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Report

Uptake of Colchicine, a Microtubular System Disrupting Agent, by Isolated Rat Hepatocytes

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The possible mechanism of hepatic uptake of colchicine (CLC), a microtubule system disrupting agent, was examined using isolated rat hepatocytes. The existence of a carrier-mediated active transport system for CLC was demonstrated. This transport system is saturable, is affected by metabolic inhibitors (dinitrophenol, KCN) and a SH-group blocker (*p*-hydroxymercuribenzoic acid but not *N*-ethylmaleimide), and is sensitive to temperature. Ouabain, an inhibitor of Na^+, K^+-ATPase, does not affect the transport system of CLC.

KEY WORDS: colchicine; hepatic uptake; isolated hepatocyte; microtubule.

INTRODUCTION

Colchicine (CLC) is a disrupting agent of microtubules which causes metaphase arrest. CLC binds specifically to free tubulin, a structural subunit of the microtubules, and further, preformed tubulin–CLC complex interacts with microtubules. In consequence, the elongation of microtubules is inhibited, while at the other end of the polymer, the process of microtubule assembly occurs (1). Finally, CLC-induced microtubule depolymerization inhibits cell mitosis.

Intracellular and membrane transport of several substances is mediated by the microtubular system (2–7), and CLC or other microtubular poisons are used as a probe to study these transport phenomena. The effect of CLC on the intracellular disposition and excretion through the bile canalicular membrane of several compounds has been studied in the perfused rat liver (4–6). However, few studies exist on CLC uptake into cultured cells (8–10), and there are no data on CLC uptake by the isolated hepatocytes. CLC uptake into Chinese hamster ovary cells is nonsaturable and unaffected by the sulfhydryl reagents and ouabain, which are potent inhibitors of various mediated transport systems (9). On the other hand, some metabolic inhibitors, such as cyanide, azide, and dinitrophenol, stimulate CLC uptake into the Chinese hamster ovary cells. This finding indicates the presence of an energy-dependent carrier-mediated drug-efflux system for the maintenance of a drug-permeability barrier.

CLC serves as a specific marker of the microtubular system, to test whether an agent is transported by a microtubule-mediated system. The purpose of our study was to elucidate the possible mechanism of hepatic uptake of CLC using isolated rat hepatocytes.

MATERIALS AND METHODS

^{3}H-Colchicine (CLC) (32.5 Ci/mmol) was purchased from the New England Nuclear Co. (Boston, Mass.). Unlabeled CLC was purchased from Sigma Chemical Co. (St. Louis, Mo.). Phthalic acid diisobutyl ester (d = 1.041) was obtained from Tokyo Kasei Kogyo Co., Tokyo. Collagenase (150–300 U/mg) was obtained from the Wako Pure Chemical Industries, Ltd., Osaka, Japan. All other reagents were of analytical grade.

Isolated hepatocytes were prepared by collagenase perfusion according to the procedure of Berry and Friend (12) as modified by Baur *et al.* (13). Adult male, Wistar rats, 180–200 g, were used as liver donors. Animals were maintained in a controlled environment. After isolation, hepatocytes were suspended in ice-cold Hanks buffer (8 g NaCl, 0.4 g KCl, 0.14 g $CaCl_2$, 0.213 g $MgCl_2$ $6H_2O$, 0.205 g $MgSO_4$ $7H_2O$, 0.078 NaH_2PO_4, 0.151 g Na_2HPO_4, 0.35 g $NaHCO_3$, and 0.9 g glucose per liter of distilled water), oxygenated with 95% O_2–5% CO_2, pH 7.4. Viability was assessed by trypan blue exclusion, and only hepatocytes with greater than 90% viability were used for experiments.

Isolated hepatocytes were suspended in the incubation buffer at a final cellular protein concentration of approximately 2 mg/ml. After preincubation at 37°C for 10 min, uptake was initiated by the addition of radiolabeled substrate (tracer experiments) or by the addition of an aliquot of hepatocyte suspension to the prewarmed (37°C) buffer solutions of CLC, containing a wide range of CLC concentrations (0.1 to 100 μM). One hundred-microliter aliquots of cell suspension were removed at 20, 40, 60, and 120 sec and 15 and 30 min and were transferred to 400-μl microcentrifuge

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tubes containing 50 μl of 3 *M* KOH and 100 μl of oil layer (phthalic acid disobutyl ester; $d = 1.041$). The tubes were centrifuged at 10,000*g* for 15 sec in a tabletop microfuge (Beckman Instruments, Fullerton, Calif.). After standing overnight, the tubes were cut at the lower part of the oil layer. The radioactivity in both upper and lower layers was determined in a Packard Tri-Carb counter (Model 3255, Packard Instruments, Downers Grove, Ill.) in 10 ml of liquid scintillation cocktail (0.1 g of POPOP, 4.0 g of DPO, 500 ml of Triton X-100, and 1 liter of toluene). The pellet fraction was neutralized with 1 *N* HCl before scinitillation counting.

In inhibition experiments, following temperature equilibration, hepatocytes were preincubated at 37°C for 3 min with the metabolic inhibitors [1 m*M* KCN, 1 m*M* dinitrophenol (DNP)], Na^+, K^+-ATPase inhibitor (1 m*M* ouabain), or SH-group blockers [0.1 m*M* *N*-ethylmaleimide (NEM), 0.1 m*M* *p*-hydroxymercuribenzoic acid (PHMB)], then CLC tracer (0.1 μCi) was added and the initial velocities as well as the total uptake at 30 min were determined.

The initial uptake rate (v_0) was calculated from the slope of the linear regression line obtained during the 60-sec incubation period, where the slope corresponds to the v_0 and the value extrapolated to "zero time" relates to a nonspecific binding to the cell surface. The relation between the v_0 and the extracellular concentration of CLC (C) was analyzed according to the Michaelis–Menten-type equation:

$$v_0 = \frac{V_{max} \times C}{K_m + C} \quad (1)$$

The analysis was performed by the nonlinear iterative least-squares method (14). The initial estimates of the parameters were obtained by an Eadie–Hofstee-type plot derived from Eq. (1).

The intracellular drug concentration was calculated based on the reported intracellular volume of 2.5 μl/mg protein (15). The CLC binding to the rat liver 100,000*g* cytosol, prepared from 33% liver homogenates (16), was determined by the activated charcoal method on the day of preparation (17). Tracer CLC was added to an aliquot of 100,000*g* liver cytosol to the final radioactivity concentration of 0.2 μCi/50 μl of cytosol, then an aqueous solution of unlabeled CLC (10 μl) was added to a final concentration ranging from 10^{-8} to 10^{-4} *M*. The samples were incubated for 60 min (equilibrium was reached at that time) at 37°C. The reaction was stopped with the addition of 400 μl of 0.25% distilled water-activated charcoal suspension and vigorous mixing. Ten minutes later, the samples were centrifuged, and the concentration of the bound CLC in the supernatant was determined. Estimates of the apparent binding parameters for the CLC binding to the liver cytosol were made by a nonlinear iterative least-squares method according to the following equation (14):

$$C_{bound} = \frac{n(P) \times C_{free}}{K_D + C_{free}} \quad (2)$$

where C_{free} and C_{bound} are the unfound and bound concentrations of CLC, respectively, $n(P)$ is the binding capacity, and K_D is the dissociation constant. The protein concentration was determined by Bio-Rad assay kit (Bio-Rad Laboratories, Richmond, Calif.). Bovine serum albumin was used as the standard.

All experiments were performed in three to five separate liver-cell preparations. Statistical significance was analyzed by Student's t test.

RESULTS AND DISCUSSION

The time course of tracer CLC uptake into the isolated rat hepatocytes is presented in Fig. 1. The uptake rate of CLC is linear up to 60 sec. Incubation of hepatocytes at a low temperature (0°C) dramatically decreased the uptake of CLC. When the incubation time exceeds 60 sec, the transport of CLC is no longer linear (Fig. 1b). Therefore, the values of v_0 were estimated from the slope of the linear regression line obtained during the 60-sec incubation period with simultaneous correction for the nonspecific binding to the cell surface ("zero-time" uptake). The relationship between the v_0 and the substrate concentration, presented as an Eadie–Hofstee plot (Fig. 2), shows that the uptake of CLC is a saturable process. The K_m and V_{max} values for that process were 28.0 ± 5.7 μ*M* and 1670 ± 200 pmol/min/mg, respectively.

Equilibrium binding experiments were performed with rat liver 100,000*g* cytosols (Fig. 3), to determine the binding parameters of CLC to its specific intracellular binder, tubu-

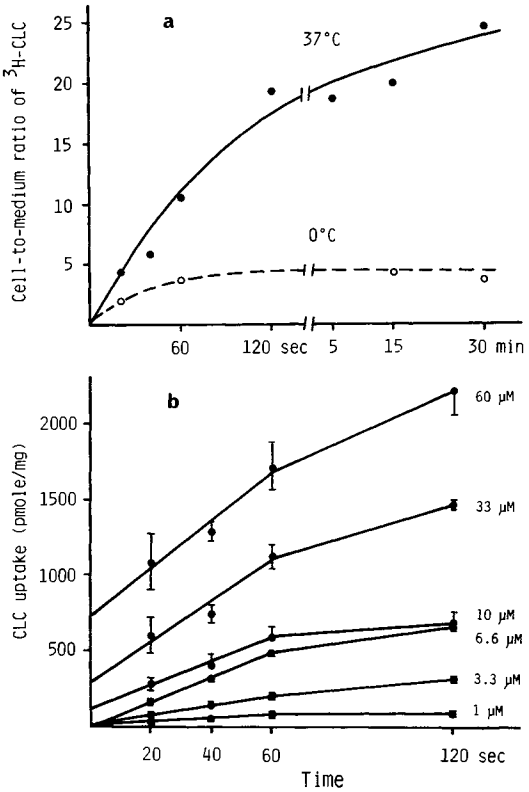

Fig. 1. Time course of the uptake of ^{3}H-colchicine by isolated rat hepatocytes. (a) The tracer CLC concentration was used. The uptake was measured at 37 and 0°C and was expressed as the cell-to-medium concentration ratio. (b) Concentration-dependent uptake of CLC. Each point and vertical bar represent the mean ± SE of three to five independent experiments. The uptake is expressed as the amount taken up per milligram of protein.

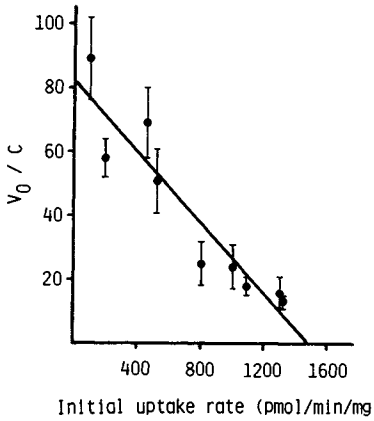

Fig. 2. Concentration dependency of the initial uptake rate (v_0) of CLC by isolated rat hepatocytes. The v_0 was determined using the data at 20, 40, and 60 sec by least-squares regression analysis. Data are presented as an Eadie–Hofstee plot. Each point and vertical bar represent the mean ± SE of three to five independent experiments.

lin. The results are presented in Table I together with the uptake data for comparison. The tissue-to-medium concentration ratio, calculated by considering the dilution factor of the cytosol, is approximately 4, which is six times lower (Fig. 1a) than the observed value. As discussed later, this discrepancy may result from active CLC transport. CLC was first considered a highly specific marker for the involvement of microtubular system in transport. However, Mizel and Wilson (18) have demonstrated a competitive inhibition of nucleoside transport in a number of mammalian cell lines. This effect of CLC does not seem to be due to the disruption of microtubules, since lumicolchicine, an analogue of CLC that does not bind to tubulin and does not prevent microtubule assembly, was as potent as CLC in the inhibition of nucleoside transport. Although tubulin is a main binder of CLC (its binding to other macromolecules is very low), the high cell-to-medium concentration ratio and its saturable uptake by hepatocytes cannot be explained by its binding to tubulin. One may expect that this compound is transported to the intracellular space of hepatocytes by a carrier-mediated active transport system.

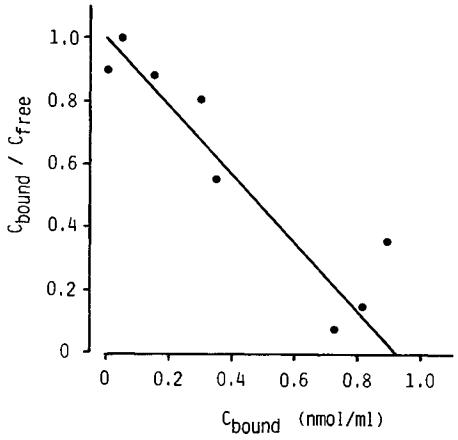

Fig. 3. Scatchard plot of the CLC binding to rat liver 100,000g cytosol determined with the activated charcoal method.

Table I. Kinetic Parameters of the Initial Uptake Rate (v_0), by Isolated Rat Hepatocytes and Specific Binding of CLC to Rat Liver Cytosol

Experiment	Parameter[a]
Initial uptake rate (v_0)	K_m = 28.0 ± 5.7 μM
	V_{max} = 1670 ± 200 pmol/min/mg
Binding to 100,000g rat liver cytosol	K_D = 0.92 ± 0.09 μM
	$n(P)$ = 0.92 ± 0.13 nmol/ml[b]

[a] Mean ± computer-estimated SD value.
[b] Binding capacity [$n(P)$] is expressed as nanomoles of CLC bound to 1 ml of 100,000g liver cytosol (33%); this corresponds to 2.79 ± 0.39 nmol/g of liver.

To examine the above hypothesis, several inhibitors of the membrane transport were used. The v_0 of CLC was significantly reduced by PHMB and DNP, and the effect of KCN, ouabain, and NEM was less pronounced (Fig. 4a). Under pseudoequilibrium conditions (30 min), the total uptake was sensitive to the same inhibitors (Fig. 4b). The inhibitory effect of DNP and KCN indicates that CLC is taken up by isolated rat hepatocytes by a carrier-mediated active transport system. Contrary to the results obtained by See *et al.* (11), who showed the DNP and KCN in the absence of glucose stimulated the uptake of CLC into Chinese hamster ovary cells, the effect of these metabolic inhibitors on CLC uptake by isolated rat hepatocytes was completely different. The increased uptake of CLC by Chinese hamster ovary cells in the presence of DNP and KCN was probably the result of the inhibition of an energy-dependent efflux system existing in these cells. Two SH-group blocking agents (PHMB and NEM) used in this study are known to be able to disrupt microtubules (16,19). However, only PHMB exerted a pronounced effect on the uptake of CLC to isolated rat hepatocytes, reflecting a different mechanism of action of these two SH-group blockers.

CLC, as a specific inhibitor of transport phenomena involving microtubules, should be employed with caution, since other substances may share the same carrier system.

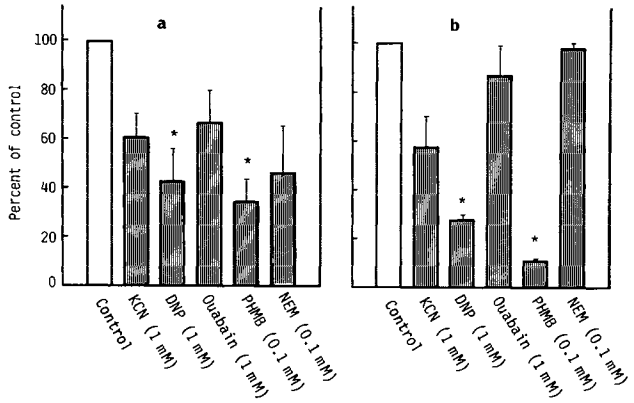

Fig. 4. Effect of metabolic inhibitors and sulfhydryl reagents on the initial uptake rate (v_0) (a) and the uptake at 30 min (b) of tracer CLC. Data are expressed as a percentage of the control. Each point and vertical bar represent the mean ± SE of four independent experiments. (*) Statistically significant ($P < 0.05$) compared to the control.

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