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THE ROLE OF MICROFILAMENTS AND OF MICROTUBULES IN TAUROCHOLATE UPTAKE BY ISOLATED RAT LIVER CELLS

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Summary

The role of microfilaments and microtubules on bile salt transport was studied by investigating the influence of a microfilament and a microtubule inhibitor, cytochalasin B and colchicine, respectively, on taurocholate uptake by isolated hepatocytes in vitro. Hepatocytes were prepared by the enzyme perfusion method and [^{14}C]taurocholate uptake velocity was determined by a filtration assay. Taurocholate uptake obeyed Michaelis-Menten kinetics, maximal uptake velocity and apparent half-saturation constants averaging 0.87 ± SD 0.05 nmol · $s^{-1} \cdot 10^{-6}$ cells and 10.9 ± 1.8 μM, respectively. Cytochalasin B (4.2—420 μM) inhibited taurocholate uptake in a competitive fashion; K_i being 33 ± 7 μM. At concentrations above 100 μM the compound decreased ^{36}Cl membrane potential and intracellular K^+ concentration. Other parameters of cell viability were not affected by cytochalasin B. Colchicine (0.1—1.0 mM), by contrast, inhibited taurocholate uptake non-competitively, K_i being 0.47 ± 0.07 mM. The inhibition brought about by colchicine was considerably smaller than that induced by cytochalasin B. None of the parameters of cell viability tested was affected by colchicine. These results suggest that microfilaments may be involved in the carrier-mediated hepatocellular transport of bile salts. This could, at least in part, account for cytochalasin B-induced cholestasis. The contribution of the microtubular system, if any, is less important quantitatively. The mechanisms whereby these two components of the cytoskeleton partake in bile salt transport remain to be elucidated.

Microfilaments and microtubules, two major components of the cytoskeleton [1] have been shown to be involved in different hepatocellular functions. Thus, microfilament inhibition by cytochalasin B has been shown to result in

cholestasis [2], while inhibition of microtubules by colchicine reduces biliary lipid excretion [3]. Since bile salt transport presumably is a major determinant of both bile flow and biliary lipid excretion, we studied the effect of cytochalasin B and of colchicine on taurocholate uptake by isolated hepatocytes in vitro.

Materials and Methods

Preparation of isolated hepatocytes

Male Sprague-Dawley rats (Taconic Animal Farms, Germantown, NY) of mean body weight 299 ± 16 g were maintained on a standard rat diet and tap water ad libitum. Hepatocytes were prepared by a modification of the enzyme perfusion method of Berry and Friend [4]. Fed rats were anesthesized with sodium pentobarbital (80 mg/kg body wt.) intraperitoneally and heparinized (500 I.U./kg body wt.) intravenously prior to cannulation of the portal vein and vena cava. The portal vein was cannulated with a sixteen gauge needle and the liver was washed blood-free with 50 ml of Ca^{2+}-free Krebs-Henseleit buffer which had been equilibrated to pH 7.40 with 95% O_2/5% CO_2. Thereafter the liver was perfused in a recirculating system with Krebs-Henseleit buffer containing 0.3% (w/v) collagenase type II (Worthington Biochemical Co., Freehold, NJ) and 0.1% (w/v) hyaluronidase (Boehringer-Mannheim, Indianapolis, IN) for 25 min at 37°C. At the conclusion of the perfusion the liver was excised, minced with scissors and filtered three times through a nylon mesh (0.3 × 0.5 mm). The cell suspension was then centrifuged for two minutes at 50 × *g*. The supernatant was discarded, the pellet resuspended in 40 ml of Krebs-Henseleit buffer containing 2.5% (w/v) bovine albumin (Reheis Chemical Company, Chicago, IL) and recentrifuged twice. The stock solution of hepatocytes was adjusted to a cell concentration of approx. 10^7 cells/ml in Krebs-Henseleit buffer containing 2.5% (w/v) bovine albumin and 0.1% glucose and stored at 4°C for up to 3 h.

Determination of taurocholate uptake velocity

1 ml of the cell solution was diluted in 8 ml buffer and incubated for 30 min at 37°C in a shaking water bath under a low flow of O_2 (95%) and CO_2 (5%). Cytochalasin B and colchicine (Sigma Chemical Company, St. Louis, MO) were added at concentrations ranging from 4.2 to 420 μM and 0.03 to 1 mM, respectively. Cytochalasin B was dissolved in dimethylsulfoxide to a final concentration of 0.2% (v/v). Colchicine was dissolved in Krebs-Henseleit buffer. Appropriate concentrations of the solvents were employed as controls. After the 30-min incubation period 1 ml of ^{14}C-labeled taurocholate (spec. act. 54 mCi/mmol; Amersham Corporation. Arlington Heights, IL) was rapidly added and mixed. Final taurocholate concentrations ranged from 1 to 50 μM. Thereafter, cells were separated from surrounding medium by rapid filtration on Whatman GF/D glass fiber filters (Fisher Scientific Corporation, Rockville, MD) at 10-s intervals up to 50 s. The filters were rapidly washed with 5 ml of 102 mM $MgCl_2$. They were then placed in glass liquid scintillation vials and retained cells were lysed with 1 ml of 1.7% (w/v) trichloroacetic acid. Radioactivity was counted using Aquasol® (New England Nuclear, Boston, MA) as a scintillator.

Quench correction was performed by internal standardization using [^{3}H]- and [^{14}C]toluene successively.

To determine the time-course of cytochalasin B and colchicine binding to hepatocytes, different concentrations of [^{3}H]cytochalasin B (spec. act. 10 mCi/mmol;Amersham) or [^{3}H]colchicine (spec. act. 3 mCi/mmol; Amersham) were incubated with 10^6 hepatocytes for 1—60 min and the amount bound was determined as described above. To determine the influence of taurocholate on cytochalasin B and colchicine binding, varying concentrations of [^{3}H]cytochalasin B (4.2—420 μm) or colchicine (100—1000 μM) were incubated either alone or with 1 or 50 μM taurocholate (Calbiochem, La Jolla, CA) for 30 min. Then the amount bound was determined as described above.

Assay of viability of hepatocytes

Hepatocytes were tested throughout the experiments for trypan blue exclusion. Intracellular ATP levels were assayed using standard procedures [5] immediately after preparation and after 30 min incubation with the different agents. Cell membrane potentials were estimated using ^{36}Cl (spec. act.5 mCi/mmol; Amersham); membrane potentials were calculated using the Nernst equation [6]. Intracellular potassium concentrations were measured with ^{42}K (New England Nuclear) in the incubation medium. Total potassium concentration was measured by flame photometry. Cellular volume was determined by centrifuging the cell suspension in microhematocrit tubes and by estimating ^3H_2O space by the filtration assay.

In all filtration assays extracellular medium trapped on the filters was determined by inclusion of an appropriate extracellular marker [^{3}H]- or [^{14}C]dextran, M_r 70 000; spec. act. 250 and 2.2 mCi/g, respectively; New England Nuclear). It averaged 0.36 ± 0.12%.

Analysis of data

The fraction of taurocholate retained on each filter was corrected for extracellular contamination and plotted against time. Fractional taurocholate uptake was then graphically estimated from this plot and taurocholate uptake velocity calculated as the product of taurocholate concentration and fractional uptake. Kinetic analysis of the relationship between uptake velocity (v) and taurocholate concentration (S) was performed using the Michaelis-Menten equation [7]:

$$v = \frac{V \cdot S}{S + K_m} \tag{1}$$

where V stands for maximal uptake velocity and K_m for the apparent half-saturation constant. The data were fitted to Eqn. 1 in nonlinear, weighted form [8]. To compare different sets of kinetic data, a linear transformation of Eqn. 1 was employed:

$$\frac{S}{v} = \frac{1}{V} + S \cdot \frac{K_m}{V} \tag{2}$$

Regression analysis was performed using the methods of least squares [9]. The means of two samples were compared by Student's t-test after testing the equality of variances by an F-test [9]. If the variances were unequal, a modi-

fied *t*-test was employed [10]. Unless otherwise indicated, all results are expressed as mean ± 1 S.D. $P \leqslant 0.05$ was considered statistically significant.

Results

Preparation of isolated hepatocytes resulted in more than 95% viable cells up to 4 h after preparation as judged by trypan blue exclusion. Intracellular ATP levels averaged 3.42 ± 0.18 μmol/g liver. Neither of these parameters was affected by cytochalasin B or colchicine treatment. Results of the assay of membrane potentials and intracellular K^+ concentrations are given in Table I.

Binding of 126 μM cytochalasin B to hepatocytes averaged 5.8, 19.3, 20.6, 20.8 and 19.6%/10^6 cells at 1, 5, 10, 30 and 60 min, respectively. The corresponding values for 100 μM colchicine were 2.4, 3.6, 11.8, 12.4 and 12.2%/10^6 cells. The time-course of both cytochalasin B and colchicine binding was similar at the other concentrations studied (data not shown).

Taurocholate uptake was linear with time at all concentrations studied, fractional uptake decreasing with increasing taurocholate concentration. The relationship between taurocholate uptake velocity and concentration could adequately ($F = 592$; d.f.1 = 2, d.f.2 = 38) be described by the Michaelis-Menten equation. Apparent half-saturation constant and maximal uptake velocity averaged 10.9 ± 1.8 μM and 0.87 ± 0.05 nmol · s^{-1} · 10^6 cells, respectively (Fig. 1).

Cytochalasin B led to a dose-dependent decrease in fractional taurocholate uptake at cytochalasin concentrations higher than 4.2 μM. The effect of the different cytochalasin B concentrations on half-saturation constant and maximal uptake velocity of taurocholate uptake are given in Table II. It is evident that cytochalasin B decreased the half-saturation constant in a dose-dependent fashion without affecting maximal uptake velocity; this suggests a competitive type of inhibition. Such a type of inhibition could be confirmed by kinetic analysis, K_i of cytochalasin B being 33 ± 7 μM.

TABLE I

THE EFFECT OF CYTOCHALASIN B AND OF COLCHICINE ON $^{36}Cl^-$ MEMBRANE POTENTIALS AND INTRACELLULAR ^{42}K CONCENTRATION IN ISOLATED RAT LIVER CELLS

Results are expressed as mean ± S.D. ($n = 4$).

Inhibitor (μM)		$^{36}Cl^-$ Membrane potential	Intracellular [$^{42}K^+$]
None (control)		36.7 ± 1.2	117 ± 6
Cytochalasin B	42	36.8 ± 1.2	118 ± 7
	10	35.4 ± 1.4	116 ± 10
	42	34.6 ± 1.5	114 ± 5
	100	32.2 ± 1.5 *	108 ± 41
	420	30.1 ± 2.1 **	102 ± 61
None (control)		35.8 ± 1.8	116 ± 10
Colchicine	100	35.3 ± 1.4	114 ± 8
	300	34.7 ± 1.7	115 ± 7
	1000	35.4 ± 1.3	114 ± 9

* $P < 0.05$.
** $P < 0.005$.

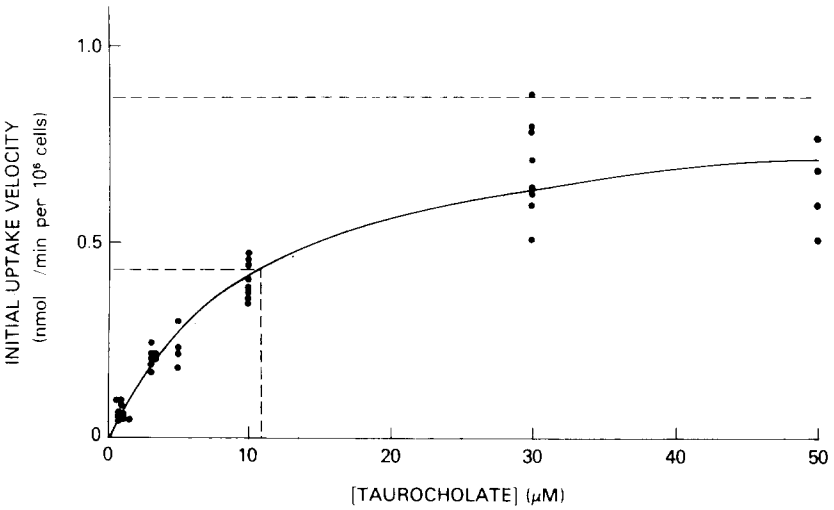

Fig. 1, Saturability of taurocholate uptake by isolated hepatocytes. The relationship between initial uptake velocity and taurocholate concentration could adequately ($F = 592$; d.f.1 = 2; d.f.2 = 38, solid line) be described by the Michaelis-Menten equation. Maximal uptake velocity (upper dotted line) averaged 0.87 ± 0.05 nmol · s^{-1} · 10^6 cells and the half-saturation constant (intersection of vertical dotted line with abscissa) 10.9 ± 1.8 μM.

TABLE II

EFFECT OF DIFFERENT CONCENTRATIONS OF CYTOCHALASIN B ON TAUROCHOLATE UPTAKE BY ISOLATED RAT LIVER CELLS

Mean ± S.D. are given.

Cytochalasin B (μM)	V	K_m	F	N
None (control	0.87 ± 0.05	10.9 ± 1.8	592.1	40
4.2	0.82 ± 0.17	11.4 ± 3.5	168.5	24
12.6	0.82 ± 0.14	39.4 ± 17.2 **	170.3	34
42	0.90 ± 0.14	67.0 ± 4.1 **	146.4	34
126	0.87 ± 0.14	113.0 ± 24.9 **	1783.3	12
420	0.82 ± 0.06 *	109.0 ± 12.0 **	66.6	12

* $P < 0.01$, significantly different from control.
** $P < 0.001$, significantly different from control.

TABLE III

EFFECT OF DIFFERENT CONCENTRATIONS OF COLCHICINE ON TAUROCHOLATE UPTAKE BY ISOLATED RAT LIVER CELLS

Mean ± S.D. are given.

Colchicine (μM)	V	K_m	F	N
None (control)	0.94 ± 0.004	11.9 ± 2.4	1068	18
30	0.92 ± 0.09	12.6 ± 2.1	67.8	12
100	0.74 ± 0.08 *	12.4 ± 1.6	384.3	12
300	0.72 ± 0.07 *	13.6 ± 2.4	288.9	12
1000	0.38 ± 0.02 *	13.1 ± 3.5	1226.0	12

* $P < 0.001$, significantly different from control.

TABLE IV

BINDING OF ^{3}H-CYTOCHALASIN AND ^{3}H-COLCHICINE IN THE PRESENCE OF DIFFERENT TAUROCHOLATE CONCENTRATIONS

Results are given as % of radioactivity retained per 10^6 cells and are expressed as mean ± S.D. (n = 3).

		Taurocholate (μM)		
		0	1	50
DMSO		28.7 ± 1.6	27.4 ± 1.4	28.3 ± 0.9
Cytochalasin B	42 μM	29.6 ± 2.3	28.3 ± 1.9	29.4 ± 1.2
	126 μM	20.8 ± 4.2	19.6 ± 1.2	20.2 ± 1.3
	420 μM	21.4 ± 1.8	20.9 ± 1.6	21.2 ± 1.0
Control		11.8 ± 0.9	12.1 ± 1.2	11.9 ± 1.7
Colchicine	0.1 mM	12.4 ± 1.1	12.3 ± 3.4	12.4 ± 1.9
	1.0 mM	10.9 ± 2.4	11.6 ± 1.6	11.4 ± 1.7

Colchicine also decreased fractional taurocholate uptake in a dose-dependent fashion, a significant effect appearing at colchicine concentrations higher than 30 μM. The effect of different colchicine concentrations on half-saturation constant and maximal uptake velocity are shown in Table III. In contrast to cytochalasin B, colchicine reduced the maximal taurocholate uptake velocity without affecting the half-saturation constant. Kinetic analysis confirmed this non-competitive type of inhibition, K_i being 0.47 ± 0.07 mM.

Binding of cytochalasin B to hepatocytes was dose-dependent, decreasing from 49.3 ± 5.2% to 21.4 ± 6.3% at 4.2 and 420 μM, respectively. Taurocholate did not affect cytochalasin B binding to hepatocytes (Table IV).

Discussion

Bile acid transport in the intact liver is a carrier-mediated, sodium-dependent mechanism [11]. This property is maintained by isolated hepatocytes [12], suggesting that this model is a useful tool in transport studies. Hexoses [13] and amino acids [14] are substrates transported by similarly efficient, highly differentiated mechanisms. The transport of hexoses has been shown to be influenced by the microfilament system [15] in a fashion similar to that described in the present investigation. Hepatocytes are rich in microfilaments [16] and their inhibition by cytochalasin B has been shown to result in cholestasis [2]. Since bile acid excretion is a major determinant of bile flow [17], it is conceivable that decreased bile salt uptake is at least in part responsible for the cytochalasin B-induced cholestasis. The competitive type of inhibition of taurocholate uptake by cytochalasin B could be due to different mechanisms. It has been demonstrated that microfilaments are responsible for the spatial arrangement of receptors on the cell surface [18]. Clustering of bile salt receptors may lead to site-site interactions and thereby result in a decrease in the apparent K_m as demonstrated in the present investigation. An alternate possibility is that cytochalasin B competes directly with taurocholate for binding to the bile salt carrier. The finding that taurocholate did not affect cytochalasin B binding militates against this hypothesis. It has to be acknowledged, however, that the cytochalasin B binding data represent steady-state and not initial

conditions. Cytochalasin B binding by intact cells presumably is a complex phenomenon, representing binding to the target organ (the microfilaments), binding to plasma membrane components [19] and lipid partition phenomena within the membrane [20]. It is therefore conceivable that only a small fraction of the cytochalasin B bound represents binding to bile salt receptors and that such a phenomenon had not been unveiled by this rather crude binding assay. A third alternative which has to be considered is inhibition of Na^+-transport by cytochalasin B. Such an effect would have to result in an inhibition of bile salt transport, since the latter is sodium dependent [11,12]. The finding of a slightly reduced membrane potential and decreased intracellular K^+ concentration at high cytochalasin B concentration makes an alteration of the electrical potential as a cause of reduced taurocholate uptake appear possible. However, cytochalasin B was an effective inhibitor of taurocholate uptake already at concentrations where no effects on membrane potential or intracellular K^+ concentrations were apparent. Moreover, abolition of the Na^+ gradient in the perfused rat liver [11] or isolated hepatocytes [12] led to a noncompetitive type of inhibition rather than the competitive type of inhibition induced by cytochalasin B. A last possibility, namely that the inhibition of taurocholate uptake by cytochalasin B is due to a direct toxic effect of cytochalasin B, has to be considered. Although none of the parameters of viability tested was affected by cytochalasin B, it is conceivable that a toxic effect might occur without being recognized by the viability assays used in the present investigation.

Another component of the cytoskeleton is represented by the microtubules [1] which are involved in protein [21] and biliary lipid [3] secretion. Since biliary lipid secretion is closely related to bile salt excretion [22] it seemed of interest to investigate whether the microtubule inhibitor colchicine exerts any effect on taurocholate uptake. It had already been shown that bile salt excretion is diminished after colchicine administration, albeit to a lesser extent than lipid excretion [3]. In contrast to cytochalasin B, colchicine reduced maximal uptake velocity, leaving the half-saturation constant unaffected. Furthermore, relatively high concentrations of colchicine were required before any effect on taurocholate uptake became noticeable. None of the parameters of viability tested was affected by this drug; therefore, it seems unlikely that the high concentrations required to reduce taurocholate transport are due to toxic effects of colchicine, although the same restrictions as for cytochalasin B apply. The effect of colchicine on taurocholate uptake is similar to the effect of microtubule inhibitors described on amino acid transport by Ehrlich ascites tumor cells [23]. As in the latter system, the contribution of the colchicine inhibited part to total taurocholate uptake is relatively small and is achieved without alteration in membrane potentials or intracellular K^+ concentration.

In summary, the present investigation demonstrates that both the microfilament inhibitor cytochalasin B and the microtubule inhibitor colchicine interfere with taurocholate uptake by isolated rat hepatocytes. While relatively low doses of cytochalasin B achieve significant inhibition of taurocholate transport, relatively high concentrations of colchicine are required to impair bile salt transport. In addition to these quantitative differences, the two agents differ also qualitatively in their effect on taurocholate uptake, cytochalasin B and

colchicine leading to competitive and non-competitive types of inhibition, respectively. The mechanisms by which microfilaments and microtubules are related to bile salt transport remain to be elucidated.

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