Mechanisms of Taurocholate Transport in Canalicular and Basolateral Rat Liver Plasma Membrane Vesicles

EVIDENCE FOR AN ELECTROGENIC CANALICULAR ORGANIC ANION CARRIER

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The driving forces for taurocholate transport were determined in highly purified canalicular (cLPM) and basolateral rat liver plasma membrane (LPM) vesicles. Alanine transport was also examined for comparison. Inwardly directed Na⁺ but not K⁺ gradients transiently stimulated [³H]taurocholate (1 μM) and [³H]alanine (0.2 mM) uptake into basolateral LPM 3-4 fold above their respective equilibrium values (overshoots). Na⁺ also stimulated [³H]taurocholate countertransport and tracer exchange in basolateral LPM vesicles whereas valinomycin-induced inside negative K⁺ diffusion potentials stimulated alanine efflux but had no effect on taurocholate uptake. Efflux of [³H]taurocholate from cLPM was also independent of Na⁺ and could be trans-stimulated by extravesicular taurocholate. Furthermore, an inside negative valinomycin-mediated K⁺ diffusion potential inhibited taurocholate uptake into and stimulated taurocholate efflux from the cLPM vesicles. These studies provide direct evidence for a "carrier mediated" and potential-sensitive conductive pathway for the canalicular excretion of taurocholate. In addition, they confirm the presence of a possibly electroneutral Na⁺-taurocholate cotransport system in basolateral membranes of the hepatocyte.

Bile acids are a major determinant of bile secretion and undergo an efficient enterohepatic circulation (1, 2). In order for bile acids to be cleared from portal blood and excreted into bile, the liver must transport these anions across both sinusoidal and canalicular domains of the cell where both concentrative as well as vectorial transport mechanisms are presumed to exist. Several previous studies in intact animals (3), the isolated perfused rat liver (4) and with isolated rat hepatocytes (5, 6) have suggested that the hepatocellular uptake of bile acids across sinusoidal and lateral (e.g. basolateral) plasma membranes is a "carrier-mediated" process which for taurocholate is driven by the inwardly directed Na⁺ gradient that is maintained by the basolateral membrane enzyme Na⁺K⁺-ATPase (7, 8). More recently, this concept has been more clearly defined by direct evidence for a basolateral Na⁺-taurocholate cotransport system in isolated rat liver plasma membrane vesicles (9-11). In contrast, the mechanisms and driving forces for bile acid transport across the canalicular or secretory pole, the rate-limiting step in overall transport from blood to bile, remain to be precisely delineated.

In the present study we have directly evaluated and compared the driving forces for taurocholate transport across the two polar membrane domains of hepatocytes. Highly purified cLPM and bLPM rat liver plasma membrane vesicles were simultaneously isolated from the same homogenate by rate zonal and discontinuous sucrose density centrifugation techniques (12). Since some of the characteristics of the basolateral carrier for taurocholate, have been previously defined (9-11) emphasis will be directed toward the canalicular transport system and studies in bLPM vesicles will be included only to the extent that are required for clear demonstration of differences between the taurocholate transport mechanisms in the two isolated LPM subfractions. Experiments with the neutral amino acid alanine, that is transported across bLPM by an electrogenic Na⁺-alanine cotransport system (13), will also be included to control for both the functional integrity of the isolated LPM subfractions and the effects of artificially induced transmembrane potential changes (e.g. valinomycin-induced K⁺ diffusion potentials) on taurocholate transport. Part of this work has previously been presented in preliminary form (14).

EXPERIMENTAL PROCEDURES

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 ad libitum and were housed in a constant temperature, humidity environment with alternating 12-h light (7 a.m. to 7 p.m.) and dark cycles. Fed animals were regularly killed by decapitation between 7:30 and 8:30 a.m.

Isolation of Canalicular and Basolateral Liver Plasma Membrane Vesicles

The methods for isolation of the cLPM and bLPM subfractions as well as their morphologic and biochemical characterization are described in detail elsewhere (12). In brief, a canalicular enriched "mixed LPM" subfraction was first separated out of a "crude nuclear fraction" by rate zonal flotation (44/35%, w/w, sucrose density interface) in the T2-28 (Sorvall) zonal rotor. After tight homogenization (Type B Dounce homogenizer, 50 up and down strokes) the vesicles

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luted cLPM and blLPM were separated by high speed centrifugation (195,300 x g, for 3 h) of the mixed LPM through a 3-step sucrose gradient (31, 34, and 38%, w/w). The membranes were collected at 105,000 x g for 60 min and, except where stated otherwise, were resuspended in a standard membrane suspension medium consisting of 0.25 M sucrose, 0.2 mM CaCl₂, 5 mM MgSO₄, 10 mM Hepes/Tris, pH 7.5. Routinely, the membranes were frozen and stored in liquid nitrogen (–70 °C, protein concentration >3 mg/ml) for up to 2 weeks without loss of transport function for taurocholate or alanine.

Characterization of the Isolated LPM Subfractions

The degree of purification of cLPM and blLPM was extensively analyzed by intracellular and plasma membrane marker enzyme activities (12). These studies indicated minor contamination of both LPM subfractions by intracellular organelles and virtually complete separation of blLPM from cLPM as reflected by the absence of Na⁺K⁺-ATPase, and glucagon-stimulatable adenylate cyclase activities, or intact secretory component in cLPM (12). In contrast, the blLPM subfraction was contaminated with cLPM by approximately 10% (12). Transmission electron microscopy revealed that both cLPM and blLPM are composed of membrane vesicles. Although blLPM still contain some unbroken lateral membrane sheets (12). All cLPM subfractions in this study were routinely tested for Na⁺K⁺-ATPase activity (15) and only those cLPM preparations without any detectable Na⁺K⁺-ATPase activity were used for the transport studies (approximately four out of five membrane preparations). Protein was determined according to Lowry et al. (16) using bovine serum albumin as a standard.

Freeze Fracture Analysis

In Vitro—To define the particle density on the P-face and E-face surface of canaliculi microvilli, freeze fracture replicas of bile canaliculi were prepared as previously described in specimens of liver of untreated rats (17) using unidirectional shadowing at an angle of 45°. In Vitro—50 µg of frozen, quick thawed (37 °C), and remethylene cLPM vesicles (30 up and down strokes with a tight Dounce homogenizer) were fixed in 1% glutaraldehyde/protoprotected with glycerol to a final concentration of 25% (v/v) at 4 °C. After 1.5 h at 4 °C, the samples were frozen in Freon 22 cooled by liquid nitrogen at its melting point and stored in a liquid nitrogen (–70 °C, protein concentration >3 mg/ml) for up to 2 weeks before vesiculation of the thawed membranes. After tight homogenization, cLPM vesicles were contaminated with blLPM by approximately 10% (12). In contrast, the blLPM subfraction was contaminated with cLPM by approximately 10% (12). Transmission electron microscopy revealed that both cLPM and blLPM are composed of membrane vesicles. Although blLPM still contain some unbroken lateral membrane sheets (12). All cLPM subfractions in this study were routinely tested for Na⁺K⁺-ATPase activity (15) and only those cLPM preparations without any detectable Na⁺K⁺-ATPase activity were used for the transport studies (approximately four out of five membrane preparations). Protein was determined according to Lowry et al. (16) using bovine serum albumin as a standard.

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Transport Studies

Frozen membrane suspensions were quickly thawed by immersion of the tubes in a 37 °C water bath, diluted to the desired protein concentration and again vesiculated with a tight Dounce homogenizer (Type B; 30 up and down strokes), and placed on ice. In experiments where preloading of the vesicles with taurocholate was required, the unlabeled (Table II) or labeled (Figs. 7 and 8) compound was added before vesiculation of the thawed membranes. After tight homogenization these membranes were preincubated at 25 °C for 20-30 min before placing them on ice to ensure maximal intravesicular concentrations of taurocholate. Transmembrane transport of [3H]taurocholic acid and [3H]alanine were measured by a rapid Millipore filtration technique (Millipore Corp., Bedford, MA). Membrane suspensions (40–80 µg of protein in 20 µl) were preincubated at 25 °C for at least 5 min. Uptake studies were initiated by the addition of 80 µl of incubation medium whereas tracer efflux was determined by addition of 180 µl of incubation medium. The exact composition of the media is given in the figure and table legends of the individual experiments. After incubation for the indicated time intervals, transport was terminated by the addition of 3.5 ml of ice-cold stop solution (100 mM KCl, 100 mM sucrose, 0.2 mM CaCl₂, 5 mM MgSO₄, 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 (HAWP) which had been precooled in cold deionized water and in the case of taurocholate, additionally prefiltered with 3 ml of 1 mM taurocholate to diminish nonspecific filter binding. The filter was washed twice with 3.5 ml of stop solution, dissolved in 6 ml of RSB (Beckman Instruments, Inc.) and counted in a Beckman LS 7000 liquid scintillation counter. Nonspecific binding to the membranes was determined in each experiment by addition of cold incubation solution and cold stop solution to 30 µl of membrane suspension kept at 0–4 °C. This blank was subtracted from all determinations except for efflux studies where the blank values represented 100%. Unless otherwise indicated all incubations were performed in triplicates and all observations confirmed with two or more separate membrane preparations.

Reagents

[6-3H]Taurocholic acid (6.6 Ci/mmole), L-[3-3H]alanine (75.0 Ci/mmole), and D-[1-14C]glucose (4.4 µCi/mmole) were obtained from New England Nuclear. All other chemicals and reagents (analytical grade) were purchased either from Sigma, P-L Biochemicals, or Calbiochem-Behring Corp. All inorganic chemicals were of reagent grade or of the highest purity available.

RESULTS

Sidedness of cLPM Vesicles

The orientation of the isolated cLPM vesicles was determined by freeze fracture analysis (19) in order to know whether the direction of the flux observed in vivo is identical with that occurring in vitro. Following the general principle that the P-face of cellular membranes exhibit a higher particle density than the external membrane leaflet (E-face; 20), we first defined the particle densities on P- and E-faces of canaliculi microvilli in vivo (Fig. 1A). Since in situ the microvilli all exhibit right-side out configuration, P- and E-faces are represented by convex-particle enriched and concave-particle poor membrane areas, respectively. A circular test system of known area (70 nm in diameter; Fig. 1A) was randomly assigned to a total of 250 cast shadow-free canaliculi microvilli and the enclosed number of particles determined (18). This analysis revealed 2538 ±764 particles per µm² (mean ± S.D.) on P-faces and 398 ±427 particles per µm² on E-faces. Based on two standard deviations, P-faces were counted as every fractured cLPM vesicle exhibiting a particle density exceeding 1250 per µm² (upper limit for E-faces). Using these criteria, P-faces represented about 42% of all cast shadow-free vesicles (Table I), which is close to the theoretically expected value of 50%. The particle density on these P-faced vesicles was then analyzed (see "Experimental Procedures") and the convex- and concave-oriented vesicles were defined as "right-side out" or "inside out" vesicles, respectively. By these criteria 77% of the vesicles in the cLPM subfraction demonstrate right-side out configuration in which the extravesicular membrane face corresponds to the bile luminal surface in vivo. This predominantly right-side out configuration of the cLPM vesicles is in agreement with the sidedness of cLPM vesicles recently isolated and determined by other techniques (21). Thus, canaliculi rat liver microvilli are also predominantly oriented right-side out when fragmented into membrane vesicles like brush-border membranes from rat kidney cortex and rat small intestine (19).

Effects of Na⁺ and K⁺ on Alanine and Taurocholate Uptake into blLPM and cLPM Vesicles

To test for the functional integrity of solute transport we first measured the uptake of alanine and taurocholate into both blLPM and cLPM vesicles. In agreement with recently
applying a circular test system of known area determination of the sidedness of cLPM vesicles.

Inside-out depending on their convex or concave orientation, respectively.

rate vesicle preparations.

contrast, 5-6-fold lower initial rates of uptake were observed in plasma membranes for the transport of both alanine and taurocholate. These findings establish that true Na+ ion-equilibrated conditions, initial uptake rates of both alanine and taurocholate were consistently higher in the presence of Na+ than with K+.

librated on both sides of the membranes. However, even under directed Na+ gradient (3-4-fold intravesicular accumulation of [3H]taurocholate), reported findings (9-11, 13) both alanine and taurocholate uptakes into bLPM were markedly stimulated by an inwardly directed Na+ gradient (3-4-fold intravesicular accumulation above equilibrium values; Fig. 2, A and B; bLPM). In contrast, 5-6-fold lower initial rates of uptake were observed in the presence of K+ gradients or when Na+ and K+ were equilibrated on both sides of the membranes. However, even under ion-equilibrated conditions, initial uptake rates of both alanine and taurocholate were consistently higher in the presence of Na+ than with K+. These findings establish that true Na+ coupled cotransport systems exist in basolateral rat liver plasma membranes for the transport of both alanine and taurocholate. Furthermore, the data demonstrate that these bLPM remained functionally intact during the isolation and storage conditions adopted in this study.

When the cLPM vesicles were studied in similar fashions, inwardly directed Na+ gradients also stimulated alanine and taurocholate uptake in contrast to K+ gradients or Na+- and K+-equilibrated conditions (Fig. 2, A and B, cLPM). However, the inwardly directed Na+ gradient did not stimulate uptake values above equilibrium values for alanine whereas taurocholate was transiently accumulated 1.5-fold above equilibrium values. As previously observed with a mixed LPM fraction (11) the extent of the Na+-stimulated taurocholate uptake was proportional to the size of the imposed Na+ gradients (25, 50, and 100 mM in Fig. 2B, cLPM). However, in contrast to the studies with bLPM, taurocholate transport into cLPM was not stimulated by Na+ when the ion was equilibrated on both sides of the membrane. Higher equilibrium uptakes (60 min) were observed for both alanine and taurocholate in cLPM compared to bLPM vesicles (Fig. 2). This is explained by differences in intravesicular volumes in the two LPM subfractions, which are approximately 2-fold higher in cLPM (1.9 ± 0.2 μl × mg⁻¹ of protein; mean ± S.D., n = 6) than bLPM (0.9 ± 0.2 μl × mg⁻¹ of protein) as calculated from equilibrium (60 min) uptakes of [14C]glucose. Comparable values for intravesicular volumes were also obtained with alanine (2.3 ± 0.2 μl × mg⁻¹ of protein and 1.3 ± 0.1 μl × mg⁻¹ of protein for cLPM and bLPM, respectively) whereas the apparent intravesicular volume of distribution for taurocholate was considerably higher, amounting to 15.5 ± 2.9 μl × mg⁻¹ of protein and 5.5 ± 0.5 μl × mg⁻¹ of protein in cLPM and bLPM respectively, confirming a high degree of taurocholate binding to or within the isolated membrane vesicles (10, 11).

Bind of Taurocholate to cLPM Vesicles

The extent of taurocholate binding was first evaluated by determining the effect of the medium osmolarity on taurocholate uptake at equilibrium (60 min). Alanine and D-glucose were also included for comparison. As illustrated in Fig. 3 the equilibrium uptakes of all three substrates decreased linearly as the osmolality (concentration of cellobiose) increased and the vesicles diminished in size. Only the extrapolated regression line of D-glucose demonstrated zero uptake at infinite osmolality indicating that D-glucose does not bind significantly to cLPM vesicles. In contrast, in three separate experiments 15-25% of the alanine and 45-65% of taurocholate was displaced by a 100-fold excess of cold taurocholate in the stop medium. This strongly suggests that taurocholate binds predominantly to the inside of the membrane vesicles, e.g. after uptake of taurocholate into the intravesicular space, a conclusion supported by the findings that taurocholate uptake did not occur at 0 °C whereas at 25 and 37 °C, intravesicular taurocholate increased linearly with time (Fig. 4).

Temperature Dependency of Taurocholate Uptake in cLPM Vesicles

Increasing the temperature from 0 to 37 °C increased initial uptake rates for taurocholate in the presence of both NaCl and KCl gradients (Fig. 4). Except at 0 °C, NaCl gradients promoted approximately 4-6-fold higher taurocholate uptake.
rates than KCl gradients as expected. Furthermore, maximal intravesicular accumulation of taurocholate occurred earlier at 37 °C (9 s) then at 25 °C (>15 s) suggesting that initial rates for taurocholate efflux are temperature-sensitive as well (Fig. 7).

Cation Specificity of Taurocholate Uptake into cLPM Vesicles

Fig. 5 illustrates the effect of various cation gradients on taurocholate uptake in cLPM. As in the mixed LPM (11) only a Na+ gradient clearly sustained an overshoot phenomenon. However, Cs+, K+, and Li+ each had an intermediate stimulatory effect in cLPM. In contrast tetramethylammonium and choline had minimal if any stimulatory effects presumably because of their relatively low ability to permeate the canalicular plasma membrane. Initial uptake rates of taurocholate exhibited saturability in the presence of both Na+ and K+ gradients indicating carrier-mediated canalicular transport of bile acids (data not shown). However, neither these kinetic data nor the effects of the Na+ and other cation gradients on stimulating taurocholate uptake distinguish whether the cations create a vesicle inside positive diffusion potential or whether there is a cation selective cotransport system or charge barrier within the canalicular membrane. In order to examine these possibilities we first looked for evidence for taurocholate countertransport and tracer exchange in both bLPM and cLPM.

Countertransport (Trans-stimulation) of Taurocholate Uptake into bLPM and cLPM Vesicles

The membrane vesicles of both subfractions were preloaded with 20 μM unlabeled taurocholate as described under “Experimental Procedures.” Initial uptake rates of 5 μM [3H]taurocholate were determined in the presence of out to in Na+ and K+ gradients. NO3− was used as a highly permeable anion.
A) Countertransport (NaNO₃ and KNO₃ out to in gradients; potential differences which might exert stimulatory or inhibitory effects on taurocholate uptake. As demonstrated in Table II (part A), in bilPM vesicles the intravesicular taurocholate (20 µM) stimulated tracer taurocholate uptake (countertransport) only in the presence of an inwardly directed Na⁺ gradient. In contrast, in cLM vesicles the presence of Na⁺- and K⁺-gradients suggesting the presence of a Na⁺ independent canalicular taurocholate anion "carrier.

**Tracer Exchange of Taurocholate in bilPM and cLM vesicles**

If transmembrane taurocholate transport is directly coupled with the transport of Na⁺ (Na⁺-taurocholate cotransport), uptake across the vesicular membrane should occur at faster rates when Na⁺ rather than K⁺ is equilibrated on each side of the vesicle membrane (tracer exchange). The data in Table II (part B) demonstrate that under Na⁺- and K⁺-equilibrated conditions, Na⁺ indeed stimulated the uptake of the extravesicular tracer into taurocholate preloaded (20 µM) bilPM vesicles. In contrast, in identical experiments no differences in taurocholate tracer exchange rates between Na⁺ and K⁺ were observed in cLM vesicles. Thus, the "Na⁺ effect" on uptake rates of taurocholate in cLM cannot be attributed to a coupled Na⁺-taurocholate cotransport system. Rather, it appears that the observed stimulation of taurocholate transport into cLM vesicles by Na⁺ and other cation gradients (Figs. 2, 4, and 5) might result from creation of positive diffusion

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**TABLE II**

Countertransport (A) and tracer exchange (B) of taurocholate in bilPM and cLM vesicles

| LPM subfraction     | Experimental conditions | Concentration of taurocholate (in/out) µM | Tracer taurocholate uptake: incubation time 5 s | 15 s | 60 min | dpm |
|---------------------|-------------------------|-------------------------------------------|-----------------------------------------------|------|--------|-----|
| A) Countertransport (NaNO₃ and KNO₃ out to in gradients; 80 mM) | bilPM | NaNO₃ | 5/20 | 1497 ± 228 | 2112 ± 125 | 1765 ± 238 |
|                     |                        | KNO₃ | 5/20 | 1206 ± 77 | 1500 ± 328 | 1571 ± 295 |
|                     |                        |      | 5/0  | 570 ± 120 | 885 ± 126 | 1363 ± 283 |
|                     |                        | NaNO₃ | 5/0  | 705 ± 292 | 1103 ± 252 | 1563 ± 205 |
|                     |                        | KNO₃ | 5/0  | 4108 ± 350 | 6423 ± 570 | 5023 ± 500 |
|                     |                        |      | 5/0  | 3070 ± 648* | 5555 ± 275 | 6188 ± 825 |
|                     |                        | NaNO₃ | 5/0  | 796 ± 352 | 1416 ± 377 | 4395 ± 832 |
|                     |                        | KNO₃ | 5/0  | 430 ± 197** | 975 ± 302** | 5486 ± 427 |
| B) Tracer Exchange (NaNO₃ and KNO₃ equilibrated; 100 mM) | bilPM | NaNO₃ | 20/20 | 645 ± 235 | 858 ± 92 | 2215 ± 312 |
|                     |                        | KNO₃ | 20/20 | 307 ± 108* | 360 ± 238* | 2017 ± 312 |
|                     |                        | NaNO₃ | 20/20 | 645 ± 400 | 1445 ± 393 | 5458 ± 452 |
|                     |                        | KNO₃ | 20/20 | 626 ± 348 | 1456 ± 543 | 5355 ± 353 |

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**FIG. 4** (left). Temperature dependency of initial taurocholate uptakes into cLM vesicles. Membrane vesicles were incubated in 100 mM sucrose, 0.2 mM CaCl₂, 5 mM MgSO₄, 10 mM Hepes/Tris, pH 7.5, and 100 mM NaCl (●, □, △) or KCl (○, □, △) cut to in gradients. The concentration of taurocholate was 1 µM. Values represent the mean ± S.D. of six determinations in two independent experiments.

**FIG. 5** (right). Cation specificity of taurocholate uptake into cLM vesicles. Membrane vesicles were incubated in 100 mM sucrose, 0.2 mM CaCl₂, 5 mM MgSO₄, 10 mM Hepes/Tris, pH 7.5, in the presence of the indicated salt gradients (100 mM, out > in). TMA, tetramethylammonium.
potentials inside the vesicles or from effects of Na\(^+\) on negatively charged barriers within the membrane. Therefore, we next investigated the effects of membrane potential changes on the transmembrane taurocholate transport in cLPM.

Effects of the Electrical Membrane Potential on Taurocholate Transport

Previous studies with plasma membrane vesicles from rat liver have demonstrated that the hepatic uptake of alanine is Na\(^+\) dependent and stimulated by a negative intravesicular electrical membrane potential (13). Therefore, alanine uptake was included in this part of the study as a reference for assessment of the effectiveness of either anions or the K\(^+\) ionophore valinomycin in inducing transmembrane potential changes.

Effects of Anions on Na\(^+\)-stimulated Taurocholate Uptake into cLPM Vesicles—Variable changes in the transmembrane potential may be induced by selecting anions with different membrane permeability characteristics. As demonstrated in Table III initial uptakes of alanine in the presence of an inwardly directed Na\(^+\) gradient decreased with decreasing membrane permeation by the accompanying anions (SCN\(^-\) > NO\(_3^\) > SO\(_4^{2-}\) > gluconate\(^-\)). Since thiocyanate usually diffuses into vesicles more rapidly and gluconate more slowly than Na\(^+\), these data indicate that uptake of alanine is stimulated by the relatively more negative intravesicular membrane potential. In contrast, using identical conditions, the Na\(^+\)-stimulated taurocholate uptake is inhibited in the presence of the more permeant anions thiocyanate and nitrate compared to the less permeant anions sulfate and gluconate. As previously observed in the mixed LPM (11), highest initial uptake rates were consistently found with chloride suggesting that this anion exerts an additional, but as yet undetermined stimulatory effect on transmembrane transport of taurocholate. Nevertheless, the overall comparison of the effect of anion substitution on alanine and taurocholate uptakes in cLPM indicate that in the presence of Na\(^+\), uptake of alanine occurs as a cation whereas under similar conditions taurocholate is transported as an anion.

Effects of Valinomycin-induced K\(^+\)-diffusion Potentials (Inside Negative) on Alanine and Taurocholate Uptake into bILPM and cLPM Vesicles—To more directly investigate the effects of the electrical membrane potential on alanine and taurocholate transport, bILPM and cLPM were preloaded with K gluconate and uptake rates of both solutes were studied in the presence and absence of the K\(^+\) ionophore valinomycin. Under these conditions and without any K\(^+\) present outside the vesicles (e.g. in the incubation medium) valinomycin permits a rapid out-diffusion of K\(^+\) thereby creating a transient negative potential within the vesicles. As shown in Fig. 6A, an inside negative membrane potential enhances the uptake of alanine into bILPM above control values as expected in the presence of an inwardly directed Na\(^+\) gradient (13). However, the same phenomenon was also seen in cLPM indicating the occurrence of an electrogenic Na\(^+\)-alanine cotransport in canalicular plasma membranes as well. In the absence of Na\(^+\) (tetramethylammonium substitution) the transport of the neutral alanine was diminished and not affected by the valinomycin-induced transmembrane potential changes. In contrast, under Na\(^+\)-free conditions, the uptake of the anionic taurocholate was consistently inhibited by the negative intravesicular membrane potential both in bILPM as well as cLPM (Fig. 6B). However, in the presence of inwardly directed Na\(^+\) gradients, taurocholate uptake was distinctly different in the two LPM subfractions. In these experiments, uptake of taurocholate into bILPM vesicles was unchanged by valinomycin whereas its transport into cLPM vesicles was inhibited (Fig. 6B). These results are consistent with the anion substitution experiments (Table III) and indicate that, whereas the basolateral Na\(^+\)-taurocholate co-
transport appears electroneutral (11), taurocholate is invariably transported as an anion across the bile canalicular membrane whether Na⁺ is present or absent in the incubation system. The latter findings complement preliminary reports by others demonstrating stimulation of taurocholate uptake into cLPM vesicles by valinomycin-induced inside positive K⁺ diffusion potentials (22).

Efflux of Taurocholate from cLPM Vesicles

Since the majority of cLPM vesicles exhibit right-side out configuration (Table I), efflux from the vesicles rather than uptake represents the physiologic direction of transcanalicular solute transport. We therefore measured taurocholate efflux from cLPM vesicles to determine if we could verify the conclusions drawn from the uptake studies. As demonstrated in Fig. 7A efflux rates from taurocholate preloaded (10 μM) cLPM vesicles were temperature-sensitive, unchanged in the presence of in to out gradients of either Na⁺ or K⁺ and stimulated by the simultaneous presence of 50 μM of unlabeled taurocholate on the outside of the vesicles. This countercurrent transport of [³H]taurocholate efflux was also observed at similar rates with either in to out Na⁺ or K⁺ gradients. Furthermore, taurocholate efflux was directly stimulated by a valinomycin-induced intravesicular negative K⁺ diffusion potential (Fig. 7B). The alternative possibility of an alkalization of the extravesicular medium through a K⁺/H⁺ exchange, which then could stimulate taurocholate efflux through an additional OH⁻/taurocholate anion exchange mechanism, was excluded by the findings that an outside alkaline pH gradient (pH 7.6 out/6.0 in) did not stimulate taurocholate efflux (data not shown). The data therefore are consistent with a conductive carrier-mediated pathway for the canalicular excretion of the taurocholate anion.

DISCUSSION

In the present study we used simultaneously isolated and highly purified basolateral and canalicular rat liver plasma membrane vesicles to directly evaluate the driving forces for taurocholate transport across these two polar membrane domains of hepatocytes. The data obtained with bLPM confirm that the sinusoidal or basolateral uptake of taurocholate into hepatocytes is a secondary active transport process driven by the out to in Na⁺ gradient (Fig. 2) and mediated by an apparently electroneutral Na⁺-taurocholate cotransport system (Fig. 6; Table II; Refs. 9-11). In contrast, excretion of taurocholate from the cells into bile canalici, a process that is directly mimicked by taurocholate efflux from the isolated right-side out cLPM vesicles (Table I), is mediated by a Na⁺ independent anion carrier and appears to be driven by the physiologic intracellular negative membrane potential (Fig. ?). Finally, the present study also shows evidence for an electrogenic Na⁺-alanine cotransport system in cLPM (Figs. 1 and 6) that may reflect a potentially important mechanism for back reabsorption of metabolically precious neutral amino acids from bile canalici.

A high degree of purification of the two separated vesicular LPM subfractions is crucial for the correct assignment of the presented in vitro data to the two surface domains of hepatocytes. This question has been dealt with extensively in a previous validation of the subcellular fractionation procedure adopted in the present investigation (12). These earlier studies have shown that the presence of Na⁺K⁺-ATPase activity in

![Fig. 7. Efflux of taurocholate from cLPM vesicles](image-url)

The membranes were preloaded with 100 mM sucrose, 0.2 mM CaCl₂, 5 mM MgSO₄, 10 mM Hepes/Tris, pH 7.5, and either 100 mM NaCl (●) or 100 mM KCl (○). The vesicles were preloaded with 10 μM [³H]taurocholate and tracer efflux determined at 0 and 25 °C as described under “Experimental Procedures” (e.g. 10-fold dilution). The incubation media contained 100 mM tetramethylammonium chloride instead of NaCl and KCl and plus (——) or minus (—) 50 μM unlabeled taurocholate (TC). The data represent the mean ± S.D. of six determinations in two separate membrane preparations. B, membranes were resuspended in 100 mM K gluconate, 100 mM sucrose, 0.2 mM CaCl₂, 5 mM MgSO₄, 10 mM Hepes/Tris, pH 7.5. The vesicles were preloaded with 10 μM [³H]taurocholate, treated with valinomycin and incubated in the presence of 100 mM K (○) or tetramethylammonium gluconate (△) in the extravesicular medium. Data represent the mean ± S.D. of six determinations in two separate experiments.
Taurocholate Transport in Rat Liver Plasma Membrane Vesicles

Taurocholate transport is a sensitive criterion to test for eventual cross-contamination with basolateral membrane fragments and indicated that cLPM with undetectable Na⁺K⁺-ATPase activity are maximally contaminated with bLPM to an extent of 0.7%. Since in the present study only Na⁺K⁺-ATPase activity free LPM preparations were used for functional studies, it can be assumed that the results obtained with this LPM subtraction indeed reflect bile canalicular transport processes in vivo. In contrast, the bLPM subtraction is consistently contaminated to an extent of approximately 10% with canalicular membrane components. Since the cLPM vesicles exhibit a 2-fold higher intravesicular volume per milligram of protein compared to bLPM vesicles (see “Results”) it must be assumed that around 20% of the total intravesicular space in bLPM is enclosed by bile canalicular membranes. Nevertheless, because cLPM and bLPM are simultaneously isolated from the same homogenate and cLPM are virtually free of basolateral contaminants, functional phenomena that are exclusively present in bLPM can be correctly assigned to the basolateral pole of hepatocytes in vivo.

Recent studies from this and other laboratories have demonstrated direct evidence for a basolateral Na⁺-taurocholate cotransport system in sinusoidal rat liver plasma membrane vesicles isolated by different techniques (9-11). The present findings of a Na⁺ gradient induced by intravesicular accumulation of taurocholate within the bLPM vesicles (Fig. 2) as well as the Na⁺-stimulated countertransport (trans-stimulation) and tracer exchange of taurocholate (Table II) confirm these earlier reports and establish that the basolateral uptake of taurocholate into hepatocytes is driven by the out to in electrochemical Na⁺ gradient via an obligatory coupled Na⁺-taurocholate symport system. However, similar to rat intestinal brush-border membrane vesicles (23, 24), the rheogenicity of this hepatocellular Na⁺-taurocholate symport remains controversial (9-11). Thus, no effects of valinomycin-induced K⁺ diffusion potentials (inside negative) on Na⁺-stimulated taurocholate uptake were observed in bLPM isolated in this study (Fig. 6) as well as in our earlier studies with the mixed LPM fraction (11), suggesting electroneutral Na⁺-coupled taurocholate uptake, whereas others have provided evidence for an electrogenic (positively charged) Na⁺-taurocholate cotransport in sinusoidal plasma membrane vesicles (9, 10). Since our bLPM subtraction is still contaminated with cLPM (see above) an electrogenic basolateral transport system for taurocholate might be masked. However, the data clearly demonstrate that in bLPM the Na⁺-stimulated taurocholate uptake can overcome an inside negative membrane potential (Fig. 6) indicating that the physiologic output to in Na⁺ gradient can drive uphill transport of the anionic taurocholate against an otherwise unfavorable electrochemical gradient (intravesicular negativity; [taurocholate]intravesicle > [taurocholate]portal blood).

In contrast, in cLPM the Na⁺-stimulated taurocholate uptake was clearly inhibited by an intravesicular negative membrane potential created either by permeant anions (Table III) or by valinomycin-induced rapid outdiffusion of K⁺ (Fig. 6). Furthermore, efflux of taurocholate from the cLPM vesicles was stimulated by an artificially imposed intravesicular negativity (Fig. 7). Thus, transfer of taurocholate across the bile canalicular membrane appears to be invariably accompanied by transfer of a negative charge. Since efflux from cLPM vesicles represents the physiologic direction of solute movement in vivo (Fig. 1, Table I), these findings strongly indicate that the physiologic intravesicular negative membrane potential of approximately -30 to -40 mV (25, 26) can energize the canalicular excretion of the anionic taurocholate and most probably also of other bile acids. However, calculations based on the Nernst equation suggest that the electrical potential cannot be the only driving force for the movement of bile acids across the canalicular surface since it would account for only a 3- instead of a 10-fold concentration difference (1, 2). Additional driving forces might include extrusion of bile acid loaded vesicles into the canalicular lumen through exocytosis (27) and/or other direct energy dependent carrier-mediated transport mechanisms (28).

Our present observations that [3H]taurocholate uptake in cLPM exhibits saturation while efflux demonstrates countertransport (Fig. 7) strongly suggest that an integral bile acid anion carrier is present within the bile canalicular membrane. This conclusion is consistent with binding studies and photoaffinity labeling of isolated bile canalicular enriched plasma membrane subfractions using either bile acids (29) or photolabile bile acid analogues (30), respectively. Similar bile acid binding proteins have been demonstrated in basolateral as well as canalicular plasma membranes (30) supporting our findings that Na⁺ stimulates initial taurocholate uptake in both bLPM and cLPM (Figs. 2 and 5) despite intrinsic biochemical differences in the two transport systems as discussed above. We therefore speculate that a similar but differentially functioning bile acid carrier may be present at the two polar domains of hepatocytes. Further experimental validation must include isolation and functional reconstitution of the putative transport protein(s) present in basolateral (31, 32) as well as canalicular rat liver plasma membranes.

Our studies also provide evidence for electrogenic Na⁺-dependent alanine uptake into cLPM vesicles (Figs. 2 and 7, Table III). Preliminary observations suggest that this canalicular Na⁺-stimulated alanine uptake exhibits countertransport and is not inhibitable by the amino acid analog 2-methylaminoisobutyric acid. These criteria have been reported to be characteristic for the so called “ASC-system” in intact cells including rat hepatocytes (33, 34). Thus, the ASC-system rather than the “A-system” may be present at hepatic canalicular liver plasma membranes, where it could primarily serve to reabsorb cysteine, a breakdown product of canalicular secreted reduced glutathione. The intriguing possibility that the various amino acid transport systems are differentially distributed over the two polar surfaces of hepatocytes is under further investigation in this laboratory.

In summary, our findings demonstrate that transepithelial excretion of taurocholate is a secondary active transport driven by the electrochemical potential difference for Na⁺ across the basolateral membranes (Na⁺-taurocholate cotransport) and by the electrical potential difference across the bile canalicular membranes. The canalicular excretory step is mediated by an anion carrier the molecular characteristics of which remain to be exactly defined. The presented biochemical differences between bLPM and cLPM extend the previously reported evidence for functional polarity of the various hepatocellular surface domains (12) and confirm that the isolated basolateral and canalicular LPM subfractions are suitable for further evaluation of the physiologic polarization of the liver cell.

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