Evidence for Carrier-mediated Chloride/Bicarbonate Exchange in Canalicular Rat Liver Plasma Membrane Vesicles

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Abstract

To determine whether anion exchangers might play a role in hepatic bile formation, we looked for the presence of Cl^-:OH^- and Cl^-:HCO_3^- exchange in highly purified canalicular (c) and basolateral (bl) rat liver plasma membrane (LPM) vesicles. In cLPM vesicles, a pH gradient (7.7 in/6.0 out) stimulated $^{36}Cl^-$ uptake twofold above values obtained during pH-equilibrated conditions (7.7 in = out). When 50 mM HCO_3^- was also present inside the vesicles, the same pH gradient (7.7 in/6.0 out) resulted in Cl^- uptake to levels fourfold above pH- and HCO_3^--equilibrated controls and two- to threefold above Cl^- equilibrium (overshoot). Initial rates of both pH and HCO_3^- gradient-stimulated Cl^- uptake were completely inhibited by 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS). A valinomycin-induced K^+ diffusion potential (inside positive) also stimulated Cl^- uptake in cLPM, but this conductive Cl^- pathway was insensitive to DIDS. The DIDS-sensitive, pH and HCO_3^- gradient-stimulated Cl^- uptake demonstrated: (*a*) saturation with Cl^- ($K_m \sim 6.3$ mM; $V_{max} \sim 51$ nmol · mg^{-1} · min^{-1}); (*b*) partial inhibition by bumetanide (26%), furosemide (33%), probenecid (37%), and 4-acetamido-4'-isothiocyano-2,2'-disulfonic acid stilbene (49%); (*c*) *cis*-inhibition by chloride and nitrate but not by sulfate and various organic anions, and (*d*) independence from the membrane potential. These data demonstrate the presence of an electroneutral Cl^-:OH^- and Cl^-:HCO_3^- exchanger in rat liver canalicular membranes that favors Cl^-:HCO_3^- exchange. In contrast, no evidence was found for the presence of a Cl^-:HCO_3^- (OH^-) exchange system in blLPM vesicles. Furthermore, neither blLPM nor cLPM vesicles exhibited Na^+-stimulatable Cl^- uptake, indicating the absence of a NaCl co-transport system in either LPM subfraction. These findings are consistent with a functional role for a Cl^-:HCO_3^- (OH^-) exchanger in canalicular bile formation.

Introduction

Bile formation is a major function of the liver. According to current views canalicular bile flow is mainly an osmotic process in which water and electrolytes diffuse either from the cell or paracellularly from plasma into bile canaliculi along osmotic gradients resulting from the active hepatocellular secretion of inorganic and organic solutes into the canalicular lumen (for reviews, see references 1–6). Conceptually, canalicular bile formation is generally divided into bile acid-dependent bile flow (BADF)[1] and bile acid-independent bile flow (BAIF) components. Although these two fractions of canalicular bile flow may not be totally independent from each other, bile acids represent the most potent endogenous stimulants of bile secretion. Recent studies with plasma membrane vesicles selectively isolated from the basolateral (i.e., sinusoidal and lateral) and canalicular pole of rat hepatocytes have indicated that transhepatocyte excretion of taurocholate, the most abundant conjugated bile acid in the rat, is a secondary active transport process driven by the electrochemical potential difference for Na^+ across the basolateral membranes (Na^+-taurocholate co-transport) and by the electrical potential difference across the bile canalicular membranes (facilitated diffusion; 7, 8). In contrast, the mechanisms responsible for BAIF are more poorly understood. Indirect studies in the isolated perfused rat liver have suggested that active canalicular secretion of anions such as chloride (5), bicarbonate (9–11), and/or acidic oligopeptides and amino acid conjugates (5, 12) could account for the formation of BAIF.

In the present study we tested the hypothesis that anion exchange mechanisms (13–15) may play a primary role in transhepatocytic excretion of anions. Specifically we investigated mechanisms of chloride (Cl^-) and bicarbonate (HCO_3^-) transport in highly purified canalicular (c) and basolateral (bl) rat liver plasma membrane (LPM) vesicles. The data provide direct evidence for the presence of an electroneutral Cl^-:HCO_3^- exchange in cLPM but not in blLPM vesicles. In addition, a separate Cl^- conductive channel could also be identified in cLPM vesicles. These findings permit a unifying hypothesis to be proposed for the genesis of BAIF in rat liver and provide definitive support for models based on indirect observations in the isolated perfused rat liver (5, 9–11), and in isolated hepatocytes (16). Furthermore, the basic mechanisms involved in hepatic bile formation appear similar to anion transport processes initially observed in intestinal and renal epithelial cells (17–21). Part of this work has been presented in preliminary form (22).

Methods

Materials. Chlorine-36 ($^{36}Cl^-$) was obtained as a 0.2–3.0 M HCl solution from New England Nuclear (Boston, MA). This radioactive

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1. *Abbreviations used in this paper:* BADF, bile acid-dependent bile flow; BAIF, bile acid-independent bile flow; blLPM (vesicles), basolateral liver plasma membrane (vesicles); cLPM (vesicles), canalicular liver plasma membrane (vesicles); DIDS, 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone; SIM, standard incubation medium; SITS, 4-acetamido-4'-isothiocyano-2,2'-disulfonic acid stilbene; SMS, standard membrane suspension; TMA^+, tetramethylammonium.

HCl solution was neutralized with tetramethylammonium hydroxide (TMA) to a pH of 7.5 before use. 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS) and 4-acetamido-4'-isothiocyano-2,2'-disulfonic acid stilbene (SITS) were purchased from Pierce Chemical Co. (Rockford, IL). Carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone (FCCP) was from Aldrich Chemical Co., Milwaukee, WI. Furosemide and amiloride were gifts from Hoechst-Roussel Pharmaceuticals (Summerville, NJ) and the Merck Sharp & Dohme Research Laboratories (Rahway, NJ), respectively. All other chemicals and reagents were obtained either from Sigma Chemical Co. (St. Louis, MO), Baker Chemical Co. (Phillipsburg, NJ), or Schwarz/Mann Inc. (Spring Valley, NY).

Animals. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) weighing 200–250 g were used throughout this study. The animals had free access to water, were fed Purina Rodent Chow (Ralston Purina Co., St. Louis, MO) ad lib. and were housed in a constant temperature-humidity environment with alternating 12-h light (7 a.m.–7 p.m.) and dark cycles. Fed animals were regularly killed by decapitation between 7:30 and 8:30 a.m.

Isolation of cLPM and blLPM vesicles. The methods for isolation of the cLPM and blLPM subfractions as well as their morphologic and biochemical characterization are described in detail elsewhere (23). In brief, a canalicular enriched "mixed LPM" subfraction was first separated out of a "crude nuclear pellet" by rate zonal floatation (44%: 36.5%, wt/wt, sucrose density interface) in the TZ-28 (DuPont Instruments, Sorvall Biomedical Div., Newtown, CT) zonal rotor. After tight homogenization (type B Dounce homogenizer, 50 up and down strokes) the vesiculated cLPM and blLPM were separated from the "mixed LPM" by high-speed centrifugation (195,200 g_{ave} for 3 h) through a three-step sucrose gradient (31%, 34%, and 38%, wt/wt). cLPM were recovered from the top of the 31% sucrose layer and blLPM were harvested from the 34%:38% sucrose density interface. Both LPM subfractions were collected at 105,000 g_{ave} for 60 min and resuspended in the appropriate membrane suspension buffers (see below).

Characteristics of the isolated LPM subfractions. The degree of purification of cLPM and blLPM vesicles was extensively analyzed by intracellular and plasma membrane marker enzyme activities. These studies indicated minor contamination of both LPM subfractions with intracellular organelles and virtually complete separation of blLPM from cLPM vesicles as reflected by the absence of Na^+K^+-ATPase- and glucagon-stimulatable adenylate cyclase activities or intact secretory component in cLPM (23). In contrast, the blLPM subfraction is contaminated with cLPM by ~10%. Transmission electron microscopy revealed that both cLPM and blLPM are composed of membrane vesicles, although blLPM vesicles still contain unbroken lateral membrane sheets (23). Correspondingly, intravesicular volumes are approximately twofold higher in cLPM (~2 $\mu l \cdot mg^{-1}$ protein) than in blLPM (~1 $\mu l \cdot mg^{-1}$ protein) as calculated from equilibrium uptakes of [^{14}C]glucose that did not bind to any extent to the isolated membrane vesicles (8). Finally, freeze fracture analysis revealed that ~80% of the cLPM vesicles exhibit "right side out" configuration, in which the extravesicular membrane face corresponds to the bile luminal surface in vivo (8).

Chloride ($^{36}Cl^-$) uptake studies. The experimental design used in this study to determine the effect of pH and HCO_3^- on Cl^- transport closely paralleled studies previously performed in rabbit ileal and *Necturus* renal brush border vesicles (17, 24). In order to preload the vesicles with HCO_3^- (50 mM), freshly isolated membrane vesicles were resuspended by tight homogenization (10 up and down strokes, type B glass-glass Dounce homogenizer) in 150 mM sucrose, 100 mM TMA gluconate, 50 mM choline bicarbonate, 35 mM Tris, 35 mM Hepes, 0.2 mM Ca-gluconate, and 5 mM Mg-gluconate, pH 7.7. This buffer system will be referred to as "bicarbonate standard membrane suspension" (HCO_3^--SMS, pH 7.7) medium throughout this study. In experiments, where the vesicles were preloaded differently, the composition of the membrane resuspension buffers will be detailed in the corresponding figure legends. Routinely, the freshly isolated membrane vesicles were frozen and stored in liquid nitrogen (−70°C; protein concentration > 5 mg/ml) for up to 4 d without loss of transport function for $^{36}Cl^-$.

For measuring uptake of $^{36}Cl^-$, the frozen vesicle suspensions were quickly thawed in a 37°C water bath, diluted to the desired protein concentration and revesiculated by repeated (10 times) passage through a 25-gauge needle. 10 μl (45–70 μg of protein) of this membrane vesicle suspension was then incubated at 25°C in 90 μl of incubation medium. In the presence of an outwardly directed simultaneous pH and HCO_3^- gradient, the incubation medium consisted of a HCO_3^--free buffer, pH 6.0, containing 184 mM sucrose, 100 mM TMA gluconate, 30 mM Tris, 14 mM Hepes, 90 mM morpholino-ethane-sulfonic acid, 0.2 mM Ca-gluconate and 5 mM Mg-gluconate. This solution will be called "standard incubation medium," pH 6.0 (SIM, pH 6.0) throughout this study. Chloride in the desired concentrations was added to all incubation media as a neutral TMA $^{36}Cl^-$ solution. Routinely, the reaction mixtures were gassed with 95% N_2, 5% CO_2 to maintain the pH during the whole incubation period. If no HCO_3^- was present in the reaction mixture gassing was done with 100% N_2. After incubation of the vesicles for the indicated time intervals, uptake of $^{36}Cl^-$ was terminated by the addition of 3.5 ml of ice-cold stop solution consisting of 204 mM sucrose, 150 mM K-gluconate, 0.2 mM Ca-gluconate, 5 mM Mg-gluconate, 10 mM Hepes/Tris, pH 7.5. Membrane vesicle-associated ligand was separated from free ligand by immediate rapid filtration (1 ml/s) through a 0.45-μm Millipore filter (Millipore/Continental Water Systems, Bedford, MA), which had been presoaked in cold deionized water. The filter was washed twice with 3.5 ml of stop solution, dissolved in Redisolv HP (Beckman Instruments, Inc., Palo Alto, CA) and counted in a Beckman LS 2000 liquid scintillation counter. Nonspecific binding to the filter and the membrane vesicles was determined in each experiment by addition of cold incubation solution and cold stop solution to 10 μl of membrane suspension kept at 0–4°C. This membrane/filter blank was subtracted from all determinations. Unless otherwise indicated all experimental data were obtained from triplicate analysis of two or more separate membrane preparations. Statistical significance was determined by analysis of variance with two-way layout.

Results

Direct evidence for Cl^-:HCO_3^- and Cl^-:OH^- exchange in cLPM vesicles. The effects of the gradients of in to out pH and HCO_3^- on uptake of Cl^- (5 mM) into cLPM vesicles are demonstrated in Fig. 1. With the vesicles preloaded with pH 7.7 buffers, the uptake of Cl^- was stimulated twofold when incubated at pH 6.0 (7.7 in/6.0 out) as compared with pH-equilibrated conditions (7.7 in/7.7 out). If 50 mM HCO_3^- was additionally present within the vesicles, the same in to out pH gradients (7.7, 50 mM HCO_3^- in/6.0, 0.9 mM HCO_3^- out) further stimulated Cl^- uptake up to three- to fourfold above values obtained under pH- and HCO_3^--equilibrated conditions (7.7, 50 mM HCO_3^- in = out). Furthermore, Cl^- was transiently accumulated within the vesicles to ~2.5 times above Cl^- equilibrium values at 180 min (overshoot phenomenon). No significant differences in Cl^- uptake were observed between the two pH-equilibrated conditions 7.7/7.7 or 6.0/6.0 (data not shown). Thus, it is the pH gradient rather than the pH per se that markedly accelerated the uptake of Cl^-. The additional stimulatory effect of an in to out HCO_3^- gradient indicates that HCO_3^- is favored over OH^- for exchange with extravesicular Cl^-. This conclusion is not invalidated by the observation that HCO_3^- did not stimulate Cl^- uptake under pH-equilibrated conditions (○ in Fig. 1) inasmuch as the high extravesicular concentration of HCO_3^- (50 mM) might have effectively competed with chloride for uptake.

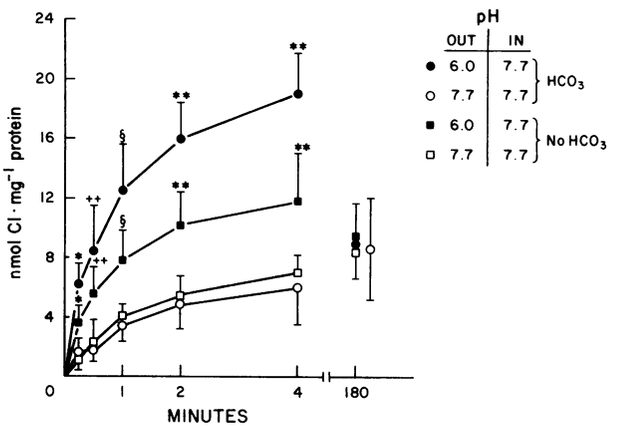

Figure 1. Effects of outwardly directed pH and HCO_3^- gradients on chloride uptake into cLPM vesicles. The vesicles were preloaded with a pH 7.7 buffer either in the presence of 50 mM HCO_3^- (resuspension in HCO_3^--SMS) or in the absence of HCO_3^- (resuspension in 150 mM sucrose, 115 mM TMA gluconate, 70 mM Tris, 70 mM Hepes, 0.2 mM Ca-gluconate, and 5 Mg-gluconate) as described in Methods. Chloride (5 mM) uptake was determined at 25°C by diluting the vesicles 10-fold into a gassed (95% N_2, 5% CO_2 in the presence of HCO_3^-; 100% N_2 in the absence of HCO_3^-) incubation medium containing $^{36}Cl^-$. Under pH (pH 7.7 in = out; □) or pH and HCO_3^- (pH 7.7, 50 mM HCO_3^- in = out; ○) equilibrated conditions, the composition of membrane suspension media and incubation media was identical (except for $^{36}Cl^-$). In the presence of outwardly directed pH (7.7 in/6.0 out; ■) or pH and HCO_3^- (pH 7.7, 50 mM HCO_3^- in/pH 6.0, 0.91 mM HCO_3^- out; ●) gradients the vesicles were incubated in HCO_3^- free "standard incubation medium" of pH 6.0 (SIM, pH 6.0), the composition of which is given in Methods. Data represent the mean±SD of nine determinations in three separate membrane vesicle preparations. Levels of significant differences are $P < 0.025$ (*), $P < 0.05$ (++), $P < 0.005$ (§), and $P < 0.001$ (**), respectively.

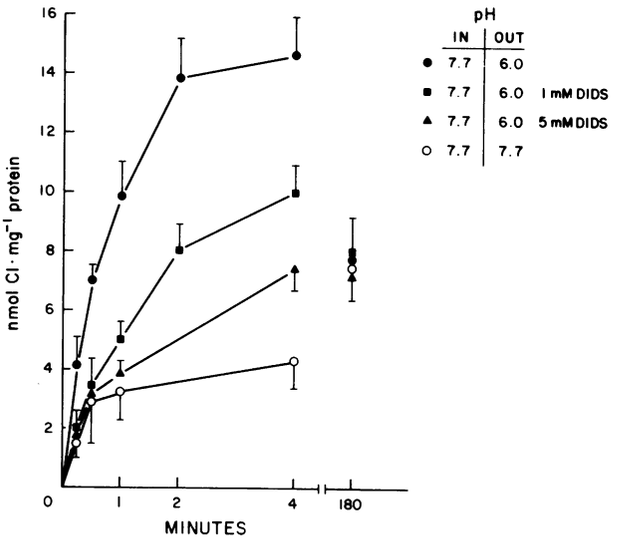

Figure 2. Effect of DIDS on pH- and HCO_3^--stimulated chloride uptake in cLPM vesicles. HCO_3^- (50 mM) preloaded cLPM vesicles (HCO_3^--SMS, pH 7.7) were incubated in gassed (95% N_2, 5% CO_2) SIM, pH 6.0 in the presence (■, ▲) and absence (●) of 1 and 5 mM DIDS. The concentration of Cl^- in the reaction mixture was 5 mM. Uptake of $^{36}Cl^-$ in the absence of a pH and HCO_3^- gradient (○) was determined by incubating the vesicles in HCO_3^--SMS, pH 7.7. Because of DIDS sensitivity to light, all experiments using this compound were performed with minimal exposure to light. Data represent the mean±SD of six determinations in two separate membrane preparations.

The presence of a Cl^-:HCO_3^- and Cl^-:OH^- exchanger in cLPM vesicles is further supported by the finding that DIDS, the most potent inhibitor of inorganic anion exchange in erythrocytes (25) and intestinal brush border membranes (17), completely inhibited the initial pH- and HCO_3^--stimulated uptake rates of Cl^- (Fig. 2). However, after 30 s the inhibition of Cl^- uptake was incomplete even with 5 mM DIDS. These data suggest that, whereas initial uptake rates of Cl^- were mainly due to DIDS-sensitive Cl^-:HCO_3^- and Cl^-:OH^- exchange, uptake at later time points proceeded to a significant extent via a DIDS-insensitive, most likely conductive, pathway. Indeed when Cl^- uptake was investigated at pH equilibrium (7.5/7.5), the generation of an inside-positive K^+ diffusion potential by valinomycin (K^+ out > in; 100 mM) significantly stimulated Cl^- uptake compared with K^+-equilibrated conditions (K^+ in = out, 100 mM; Fig. 3). However, this potential driven Cl^- uptake could not be inhibited by DIDS. Thus, the previously demonstrated pH and HCO_3^- gradient-stimulated, DIDS-sensitive Cl^- uptake (Fig. 2) can be attributed to Cl^-:HCO_3^- and Cl^-:OH^- exchange mechanisms. Because HCO_3^- appears to be favored over OH^- for transcanalicular exchange with Cl^- (Fig. 1), the term Cl^-:HCO_3^- exchange will be preferentially used subsequently.

Characteristics of the canalicular Cl^-:HCO_3^- exchange. As demonstrated in Fig. 4 the pH and HCO_3^- gradient-stimulated initial Cl^- uptake rates were temperature sensitive. Furthermore, the DIDS-sensitive portions of Cl^- uptake exhibited linearity with incubation time up to ~6 s. Based on these results 4-s uptake values were chosen for the kinetic studies.

The dependence of the pH and HCO_3^- gradient-driven initial Cl^- uptake rates on increasing concentrations of Cl^- is illustrated in Fig. 5. A large component of Cl^- uptake, especially at higher Cl^- concentrations was DIDS insensitive and nonsaturable. However, when the DIDS-insensitive uptake (○) was subtracted from total uptake (●), Cl^- uptake resolved into a

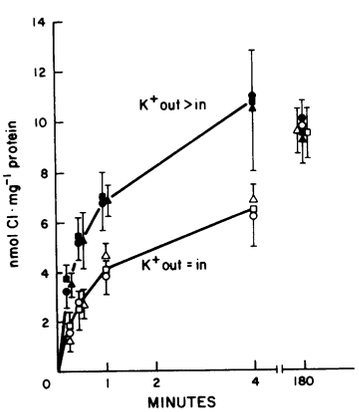

Figure 3. Effect of a valinomycin-induced K^+ diffusion potential (inside positive) on chloride uptake into cLPM vesicles. The vesicles were resuspended (preloaded) in 150 mM sucrose, 0.2 mM Ca-gluconate, 5 mM Mg-gluconate, 70 mM Hepes-TMA OH^- (pH 7.5) and either 100 mM TMA-gluconate (●, ■, ▲) or 100 mM K-gluconate (○, □, △). Valinomycin (10 μg/mg protein) was added to all vesicle suspensions 2–5 min before the reactions were started. Incubations were done in 140 mM sucrose, 0.2 mM Ca-gluconate, 5 mM Mg-gluconate, 70 mM Hepes-TMA-OH^- (pH 7.5), 100 mM K-gluconate, 5 mM $^{36}Cl^-$ and either 0 (○, ●), 1 (□, ■), or 5 (△, ▲) mM DIDS. Data represent the mean±SD of six determinations in two separate membrane preparations.

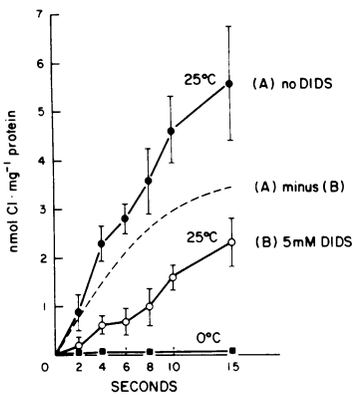

Figure 4. Initial rates of pH- and bicarbonate-stimulated chloride uptake in cLPM vesicles. HCO_3^- preloaded vesicles (HCO_3^--SMS, pH 7.7) were incubated in HCO_3^--free SIM, pH 6.0, containing $^{36}Cl^-$ (5 mM) and either none (•) or 5 mM (○) DIDS (mean±SD; n = 6). The dotted line represents the DIDS-sensitive initial uptake rates at 25°C that are due to Cl^-:HCO_3^- (and Cl^-:OH^-) exchange. For comparison the pH and HCO_3^--stimulated $^{36}Cl^-$ uptake was also determined at 0°C (■) in one experiment.

saturable function of the Cl^- concentration (•, ---). A Lineweaver–Burke plot analysis of the DIDS-sensitive component of Cl^- uptake revealed an apparent K_m of 6.3 mM and a V_{max} of 51 nmol · min^{-1} · mg^{-1} protein.

The effects of various inhibitors of carrier-mediated transport on pH and HCO_3^- gradient-stimulated Cl^- uptake into cLPM vesicles are summarized in Table I. DIDS and SITS were the most effective inhibitors (as in erythrocytes and intestinal brush border; 17, 25). Probenecid, the "standard" inhibitor of carrier-mediated organic anion transport in the kidney (26) was less effective, suggesting that the canalicular Cl^-:HCO_3^- exchanger does not primarily function as an organic anion transporter. Amiloride, a competitive inhibitor of renal and intestinal Na^+:H^+ exchange (27, 28), had no effects as expected. Although their exact mechanisms of action are unclear at present, the observed partial inhibitory effects of furosemide and bumetanide are consistent with their effects on carrier-mediated Cl^- transport in intestinal brush border membranes (17) and erythrocytes (29). None of the tested compounds affected equilibrium Cl^- uptake at 180 min, indicating the absence of nonspecific toxic membrane injury under all conditions studied. In summary, the observed effects of the various inhibitors of pH- and HCO_3^--stimulated Cl^- uptake into cLPM vesicles further support the concept of a carrier-mediated Cl^-:HCO_3^- exchange in bile canalicular membranes of rat liver.

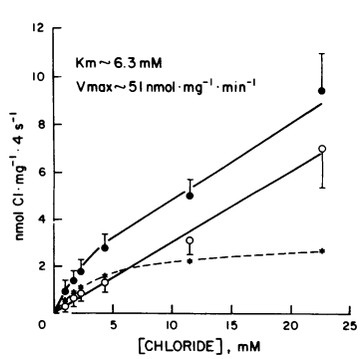

Figure 5. Kinetics of Cl^-:HCO_3^- exchange in cLPM vesicles. 4-s uptakes of increasing concentrations of chloride into HCO_3^- (50 mM) preloaded cLPM vesicles (HCO_3^--SMS, pH 7.7) were determined in gassed (95% N_2, 5% CO_2) SIM, pH 6.0 in the presence (○) and absence (•) of 5 mM DIDS. The DIDS-sensitive initial uptake rates exhibited saturation with increasing concentrations of Cl^- (• --- •). The data represent the mean±SD of eight determinations in two separate membrane preparations. The lines through the various points represent a visual fit.

*Table I. Effects of Membrane Transport Inhibitors on pH- and HCO_3^--stimulated Cl^- Uptake in cLPM Vesicles**

	$^{36}Cl^-$ uptake		
	10 s	%	180 min
	nmol · mg^{-1} protein		*nmol · mg^{-1} protein*
Controls	4.3±0.8	(100)	9.8±0.9
Amiloride	4.4±1.1‡	(102)	10.0±1.4
Bumetanide	3.2±0.5*	(74)	10.7±2.0
Furosemide	2.9±0.4‖	(67)	10.5±0.9
Probenecid	2.7±0.4‖	(63)	10.5±1.0
SITS	2.2±0.9‖	(51)	9.7±1.6
DIDS	1.3±0.3‖	(30)	9.7±1.3

* The vesicles were preloaded with HCO_3^--SMS, pH 7.7, and incubated in SIM, pH 6.0, containing $^{36}Cl^-$ (5 mM). The various inhibitors (1 mM) were directly added to the membrane suspensions from stock solutions prepared in HCO_3^--SMS. Data represent the mean±SD of eight determinations in two different membrane preparations. Levels of significance with respect to controls are: not significant (‡), $P < 0.005$ (§), and $P < 0.001$ (‖), respectively. In addition, the effects of DIDS, but not those of SITS, are significantly different from probenecid ($P < 0.001$).

The effects of various organic and inorganic anions on pH- and HCO_3^--stimulated Cl^- uptake into cLPM vesicles are given in Table II. Neither sulfate nor *p*-aminohippuric acid, lactate, aspartate, and glutamate (20 mM) had any *cis*-inhibitory effect on initial (8 s) uptake rates of Cl^-. Although significantly different from controls, the effects of acetate are too small for being attributable to co-transport under the given experimental conditions (30). In contrast, chloride demonstrated *cis*-inhibition to the extent that was expected theoretically (30). In addition, nitrate may be a co-substrate for the canalicular Cl^-:HCO_3^- exchange system as has also been observed in the

*Table II. Effects of Various Organic and Inorganic Anions on pH- and HCO_3^--stimulated Cl^- Uptake in cLPM Vesicles**

	$^{36}Cl^-$ uptake
	%
Controls	100
p-Aminohippuric acid	102±23‡
Lactate	100±23‡
Aspartate	109±21‡
Sulfate	98±18‡
Glutamate	93±16‡
Acetate	85±12§
Chloride	41±11‖
Nitrate	45±8‖

* HCO_3^--SMS, pH 7.7, preloaded vesicles were incubated in SIM, pH 6.0, containing either 5 mM $^{36}Cl^-$ alone (= controls) or, in addition, 20 mM of the indicated anions. Uptake of $^{36}Cl^-$ was determined at 8 s. Data represent the mean±SD of eight determinations in two separate membrane preparations. No effects of the various anions on equilibrium $^{36}Cl^-$ uptake at 180 min. were observed. Levels of significance with respect to controls are: not significant (‡), $P < 0.01$ (§), and $P < 0.001$ (‖), respectively.

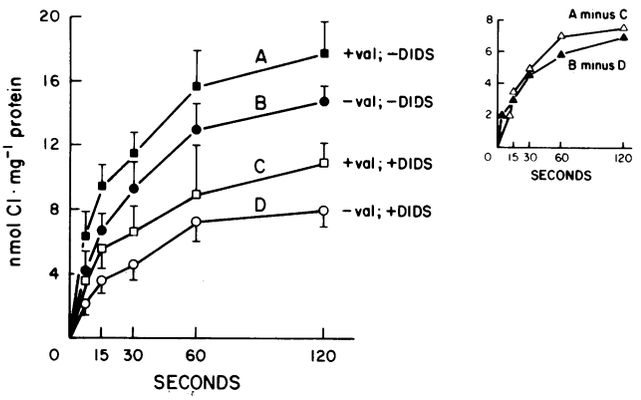

Figure 6. Effect of valinomycin-induced membrane potential changes on canalicular Cl^-:HCO_3^- exchange. HCO_3^--SMS, pH 7.7, preloaded cLPM vesicles were treated with valinomycin in ethanol (10 μg/mg protein; □, ■) or the equivalent amount of ethanol (0.3%) only (○, ●). The vesicles were then incubated in SIM, pH 6.0 containing 100 mM K-gluconate instead of TMA-gluconate. $^{36}Cl^-$ (5 mM) uptake was determined in the presence (○, □) and absence (●, ■) of 5 mM DIDS. Data represent the mean±SD of six determinations in two separate membrane preparations. Inset demonstrates that valinomycin has no effect on the DIDS-sensitive portion of pH and HCO_3^--driven Cl^- uptake.

erythrocyte (25) and the intestine (17). These studies complement the data obtained with the various membrane transport inhibitors (Table I) and indicate that, unlike in the dog renal microvillus membrane (20) but similar to the rabbit ileal brush border (17), the canalicular Cl^-:HCO_3^- exchanger in rat liver transports neither sulfate nor organic anions. Parenthetically, the addition of taurocholate or ursodeoxycholate (100 μM) to the reaction mixture had no effects on the pH and HCO_3^- gradient-stimulated uptake of Cl^- into cLPM vesicles (data not shown).

Finally, we investigated the electrogenicity of the DIDS-sensitive component of the pH- and HCO_3^--stimulated Cl^- uptake in cLPM vesicles. As demonstrated in Fig. 6, when a valinomycin-induced inside-positive K^+ diffusion potential was superimposed upon an outwardly directed pH and HCO_3^- gradient, overall Cl^- uptake was stimulated (curves *A* and *C*) compared with the absence of valinomycin (curves *B* and *D*). However, the DIDS-sensitive portion of the pH- and HCO_3^--stimulated Cl^- uptake was not affected by the imposed membrane potential changes (Fig. 6, *inset;* △ compared with ▲). These data are consistent with an electroneutral 1:1 exchanger of Cl^- and HCO_3^- across the canalicular membrane.

Distribution of Cl^-:HCO_3^- exchange on the various surface domains of hepatocytes. To investigate whether the identified rat liver Cl^-:HCO_3^- exchanger is selectively confined to the canalicular pole or present on all surface domains of hepatocytes, we directly compared the pH and HCO_3^- gradient-driven Cl^- uptake in cLPM and blLPM isolated simultaneously from the same homogenate. In contrast to cLPM, intravesicular Cl^- did not overshoot the equilibrium values in blLPM although there was a constant, albeit small, stimulation of Cl^- uptake by the in to out pH and HCO_3^- gradient (Fig. 7). These data are in contrast to those of our previous studies with alanine and taurocholate where transport systems are present in both LPM subfractions and initial solute uptake rates therefore were similar in cLPM and blLPM despite the twofold differences in their intravesicular volumes (8). Based on these earlier studies with different substrates as well as the fact that ~20% of the total intravesicular space of blLPM represents contamination with cLPM vesicles (23), the data presented in Fig. 7 suggest that the Cl^-:HCO_3^- exchange system is not present in the basolateral domain of rat hepatocytes.

Effects of inwardly directed Na^+ gradients on Cl^- uptake in cLPM and blLPM vesicles. Fig. 8 summarizes results of studies designed to determine whether sodium-chloride co-transport mechanisms exist in canalicular and/or basolateral rat liver plasma membranes. In these experiments the intravesicular uptake of chloride was compared in the presence of inwardly directed Na^+, K^+, Na^+ and K^+, or TMA^+ gradients. To control for the influence of the electrical membrane potential, the incubations were done under pH-equilibrated conditions (high ionic strength buffer) and in the presence of the proton ionophore FCCP. As shown in Fig. 8, under these conditions neither Na^+ nor K^+ nor both together exhibited any stimulation of initial Cl^- uptake rates when compared with TMA^+. These results were the same in cLPM and blLPM. Hence, no evidence was found for the presence of a sodium-chloride co-transport system in rat liver plasma membranes. These findings are in agreement with recent studies in the isolated perfused liver and with cultured hepatocytes (31).

Discussion

Recent indirect studies in the isolated perfused liver have suggested that active transhepatocyte secretion of both Cl^- and

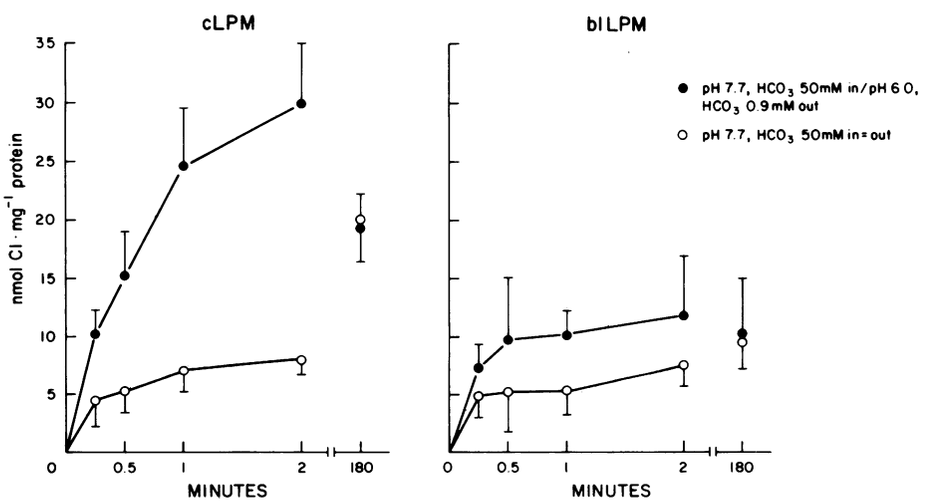

Figure 7. Comparison of pH- and HCO_3^--driven chloride uptake between cLPM and blLPM vesicles. The vesicles were preloaded with HCO_3^--SMS, pH 7.7, and incubated either in HCO_3^--free SIM, pH 6.0 (●), or HCO_3^--SMS, pH 7.7 (○). Cl^- was present in a concentration of 11.4 mM. Data represent the mean±SD of four determinations in two separate membrane preparations.

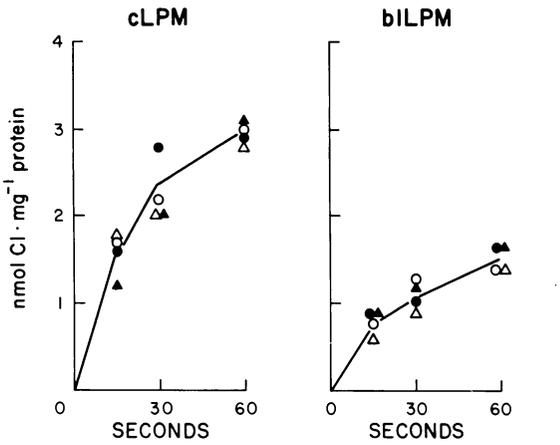

Figure 8. Effect of inwardly directed cation gradients on chloride uptake in cLPM and blLPM vesicles. Membrane vesicles were prepared in 250 mM sucrose, 0.2 mM Ca-gluconate, 5 mM Mg-gluconate, 50 mM Tris, and 80 mM Hepes (pH 7.5). Uptake of $^{36}Cl^-$ (5 mM) was assayed in the presence of 80 μM FCCP and 70 mM sucrose, 0.2 mM Ca-gluconate, 5 mM Mg-gluconate, 50 mM Tris, 80 mM Hepes (pH 7.5), and 100 mM Na^+ (●), 100 mM K^+ (○), 50 mM Na^+ and 50 mM K^+ (▲), or 100 mM TMA^+ (△). Equilibrium Cl^- uptake values at 180 min were 9.5±0.6 nmol · mg^{-1} protein for cLPM and 4.3±0.5 nmol · mg^{-1} protein for blLPM vesicles (mean±SD). Data represent the mean of three and six determinations in cLPM and blLPM vesicles, respectively.

HCO_3^- plays an important role in the generation of BAIF in rat liver (9–11). Furthermore, studies with isolated hepatocytes in suspension have provided evidence for the existence of inorganic anion (e.g., Cl^-, HCO_3^-, and sulfate) transport systems on the surface membrane of normal liver cells (16). However, in that hepatocytes loose their structural and functional polarization during the course of their isolation, the physiologic distribution of these transport systems along the basolateral and canalicular surface domains are not known. Furthermore, studies with isolated hepatocytes do not distinguish between membrane transport processes and intracellular (e.g., cytosolic) metabolic events. In the present study, therefore, we used selectively isolated and highly purified cLPM and blLPM vesicles (23) to investigate the mechanisms and driving forces for transmembrane transport of Cl^- across the two major surface domains of hepatocytes.

The results of these studies provide direct evidence for the existence of a carrier-mediated electroneutral Cl^-:HCO_3^- (OH^-) exchange in cLPM but not in blLPM vesicles (Figs. 2, 6, and 7). Because HCO_3^- gradients resulted in greater initial rates of ^{36}Cl uptake than OH^- gradients, the exchanger favors HCO_3^- over OH^- anions at identical pH gradients. This preferential stimulation of Cl^- uptake by HCO_3^- could not be explained by an increase in buffering capacity because the concentration of Tris-Hepes was reduced by 50% when HCO_3^- was added (Fig. 1), and stimulation by HCO_3^- was observed at the earliest time points when buffering capacity should be adequate (Figs. 1 and 4). Cl^-:HCO_3^- exchange was most effectively inhibited by DIDS (70%) and to a lesser extent also by equimolar amounts of SITS (49%), probenecid (37%), furosemide (33%), and bumetanide (26%; Table I, and Fig. 2). This pattern of inhibition is analogous to the effect of these compounds on Cl^- transport in erythrocytes (29, 32), dog renal microvillus membrane vesicles (33), as well as rabbit ileal brush border membrane vesicles (17), and further suggests that at least a portion of transcanalicular Cl^- movement is a carrier-mediated exchange with HCO_3^-. Although a valinomycin-induced inside-positive K^+ diffusion potential also stimulated overall Cl^- uptake both in the presence (Fig. 6) and absence (Fig. 3) of outwardly directed OH^- and HCO_3^- gradients, the DIDS-sensitive portion of the pH and HCO_3^- gradient-stimulated Cl^- uptake was not affected by the artificially imposed transmembrane potential changes (Fig. 6), suggesting an electroneutral Cl^-:HCO_3^- exchange in cLPM. Altogether, this data indicates that in addition to a voltage-insensitive Cl^-:HCO_3^- exchange pathway, a physiologic conductive leak pathway for Cl^- may exist in canalicular rat liver plasma membranes as well.

In contrast to the recently reported anion exchange mechanisms in rat renal basolateral (19) and dog renal microvillus (20, 21) membrane vesicles, the rat liver canalicular Cl^-:HCO_3^- exchange pathway, described in the present study, is not shared by other anions such as sulfate, *p*-aminohippurate, lactate, or acetate (no *cis*-inhibition of pH- and HCO_3^--stimulated Cl^- uptake, Table II). Furthermore, we found no effect of pH and HCO_3^- gradients on transcanalicular transport of taurocholate (8) nor was the pH and HCO_3^- gradient-driven Cl^- uptake affected to any degree by the addition of 100 μm of taurocholate to the reaction mixture (data not shown). In contrast, nitrate may also be able to exchange with HCO_3^- (Table II). Thus, there appears to be a certain selectivity of the canalicular rat liver Cl^-:HCO_3^- exchange for inorganic anions rather than organic anions, as recently observed in rabbit brush border membranes (17). This interpretation is further substantiated by the greater inhibition of pH and HCO_3^- gradient-stimulated Cl^- uptake by DIDS and SITS as compared with probenecid (Table I). The latter represents the standard inhibitor of organic anion transport in the kidney (26) and has been shown to preferentially inhibit pH gradient stimulated urate and *p*-aminohippurate transport in dog renal microvillus vesicles (33). Future studies will be required to determine whether similar or different (albeit closely related) membrane components are responsible for the transcanalicular transfer of chloride, bicarbonate, and organic anions.

By utilizing highly purified and selectively isolated blLPM and cLPM vesicles, the present study resolves two major current controversies with respect to the hepatocellular transport of Cl^-. First, the selective localization of the identified Cl^-:HCO_3^- exchange to the canalicular pole of hepatocytes (Fig. 7) explains why evidence for such an exchange mechanism has been found up to now only in isolated hepatocytes in suspension, where the canalicular surface is directly exposed to the surrounding medium (16). In contrast, Cl^-:HCO_3^- exchange was not found in the isolated perfused rat liver, probably because the canalicular membrane does not have direct contact with the perfusate in this experimental model (34). Secondly, we find no evidence for the existence of a Na^+:Cl^- co-transport system, either in blLPM or in cLPM vesicles (Fig. 8). In accordance with some (31) but not all (9) studies in the isolated perfused liver and cultured hepatocytes, these findings indicate the absence of a sodium coupled secondary active transport process for transhepatocyte excretion of Cl^-. Thus, our data support the concept of a passive distribution of Cl^- across the various domains of the hepatocellular plasma membrane according to the physiologic electrochemical membrane potential (4, 5, 35).

Fig. 9 integrates the identified canalicular Cl^-:HCO_3^- exchange as well as the Cl^- conductive leak pathway into a new model for the formation of BAIF. According to this concept the intracellular negativity, that is maintained by the basolateral

Na^+K^+-ATPase activity, leads to a passive (electrodiffusional) concentration of Cl^- within the bile canalicular lumen compared with the cell interior. The resulting transcanalicular out to in Cl^- concentration gradient can then drive electroneutral exchange for intracellular HCO_3^-, the constant availability of which is guaranteed by metabolic formation of CO_2 and the coordinated actions of cytosolic carbonic anhydrase (36) and basolateral Na^+:H^+ exchange (10, 37, 37a). The latter pump will also maintain intracellular HCO_3^- above its electrochemical equilibrium, thereby providing an additional driving force for the Cl^-:HCO_3^- exchanger. Because the canalicular Cl^- concentration remains constant as long as the physiologic intracellular negative potential is maintained, these processes result in a net accumulation of HCO_3^- within the bile canalicular lumen. Theoretically, this chloride driven out to in HCO_3^- gradient could now energize the canalicular excretion of certain organic anions via a second anion exchange process. Although the existence of such a canalicular HCO_3^-: organic anion exchange mechanism is hypothetical at present, pH- and HCO_3^--dependent organic anion exchangers with very broad substrate specificities have been recently identified in dog (20, 33) and rat (21) renal microvillus as well as rat renal basolateral membrane vesicles (18, 19). Inasmuch as kidney and liver excrete similar organic anions (38–41), it appears reasonable to propose that the two organs may also possess similar membrane transport processes.

The model presented in Fig. 9 satisfies several observations concerning the subcellular mechanisms involved in overall hepatic bile formation. First, the findings that the canalicular excretion of taurocholate (7, 8) as well as chloride (this study, Fig. 3) are both driven by the intracellular negative potential strengthen the concept that the activity of basolateral Na^+K^+-ATPase and the outward conductive diffusion of K^+ provide a major "driving force" for both components of canalicular bile flow, e.g., BADF and BAIF (1, 5, 42). Second, the absence of Na^+:Cl^- co-transport in blLPM vesicles (Fig. 8) in conjunction with the presence of a conductive leak pathway for Cl^- in cLPM (Fig. 3) suggest that the passive electrodiffusion of intracellular Cl^- into the canalicular lumen plays an important role in the generation of BAIF (5, 9). Indeed, inhibition of Na^+K^+-ATPase (membrane depolarization) results in intracellular sequestration of Cl^-, Na^+, and water (43); whereas, hyperpolarization of the cell membrane is associated with an increased hepatocellular efflux of Cl^- into bile and a transient rise in bile flow (44). Third, an electroneutral cLPM Cl^-:HCO_3^- exchanger (Figs. 1, 2, and 6) can also explain why omission of perfusate HCO_3^- from rat liver perfusate results in a decrease of BAIF in the isolated perfused rat liver (9–11). In one study an additive decrease in BAIF was also observed after sequential omission of Cl^- and HCO_3^- from the perfusate (9), findings consistent with a high intraluminal Cl^- concentration stimulating the canalicular excretion of intracellular HCO_3^- (Fig. 9).

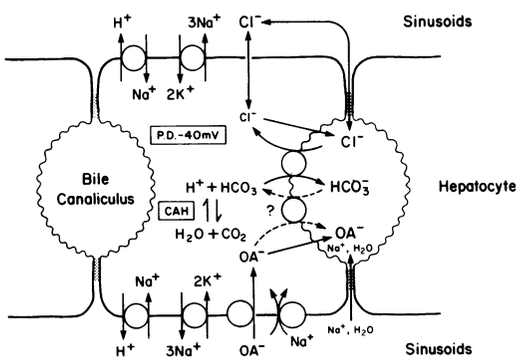

Figure 9. Model for a role of the identified electroneutral canalicular Cl^-:HCO_3^- exchange in bile acid-independent bile formation in rat liver. For discussion of the model see text. P.D., potential difference with respect to the extracellular space; CAH, cytosolic carbonic anhydrase; OA^-, organic anions (e.g., taurocholate; 7, 8).

Our data does not exclude the possibility of a separate conductive leak pathway for HCO_3^- in cLPM, which also could generate net HCO_3^- excretion, particularly if a basolateral Na^+:H^+ exchanger maintains intracellular HCO_3^- above its electrochemical equilibrium. Functional coupling between basolateral Na^+:H^+ exchange and canalicular Cl^-:HCO_3^- exchange may also exist in the liver cell, and Na^+:H^+ exchange could be the major driving force rather than the cLPM Cl^- gradient. The resistance of biliary HCO_3^- secretion to replacement of Na^+ by Li^+ in the perfused rat liver (9–11) could also be explained by such functional coupling, since Li^+ can effectively replace Na^+ for exchange with H^+ in the liver as well as the kidney, and intestine (13, 28, 37a). Finally the identification of a canalicular Cl^-:HCO_3^- exchanger provides an additional mechanism to duct HCO_3^- excretion for enriching bile bicarbonate content in many animal species (10, 45).

In summary, the present study provides direct evidence for the existence of an electroneutral Cl^-:HCO_3^- exchanger as well as a conductive leak pathway for Cl^- in canalicular plasma membrane vesicles of rat liver. Although these findings have been discussed with respect to their possible physiologic significance in bile acid-independent bile formation, it is realized that Cl^- and HCO_3^- transport processes might also be involved in the control of cell volume and the regulation of intracellular pH (43, 46, 47). Thus, it is possible that in the intact liver a dynamic relationship exists between the canalicular excretion of Cl^- and HCO_3^- and Na^+/H^+ exchange and the regulation of cell volume and intracellular pH. Elucidation of these functional interdependencies will require studies in intact cells including micropuncture of bile canaliculi in isolated couplets of hepatocytes (48).

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