Two Distinct Mechanisms for Bilirubin Glucuronide Transport by Rat Bile Canalicular Membrane Vesicles

Demonstration of Defective ATP-dependent Transport in Rats (TR−) with Inherited Conjugated Hyperbilirubinemia

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Abstract

Bilirubin is conjugated with glucuronic acid in hepatocytes and subsequently secreted in bile. The major conjugate is bilirubin diglucuronide. Using sealed vesicles which are primarily derived from the canalicular (CMV) and sinusoidal (SMV) membrane vesicle domains of the plasma membrane of hepatocytes, we demonstrated that bilirubin glucuronides are transported by CMV by both ATP- and membrane potential–dependent transport systems. In CMV from normal rats, these processes are additive. In CMV from TR− rats, which have an autosomal recessively inherited defect in biliary secretion of nonbile acid organic anions, ATP-dependent transport of bilirubin diglucuronide was absent whereas the membrane potential driven system was retained. Other canalicular ATP-dependent transport systems, which were previously described for organic cations and bile acids, are functionally retained in TR− rats. Our study indicates that bilirubin glucuronides are primarily secreted into the bile canaliculus by an ATP-dependent mechanism which is defective in an animal model of the human Dubin-Johnson syndrome. (*J. Clin. Invest.* 1992. 90:2130–2135.) Key words: ATP-dependent transport • bilirubin glucuronides • canalicular transport • inheritable jaundice (Dubin-Johnson syndrome)

Introduction

Bilirubin is primarily secreted in bile as mono- and diglucuronide conjugates (BMG and BDG)[1] (1). Inheritable and acquired defects in bilirubin secretion result in predominantly conjugated hyperbilirubinemia (1). An autosomal recessively inherited defect in hepatocellular secretion of bilirubin glucuronides and other nonbile acid organic anions characterizes the Dubin-Johnson syndrome in humans (2, 3) and a phenotypically similar defect in mutant Corriedale sheep (4), Golden Lion Tamarin monkeys (Schulman et al., manuscript submitted for publication), and TR- rats (5–7).

Unconjugated bilirubin in serum is bound to albumin and subsequently transferred into the hepatocyte where it becomes conjugated with glucuronic acid in a reaction catalyzed by UDP glucuronyl transferase (bilirubin) (8). BMG and BDG are subsequently secreted into the bile canaliculus. BDG is the predominant bilirubin conjugated which is secreted. Bilirubin glucuronide secretion has been proposed to be the rate-limiting step in the overall transfer of bilirubin from blood to bile (9).

Mutant Wistar (TR−) rats manifest predominantly conjugated hyperbilirubinemia and reduced biliary secretion of BMG, BDG, leukotriene C4 and other non–bile acid organic anions and are a model of the Dubin-Johnson syndrome in humans (5–7, 10–12). Studies with purified rat liver canalicular membrane vesicles (CMV) reveal that TR− rats lack ATP-dependent transport of BSP and BSPGSH but not taurocholate, other bile acids, or various organic cations (11, 12). BDG competitively inhibited ATP-dependent transport of BSP in CMV from normal rats (11). These results in normal and TR− rats suggest that bilirubin glucuronides may be transported into the bile canaliculus by an ATP-dependent process. However, a recent study that used rat liver canalicular membrane vesicles, suggested that BDG transport is solely driven by membrane potential but not by ATP (13). Thus, the mechanism of transport of bilirubin glucuronides across the bile canaliculus has not been resolved.

We have examined the role of ATP and membrane potential on BMG and BDG transport in CMV from normal and TR− rats. The results indicate that ATP-dependent transport is a major physiologic mechanism in bilirubin glucuronide transport across the bile canaliculus and is functionally absent in TR− rats. Membrane potential–driven transport of BDG was unaffected in CMV from TR− rats. Kinetic and genetic evidence suggests that the putative carrier protein for the ATP-dependent system may differ from that for the system which is driven by the membrane potential although definitive evidence awaits purification of the transporters.

Methods

Materials

Delta-[2,3-^{3}H]aminolevulinic acid (30 Ci/mol) was purchased from Schwarz/Mann, Div. of Becton Dickinson & Co., Orangeburg, NY; [^{3}H(G)]taurocholate (2.1 Ci/mmol), [^{3}H(G)]daunomycin (1.6 Ci/

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Received for publication 19 May 1992 and in revised form 21 August 1992.

1. *Abbreviations used in this paper:* BMG and BDG, bile mono- and diglucuronide conjugates; BSP, sulfobromphthalein; BSPGSH, sulfobromphthalein glutathione; CMV and SMV, canalicular and sinusoidal membrane vesicle domains; GSSG, oxidized glutathione.

J. Clin. Invest.

0021-9738/92/11/2130/06 $2.00
Volume 90, November 1992, 2130–2135

mmol), and [glycine-2-^{3}H] oxidized glutathione (GSSG) (0.24 mCi/mmol) were purchased from DuPont-New England Nuclear, Boston, MA. Adenylyl imidodiphosphate (AMPPNP), b,γ-methylene adenosine 5′ triphosphate (AMPPCP), doxorubicin, and nucleotides were obtained from Sigma Chemical Co., St. Louis, MO.

Animals

Male Wistar rats were obtained from Charles River Breeding Laboratories, Wilmington, MA, and TR− rats were kindly provided by Dr. Peter L. M. Jansen (Academic Medical Center, Amsterdam, The Netherlands).

Methods

Bilirubin glucuronides were prepared as follows. A male Wistar rat (250 g) was anesthetized with diethylether and the bile duct was cannulated with a PE 10 catheter. The rat was placed in a restraining cage and, after regaining consciousness and return of body temperature to normal, [^{3}H]δ-aminolevulinic acid, 250 μCi was injected into the jugular vein. Bile was collected for 6 h on ice in a darkened room and frozen in 1-h aliquots. A Waters Sep-pak cartridge (Millipore Corp., Milford, MA) containing C18-coated silica gel was equilibrated with 0.1 M ammonium acetate/acetic acid, pH 4.0. The bile was mixed with an equal volume of buffer and 1 ml of the mixture was applied per cartridge. The cartridge was washed with 2 ml of buffer and pigments were eluted with methanol which was evaporated in reduced pressure and the pigments were redissolved in methanol/buffer (3:7). HPLC was performed in a Waters high-pressure liquid chromatograph as described by Spivak and Carey (14). BMG and BDG fractions were collected and evaporated to dryness under reduced pressure, and the pigments were redissolved in methanol. Pigment purity was determined by analytical HPLC (14) and ultraviolet-visible spectral analysis. The pigment concentration was determined by ethylanthranilate diazo analysis. The specific radioactivity in the aliquots was 96–159 μCi/μmol.

Preparation of membrane vesicles. CMV and sinusoidal membrane vesicles (SMV) were separately prepared from male normal and TR− rats which weighed 230–260 g, CMV preparation was based upon nitrogen cavitation and calcium precipitation as previously described (15, 16). As compared with homogenates, these preparations were enriched 64- and 53-fold in the activities of γ-glutamyl transpeptidase and leucine aminopeptidase, respectively, which served as enzymatic canalicular markers. Enrichment of the activity of ouabain-inhibitable Na^+ K^+ ATPase, a predominantly basolateral membrane enzyme marker, was negligible. Only preparations of CMV, which manifested ATP-dependent transport of daunomycin (17), were used for the pres-

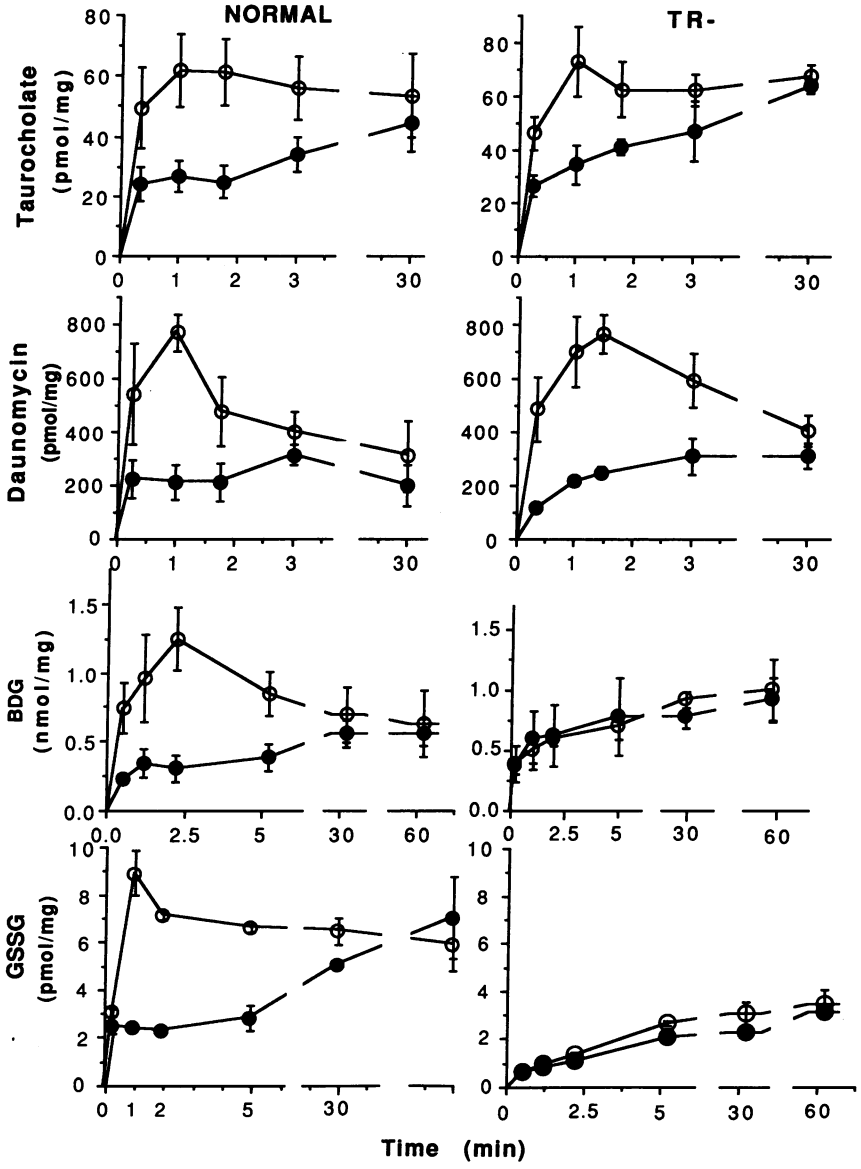

Figure 1. ATP-dependent transport of organic compounds by CMV from normal and TR− rats. CMV (0.5 mg protein/ml) from normal (*left*) and TR− (*right*) rats were incubated with 10 μM [^{3}H]taurocholate, 10 μM [^{3}H]-daunomycin, 20 μM [^{3}H]BDG, or 1 μM [^{3}H]-GSSG in the presence (○) and absence (●) of 3 mM ATP and an ATP-generating system. At the indicated times, vesicle-associated radioactivity was measured in duplicate. Values are expressed as mean±SD (n = 3).

ent studies. SMV were isolated from homogenates using differential and sucrose-Ficoll density gradient centrifugation (18); ouabain-inhibitable Na^+K^+ATPase activity was enriched 19-fold in these preparations and leucine aminopeptidase activity was negligible. CMV and SMV were stored in buffer A (10 mM Hepes-Tris buffer, pH 7.4, 0.25 M sucrose, and 0.2 mM $CaCl_2$) at −70°C until used.

Marker enzyme activities were assayed as previously described (15–19). Protein concentration was determined by the method of Lowry et al. using bovine serum albumin as a standard (20).

Transport studies. BDG transport by CMV and SMV was quantitated using a rapid filtration method (15–18). ATP-dependent transport was measured in an incubation medium containing 40–80 μg of protein of SMV or CMV, 20 μM BDG or BMG, 3 mM ATP, and an ATP-generating system (6 mM creatine phosphate and 100 μg/ml creatine kinase) in buffer B (10 mM Hepes-Tris buffer, pH 7.4, 0.25 M sucrose and 10 mM $MgCl_2$). A membrane potential was generated in CMV using valinomycin-induced K^+ diffusion potential which consisted of 100 mM KCl and 10 μM valinomycin. Transport was initiated by adding 20 μl CMV (40–80 μg protein) suspended in buffer A to 0.1 ml of the incubation medium which had been maintained for 10 min at 37°C. In similar preparations and buffers, 10 μM [^{3}H]daunomycin or 10 μM [^{3}H]taurocholate were separately incubated with or without ATP as previously described (17, 20). The effect of various nucleotides and putative inhibitors on BDG transport by CMV was studied using concentrations as described in the legends of the tables and figures in this article. The effect of GSDNP on membrane potential–mediated transport of BDG by CMV was studied in further detail. Aliquots of 20 μl were removed at indicated intervals and diluted to 1 ml with ice-cold buffer B. Vesicles were filtered through glass microfiber filters (Whatman, Inc., Clifton, NJ; 0.45 μm pore size), which were washed twice with 10 ml of ice-cold buffer B. Radioactivity on the filters was measured in a liquid scintillation spectrometer (model LS 1801, Beckman Instruments, Inc., Palo Alto, CA). Results were expressed as mean±SD after correction for radioactivity on filters in the absence of membrane vesicles.

Results

In CMV from control rats, daunomycin, taurocholate, BDG, and GSSG were transported by an ATP-dependent mechanism (Fig. 1). In contrast, CMV from TR− rats transported daunomycin and taurocholate but not BDG or GSSG, by an ATP-dependent process (Fig. 1). These ATP-dependent transport processes showed maximum uptake within a few minutes, equilibrated within 30–60 min, and saturated at an apparent K_m for ATP of 0.37±0.08 mM (n = 3) (Fig. 2). Neither BMG nor BDG transport by SMV were stimulated by addition of 1 mM ATP (data not shown).

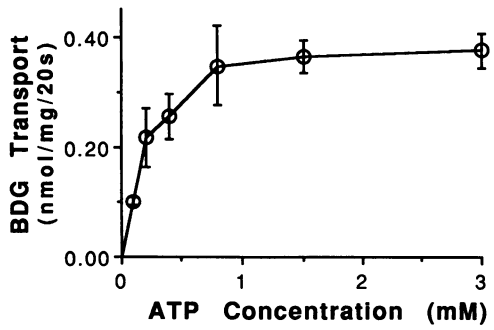

Figure 2. Effect of ATP concentration on ATP-dependent BDG transport by CMV. The incubation medium contained 20 μM BDG and various concentrations of ATP plus an ATP-generating system. Transport was started by adding vesicles into the incubation medium. After 20 s of incubation at 37°C, vesicle-associated radioactivity was measured in duplicate. ATP-dependent BDG transport was calculated by subtracting values obtained in the absence of ATP. Values are expressed as mean±SD (n = 3).

Table I. Effect of Nucleotides and ATP Analogues on BDG Transport by CMV

Nucleotides	Transport	Percent
	nmol/mg per 20 s	
ATP	4.92±0.85	100
GTP	0.15±0.32	3±6
CTP	0.13±0.23	3±6
TTP	0.10±0.35	2±7
UTP	0.04±0.46	1±9
ITP	0.18±0.15	4±3
ADP	0.61±0.05	13±3
AMP	0.05±0.10	1±2
AMPPCP	0.22±0.45	6±1
AMPPNP	0.01±0.12	1±2

Transport was measured in incubation medium containing 20 μM BDG and various agents (3 mM) without an ATP-regenerating system. After 20 s of incubation, vesicle-associated radioactivity was measured as described. Nucleotide-dependent BDG transport was determined in duplicate preparations from the difference in the presence and absence of nucleotide. Results are expressed as mean±SD (n = 3).

Of the various substrates which were studied, only ATP enhanced the transport of BDG by CMV. Neither nonhydrolyzable ATP analogues nor other nucleotides stimulated BDG transport by CMV from normal rats (Table I). As shown in Table II, ATP-dependent BDG transport was inhibited by vanadate (50 μM) but not by oligomycin, *N*-ethylmaleimide, or chlorophenylhydrazone.

As shown in Fig. 3, BDG transport by CMV from normal rats increased after addition of ATP or increasing membrane potential. The effects were additive in CMV from normal rats. In contrast, CMV from TR− rats lacked ATP-dependent BDG transport but retained membrane potential-dependent transport which was not enhanced by addition of ATP. Similar results were observed using BMG (not shown).

Both ATP-dependent and membrane potential-dependent BDG transport were temperature-sensitive (Fig. 4) and de-

Table II. Effect of Inhibitors on ATP-dependent BDG Transport by CMV

Inhibitor	Transport	Percent	*P*
	nmol/mg per 20 s		
Control	6.11±0.70	100	
Oligomycin (10 μg/ml)	4.87±0.34	80±7	NS
Carbonyl cyanide *m*-chlorophenylhydrazone (10 μg/ml)	5.96±0.23	99±10	NS
N-ethylmaleimide (20 μM)	5.26±0.43	88±5	NS
Vanadate (50 μM)	3.17±0.20	52±4	<0.01

Results are expressed as mean±SD (n = 3). Significance was determined by Student's t test.

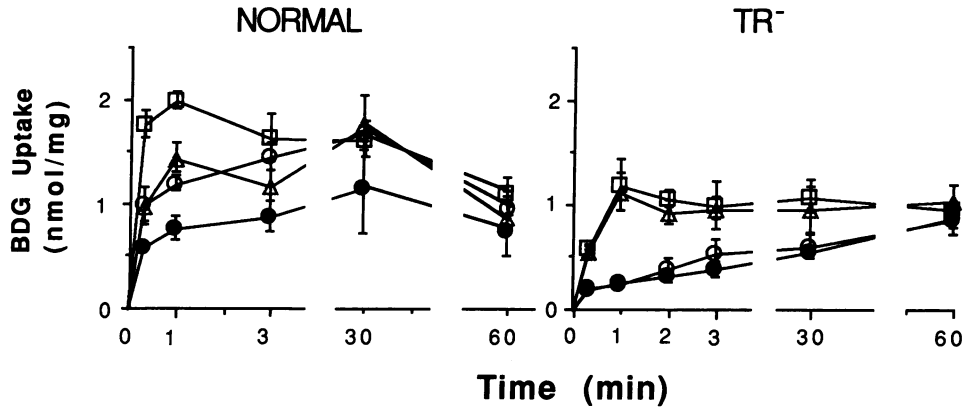

Figure 3. ATP- and membrane potential–dependent BDG transport in CMV from normal and TR− rats. CMV from normal (*left*) and TR− (*right*) rats were incubated with 20 μM BDG and 100 mM KCl (n = 3). Additions to the incubation medium were as follows: (●) no addition; (○) 3 mM ATP plus an ATP-generating system; (△) 10 μM valinomycin; (□) 3 mM ATP plus ATP-generating system and 10 μM valinomycin. Values are expressed as mean±SD (n = 3).

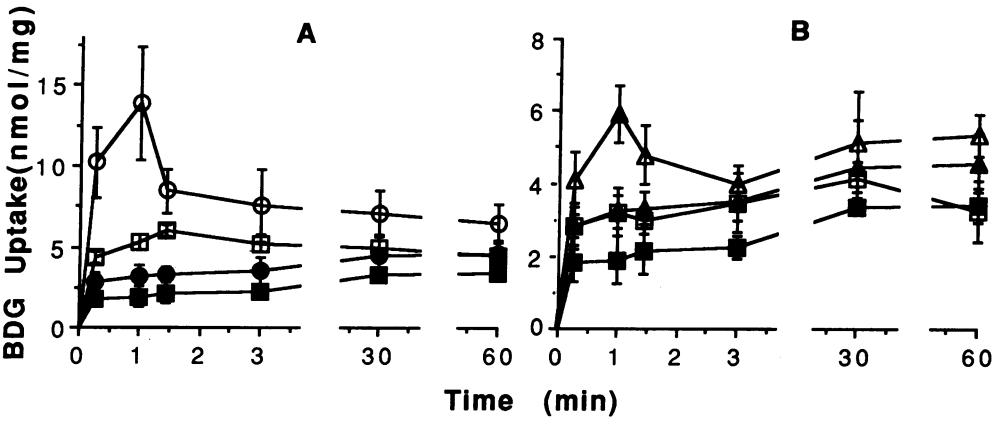

Figure 4. Temperature dependence of BDG transport by CMV from normal rats. BDG transport by CMV was measured in duplicate in incubation medium containing 20 μM BDG and 100 mM KCl at 5°C (*squares*) and at 37°C (*circles* and *triangles*). Additions to incubation medium were as follows: (*A*) (■, ●) no addition; (□, ○) 3 mM ATP plus an ATP-regenerating system. (*B*) (■, ▲) no addition; (□, △) 10 μM valinomycin. Values are expressed as mean±SD (n = 3).

creased when CMV were incubated in increasing concentrations of raffinose which increase extravesicular osmolarity and collapse the vesicles (Fig. 5). These results confirm that BDG transport was occurring rather than only binding of BDG to CMV. Similar results were observed using BMG (not shown).

In CMV from normal rats, ATP-dependent and membrane potential–dependent transport of BDG exhibited saturation kinetics with respect to BDG (Fig. 6). Apparent K_m values for BDG of ATP-dependent and membrane potential–dependent transport were 71±18 and 26±10 μM, respectively. The corresponding V_{max} values were 17±3 and 1.8±0.3 nmol/mg per 20 s, respectively. The transport activity of the ATP-dependent process was 10 times greater than that of the membrane potential stimulated system. The effect of ATP and increased membrane potential on BDG transport was additive at different concentrations (V_{max}, 19±2 nmol/mg per 20 s). The apparent K_m in the presence of ATP and increased membrane potential was 65 + 15 μM. As shown in Table III, DIDS and GSSG inhibited both transport mechanisms and probenecid and re-

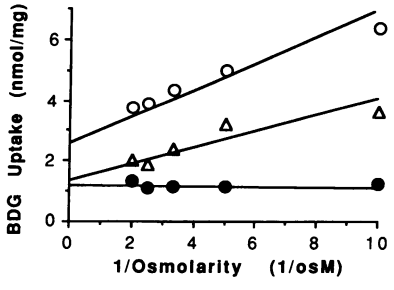

Figure 5. Effect of osmolarity of the medium on BDG transport by CMV. CMV were incubated in 0.1–0.5 M raffinose in buffer B for 30 min at 25°C. BDG transport was measured in duplicate in medium adjusted to the osmolarity of the vesicle-containing buffer. Additions to incubation medium were as follows: (●) 100 mM KCl; (△) 100 mM KCl and 10 μM valinomycin; (○) 3 mM ATP plus an ATP-regenerating system. Values are expressed as mean±SD (n = 3).

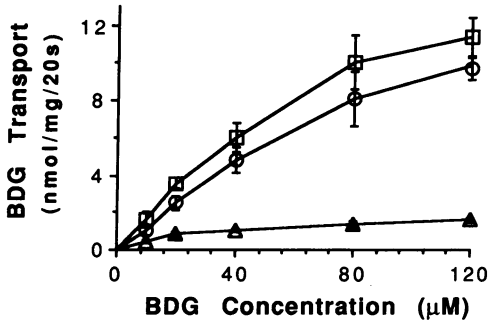

Figure 6. Additive effect of ATP and membrane potential on BDG transport by CMV. CMV (40–60 μg of protein) was incubated at 37°C with various concentrations of BDG in medium (0.12 ml final volume) which contained 100 mM KCl. After 20 s, vesicle-associated radioactivity was measured in duplicate preparations. Membrane potential-dependent (△) and ATP- dependent (○) BDG transport were calculated from the difference in transport in the presence and absence of 10 μM valinomycin and 3 mM ATP plus an ATP-regeneration system, respectively. BDG transport in the presence of increased membrane potential and ATP (□) was calculated by subtracting values obtained in the absence of ATP and valinomycin. Results are expressed as mean±SD (n = 3).

Table III. Effect of Inhibitors on ATP-dependent and Membrane Potential-dependent BDG Transport by CMV

	ATP-dependent	P	Potential-dependent	P
	nmol/mg per 20 s		*nmol/mg per 20 s*	
Control	1.08±0.12		0.98±0.20	
Probenecid (1 mM)	2.16±0.45	<0.01	0.61±0.24	<0.05
DIDS (0.1 mM)	0.32±0.10	<0.01	0.40±0.13	<0.01
GSH (5 mM)	0.84±0.02	<0.03	0.53±0.08	<0.01
GSSG (0.1 mM)	0.55±0.03	<0.01	0.45±0.15	<0.01
Control	2.09±0.16		0.28±0.10	<0.03
BSP (50 μM)	1.36±0.14	<0.01	0.18±0.03	<0.03
GSDNP (50 μM)	1.30±0.35	<0.01	0.14±0.10	<0.01
TCA (50 μM)	1.82±0.41	NS	0.37±0.14	NS
TCA (500 μM)	1.35±0.36	<0.03	0.05±0.08	<0.05
GCA (50 μM)	2.38±0.23	NS	0.18±0.06	NS
Doxorubicin (50 μM)	1.86±0.36	NS	0.13±0.03	<0.05

Results are expressed as mean±SD (n = 3). NS, not statistically significant ($P > 0.05$).

duced glutathione selectively inhibited the membrane potential-stimulated transport system. Both transport systems were inhibited by BSP, GSDNP, and high concentrations of taurocholate (500 μM) but not by physiologic concentrations of taurocholate or glycocholate (50 μM). Doxorubicin (50 μM) did not affect ATP-dependent transport but decreased membrane potential-dependent transport. GSDNP appeared to inhibit competitively membrane potential-mediated BDG transport by CMV from normal rats (Fig. 7).

Discussion

In previous studies, we demonstrated that CMV prepared by the method used in the present study are 75–80% right side out (16) and that ATP and membrane potential-dependent transport of bile acids, daunomycin and nonbile acid organic anions occurs only in inside-out vesicles (16, 17, 20, 21, Nichida et al., manuscript submitted for publication). Therefore, it is reasonable to assume that the ATP-dependent and membrane potential-dependent transport of BDG and BMG observed in the present study reflect transport by inside-out CMV, and that transport in hepatocytes occurs from the intracellular domain across the plasma membrane into the bile canaliculus.

Adachi et al. (13) did not find ATP-dependent transport of BDG or BMG in CMV which were prepared by essentially the same method used in the present studies. The most likely explanation for the different results is the relative proportion of inside-out vesicles in each study. We have observed that the proportion of inside-out vesicles can range from 0% to 20% depending on seemingly minor methodologic details including Dounce homogenization and nitrogen cavitation. Each CMV preparation used in the present study was initially demonstrated to have ATP-dependent transport of daunomycin (17); preparations lacking this transport activity were discarded.

ATP-dependent transport of BDG and BMG was temperature and osmotically dependent; saturable; inhibited by vanadate, DIDS, GSSG, BSP, and GSDNP but not by daunomycin or physiologic concentrations of taurocholate or glycocholate, followed Michaelis-Menten kinetics; and specifically required hydrolyzable ATP (Figs. 1–6, Tables I–III). In addition, CMV from TR− rats lacked ATP-dependent transport of BDG and other nonbile acid organic anions (Fig. 1).

BDG transport by CMV from normal and TR− rats increased when the membrane potential was enhanced by incubation in a potassium gradient and the potassium ionophore, valinomycin. In CMV from normal rats, ATP and increased membrane potential showed additive effects. In CMV from TR− rats, the effect of increasing membrane potential increased BDG transport to the approximately same level as that observed in CMV from normal rats under similar experimental circumstances. These results in normal and TR− rats demonstrate that ATP-dependent transport is a major mechanism for BDG transport in CMV and suggest that, at the functional level, there are likely to be separate mechanisms for ATP-dependent and membrane potential-dependent transport of BDG across the bile canaliculus. GSDNP appears to inhibit BDG competitively when the driving force for secretion is the membrane potential (Fig. 7). More definitive testing of this hypothesis awaits purification and characterization, which is in progress in our laboratory, of the putative transport protein(s).

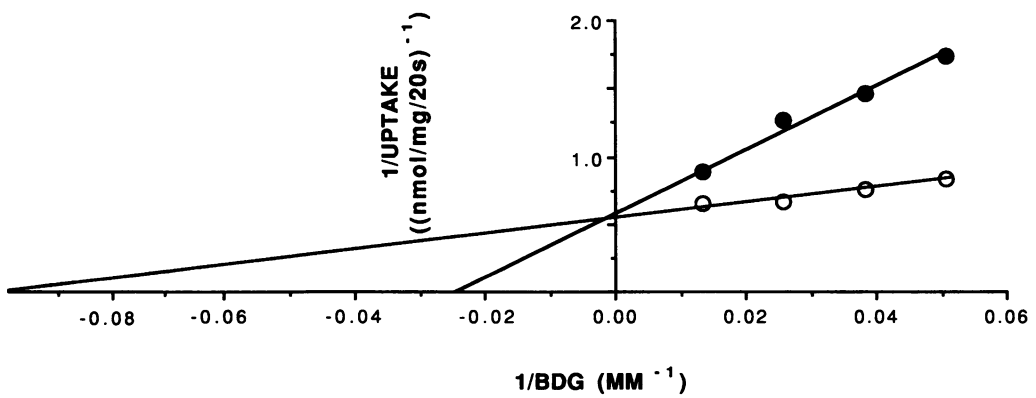

Figure 7. Inhibition of potential-mediated transport of BDG by GSDNP in CMV from normal rats. Varied concentrations of [^{3}H]BDG were added to medium which included 10 μM valinomycin and 100 mM KCl. BDG transport was measured at 20 s in the absence (○) and presence (●) of GSDNP (50 μM). The double reciprocal plot of transport at 20 s and BDG concentration was linear. GSDNP appears to inhibit BDG transport competitively. Results are expressed as the mean of two experiments which were performed in duplicate.

The transport defect in the Dubin-Johnson syndrome in humans and the comparable disorder in TR− rats, mutant Corriedale sheep, and mutant Golden Lion Tamarin monkeys is phenotypically manifested by mild conjugate hyperbilirubinemia and impaired biliary secretion of several nonbile acid organic anions but not bile acids or organic cations (1–8). The functional defect in TR− rats involves virtual absence of ATP-dependent transport of nonbile acid organic anions, including BDG (11, 12, this report). We speculate that the comparatively mild degree of conjugated hyperbilirubinemia and the presence of bilirubin glucuronides in the bile of the human, sheep, rat and monkey mutants probably results from the transport of bilirubin glucuronides by a membrane-potential driven canalicular system.

Acknowledgments

This study was supported in part by grants (DK-35652, DK-34926 to IMA, DK-39137, DK-41296 to JR) from the National Institutes of Health. Dr. Nishida was recipient of a Postdoctoral Fellowship award from the American Liver Foundation.

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