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Relationship of hepatic cholate transport to regulation of intracellular pH and potassium

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Modulation of hepatic cholate transport by transmembrane pH-gradients and during interferences with the homeostatic regulation of intracellular pH and K^+ was studied in the isolated perfused rat liver. Within the concentration range studied uptake into the liver was saturable and appeared to be associated with release of OH^- and uptake of K^+. Perfusate acidification ineffectually stimulated uptake. Application of NH_4Cl caused intracellular alkalinization, release of K^+ and stimulation of cholate uptake, withdrawal of NH_4Cl resulted in intracellular acidification, regain of K^+ and inhibition of cholate uptake. Inhibition of Na^+/H^+-exchange with amiloride reduced basal release of acid equivalents into the perfusate, initiated K^+-release, and inhibited both, control cholate uptake and its recovery following intracellular acidification. K^+-free perfusion caused K^+-release and inhibited cholate uptake. K^+-readmission resulted in brisk K^+-uptake and recovery of cholate transport. Both effects were inhibited by amiloride. Interference with cholate transport through modulation of pH homeostasis by diisothiocyanostilbenedisulfonate (DIDS) could not be demonstrated because DIDS affected bile acid transport directly. Biliary bile acid secretion was stimulated by intracellular alkalinization and by activation of K^+-transport. Uncoupling of the mutual interference between pH-dependent cholate uptake and K^+-transport by amiloride indicates tertiary active transport of cholate. In this, Na^+/K^+-ATPase provides the transmembrane Na^+-gradient to sustain Na^+/H^+-exchange which maintains the transmembrane pH-gradient and thus supports cholate uptake. Effects of canalicular bile acid secretion are consistent with a saturable, electrogenic transport.

Introduction

Uptake of bile acids across the sinusoidal liver cell membrane from portal venous blood provides for their continuous supply for bile canalicular secretion. Cholic acid represents a major fraction of bile acids returning to the liver by enterohepatic circulation (approx. 50 μM in rat portal venous blood [1]) and is avidly excreted by the organ after its intracellular conjugation [2].

The mechanism of hepatic uptake has been studied in the isolated perfused rat liver, in isolated hepatocytes and in isolated plasma membrane vesicles. From these kinetic studies a Na^+-dependent and a Na^+-independent saturable and energy dependent, carrier mediated transport component have been identified [3–11]. Both transport systems are shared by other bile acids and other substrates [12]. Furthermore, a non-saturable diffusional component has been identified in isolated cells and cell membrane vesicles [8,13,14]. A more recent study by Blitzer et al. [15] showed that cholate uptake in isolated membrane vesicles depends on the transmembrane pH-gradient suggestive of a carrier mediated cholate/OH^--exchange mechanism. Similar observations were made by Hugentobler and Meier [16] but appeared to be entirely explained by non-ionic diffusion of the bile acid [17].

The aim of this study was to investigate whether cholate uptake is mediated by pH-gradient dependent transport in intact liver. Using the isolated perfused rat liver, we studied the saturability of uptake, the effects of variations of intracellular and extracellular pH, and the effects of maneuvers that interfere with intracellular pH-regulation. These latter experiments included inhibition of Na^+/H^+-exchange by amiloride,

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effects of diisothiocyanostilbenedisulfonate (DIDS), an inhibitor of anion transport systems, and disturbances of the transmembrane Na^+-concentration gradient by removal of external K^+ and inhibition of Na^+/K^+-ATPase.

The data show that a fraction of cholate uptake is dependent on the transmembrane pH-gradient and that interferences with pH-regulation that increase or decrease intracellular pH activate or inhibit cholate uptake, respectively. Association of pH-dependent cholate uptake with changes of K^+-transport indicate that Na^+/H^+-exchange is linked to Na^+/K^+-ATPase activity and provides for intracellular alkalinization that supports cholate uptake. This pH-dependent transport may coexist with Na^+-driven uptake operating at low bile acid concentrations with low transport capacity.

Furthermore, we noticed that maneuvers known to affect the transmembrane electric potential (intracellular pH (pH_i) and transmembrane K^+-concentration gradient) led to changes in bile acid secretion, an observation consistent with the notion that canalicular secretion of bile acids is accomplished by an electrogenic transport system.

Materials and Methods

Male Louvain rats (Versuchstierzucht und -haltung der Universität Wien, Himberg, Austria), fed ad libitum and weighing between 200 and 250 g, were used as liver donors. Surgical isolation of the liver was done as previously described [18] and livers were perfused at 37°C in a non-recirculating system with control Krebs-Henseleit buffer containing 120 mM NaCl, 4.75 mM KCl, 2.57mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.18 mM $MgSO_4$, and 25 mM $NaHCO_3$, gassed with 95% O_2/5% CO_2. For HCO_3^--free perfusion $NaHCO_3$ was replaced by 10 mM Hepes/NaOH and solutions were gassed with 100% O_2. For K^+-free perfusion K^+-salts were replaced by the corresponding Na^+-salts and, in experiments performed to test for the hypothesis that SO_4^{2-} may inhibit cholate transport, 120 mM NaCl was substituted by 60 mM Na_2SO_4 and 60 mM mannitol. A perfusate with low buffer capacity (25 mM $NaHCO_3$ replaced by 3 mM NaH_2PO_4/Na_2HPO_4, 142 mM NaCl, 1.5 mM $CaCl_2$) was used to measure H^+ or OH^- uptake by the organ. Perfusate flow-rate was constant in individual experiments and ranged between 3 to 3.5 ml/g liver per min. A peristaltic pump (model PA-SF, IKA-Labortechnik, Straufen i. Br., Germany) was used.

The bile duct was cannulated with PE 10 tubing, bile was collected drop by drop and bile flow was measured by the frequency and weight (8 to 9 mg) of bile drops. Radiolabeled [^{14}C]cholic acid (Fluka AG, Buchs, Switzerland/Amersham Intl., Amersham, UK) was added to the inflowing perfusate at concentrations ranging from 10 to 150 μM and uptake was measured by the porto-caval concentration difference. Effluent perfusate samples were collected in 10 to 60 s intervals and radioactivity was determined in a liquid scintillation counter (model LS 230, Beckman Instr., Palo Alto, CA, USA). Quenching was corrected by internal and external standardization and correction was of particular significance in experiments employing DIDS.

Perfusate pH was varied between 7.0 and 7.8 using Hepes-buffered perfusate medium. Intracellular alkalinizations and acidifications were achieved using the NH_4^+-pulse technique [19,20]: In these experiments 20 mM NaCl was replaced by 20 mM NH_4Cl for a 10 min period, resulting in intracellular alkalinization upon addition of NH_4Cl and in acidification upon its withdrawal.

Potassium was measured in a Nova 6 analyzer (Nova Biomedical, Newton, MA, USA) and the pH of the effluent perfusate was continuously monitored with a pH-electrode (Radiometer, Copenhagen, Denmark).

Biliary excretion of radioactive bile acid was determined by multiplying bile flow with biliary bile acid concentration. Due to the dead space of the biliary tree and the bile duct cannula, radioactivity in bile appeared with a time delay after onset of the stimulation of bile flow by application of cholic acid. Canalicular bile acid excretion was therefore calculated by correcting for a dead space of 20 to 30 mg of bile. Estimates for the excretion rate at given time points were obtained by linear interpolation between time points where samples were collected.

Amiloride was obtained from Merck, Sharp and Dome (West Point, PA, USA), Hepes and DIDS from Sigma Chemicals (St. Louis, MO, USA) and all other chemicals were of analytical grade (Austro-Merck, Vienna, Austria).

Saturation kinetics were calculated from average sinusoidal concentration (C_{av}) versus uptake (V). C_{av} was calculated by

$$C_{av} = (C_{in} - C_{out})/\ln(C_{in}/C_{out})$$

according to Vilstrup et al. [21]. By plotting $-C_{av}$ on the x- and V on the y-axis, respectively, each experiment was presented by a single connecting line. Intercepts of these lines give K_m and V_{max}, respectively (Cornish-Bowden plots). This method revealed significantly different values for affinity and maximal uptake rate at low (10–50 μM) and high (50–150 μM) concentration ranges. Kinetic parameters were therefore formally described by two saturable transport mechanisms

$$V = V_{max1} * C_{av}/(K_{m1} + C_{av}) + V_{max2} * C_{av}/(K_{m2} + C_{av})$$

using the Enzfitter-program (Elsevier-Biosoft, Cambridge, UK).

Results

(A) Kinetics of cholic acid uptake

Isolated livers were perfused in a single pass perfusion system alternatively fed from different reservoirs. In the experiments described below, bile acid uptake was measured continuously in order to detect small changes of uptake rates. The time dependence of uptake was therefore first established by depleting the organ for 30 min of endogenous bile acids and then shifting the single pass perfusion to a medium containing radioactive cholic acid. Radioactivity appeared in the venous effluent after a delay of 20 to 30 s. Then, venous radioactivity reached a plateau value after 50 s. At an inflow concentration of 10 μM this value remained constant for at least 20 min indicating that extraction of cholic acid stabilized at approx. 95%. At higher concentrations in the inflowing perfusate, venous concentration slowly increased after 60 s indicating that the rate of extraction decreased. Table I shows uptake rates for concentrations between 10 and 100 μM measured 1 min and 5 min after perfusion had been shifted to the bile acid containing medium. Because of the protracted equilibration of the system and the observed decrease of uptake at higher concentrations we analyzed the kinetics of uptake 60 s after bile acid admission. Uptake exhibited complex saturation kinetics: Determination of K_m at low (10–20 μM) and high concentration (50–150 μM) ranges using Cornish-Bowden plots suggested the existence of at least two different transport systems, one with high affinity ($K_m = 30 \pm 10$ μM) and a second with low affinity ($K_m = 72 \pm 7$ μM). Uptake kinetics were therefore formally described by two saturable transport systems operating in parallel. Fig. 1 shows rates of uptake as well as the theoretical concentration/uptake relationship obtained by curve fitting. Calculation of kinetic parameters from low concentrations (10–20 μM) and high concentrations (50–150 μM) revealed K_{m1} 16 μM, $V_{max1} = 108$ nmol/g liver per min and $K_{m2} = 140$ μM, $V_{max2} = 736$ nmol/g liver per min, respectively. A more detailed analysis of the low-affinity transport system appeared unfeasible because at higher bile acid concentrations we could not observe a steady-state bile acid uptake and, furthermore, bileflow decreased. The data do not fully exclude that uptake at high bile acid concentrations is not saturable.

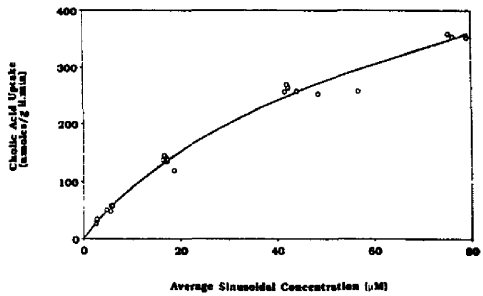

Fig. 1. Dependence of cholic acid uptake on average sinusoidal concentration. Livers were perfused with control medium and 2 min pulses of cholic acid at concentrations ranging between 10 and 150 μM were applied. Uptake was determined from the portal to hepatic venous concentration difference 1 min after application of the bile acid. Kinetic data and the theoretical concentration/uptake-relation shown were obtained by a least-square fit as described in Methods. Estimates for V_{max} and K_m are given in the text.

Perfusion with HCO_3^--free media revealed slightly lower values of K_m and V_{max} for the 'low-affinity' transport system (compare Fig. 1 and Table II). In preliminary experiments we did not observe differences of intracellular pH with HCO_3^--containing and HCO_3^--free, Hepes-buffered perfusion (compare Ref. 22). The observed reduction of uptake may therefore be attributed to the lack of HCO_3^- but other interferences of Hepes, e.g. reduced bile flow, cannot be excluded.

TABLE I

Time- and dose-dependence of cholic acid uptake

Uptake rates (nmol/g liver per min) for concentrations of cholic acid between 10 and 100 μM measured 1 and 5 min after shifting from control buffer to bile acid containing medium. The last column is the ratio of values obtained at 5 min and 1 min, respectively. Mean values ± S.D., $n = 3$.

Cholic acid concn. (μM)	Cholic acid uptake rate		Ratio
	1 min	5 min	
10	30.9 ± 3.3	30.8 ± 3.3	0.996
20	52.8 ± 4.4	52.2 ± 4.4	0.988
50	122.0 ± 9.2	116.4 ± 9.0	0.954
100	263.3 ± 5.4	223.0 ± 3.0	0.847

TABLE II

Influence of external pH on cholic acid uptake

Effect of varying perfusate-pH (pH_e) on uptake of cholic acid (nmol/g liver per min). Livers were perfused with concentrations of cholic acid of 20, 50 and 100 μM at a pH_e of 7.0, 7.4, and 7.8. Values given were determined 1 min after perfusion had been shifted from control buffer to bile acid containing medium. Mean values ± S.D., $n = 3$. Significance was tested with Student's t-test.

Cholic acid concn. (μM)	Cholic acid uptake rate		
	$pH_e = 7.0$	$pH_e = 7.4$	$pH_e = 7.8$
20	59.0 ± 2.7	58.8 ± 2.6	58.6 ± 2.8
50	137.7 ± 5.8	136.7 ± 6.9	132.9 ± 4.1
100	247.7 ± 8.5 [a]	240.0 ± 5.2	228.7 ± 5.3 [a]

[a] Significantly different from each other $P < 0.1$.

(B) Effects of transmembrane pH-gradients on transport of cholic acid

Reduction of extracellular pH (pH_e) increased uptake of cholic acid whereas extracellular alkalinization resulted in a decrease of uptake. As shown in Table II, this effect appears at higher concentrations only. Since increase of uptake by acidification may indicate that the undissociated bile acid is preferentially accepted by the transport system or may be subject to non-ionic diffusion, we calculated the relative proportions of undissociated acid/total concentration for pK values reported for free solution and in the presence of phospholipids [23], respectively. As expected, the relative concentration of undissociated acid varies within a wide range between pH 7.0 and 7.8, but this variation does not correlate with the relatively small dependence of uptake on extracellular pH. Furthermore, a change of extracellular pH by 0.4 units would alter the transmembrane H^+- or OH^--concentration gradient by a factor of 2.5 which may indicate that the dependence of cholic acid uptake on extracellular pH is not a simple function of the transmembrane pH gradient.

Intracellular pH (pH_i) was varied by using the NH_4Cl pulse technique. As previously shown in isolated hepatocytes by measurements with ion selective electrodes [19] or with the fluorescent intracellular pH-indicator 2',7'-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF) [20] application of NH_4Cl results in transient increase of pH_i by 0.5 units whereas withdrawal of NH_4Cl decreases pH_i by 0.5 units below control. pH_i then recovers to control values mainly through stimulation of Na^+/H^+-exchange. In the perfused liver, the same maneuver resulted in alkalinization and acidification of 0.2 and 0.2 pH-units, respectively [24]. In order to study the effects of pH_i on uptake of cholic acid, livers were continuously perfused with a bile acid containing medium and alterations of bile acid uptake and excretion were analyzed during and after a 10 min pulse of 20 mM NH_4Cl. As shown in Fig. 2a, intracellular alkalinization caused a small transient increase of bile acid uptake and acidification resulted in a pronounced decrease. Biliary excretion of bile acid was markedly stimulated by alkalinization (Figs. 2b and 3) but remained nearly unaffected by acidification (Fig. 2b). As seen for the variation of extracellular pH, modification of bile acid transport by variation of pH_i was more pronounced in the higher concentration range. It may be noted that Fig. 2a represents net uptake rates at points of time where considerable amounts of bile acid had accumulated in the organ (Fig. 2c). Inhibition of sinusoidal net uptake by intracellular acidification could thus result from stimulation of unidirectional efflux rather than inhibition of unidirectional influx. Furthermore, canalicular efflux is stimulated during intracellular alkalinization (Figs. 2b and 3) probably due to cell membrane hyperpolarization caused by an increase of K^+-conductance (see Fig. 4 and Discussion) [25–27]. Although this could indicate that uptake depends on the rate of

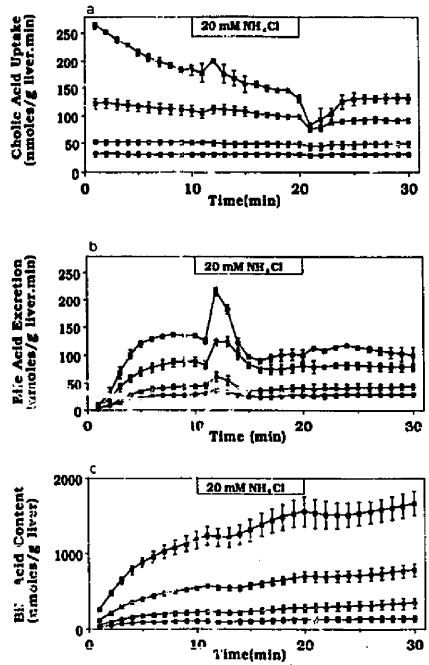

Fig. 2. Effects of changes of intracellular pH on transport of cholic acid. Transport was measured during continuous portal supply of cholic acid at concentrations of 10 (•), 20 (○), 50 (×) and 100 (■) μM (lower to upper line). pH_i was modified by addition of NH_4Cl to the perfusate for a 10 min period. Application of NH_4Cl resulted in intracellular alkalinization and its withdrawal in acidification. Mean values ± S.D., two or three experiments at each concentration. (a) Uptake of cholic acid; (b) biliary bile acid secretion; (c) hepatic bile acid content determined by continuous summation of uptake and secretion.

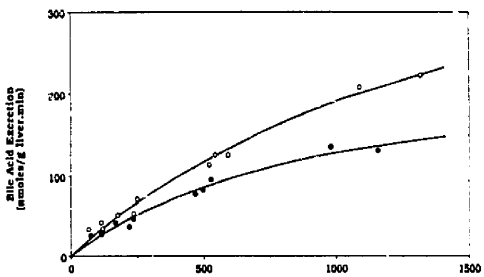

Fig. 3. Dependence of canalicular bile acid excretion on hepatic bile acid content. Data are from Figs. 2b and c, showing biliary bile acid secretion as a function of hepatic bile acid content determined 2 min before (closed circles) and 2 min after intracellular alkalinization obtained by application of NH_4Cl (open circles). Kinetic parameters obtained by curve fitting were: 'K_m'(control) 1016 nmol/g liver, V_{max}(control) 262 nmol/g liver per min; 'K_m'(alkal.) 1625 nmol/g liver V_{max}(alkal.) 501 nmol/g liver per min.

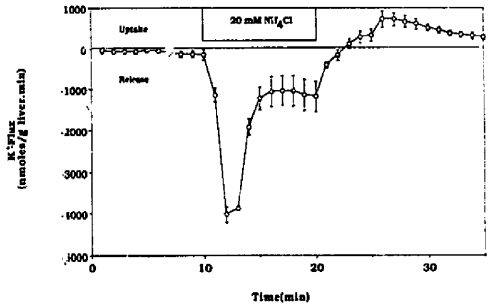

Fig. 4. Effect of changes of intracellular pH on transmembrane K^+-flux. Experimental protocol as in Fig. 2. Data show that intracellular alkalinization obtained by application of NH_4Cl is associated with a pronounced cellular release of K^+ into the perfusate whereas intracellular acidification by removal of NH_4Cl results in protracted regain of K^+. These pattern of changes of K^+-flux is comparable to variations of pH_i seen under comparable conditions (compare Ref. 25). Mean values ± S.D., $n = 3$.

canalicular extrusion we found no correlation of uptake rate to total hepatic bile acid content at perfusate concentrations below 50 μM.

In order to study whether cholic acid transport is affected by interference with mechanisms that physiologically regulate intracellular pH, we analyzed the effects of DIDS and amiloride, substances known to inhibit $NaHCO_3$ symport and Na^+/H^+-exchange, respectively.

Addition of 1 mM DIDS during continuous administration of cholic acid instantaneously and drastically reduced uptake at low and high concentrations. Neither the speed of onset nor the magnitude of inhibition of uptake by DIDS are consistent with its minimal effect in reducing pH_i as observed in isolated liver cells [22,28]. At an inflowing concentration of 20 μM cholic acid application of 1 mM DIDS reduced uptake from 57 to 12 nmol/g liver per min (single experiment). Additionally, during continuous application of 100 μM cholic acid application of DIDS actually induced net bile acid release into the effluent perfusate (Fig. 5a), an observation suggesting that DIDS could be transported by the bile acid carrier and that uptake of DIDS may cause bile acid efflux through transstimulation. In order to test this hypothesis we perfused the liver for 30 min with 100 μM cholic acid and measured venous efflux of bile acid during subsequent perfusion with cholic acid-free medium. After 5 min these bile acid loaded livers released 31.8 ± 1.6 nmol/g liver per min and subsequent application of 1 mM DIDS stimulated release to 91.7 ± 4.2 nmol/g liver per min within 3 min (Fig. 5b). Application of DIDS resulted in stimulation of bile flow (from 2.8 to 5.9 mg/g liver per min), reduced biliary bile acid concentration (from 39.2 to 34.7 mM), but augmented bile acid excretion (from 108.9 to 204.8 nmol/g liver per min). Thus, application of DIDS could not be used to further resolve the mechanism of pH-dependent cholate transport but the observations support the notion that cholate uptake, also at high concentrations, is carrier-mediated.

Application of 1 mM amiloride caused a gradual and reversible reduction of cholic acid uptake (Table III). Amiloride also intensified the inhibiting effect on cholic acid uptake produced by intracellular acidification through removal of NH_4Cl. 2 min after removal of NH_4Cl at an inflow concentration of cholic acid of 20 μM, the inhibition was 5.5 ± 2.1% and 8.5 ± 4.8% in the presence and absence of amiloride, respectively. These observations suggest that modification of intracellular pH through inhibition of Na^+/H^+-exchange affect cholic acid transport. At steady-state bile acid excretion, application of 1 mM amiloride reduced secretion from 53.7 ± 1.9 to 47.1 ± 1.4 and from 124.5 ± 9.5 to 103.7 ± 12.0 nmol/g liver per min at 20 μM and

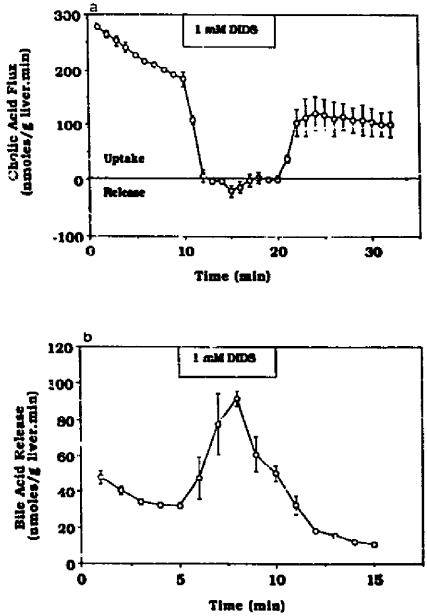

Fig. 5. Effect of DIDS on bile acid transport. (a) Effect of DIDS on uptake of cholic acid. Beginning with time zero livers were continuously perfused with 100 μM cholic acid and uptake was measured. Addition of DIDS (1 mM) to the perfusate resulted in instantaneous inhibition of uptake and in bile acid release. Uptake recovered upon removal of DIDS. Mean values ± S.D., $n = 2$. (b) Effect of DIDS on sinusoidal bile acid release. Livers were bile acid loaded by perfusion with 100 μM cholic acid for 30 min. Subsequently (time zero) hepatic bile acid release into the venous perfusate was measured during bile acid free perfusion. 5 min after shifting to cholic acid-free perfusion DIDS (1 mM) was added to the perfusion medium resulting in stimulation of bile acid release into the perfusate. Mean values ± S.D., $n = 2$.

TABLE III

Effect of the Na^+/H^+-exchange inhibitor amiloride (1 mM) on steady-state uptake (nmol/g liver per min) of cholic acid

Values were determined 2 min after shifting to the amiloride-containing medium. Mean values ± S.D., $n = 3$. Significance was tested with paired Student's t-test.

Cholic acid concn. (μM)	Cholic acid uptake rate	
	control	+ 1 mM amiloride
20	58.1 ± 3.9	56.6 ± 4.4 [a]
50	123.2 ± 5.3	111.4 ± 0.6 [a]

[a] Significantly different from control ($P < 0.05$).

50 μM inflow concentration of cholic acid, respectively (compare Ref. 29).

(C) Correlation of cholic acid transport with pH regulation and Na^+/K^+-pumping

In previous experiments [30] amiloride had been shown to cause release of liver cell K^+, probably by inhibiting Na^+-influx through Na^+/H^+-exchange and subsequent reduction of the supply of Na^+ for the Na^+/K^+-pump. Because of this functional correlation between Na^+/H^+-exchange and Na^+/K^+-pumping we hypothesized that cholic acid uptake may result in cellular OH^--release and in intracellular acidification which, in turn, would sequentially stimulate Na^+/H^+-exchange and Na^+/K^+-pumping. Conversely, inhibition of Na^+/K^+-ATPase would increase intracellular Na^+-concentration, reduce the activity of Na^+/H^+-exchange and thus result in intracellular acidification and in inhibition of uptake of cholic acid. To study this concept, experiments were carried out to answer the following questions:

(1) Is the uptake of cholic acid accompanied by cellular release of OH^-?

(2) Has the uptake of cholic acid any effect on cellular K^+-balance and is this effect modified by amiloride?

(3) Does the inhibition of Na^+/K^+-ATPase by K^+-removal influence the transport of cholic acid?

(4) Does the activation of Na^+/K^+-ATPase by K^+-readmission influence the transport of cholic acid and is this effect modified by amiloride?

Hepatic release of acid or base equivalents (see Table IV) was measured by the porto-caval pH difference at low perfusate buffer capacity (3 mM phosphate buffer). In agreement with previous observations [31], 30 min after the beginning of the perfusion experiment the liver released 1067 ± 169 nmol/g liver per min acid equivalents at a stable rate. This rate was changed by the application of cholic acid: addition at high concentrations (100 μM) resulted in a transient alkalinization of the effluent perfusate. After 5 min continuous application of cholic acid, base release ceased and subsequent removal of cholic acid resulted in an abrupt and transient acidification of the effluent perfusate.

TABLE IV

Rapid changes of H^+/OH^--fluxes in isolated perfused rat liver

The pH of the effluent perfusate was continuously monitored using a pH-sensitive electrode. Values (nmol/g liver per min) are calculated from the buffer capacity and the flow rate of the perfusate and are given as maximal changes obtained during a 5 min pulse with 100 μM cholic acid and upon its removal in the absence ($n = 4$) and presence ($n = 3$) of 1 mM amiloride, respectively. Mean values ± S.D. Significance was tested with Student's t-test.

	H^+/OH^- flux		
	control	+ 1 mM amiloride	
Addition of cholic acid	78.7 ± 19.0	122.8 ± 20.7 [a]	H^+-uptake
Removal of cholic acid	117.2 ± 21.0	not detectable [a]	H^+-release

[a] Significantly different from control ($P < 0.05$).

1 mM amiloride reduced basal acid release by 174.3 ± 42.2 nmol/g liver per min within 2 min. On top of this changed base-line value, maximal base release induced by 100 μM cholic acid was augmented and no perfusate acidification was seen after removal of cholic acid. These observations indicate that cholate uptake is associated with cellular release of OH^- (or uptake of H^+), followed by stimulation of Na^+/H^+-exchange.

Cholic acid uptake resulted in a cellular gain of K^+. After application of cholic acid K^+-uptake reached a maximum after 90 sec and returned to baseline balance within 5 min. Amiloride (1 mM) given alone induced K^+-release (compare Ref. 30) and simultaneous application of 50 μM cholic acid and 1 mM amiloride resulted in K^+-release at a rate similar to the effect on of amiloride alone (see Table V).

K^+-free perfusion of the liver decreased uptake of cholic acid. As shown in Fig. 6c, inhibition of uptake became pronounced after the liver had been depleted by approx. 10 μmol K^+/g tissue (Fig. 6b). Readmission of K^+ to the inflowing perfusate caused reuptake of K^+ (Fig. 6a) and uptake of cholic acid recovered to

TABLE V

Effect of 50 μM cholic acid and amiloride (1 mM) on transmembrane K^+-flux (nmol/g liver per min)

Values were determined 90 s after perfusion had been shifted from control buffer to a medium containing amiloride, the bile acid or both. Mean values ± S.D., $n = 5$. Values for 1 mM amiloride without bile acid are taken from Ref. 30.

	K^+-flux	
1 mM amiloride	− 186 ± 18	K^+-release
50 μM cholic acid	+ 213 ± 90	K^+-uptake
50 μM cholic acid + + 1 mM amiloride	− 200 ± 90	K^+-release

control values. In the presence of 50 μM cholic acid K^+-depletion for 15 min reduced bile flow (from 2.43 $\pm$ 0.07 to 1.93 $\pm$ 0.16 mg/g liver per min) and bile acid secretion (Fig. 6d). K^+-readmission resulted in an instantaneous transient stimulation of both, bile flow and bile acid secretion (Fig. 6d), followed by their gradual return to control values. In a second set of experiments effects of K^+-depletion and K^+-readmission were studied during the continuous presence of 50 μM cholic acid and 1 mM amiloride. Simultaneous administration of the bile acid and amiloride resulted in gradual reduction of both, uptake and secretion. Subsequent removal of K^+ further decreased cholic acid transport and K^+-readmission transiently stimulated bile acid secretion, but the recovery of uptake and reactivation of Na^+/K^+-ATPase, seen in the absence of amiloride, was blunted (Figs. 6a–d). It should be pointed out that in the intact organ amiloride at a concentration of 1 mM as used in this study does not affect the Na^+/K^+-ATPase [33].

(D) Supplementary experiments

Several additional experiments were carried out to test for the requirement of HCO_3^- and Na^+ in cholic acid transport and for possible inhibitory effect of sulfate.

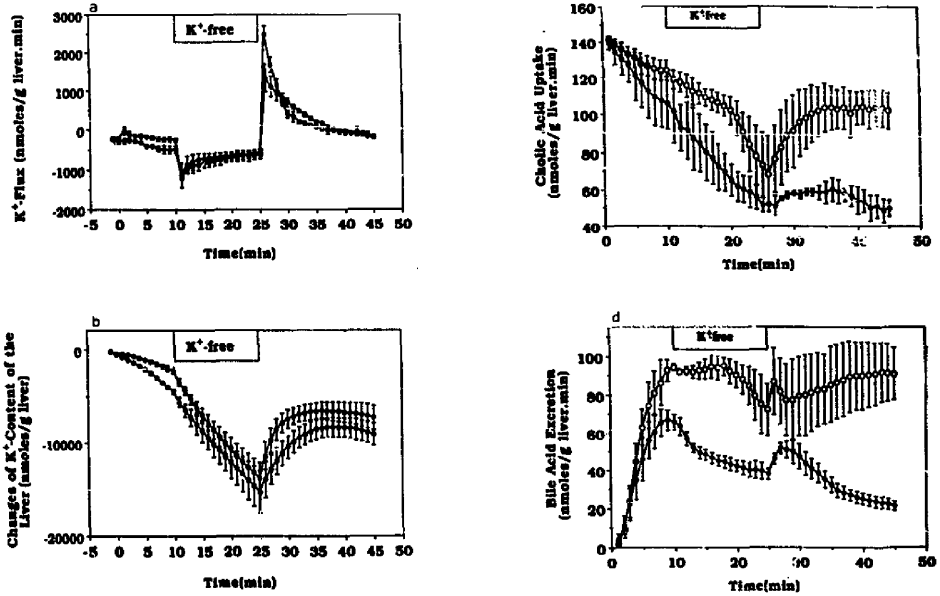

Fig. 6. Effect of K^+-depletion and K^+-readmission on transmembrane K^+-flux (a), K^+-content of the liver (b), cholic acid uptake (c) and biliary bile acid secretion (d) in the absence (open circles) and presence (closed circles) of 1 mM amiloride. (a and b) Superimposition on a small release of K^+, generally encountered in perfusion experiments, application of 50 μM cholic acid (time zero) resulted in a transient gain of K^+ in the absence but not in the presence of amiloride. Removal of K^+ from the perfusate led to a release of K^+ at a rate decreasing with time and resulted in gradual depletion of intracellular K^+ (b). Readmission of K^+ caused instantaneous uptake of K^+ probably due to both, the availability of external K^+ for K^+-pumping and the rise of intracellular Na^+-concentration that expected to occur during K^+-depletion [39]. Note that regain of K^+ is blunted if amiloride is present. This effect may be attributed to a reduced gain of Na^+ during the phase of K^+-depletion. (c) After addition of cholic acid, uptake reached a maximum (1st min) and slowly decreased thereafter (compare Table I). Simultaneous application of cholic acid and amiloride had no instantaneous effect but the decreased uptake rate was accelerated. K^+-free perfusion resulted in a further decrease of uptake, this inhibition became particularly pronounced when cell K^+ was reduced by more than 10 μmol/g liver (compare (b)). Readmission of K^+ led to recovery of uptake, this effect being blunted if amiloride was present. (d) Biliary bile acid excretion rises after administration of cholic acid reaching a maximum after 10 min. Secretion is reduced in the presence of amiloride probably due to the decrease of uptake (compare (c)). Similarly, decreasing rates of secretion during K^+-depletion in both, control experiments and during the presence of amiloride, appear to result from reduced uptake rates. K^+-readmission instantaneously increases bile acid secretion. This is followed by a second phase of recovery which is not seen in the presence of amiloride. The instantaneous increase of excretion is likely due to result from membrane hyperpolarization caused by activation of electrogenic Na^+/K^+-pumping (compare (a)), whereas the second phase of recovery appears to result from the gradual increase of uptake. The pattern of changes of bile acid secretion were accompanied by similar changes of bile flow rate. Bile flow (mg/g liver per min), measured 2 min before application of cholic acid, 10 min after application, 15 min after K^+-depletion and during maximal stimulation after K^+-readmission was 1.09 $\pm$ 0.13, 2.40 $\pm$ 0.10, 1.93 $\pm$ 0.17, 2.43 $\pm$ 0.32 and 0.93 $\pm$ 0.21, 1.85 $\pm$ 0.21, 1.17 $\pm$ 0.08, 1.61 $\pm$ 0.03 in the absence and presence of amiloride, respectively.

HCO_3^--free perfusion had no effect on the variation of cholic acid uptake induced by intracellular alkalinization and acidification during and after the NH_4Cl-pulse, but recovery of uptake after acidification was protracted. Effects of K^+-removal and K^+-readmission on cholic acid transport were also comparable to experiments performed in bicarbonate-containing media, both in the presence and absence of amiloride (single experiment each, data not shown). Complete replacement of perfusate Na^+ by Li^+ reduced cholic acid uptake. At 20 μM inflow concentration of cholic acid, shifting to Na^+-free medium resulted in a reduction of uptake from 55 ± 4 to 46.0 ± 1.7 nmol/g liver per min. Earlier observations showed that replacement of Na^+ by choline did inhibit uptake [5] whereas replacement by Li^+ had only a minor effect (this study and Ref. 12). In view of the inhibitory effect of amiloride on cholate transport, the discrepancy may be explained by the fact that replacement of Na^+ by choline inhibits Na^+/H^+-exchange, whereas this transport is insensitive to replacement by Li^+ [32]. In this case, apparent Na^+-dependence of cholate transport [5,12] could have resulted from affecting the driving force of Na^+/H^+-exchange, whereas Na^+/cholate cotransport represents a small fraction of uptake only. Partial replacement of Cl^- by SO_4^{2-} (60 mM) had no significant inhibitory effect on uptake of cholic acid (20 and 50 μM) and variations of uptake by the NH_4Cl-pulse remained unaffected. It appears thus unlikely that OH^-/SO_4^{2-}-exchange mediates cholate uptake in the intact liver (compare Ref. 16).

Discussion

This study was designed to evaluate the mechanisms responsible for the transport of cholic acid in intact rat liver. Several characteristics of transport were observed that partially support previous conclusions from transport studies in isolated liver cells or cell membrane vesicles. These characteristics are set into relation to liver cell pH-regulation and K^+-homeostasis. Results include the following: (i) transport kinetics suggest the existence of more than one transport system, one of which with high affinity and low capacity that appears Na^+-dependent, the other component has low affinity and high capacity and may include a diffusional component; (ii) both, extracellular acidification and intracellular alkalinization, stimulate cholic acid uptake in the high concentration range; (iii) interference with regulatory mechanisms of cell pH affect cholic acid uptake: inhibition of uptake by amiloride appears to result from intracellular acidification through inhibition of Na^+/H^+-exchange, whereas inhibition by DIDS appears to result from competitive inhibition; (iv) uptake of cholic acid is associated with cellular release of OH^- (or uptake of H^+). Taken together, these data indicate that uptake of cholic acid depends on the transmembrane pH gradient and that it is associated with cotransport of H^+ or countertransport of OH^- ions; (v) uptake of cholic acid is accompanied by cellular uptake of K^+ which suggests that cholic acid uptake leads to intracellular acidification that results in subsequent stimulation of Na^+/H^+-exchange and activation of Na^+/K^+-ATPase. Support of this hypothesis is obtained through the observations that (vi) application of amiloride prevents K^+-uptake; (vii) inhibition of Na^+/K^+-ATPase reduces uptake of cholic acid and (viii) activation of Na^+/K^+-ATPase stimulates uptake of cholic acid, an effect that is blunted by amiloride. Combining these observations, we tentatively interprete these results by the coupling of transport mechanisms as follows.

Cholic acid uptake is associated with intracellular acidification either through uptake of the bile acid in form of the undissociated acid that may include non-ionic diffusion or through cholate$^-$/OH^--exchange. Subsequently, intracellular acidification would stimulate Na^+/H^+-exchange resulting in a rise of intracellular Na^+-concentration which, in turn, stimulates Na^+/K^+-ATPase and thus results in uptake of K^+. Conversely, inhibition of Na^+/K^+-ATPase results in a rise of intracellular Na^+-concentration that leads to reduced activity of Na^+/H^+-exchange, intracellular acidification and inhibition of uptake of cholic acid.

The pH-dependence of cholic acid uptake and its relation to Na^+/H^+-exchange and Na^+/K^+-ATPase activity deserve specific comments.

(1) pH-dependence of cholic acid uptake

pH-dependence of cholic acid uptake had been observed in plasma membrane vesicles and isolated hepatocytes. Interpretations of these observations included the presence of a cholate$^-$/OH^--exchange system [15], some data suggested that cholate could be a substrate of the SO_4^{2-}/OH^--exchanger [16], and, since cholic acid transport is pH-dependent in lipid membranes, non-ionic diffusion could be responsible for pH-dependent transport in liver cell membranes [17]. Our data confirm the dependence of cholate uptake on the transmembrane pH gradient in the intact organ. We observe transport kinetics that are consistent with two transport systems operating in parallel, whereof the low affinity, high capacity component is pH sensitive. Uptake at these high concentrations could include a diffusional component. In the absence of HCO_3^-, we obtained lower values for uptake of cholic acid, perhaps indicating that HCO_3^- is a component of the transport system. Uptake of cholic acid at low concentrations could proceed in part through the Na^+-dependent bile acid carrier, presumably identical with a 48 kDa membrane protein recently identified [34]. This suggestion is supported by the observation that re-

placement of Na^+ by Li^+ inhibits a small fraction of cholic acid uptake [8,12]. Uptake at high concentrations was not inhibited by SO_4^{2-} and the pH-dependence of uptake was unaltered. These observations make it appear unlikely that uptake of cholate proceeds through the SO_4^{2-}/OH^--exchanger in the intact organ. Although we observe saturability of uptake in the higher concentration range we cannot exclude non-ionic diffusion on basis of this observation because apparent saturation could result from rapid intracellular accumulation of the bile acid in our system. In contrast, complete inhibition of uptake by DIDS and transstimulation of sinusoidal bile acid efflux strongly argue against diffusion being a major component of transport but suggest a carrier mediated transport system (compare Ref. 17). This notion is supported by the observation that both, cholate and DIDS, are substrates for the multispecific liver anion transport system [12]. Our data suggest that the carrier could either transport the undissociated acid or exchange cholate for OH^-. Perfusate alkalinization during cholate uptake cannot discriminate between these alternatives, but occurred in the nominal absence of HCO_3^-.

Quantitatively, transient perfusate alkalinization corresponds to a cellular release of OH^- at a rate somewhat less than cholate uptake (78.7 ± 19 nmoles/g liver.min OH^--release vs. 263.5 ± 5.5 nmol/g liver per min cholate uptake) and higher values of OH^--release were obtained in the presence of amiloride where cholate uptake proceeds at a reduced rate. These observations suggest that cholate uptake and OH^--release (or H^+-uptake) could be coupled at a stoichiometry of near 1:1, this being only apparent if any pH regulatory mechanisms, including electrogenic $Na^+(HCO_3^-)_n$-symport [35,36], are absent.

Calculated for an intracellular buffering capacity of 34 mM/pH unit [19] and assuming a stoichiometry of 1:1, cholate uptake at a rate of 260 nmol/g liver per min would result in initial acidification of the bulk cytosol at a rate of less than 0.02 pH units/min (assuming approx. 50% cell water of intact tissue weight [37]). Furthermore, this cholate uptake-stimulated OH^--release was only transient and, despite this estimated small alteration of pH_i, amiloride-sensitive K^+-uptake was readily induced. Both these observations suggest that Na^+/H^+-exchange is stimulated without substantial changes of bulk pH_i. In addition, cholate uptake is readily inhibited by amiloride before substantial changes of cytosolic pH are expected to occur [20]. This discrepancy between pH-sensitive transport rates and small changes of bulk cytosolic pH may be explained in analogy to studies of muscle transmembrane pH gradients [38,39] where membrane transport of acid equivalents appears to change pH mostly in the intimate vicinity of the membrane and, alternatively, surface pH rather than bulk pH affects membrane acid transport. Mutual interference of cholate uptake and Na^+/H^+-exchange may thus occur through local changes of pH and/or association of these membrane transporters in close vicinity. Following this line of arguing, acid extrusion by Na^+/H^+-exchange and subsequent local internal alkalinization and external acidification may also explain the observation that the trans-membrane pH gradient functions as a driving force for cholate uptake although bulk cytosolic pH is equal or more acid than perfusate pH (compare Ref. 40). Although these hypotheses may appear attractive, our data cannot exclude that a more alkaline internal pH facilitates cholate transport through allosteric effects rather than being the only direct driving force of Na^+-dependent cholate uptake.

(2) Correlation of cholic acid uptake to pH-regulation and liver cell K^+-balance

As discussed above, cholate uptake appears to result in stimulation of Na^+/H^+-exchange. Na^+-influx through this transport system or through a parallel $Na^+(HCO_3^-)_n$ symport [35,36] would result in a rise of intracellular Na^+ concentration (at least locally) and stimulate Na^+/K^+-ATPase, thus leading to K^+-uptake. As shown in Table V and Figs. 6a–d, this sequential coupling of transport systems finally results in uptake of K^+ and cholate$^-$. Support of this view is obtained through the observation that amiloride inhibits cholate-induced K^+-uptake. Functional linkage between these three transport systems is also suggested by those experiments where activity of Na^+/K^+-ATPase was altered through changes of extracellular K^+-concentration (compare Refs. 41 and 42). Inhibition of the pump by K^+-depletion resulted in inhibition of uptake of cholate whereas activation by K^+-readmission stimulated uptake. This latter stimulation was also blunted by inhibition of Na^+/H^+-exchange with amiloride. We therefore conclude that cholate uptake at high concentrations is associated with transmembrane OH^-- (or H^+)-transport and driven by the transmembrane pH gradient. The pH gradient is established by Na^+/H^+-exchange which, in turn, derives its energy from the transmembrane Na^+-concentration gradient established by Na^+/K^+-ATPase. Thus, cholate uptake operates as a tertiary active transport system. It may be of interest to note that a similar relation between pH-regulation and Na^+/K^+-ATPase activity has been proposed for regulatory liver cell volume increase following a relative hypertonic stress. There, Na^+/H^+-exchange is activated through cell shrinkage and the ensuing increase in Na^+/K^+-ATPase activity leads to a cellular gain of K^+ leading to cell volume recovery [30].

As shown in Figs. 6b, 6c and 6d, an interesting correlation is observed between bile acid transport and intracellular K^+-concentration, the cause of which needs to be elucidated. It is conceivable, however, that

K^+ is required at one ore more individual transport steps that may include segregation of the bile acid into pericanalicular transporting vesicles [43].

(3) Canalicular secretion of bile acid during variations of intracellular pH and of activity of Na^+/K^+-ATPase

Cholic acid taken up by rat liver cells is mostly secreted into bile in form of its taurine conjugate. By thin-layer chromatography we found approx. 90% as taurocholate (not determined throughout all experiments), consistent with published observations (see, for example, Ref. 44). As shown in Fig. 3, canalicular secretion of bile acid exhibited saturation kinetics with respect to intracellular bile acid content. The apparent maximal secretory rate was less than maximal uptake rate. Consequently, intracellular accumulation was observed at high uptake rates (compare Fig. 2c and Fig. 3).

K^+-depletion resulted in inhibition of bile acid secretion despite rising intracellular concentration. Conversely, activation of Na^+/K^+-ATPase by K^+-readmission stimulated bile acid secretion. Furthermore, intracellular alkalinization by the NH_4^+-pulse resulted in stimulation of bile acid secretion and application of amiloride was inhibitory. These observations may be interpreted in view of the fact that the canalicular membrane potential is a driving force for taurocholate secretion [45,46]. Inhibition of Na^+/K^+-pump results in gradual cell depolarization whereas activation of the electrogenic pump causes transient membrane hyperpolarization [42,47]. Furthermore, intracellular alkalinization leads to a rise in membrane K^+-conductance (Ref. 25, compare also K^+-release in Fig. 4) and membrane hyperpolarization. Stimulation of bile acid secretion and bileflow was thus consistently associated with cell hyperpolarization. It is possible though that, besides electrogenic bile acid secretion across the canalicular membrane, other transport systems could be affected that include segregation of bile acids into cytoplasmatic vesicles and their exocytosis [43,48].

Application of DIDS led to a substantial increase of bile flow (compare Ref. 49). Simultaneous stimulation of bile acid secretion in these experiments may therefore result from dilution of the luminal bile acid concentration and facilitation of transport due to a decreased transmembrane bile acid concentration gradient. Alternatively, DIDS may displace the bile acid from intracellular binding sites and increase the free concentration available for canalicular membrane transport.

In conclusion, the data indicate that cholate uptake by rat liver is regulated by the sinusoidal transmembrane pH-gradient which is intimately linked to Na^+/H^+-exchange and Na^+/K^+-ATPase activity. The data support the notion that canalicular bile acid secretion is driven by the intracellular negative electric potential. Transcellular bile acid transport is thus integrated in the regulation of intracellular pH, of K^+-homeostasis and of the membrane potential. These mechanisms mutually depend on each other and are components of many other functions of liver cells which may thus affect bile acid transport or may be affected through bile acid transport.

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