-inhibitory effect of TCDC-S, TLC-S and $E_2$17βG on the ATP-dependent uptake of [^{3}H]TC*

The bile acid transport systems on the bile canalicular membrane was further characterized by examining the effect of $E_2$17βG, TCDC-S and TLC-S on the ATP-dependent uptake of [^{3}H]TC by CMVs from SD rats and EHBR, and that by membrane vesicles isolated from rat Bsep-expressing Sf9 cells (Fig. 6). The uptake of [^{3}H]TC was inhibited by TCDC-S in a concentration-dependent manner with an IC_{50} of 4.6 ± 1.0 μM, 67.9 ± 16.7 μM and 58.7 ± 9.6 μM for CMVs from SD rats, EHBR and rat Bsep-expressing Sf9 vesicles, respectively (Fig. 6a). In the same manner, the IC_{50} values of TLC-S for [^{3}H]TC uptake were determined as 1.2 ± 0.3 μM, 33.0 ± 6.3 μM and 61.9 ± 19.6 μM for CMVs from SD rats, EHBR and rat Bsep-expressing Sf9 vesicles, respectively (Fig. 6b). By focusing on the fact that the sulfated bile acids give the lower IC_{50} values on Bsep-mediated transport of TC when Mrp2 is co-expressed, we prepared membrane vesicles from Sf9 cells co-expressing Bsep and Mrp2. Co-expression of Mrp2 and Bsep in membrane vesicles resulted in the shift of the IC_{50} values in a similar fashion to

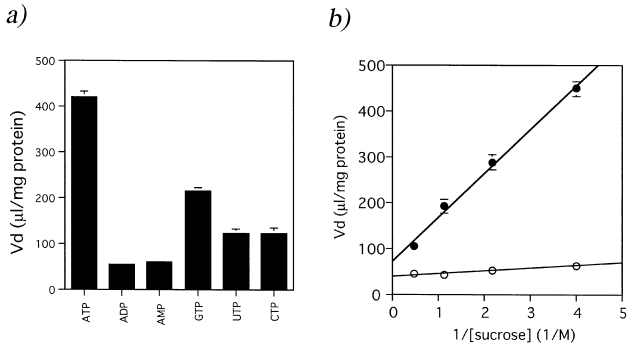

Fig. 5. Nucleotide specificity and osmotic sensitivity of [^{3}H]TLC-S transport by membrane vesicles from MRP2-expressing Sf9 cells. (a) [^{3}H]TLC-S uptake to membrane vesicles (5 μg) from MRP2-expressing Sf9 cells was measured in the presence of indicated nucleotides (5 μM) for 1 min at 37°C. No regenerating system was included in these experiments. (b) Membrane vesicles (5 μg) from MRP2 expressing Sf9 cells were preincubated at 37°C for 3 min in the medium containing different concentrations of sucrose (0.25, 0.46, 0.88, 2 and 10 mM). [^{3}H]TLC-S uptake was measured in the presence (closed) and absence (opened) of 5 mM ATP. Vertical bars represent the mean ± S.E. of three different experiments.

that of CMVs from SD rats (4.6 and 1.2 μM) (Fig. 6a,b).

Moreover, as control experiments inhibitory effect of 100 μM $E_2$17βG on the ATP-dependent uptake of [^{3}H]TC into the membrane vesicles was also characterized [21]. $E_2$17βG inhibits the uptake of [^{3}H]TC only in CMVs isolated from SD rats and in membrane vesicle isolated from Bsep/Mrp2 co-expressing Sf9 cells (16% and 36% of the control, respectively), but not significantly in CMVs from EHBR or membrane vesicles from Bsep-expressing Sf9 cells (Fig. 6c as reported previously). These results are consistent with the hypothesis that the sulfated bile acids are concentrated in the membrane vesicles due to the presence of Mrp2, and then *trans*-inhibit the Bsep-mediated transport of [^{3}H]TC (as proposed previously for $E_2$17βG [21]).

To confirm the *trans*-inhibitory effect of TCDC-S and TLC-S, sulfated bile acids were preloaded to membrane vesicles with the sulfated bile acids for 0, 1 and 3 min, and then the ATP-dependent uptake of [^{3}H]TC was measured for 2 min. The ATP-dependent transport of [^{3}H]TC into the membrane vesicle isolated from Mrp2/Bsep co-expressing Sf9 was inhibited by TCDC-S in a preincubation time-dependent manner (60–75% of control), whereas no significant inhibitory effect was observed in Bsep-expressing membrane vesicles (Fig. 7). Similarly, ATP-dependent transport of [^{3}H]TC into the Mrp2/Bsep co-expressing membrane vesicles was inhibited by the preincubation of TLC-S (70–80% of control), whereas no significant inhibitory effect was observed in Bsep-expressing membrane vesicles (Fig. 7).

4. Discussion

Previous in vivo findings suggested that sulfate-conjugated bile acids are secreted into the bile via Mrp2 [11,12]. In the present study, we have charac-

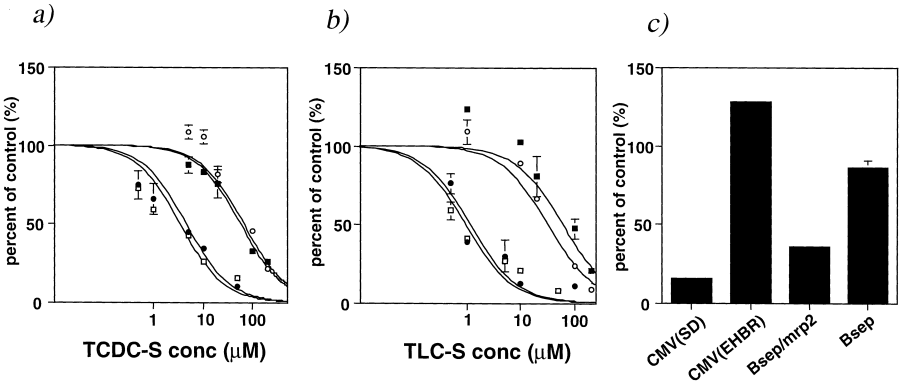

Fig. 6. Inhibitory effect of TCDC-S and TLC-S on ATP-dependent uptake of [^{3}H]TC. CMVs prepared from SD rats (●) and EHBR (○), and membrane vesicles from Bsep (■) and Bsep/Mrp2 co-expressing (□) Sf9 were incubated at 37°C for 2 min, with or without ATP and ATP-regenerating system in the medium containing [^{3}H]TC (1 μM) and various concentration of TCDC-S (a), TLC-S (b) and 100 μM $E_2$17βG. The ATP-dependent uptake was obtained by subtracting the value in the absence of ATP from that in its presence. Each point and vertical bar represent the mean ± S.E. of three different experiments.

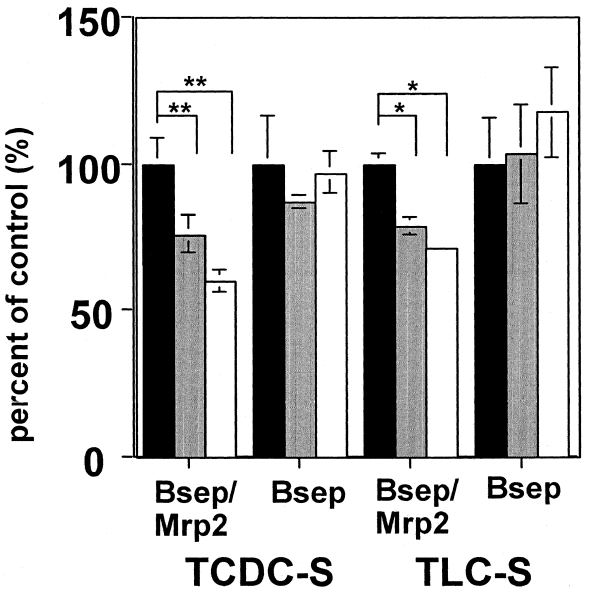

Fig. 7. Preincubation time-dependent inhibitory effect of TCDC-S and TLC-S on ATP-dependent uptake of [^{3}H]TC. Membrane vesicles isolated from Bsep/Mrp2 and Bsep expressing Sf9 cells (10 μg) were incubated in a 5-μl aliquot of transport buffer containing 5 mM ATP or 5 mM AMP, ATP-generating system and 1 μM TCDC-S or 1 μM TLC-S for 0 min (closed bar), 1 min (stippled bar) and 3 min (open bar), and then a 45-μl aliquot of transport medium containing 1 μM [^{3}H]TC, 5 mM ATP or 5 mM AMP, and an ATP-regenerating system was added to examine the uptake of [^{3}H]TC for 2 min. The ATP-dependent uptake was obtained by subtracting the value in the absence of ATP from that in its presence. ATP-Dependent uptake of [^{3}H]TC without preincubation (0 min) was defined as 100%. Each point and vertical bar represents the mean ± S.E. of three different experiments. Significantly different from control by Student *t*-test (**; $P < 0.01$, *; $P < 0.05$).

terized the transport of radiolabeled TCDC-S and TLC-S in CMVs from SD rats and EHBR and membrane vesicles expressing Bsep and Mrp2. Expression of Mrp2 and Bsep in the membrane vesicles isolated from Sf9 cells was confirmed by Western blot. The molecular mass of these transporters in Sf9 cells was smaller than that in CMVs, presumably due to the incomplete glycosylation in Sf9 cells. As shown in Fig. 1, ATP stimulated the uptake of both TCDC-S and TLC-S into CMVs from SD rats, but not from EHBR, indicating that these sulfated bile acids are substrates for Mrp2. This hypothesis was directly demonstrated by the uptake study of [^{3}H]TLC-S into membrane vesicles isolated from Mrp2-expressed Sf9 cells (Fig. 4b,d). These results are consistent with the recent report by Stieger et al. [21], performed independently. The uptake was supported to the greatest extent by ATP among the nucleotide triphosphates examined, followed by GTP > UTP = CTP (Fig. 5a), which is characteristic of transport by MRP family proteins [21]. Since the ATP-dependent uptake of [^{3}H]TLC-S was osmotically sensitive (Fig. 5b), this uptake represents transport into the intravesicular space, rather than binding to the vesicle surface. Moreover, the K_m values for the uptake of TLC-S were in the same range for CMVs and Mrp2-expressing Sf9 membrane vesicles (1.5 μM vs. 3.9 μM) (Figs. 2 and 4). These results are reasonable if we consider that there is a degree of overlap in the substrates of MRP1 and Mrp2. In fact, glucuronide conjugates of bile acids, along with TLC-S, are also substrates for MRP1 [22].

In contrast to the sulfated bile acids, the monovalent bile acids are excreted into the bile via Bsep as demonstrated by Gerloff et al. [9] and Stieger et al. [21]. These results were also reproduced in the present study. As shown in Fig. 4a, TC was taken up by membrane vesicles from Bsep-expressing Sf9 cells in an ATP-dependent manner. This ATP-dependent uptake was saturable with a K_m of 7.5 μM (Fig. 4c), which was comparable with that determined in CMVs (2.1 μM; Stieger et al. [23] and 6.0 μM; Sato et al., unpublished observation), and in membrane vesicles from Bsep-expressing Sf9 cells (5.3 μM; Gerloff et al. [9]). The fact that no ATP-dependent uptake of [^{3}H]TLC-S was observed in Bsep-expressing membrane vesicles (data not shown) is consistent with the suggestion that this sulfate conjugated bile acid is transported across the bile canalicular membrane predominantly via Mrp2.

In order to characterize the transport nature of bile acids more precisely, we also examined the inhibitory effect of TCDC-S and TLC-S on the ATP-dependent uptake of TC by CMVs and by membrane vesicles from Sf9 cells expressing cloned transporter cDNA products. Although TCDC-S and TLC-S inhibited [^{3}H]TC uptake into these membrane preparations, the IC_{50} values in CMVs from SD rats were much lower than those in membrane vesicles from Bsep-expressing Sf9 membrane vesicles (Fig. 6a,b). Then, we examined the inhibitory effect of sulfated bile acids on the transport of TC in CMVs from EHBR and found that the IC_{50} values are comparable with those determined in Bsep-expressing Sf9

membrane vesicles (Fig. 6a,b). These results indicate that the IC_{50} values of sulfated bile acids for Bsep-mediated transport of TC depend on the presence of Mrp2; the IC_{50} values in membrane vesicles expressing both Bsep and Mrp2 (CMVs from SD rats) are significantly lower than those in membrane vesicles expressing only Bsep (CMVs from EHBR and Bsep-expressing Sf9 cells) (Fig. 6a,b). In order to confirm these findings, we also determined the IC_{50} values in membrane vesicles expressing both Bsep and Mrp2, and found that the values are comparable with those determined in CMVs from SD rats (Fig. 6a,b). These findings are consistent with those reported by Stieger et al. [21] who found that the inhibition of the uptake of TC by $E_2$17βG was much greater in CMVs from Wistar rats and Bsep/Mrp2 co-expressing Sf9 membrane vesicles, compared with that in CMVs from TR^- and Bsep-expressing Sf9 cells. We were also able to confirm that 100 μM $E_2$17βG inhibits the ATP-dependent uptake of [^{3}H]TC only in CMVs from SD rats and Bsep/Mrp2 co-expressing Sf9 membrane vesicles (Fig. 6c). Stieger et al. [21] proposed a possible hypothesis to account for these results, namely, that Mrp2 substrates (such as $E_2$17βG) *trans*-inhibit the function of Bsep [21]. In the present study, in Sf9 membrane vesicles expressing both Bsep and Mrp2, TCDC-S and TLC-S are efficiently taken up into the membrane vesicles (Figs. 1 and 4), and may inhibit the function of Bsep from inside the vesicles (*trans*-inhibition). In contrast, since CMVs from EHBR and membrane vesicles from Bsep-expressing Sf9 cells do not express Mrp2, these sulfated bile acids can inhibit Bsep-mediated uptake only from outside the vesicles (*cis*-inhibition). Alternatively, sulfated bile acids could be concentrated enough to *trans*-inhibit the function of Bsep by low affinity transporter(s) expressed on the canalicular membrane of EHBR and plasma membrane of Sf9 cells at their higher concentrations.

We also found that the preincubation of the sulfated bile acids reduce the uptake of [^{3}H]TC uptake into Mrp2 and Bsep co-expressing membrane vesicles, but not into Bsep expressing membrane vesicles, in a time-dependent manner (Fig. 7). Stieger et al. also showed a time-dependent inhibitory effect of $E_2$17βG on TC uptake by Bsep/Mrp2 co-expressing Sf9 membrane vesicles [21]. The effect of $E_2$17βG was significant after a longer incubation time when a significant amount of $E_2$17βG is present in the intravesicular space [21]. Although we cannot exclude the possibility that the transport characteristics of Bsep are modulated by Mrp2 via a protein–protein interaction, the results of the present study and those by Stieger et al. [21] are consistent with the presence of *trans*-inhibition of Bsep by Mrp2 substrates. Based on the hypothesis that sulfated bile acid inhibit the function of Bsep from the extracellular domain, allosteric interaction rather than the simple competitive inhibition at TC binding site located in the intracellular domain, may be the most plausible explanation to account for the *trans*-inhibition mechanism.

However, the physiological significance for the *trans*-inhibitory effect of sulfated bile acids on the function of Bsep still remains to be clarified. As far as the effect of sulfated bile acids was concerned, Yousef et al. [14,15] indicated that infusion of sulfated bile acids including TCDC-S reduced the biliary excretion of phospholipids and cholesterol, along with the secretion of endogenous bile acids, whereas the same treatment significantly increased the bile salt-independent bile flow rate. In further studies, we plan to compare the in vivo effect of sulfated bile acids on the biliary excretion of TC in both normal SD rats and EHBR. Recently, Huang et al. [24] indicated that, after i.v. administration of the cholestatic dose of $E_2$17βG, cholestasis was induced in normal Wistar rats, but not in Mrp2-deficient TR^- rats. However, the concentration of this steroid in the bile was similar between normal and TR^- rats [24]. These results are consistent with the hypothesis that, at least for $E_2$17βG, the *trans*-inhibition mechanism may not be involved in the pathogenesis of cholestasis.

In conclusion, we have demonstrated that TCDC-S and TLC-S are transported by Mrp2. In addition, TCDC-S and TLC-S inhibit the function of Bsep with lower IC_{50} values in membrane vesicles from SD rats and Sf9 cells expressing both Bsep and Mrp2 compared with those in membrane vesicles from EHBR and Bsep-expressing Sf9. These data can be accounted for by assuming the presence of a *trans*-inhibition mechanism for Bsep function by sulfated bile acids.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research on Priority Area 'ABC proteins'/0044243 from the Ministry of Education, Science, and Culture of Japan.

References

[1] M.H. Nathanson, J.L. Boyer, Hepatology 14 (1991) 551–566.
[2] R.P. Oude Elferink, D.K. Meijer, F. Kuipers, P.L. Jansen, A.K. Groen, G.M. Groothuis, Biochim. Biophys. Acta 1241 (1995) 215–268.
[3] P.J. Meier, Am. J. Physiol. 269 (1995) G801–G812.
[4] M. Müler, P.L. Jansen, Am. J. Physiol. 272 (1997) G1285–G1303.
[5] B. Hagenbuch, H. Lubbert, B. Stieger, P.J. Meier, J. Biol. Chem. 265 (1990) 5357–5360.
[6] B. Hagenbuch, P.J. Meier, J. Clin. Invest. 93 (1994) 1326–1331.
[7] J. König, A.T. Nies, Y. Cui, I. Leier, D. Keppler, Biochim. Biophys. Acta 1461 (1999) 377–394.
[8] H. Suzuki, Y. Sugiyama, Semin. Liver Dis. 18 (1998) 359–376.
[9] T. Gerloff, B. Stieger, B. Hagenbuch, J. Madon, L. Landmann, J. Roth, A.F. Hofmann, P.J. Meier, J. Biol. Chem. 273 (1998) 10046–10050.
[10] S.S. Strautnieks, L.N. Bull, A.S. Knisely, S.A. Kocoshis, N. Dahl, H. Arnell, E. Sokal, K. Dahan, S. Childs, V. Ling, M.S. Tanner, A.F. Kagalwalla, A. Nemeth, J. Pawlowska, A. Baker, G. Mieli-Vergani, N.B. Freimer, R.M. Gardiner, R.J. Thompson, Nat. Genet. 20 (1998) 233–238.
[11] F. Kuipers, M. Enserink, R. Havinga, A.B. van der Steen, M.J. Hardonk, J. Fevery, R.J. Vonk, J. Clin. Invest. 81 (1988) 1593–1599.
[12] F. Kuipers, A. Radominska, P. Zimniak, J.M. Little, R. Havinga, R.J. Vonk, R. Lester, J. Lipid Res. 30 (1989) 1835–1845.
[13] H. Takikawa, N. Sano, T. Narita, Y. Uchida, M. Yamanaka, T. Horie, T. Mikami, O. Tagaya, Hepatology 14 (1991) 352–360.
[14] I.M. Yousef, S.G. Barnwell, B. Tuchweber, A. Weber, C.C. Roy, Hepatology 7 (1987) 535–542.
[15] I.M. Yousef, D. Mignault, B. Tuchweber, Hepatology 15 (1992) 438–445.
[16] J. Zhang, W. Griffiths, J. Jövall, J. Label. Compd. Radiopharm. 39 (1997) 159–164.
[17] N. Murata, T. Beppu, H. Takikawa, H. Otsuka, T. Kasama, Y. Seyama, Steroids 42 (1983) 575–592.
[18] K. Niinuma, O. Takenaka, T. Horie, K. Kobayashi, Y. Kato, H. Suzuki, Y. Sugiyama, J. Pharmacol. Exp. Ther. 282 (1997) 866–872.
[19] K. Ito, H. Suzuki, T. Hirohashi, K. Kume, T. Shimizu, Y. Sugiyama, Am. J. Physiol. 272 (1997) G16–G22.
[20] D.R. Hipfner, S.D. Gauldie, R.G. Deeley, S.P. Cole, Cancer Res. 54 (1994) 5788–5792.
[21] B. Stieger, K. Fattinger, J. Madon, G.A. Kullak-Ublick, P.J. Meier, Gastroenterology 118 (2000) 422–430.
[22] G. Jedlitschky, I. Leier, U. Buchholz, K. Barnouin, G. Kurz, D. Keppler, Cancer Res. 56 (1996) 988–994.
[23] B. Stieger, B. O'Neill, P.J. Meier, Biochem. J. 284 (1992) 67–74.
[24] L. Huang, J.W. Smit, D.K. Meijer, M. Vore, Hepatology 32 (2000) 66–72.