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MECHANISM OF INHIBITION OF HEPATIC BILE ACID UPTAKE BY AMILORIDE AND 4,4′-DIISOTHIOCYANO-2,2′-DISULFONIC STILBENE (DIDS)

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Abstract—The mechanisms by which amiloride and 4,4′-diisothiocyano-2,2′-disulfonic stilbene (DIDS) inhibit hepatic uptake of cholate and taurocholate (TC) were investigated in isolated rat hepatocytes. Amiloride inhibited Na^+-dependent uptake of cholate and TC only when hepatocytes were preincubated with amiloride, indicating an indirect effect of amiloride. Time-dependent studies showed that the inhibition of bile acid uptake was associated with a parallel increase in intracellular Na^+ concentration ($[Na^+]_i$). Although amiloride decreased intracellular pH, this decrease preceded amiloride-induced inhibition of bile acid uptake and increase in $[Na^+]_i$. Amiloride inhibited bile acid uptake, decreased membrane potential, and increased $[Na^+]_i$ with comparable concentration dependency. DIDS inhibited Na^+-dependent uptake of cholate and TC non-competitively. Neither DIDS nor amiloride inhibited Na^+-independent uptake of cholate and TC. These results indicate that amiloride inhibits Na^+-dependent cholate and TC uptake by decreasing the transmembrane Na^+-gradient, and further support the hypothesis that two different transporters may be involved in hepatic bile acid uptake by Na^+-dependent and Na^+-independent mechanisms.

It is now well established that taurocholate (TC†) enters hepatocytes predominantly by a Na^+-coupled cotransport mechanism [1]. Studies in perfused rat livers and isolated hepatocytes indicate that cholate uptake involves both Na^+-dependent and Na^+-independent mechanisms in addition to passive diffusion [2–4]. Since a transmembrane pH gradient ($pH_{in} > pH_{out}$) stimulated cholate uptake into plasma membrane vesicles, it was proposed that Na^+-dependent cholate uptake may involve a functional coupling between cholate/OH^- exchange and Na^+/H^+ exchange [5]. Interestingly, this pH gradient-dependent cholate uptake was not inhibited by 4,4′-diisothiocyano-2,2′-disulfonic stilbene) (DIDS), a known anion exchange inhibitor [6–9]. A recent study by Caflisch *et al.* [10], however, showed that the DIDS-insensitive, pH gradient-driven cholate uptake in isolated plasma membrane vesicles is due to nonionic diffusion. These authors also showed that an inwardly directed Na^+-gradient stimulates cholate uptake, an effect inhibited by DIDS. Additional studies showed that: (1) Na^+-dependent cholate and TC uptake are competitively inhibited by TC and cholate, respectively [2, 11]; (2) bilirubin and bromosulfophthalein inhibit Na^+-dependent cholate and TC uptake non-competitively [11, 12] and Na^+-independent cholate uptake competitively [12]; and (3) ouabain, an inhibitor of Na^+,K^+-ATPase, inhibits Na^+-dependent cholate and TC uptake to the same extent [2]. These results are consistent with the hypothesis that Na^+-dependent and Na^+-independent uptake of cholate represents two distinct mechanisms, and that cholate and TC share the same Na^+-dependent mechanism. This latter premise is also consistent with the inhibition characteristics of Na^+-dependent TC uptake by various structural analogues [11, 13].

We recently observed that amiloride inhibits uptake of cholate and TC in perfused rat livers [14]. Amiloride, a known inhibitor of Na^+/H^+ exchange [15], has also been shown to inhibit hepatic Na^+,K^+-ATPase and Na^+-coupled alanine uptake [16]. Thus, amiloride may inhibit Na^+-coupled cholate and TC uptake by decreasing intracellular pH (pH_i) and/or by decreasing the transmembrane Na^+-gradient. To distinguish between these possibilities we determined the effect of amiloride on cholate and TC uptake, pH_i, intracellular Na^+ concentration ($[Na^+]_i$) and membrane potential difference (MP) in isolated hepatocytes.

DIDS has been shown to inhibit Na^+-dependent TC uptake non-competitively [11]. However, the effect of DIDS on Na^+-independent uptake of bile acid and the type of inhibition of Na^+-dependent cholate uptake by DIDS have not been characterized. Studies with DIDS may provide further insight into the mechanism of bile acid uptake. An inhibition of Na^+-independent uptake of bile acids by DIDS may suggest that anion-exchange is involved. That Na^+-dependent uptake of bile acids is mediated by the same transporter will be further supported if DIDS also inhibits Na^+-dependent cholate uptake non-competitively. Moreover, a dissimilar effect of DIDS on Na^+-dependent and Na^+-independent uptake of

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† Abbreviations: TC, taurocholate; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic stilbene; pH_i, intracellular pH; $[Na^+]_i$, intracellular Na^+ concentration; MP, membrane potential; and [^{14}C]DMO, 5,5-dimethyl-[2-^{14}C]-4-oxazolidinedione.

bile acids could provide further support for two different transporters. Thus, we have also characterized the effect of DIDS on Na^+-dependent and Na^+-independent uptake of cholate and TC in isolated hepatocytes.

MATERIALS AND METHODS

Materials. Cholate and TC (sodium-salts) were purchased from CalBiochem (San Diego, CA). DIDS and amiloride were obtained from the Sigma Chemical Co. (St. Louis, MO). [24-^{14}C]Cholic acid (47 mCi/mmol), [24-^{14}C]taurocholic acid (56 mCi/mmol), 5,5-dimethyl-[2-^{14}C]-4-oxazolidinedione ([^{14}C]DMO; 50 mCi/mmol), ^{22}NaCl (1.08 Ci/mg) and [*methoxy*-^{3}H]inulin (80 Ci/mmol) were purchased from New England Nuclear (Boston, MA). KS^{14}CN (58 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). DIDS solutions were freshly prepared each day and kept in the dark until used. Male Wistar rats (200–300 g) obtained from Charles River Laboratories served as liver donors.

Hepatocyte preparation. Hepatocytes were isolated by a previously described collagenase perfusion method [17]. Freshly prepared hepatocytes showing the following viability criteria were used for each study: (1) less than 10% stainable by trypan blue, (2) lactate to pyruvate ratio in cell-free medium of not more than 15, and (3) spontaneous O_2 consumption of not less than 5 nmol/min/mg protein.

Bile acid uptake. The initial uptake rate of cholate and TC was determined as previously described [17]. Briefly, hepatocytes were incubated at 37° under air in an incubation medium (pH 7.4) containing 135 mM NaCl or 135 mM choline-Cl, 3 mM KCl, 2 mM KH_2PO_2, 0.8 mM $MgSO_4$, 1.0 mM $CaCl_2$ and 20 mM 4-(2-hydroxyethyl)1-1-piperazineethanesulfonic acid (Hepes). To determine the effect of HCO_3^-, hepatocytes were incubated with 24 mM $NaHCO_3$ (under 95% O_2 and 5% CO_2) instead of 20 mM Hepes. Since amiloride inhibits bile acid uptake in livers [14] perfused with HCO_3^- buffer equilibrated with 95% O_2 and 5% CO_2, studies in hepatocytes were also conducted under O_2 instead of air to stimulate conditions in perfused livers. After a 30-min incubation, hepatocytes were washed once and then resuspended in the same medium maintained at 37°. Transport was initiated by adding the cell suspension to the incubation medium (5–8 mg protein/mL of final cell suspension) containing [^{14}C]cholate or [^{14}C]TC, [^{3}H]inulin and DIDS, amiloride or solvent [dimethyl sulfoxide (DMSO), <0.3%]. Initial uptake rates were calculated from the slope of the linear portion of the time-dependent uptake curves [17], and are expressed in nanomoles per minute per milligram protein. Na^+-dependent uptake was calculated as the difference between uptake in the presence and absence of Na^+ (replaced by choline), as previously described [2]. Since bile acid uptake varies as much as 40% from one cell preparation to another, all comparative studies were done with the same cell preparation.

Intracellular pH and membrane potential difference. Intracellular pH and membrane potential difference were determined from the equilibrium distribution of [^{14}C]DMO and S^{14}CN, respectively, as previously described [18, 19]. Briefly, hepatocytes were suspended in the incubation medium described for bile acid uptake. After a 30-min preincubation at 37°, [^{14}C]DMO (0.1 μCi/mL), unlabeled DMO (15 μM) and [^{3}H]inulin (0.3 μCi/mL) were added to the cell suspension (8–10 mg protein/mL) to determine pH_i. Amiloride or DMSO (solvent) was added following a 15-min equilibration period. The effect of amiloride on the MP was determined similarly except that [^{14}C]DMO and unlabeled DMO were replaced by KS^{14}CN (0.1 μCi/mL) and unlabeled KSCN (15 μM), respectively. At indicated times, triplicate samples of 0.5 mL each were filtered under vacuum (650 mm Hg) through a dual filter arrangement consisting of a polycarbonate filter (0.4 μm pore diameter; Nucleopore Corp., Pleasanton, CA) overlaying a cellulose paper filter (Whatman No. 3MM). Filters were allowed to dry for 1 min under vacuum; then the top filter was removed and placed on a large filter paper before radioisotope counting. Intracellular radioactivity (dpm/mg protein) was calculated after correcting for residual extracellular radioactivity (2–8% of total radioactivity) estimated from [^{3}H]inulin. Binding of [^{14}C]DMO or S^{14}CN to filters determined by filtering 0.5 mL cell-free buffer containing [^{14}C]DMO or S^{14}CN was found to be less than 1% and was not corrected for. Intracellular volume (μL/mg protein) was determined in parallel experiments under identical conditions using [^{3}H]-H_2O and [^{14}C]inulin, and was used to calculate intracellular concentrations of [^{14}C]DMO and S^{14}CN.

Intracellular Na^+ *concentration* ($[Na^+]_i$). Preliminary studies showed that $^{22}Na^+$ uptake had reached steady state within 20 min. Thus, the effect of amiloride was studied by adding amiloride or DMSO (solvent) following a 30-min incubation of the hepatocyte suspension (8–10 mg protein/mL) with ^{22}NaCl (1 μCi/mL) at 37°. Results of DMSO studies served as the control. At indicated times a 1-mL sample of cell suspension was mixed with ice-cold [^{3}H]inulin (1 μCi) followed by separation of cells by centrifugation. Cell pellets were suspended in 0.8 mL saline after washing twice with 1.5 mL of ice-cold saline. A fraction (200 μl) of the suspended cell pellet was counted for radioactivity in triplicate, with the remainder used for protein determination. $^{22}Na^+$ content of hepatocytes (nmol/mg protein) was calculated after correcting for extracellular $^{22}Na^+$ (less than 10% of total count) estimated from the [^{3}H]inulin content. Intracellular volume (μL/mg protein) was determined in parallel experiments under identical conditions using [^{3}H]H_2O and [^{14}C]-inulin as previously described [19], and was used to calculate $[Na^+]_i$.

Other methods. The method of Lowry *et al.* [20] was used to determine cell protein. Each series of studies was performed in at least three different cell preparations. Student's *t*-test was used to analyze data with $P < 0.05$ considered significant.

RESULTS

Effect of amiloride on bile acid uptake. The effect of amiloride was dependent on preincubation time

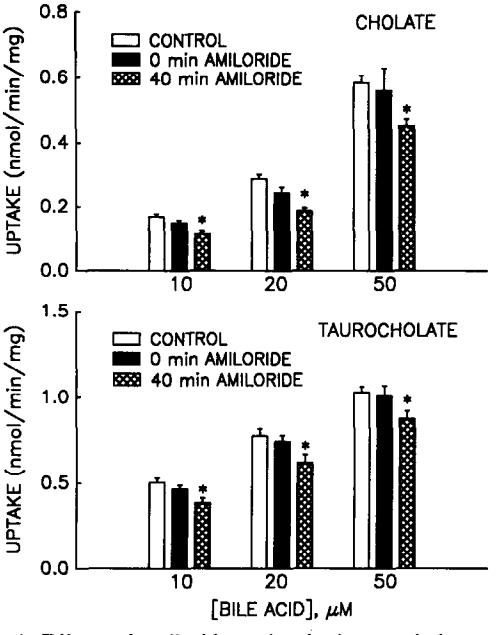

Fig. 1. Effects of amiloride preincubation on cholate and taurocholate uptake. Uptake was determined in the presence (0 min amiloride) or absence (control) of 1 mM amiloride, or following a 40-min preincubation of hepatocytes with 1 mM amiloride (40 min amiloride). Data are means ± SEM (N = 12–18). Key: (*) significantly different from respective control values ($P < 0.05$).

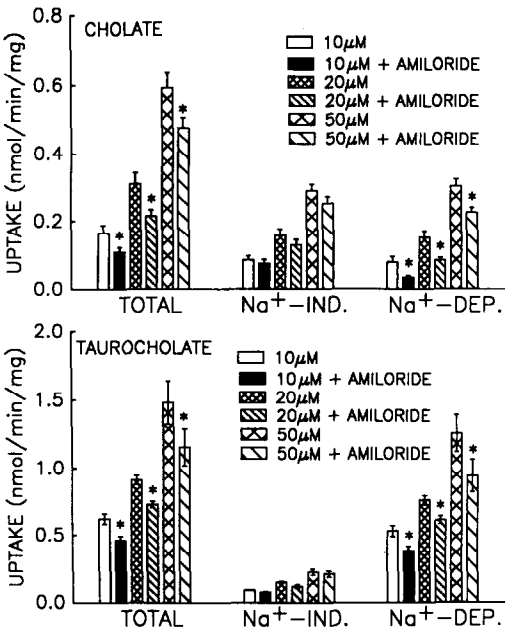

Fig. 2. Effects of amiloride on Na^+-dependent and Na^+-independent uptake of cholate and taurocholate. Hepatocytes were preincubated with 1 mM amiloride for 40 min before initiating transport studies. Na^+-dependent (Na^+-DEP.) uptake was calculated by subtracting Na^+-independent (Na^+-IND.) uptake from total uptake. Data are means ± SEM (N = 9). Key: (*) significantly different from respective control values ($P < 0.05$).

(Fig. 1). Although amiloride (1 mM) did not inhibit uptake when added simultaneously with bile acid, an inhibition of 20–30% was observed when hepatocytes were preincubated with amiloride for 40 min. A 40-min preincubation was chosen since inhibition of bile acid uptake in perfused rat livers [14] was observed after a 40-min amiloride perfusion. Since rat livers were perfused with a buffer containing 24 mM $NaHCO_3$ [14], the effect of amiloride on bile acid uptake was determined in the presence of HCO_3^- (Table 1). Bile acid uptake was not affected by the presence of HCO_3^- in the incubation medium, and amiloride inhibited bile acid uptake only when hepatocytes were preincubated with amiloride for 40 min. Thus, the time-dependent effect of amiloride was not modified by the presence of HCO_3^-. Further studies in the presence and absence of extracellular Na^+ showed that amiloride inhibited Na^+-dependent, but not Na^+-independent, uptake of cholate and TC (Fig. 2). Similar results were obtained when Na^+ was replaced by Li^+ instead of choline (data not shown).

Table 1. Effect of amiloride on bile acid uptake in the presence and absence of HCO_3^-

		Bile acid uptake (nmol/min/mg protein)			
		Preincubation time			
		0 min		40 min	
Incubation buffer	Bile acid	Control	Amiloride	Control	Amiloride
Hepes	Cholate	0.32 ± 0.03	0.29 ± 0.02	0.31 ± 0.03	0.25 ± 0.03*
	TC	1.05 ± 0.09	0.98 ± 0.05	1.15 ± 0.11	0.78 ± 0.08*
HCO_3^-	Cholate	0.34 ± 0.01	0.32 ± 0.02	0.35 ± 0.02	0.24 ± 0.04*
	TC	0.98 ± 0.07	0.94 ± 0.08	1.08 ± 0.09	0.75 ± 0.06*

Hepatocytes were incubated in an incubation medium containing either 20 mM Hepes (under air) or 24 mM HCO_3^- (under 95% O_2 and 5% CO_2). Bile acid (20 μM) uptake was determined following preincubation with 1 mM amiloride for 0 or 40 min. Hepatocytes incubated with DMSO served as controls. Data are means ± SEM (N = 6).

* Significantly different from respective control values ($P < 0.05$).

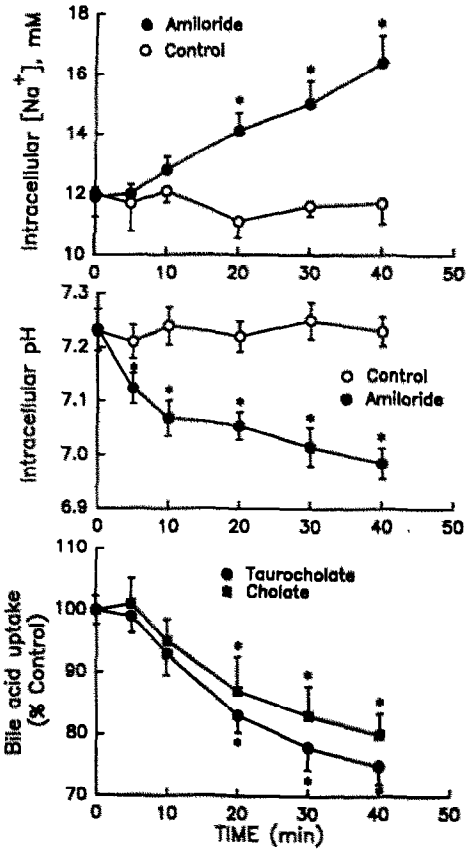

Fig. 3. Time-dependent effects of 1 mM amiloride on $[Na^+]_i$, pH_i and bile acid uptake. Following preincubations with appropriate radioisotopes (details in Materials and Methods) and determination of basal values (0 min), amiloride or DMSO (control) was added to the cell suspension and samples were taken at indicated times for the measurement of bile acid uptake (20 μM), pH_i and steady-state $^{22}Na^+$ content. Basal uptake of cholate and taurocholate and steady-state $^{22}Na^+$ content were 0.52 ± 0.081 and 1.15 ± 0.18 nmol/min/mg protein and 34 ± 1.7 nmol/mg protein, respectively. Data are means ± SEM (N = 9–12). Key: (*) significantly different from respective 0 min values ($P < 0.05$).

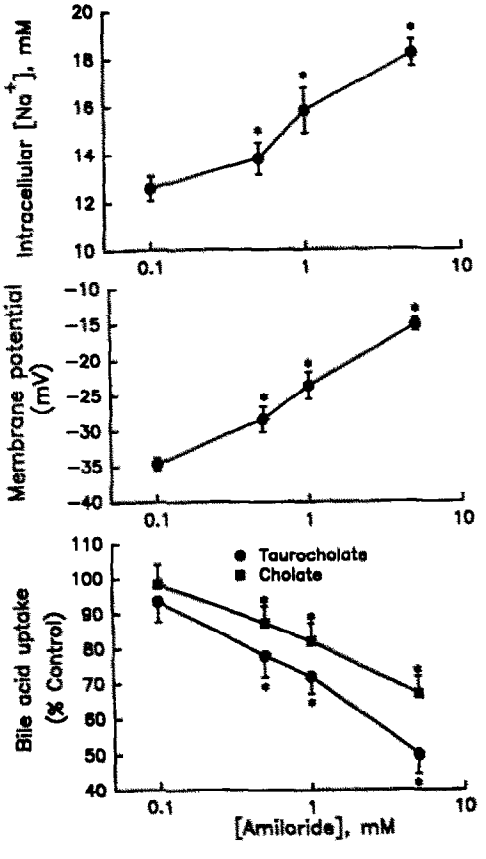

Fig. 4. Concentration-dependent effects of amiloride on $[Na^+]_i$, membrane potential, and bile acid uptake. Hepatocytes were incubated with the indicated concentration of amiloride or DMSO (control) for 40 min before determining bile acid uptake (20 μM), membrane potential and intracellular $[Na^+]$. Control values for cholate and taurocholate uptake, membrane potential and intracellular $[Na^+]$ were 0.38 ± 0.019 and 1.27 ± 0.058 nmol/min/mg protein, -35.4 ± 1.31 mV and 11.7 ± 0.46 mM, respectively. Data are means ± SEM (N = 6–12). Note that a log scale is used for the abscissas. Key: (*) significantly different from respective control values ($P < 0.05$).

Effect of amiloride on pH_i and $[Na^+]_i$. To determine if the effect of amiloride is due to inhibition of Na^+/H^+ exchange and/or Na^+,K^+-ATPase, time-dependent changes in bile acid uptake were compared with those of pH_i and $[Na^+]_i$ (Fig. 3). Similar to the inhibition of cholate and TC uptake, amiloride significantly increased $[Na^+]_i$ within 20 min. Thus, amiloride-induced decreases in cholate and TC uptake paralleled increases in $[Na^+]_i$. Amiloride also decreased pH_i indicating inhibition of Na^+/H^+ exchange. Amiloride has been shown to produce rapid inhibition of Na^+/H^+ exchange in hepatocytes [18, 21, 22]. In the present study, a significant decrease in pH_i was observed at 5 min while a decrease in bile acid uptake was not significant until 20 min. Thus, amiloride-induced inhibition of Na^+/H^+ exchange is unlikely to be the primary reason for the inhibition of bile acid uptake.

Concentration-dependent effect of amiloride. To further characterize the effect of amiloride, bile acid uptake, MP and $[Na^+]_i$ were determined in hepatocytes incubated with different concentrations of amiloride for 40 min (Fig. 4). Inhibition of bile acid uptake was concentration dependent with significant inhibition observed at 0.5 mM amiloride. Amiloride also increased $[Na^+]_i$ and induced membrane depolarization (Fig. 4) with comparable concentration dependency. Amiloride (5 mM) inhibited cholate uptake (30%) less than TC uptake (50%) which was expected since amiloride inhibited Na^+-dependent cholate uptake, and only 40–50% of cholate uptake was Na^+ dependent (Fig. 2). In separate studies, 5 mM amiloride was found to inhibit Na^+-dependent cholate and TC uptake by 63 ± 4.8 and 57 ± 5.3% (N = 6), respectively. Thus, the inhibition of Na^+-dependent bile acid uptake by

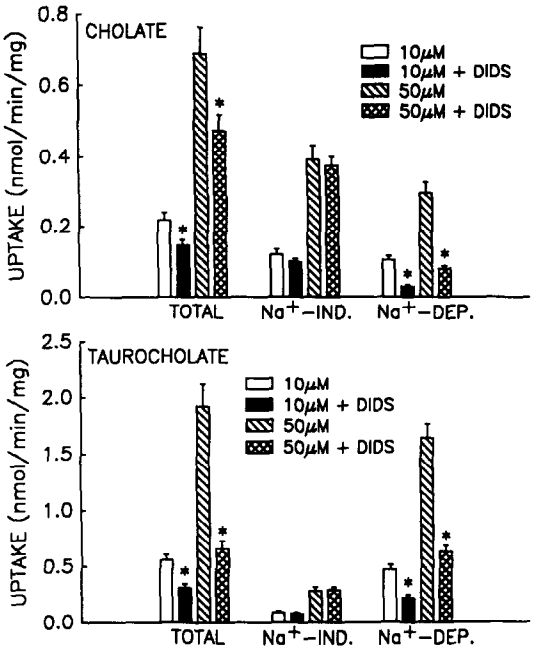

Fig. 5. Effects of DIDS on cholate and taurocholate uptake. Hepatocytes were incubated with the indicated concentrations of cholate and taurocholate in the presence or absence of 1 mM DIDS. Na^+-dependent (Na^+-DEP.) uptake was calculated by subtracting Na^+-independent (Na^+-IND.) uptake from total uptake. Data are means ± SEM (N = 9). Key: (*) significantly different from respective control values ($P < 0.05$).

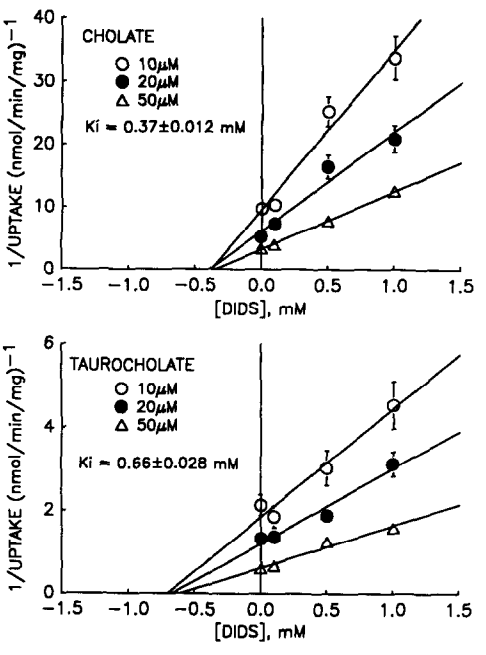

Fig. 6. Dixon plot showing non-competitive inhibition of Na^+-dependent cholate and taurocholate uptake by DIDS. Solid lines represent regression lines and data are means ± SEM (N = 9).

amiloride closely parallels its effect on MP and $[Na^+]_i$.

Effect of DIDS on bile acid uptake. Preliminary studies showed that DIDS inhibited cholate and TC uptake, irrespective of preincubation with DIDS. Thus, the effect of DIDS was evaluated by incubating hepatocytes simultaneously with DIDS and bile acid. DIDS inhibited uptake of cholate and TC in the presence, but not in the absence of extracellular Na^+ (Fig. 5). Thus, Na^+-dependent, but not Na^+-independent uptake of cholate and TC was inhibited by DIDS. To determine the type of inhibition, Na^+-dependent uptake of cholate and TC was determined at different concentrations of DIDS. A Dixon plot of the data showed that DIDS was a non-competitive inhibitor of Na^+-dependent cholate and TC uptake (Fig. 6). Non-competitive inhibition is indicated by lines intersecting each other on the X-axis.

DISCUSSION

The present study was designed to determine if the inhibition of bile acid uptake by amiloride is due to inhibition of Na^+/H^+ exchange or Na^+,K^+-ATPase. Results, as discussed below, indicate that amiloride inhibits bile acid uptake primarily by inhibiting Na^+,K^+-ATPase.

Amiloride inhibited Na^+-dependent uptake of cholate and TC (Fig. 2), and this inhibition was dependent on preincubation time (Fig. 1) indicating an indirect effect of amiloride. Amiloride is a known inhibitor of Na^+/H^+ exchange [15] and has been shown to inhibit hepatic Na^+,K^+-ATPase [16]. Thus, the indirect effect of amiloride may be mediated via its effect on pH_i (inhibition of Na^+/H^+ exchange) or the transmembrane Na^+-gradient (inhibition of Na^+,K^+-ATPase). Although amiloride decreased pH_i in a time-dependent fashion, amiloride-induced decreases in pH_i preceded its effect on bile acid uptake (Fig. 3). For example, amiloride significantly decreased pH_i from 7.25 to 7.12 within 5 min, but did not significantly inhibit bile acid uptake for up to 20 min. Moreover, amiloride also inhibited bile acid uptake in the presence of HCO_3^- (Table 1). Since hepatocyte pH_i is not decreased significantly by amiloride in the presence of HCO_3^- [23, 24], inhibition of bile acid uptake is unlikely to be due to amiloride-induced changes in pH_i. The concentration-dependent study showed that 5 mM amiloride decreased Na^+-dependent bile acid uptake by ≈50% (Fig. 4), a concentration which is one order of magnitude higher than that inhibiting Na^+/H^+ exchange half-maximally (0.3 mM) [18, 25]. Taken together, these results indicate that the amiloride-induced inhibition of bile acid uptake is not due primarily to its effect on Na^+/H^+ exchange.

Amiloride also increased $[Na^+]_i$ and produced a membrane depolarization (Fig. 4). Amiloride has been shown to inhibit rat liver plasma membrane Na^+,K^+-ATPase with a K_i value of 3 mM [16]. In the present study, 5 mM amiloride increased $[Na^+]_i$ by 55% and decreased MP by 57%. Since MP and $[Na^+]_i$ are dependent on the activity of Na^+, K^+-ATPase, these effects are most likely due to

inhibition of Na^+,K^+-ATPase. These results indicate that amiloride decreases the electrochemical gradient of Na^+ across the plasma membrane. Since amiloride-induced changes in $[Na^+]_i$ and MP closely parallel changes in bile acid uptake, it is most likely that amiloride inhibits hepatic uptake of cholate and TC by decreasing the transmembrane Na^+-gradient.

The present study also showed that DIDS inhibited Na^+-dependent uptake of cholate and TC non-competitively (Fig. 6). This result is consistent with the hypothesis that Na^+-dependent uptake of bile acids is mediated by the same transporter. Inasmuch as DIDS is known to inhibit different anion-exchange mechanisms [6–9], it may be argued that the DIDS inhibition of Na^+-dependent bile acid uptake is indicative of bile acid/OH^- exchange. However, this seems unlikely since the pH gradient-stimulated cholate uptake in plasma membrane vesicles is not inhibited by DIDS [5, 10], and Na^+-dependent TC uptake is due to Na^+/TC cotransport [1]. On the other hand, since DIDS did not inhibit Na^+-independent uptake of bile acid, it is likely that Na^+-independent uptake is also not mediated by an anion-exchanger. DIDS is transported by hepatocytes and is concentrated in bile [26, 27]. The possibility that DIDS, an organic anion, may be transported by the Na^+-coupled bile acid transporter seems unlikely since DIDS inhibited bile acid transport non-competitively (Fig. 6).

It has been argued recently that the Na^+-dependent and Na^+-independent uptake of cholate and TC may be mediated by the same carrier, a multispecific transporter [28]. Our study showed that DIDS inhibited Na^+-dependent, but not Na^+-independent uptake of cholate and TC. This specific effect of DIDS indicates that Na^+-dependent and Na^+-independent uptake of cholate and TC may be mediated by two different transporters. This conclusion is in agreement with other studies in hepatocytes [2, 12] and plasma membrane vesicles [1].

In summary, the present study shows that amiloride and DIDS inhibited Na^+-dependent uptake of cholate and TC. Amiloride inhibited bile acid uptake primarily by decreasing the electrochemical gradient of Na^+, and DIDS inhibited Na^+-dependent bile acid uptake non-competitively.

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