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PREINCUBATION ACCELERATES TAUROCHOLATE UPTAKE INTO ISOLATED LIVER CELLS

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Initial rates of taurocholate uptake into isolated hepatocytes stored at 0°C increased 3-fold during a 25 min preincubation. Concomitantly, V increased while K_m remained unaffected. There are several possible explanations for the preincubation effects, such as new synthesis of carrier protein, altered fluidity of the membrane or stimulation of the sodium-dependent taurocholate uptake via a change in the cation distribution. The experiments presented strongly favor the latter explanation as the sodium gradient as well as the uptake of the bile acid reach their steady state within 20–30 min and replacement of sodium by potassium in the medium abolished the effect.

Introduction

Many investigators store their freshly isolated hepatocytes at 0°C until transport experiments are performed [1–13]. As a consequence of the low temperature, vital cellular parameters are not at their physiological equilibrium. Cells kept in cold storage are depleted from K^+, are more alkaline [14], and contain additional Ca^{2+} [15]. When cells are transferred to incubation media at 37°C, multiple reequilibration processes take place.

Respiration is turned on, the physiological transmembrane K^+-gradient is restored [14,16], intracellular pH becomes more acidic [14], and lipogenesis improves [17]. It has been observed that concomitantly with these alterations, membrane transport processes are also altered, such as transport of amino acids [4], Ca^{2+} [18] and succinate [19].

In light of the increasing use of isolate liver cells for transport studies [13], it now seems important to know more about the influence of preincubation on transport processes. We used taurocholate as substrate in the following study, as carrier-mediated uptake of this bile acid into isolated hepatocytes has been well characterized [1].

Materials and Methods

Tauro[*carbonyl*-^{14}C]cholic acid (50 mCi/mmol) was obtained from the Radiochemical Centre (Amersham, U.K.). Taurocholate was from Serva (Heidelberg, F.R.G.), cycloheximide and collagenase were from Boehringer (Mannheim, F.R.G.) and Leibowitz L-15 medium was purchased from Seromed (Munich, F.R.G.). Silicon oils AR 20 and AR 200 for centrifugal filtration were from Wacker Chemie (Munich, F.R.G.). All other chemicals were of the highest purity available.

Liver cells were isolated from our own stock of male Sprague-Dawley rats (200 g body weight) by enzyme perfusion of the liver [20] as modified by Schwarz et al. [21]. Freshly isolated cells were stored in stock suspension (40 mg protein/ml) in Leibowitz L-15 medium at 0°C and used within 3 h unless otherwise indicated. Cell viability was tested by trypan blue exclusion (more than 92%) and by stimulation of cellular oxygen consumption with the uncoupler carbonylcyanide-*m*-chlorophenyl-hydrazone (more than 2-fold) as previously described [14]. The liver cells were transferred into Leibowitz L-15 medium and gently stirred, usually at 37°C. After various

preincubation times, [^{14}C]taurocholate was added to the suspensions.

To measure uptake, 200 μl aliquots of the cell suspensions were withdrawn at various times after addition of the substrate, and cells were separated from the extracellular medium by centrifugal filtration through a layer of silicone oil as described by Baur et al. [14]. Radioactivity in the supernatants and in the cellular sediments was measured by liquid scintillation counting in Bray's solution. The fractions of radioactivity in cells were determined. Uptake values were corrected for the small amount of medium adhering to the cells after centrifugation, which was measured with [^{14}C]dextrane and amounted to 1.6 μl/mg cell protein. Initial rates of taurocholate uptake were obtained from the slopes of the curves through the measuring points at 15, 30, 45 and 60 s after addition of [^{14}C]taurocholate. These values were used to construct the Lineweaver-Burk plot; the kinetic constants K_m and V were determined graphically.

All experiments were performed with four cellular preparations in duplicates. The coefficients of variation between the four preparations ranged from 3.2–12.7% for the initial rates of uptake. The figures show the representative results from single preparations.

Results and Discussion

The rate of taurocholate uptake into liver cells, measured immediately after transfer of the cells from the stock suspension (0°C) to the incubation medium (37°C), was considerably slower than that obtained after preincubation at 37°C (Fig. 1). During preincubation the initial rate of taurocholate uptake increased about 3-fold within 25 min. Longer preincubation times had no further effect. Results were identical in both Leibowitz L-15 medium and Hank's medium supplemented with 5 mM glucose.

To learn more about the mechanisms of this effect, the initial rates of taurocholate uptake were measured at different taurocholate concentrations. It was found that maximal rate of uptake (V) was increased during a 20 min preincubation from 1.2 to 3.2 (nmol/mg protein per min), while the apparent affinity constant (K_m 21 μM) remained unaffected (Fig. 2). This suggests that the identity of the carrier involved in uptake remains unchanged during preincubation. Consequently, either the amount of carrier molecules is increased due to protein synthesis or each carrier molecule transports at a higher rate.

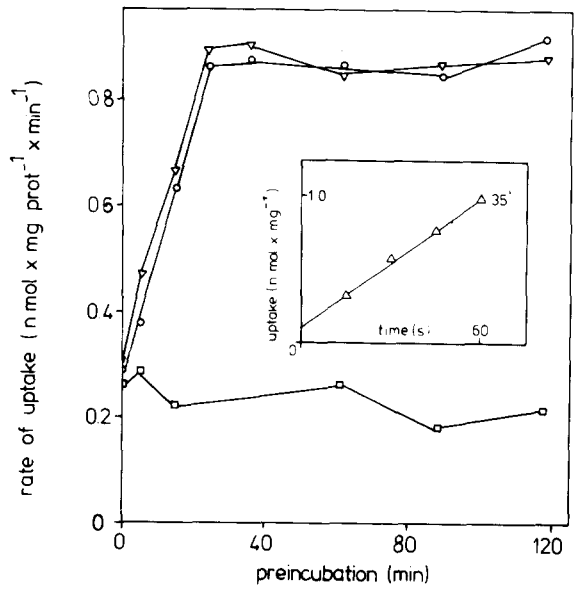

Fig. 1. Preincubation influences the rate of taurocholate uptake. Effect of cycloheximide and Na^+-depletion. Isolated liver cells (2 mg cell protein) were taken from the ice-cold stock suspension and preincubated at 37°C in 1 ml Leibowitz L-15 medium for the times indicated. Then [^{14}C]taurocholate was added to the final concentration of 10 μM. Initial rates of taurocholate uptake were determined as described previously [1] by centrifugation of cells after 15, 30, 45 and 60 s through a layer of silicon oil into 3 M KOH. Control (○); cycloheximide (5 μg/ml) during preincubation (▽); Na^+-depletion during preincubation, Na^+ in the medium was replaced by equimolar concentration of K^+ (□). Inserted figure: Initial rate of uptake of control after 35 min preincubation.

To test the first possibility, preincubations were performed in the presence of cycloheximide. This inhibitor of protein synthesis has been shown to suppress induction of amino acid transport in isolated hepatocytes [22]. However, as depicted in Fig. 1, cycloheximide did not diminish the effect of preincubation on taurocholate uptake, indicating that new synthesis of carrier molecules is not responsible for the effect of preincubation. Consequently, the turnover rate of preexisting carriers must be increased. This increase could be caused by various processes such as alteration of membrane fluidity or an increasing transmembrane Na^+-gradient, as tauro-

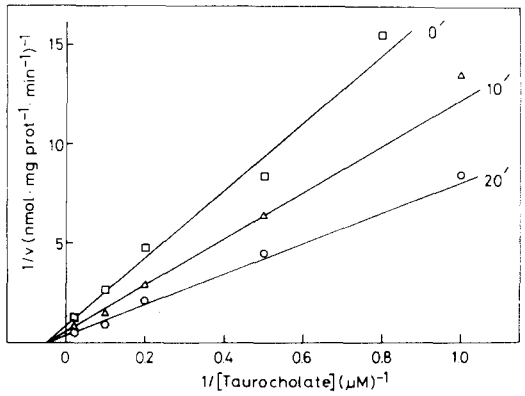

Fig. 2. Lineweaver-Burk plot demonstrating the effect of preincubation on K_m and V of taurocholate uptake. Isolated liver cells (1 mg cell protein) were taken from the ice-cold stock suspension and preincubated at 37°C in Leibowitz L-15 medium for 0 min (□), 10 min (△) or 20 min (○). The [^{14}C]taurocholate was added to each series at the concentrations of 1, 2, 5, 10 and 50 μM. Initial rates of uptake were determined from the uptake values at 15, 30, 45 and 60 s after addition of the bile acid.

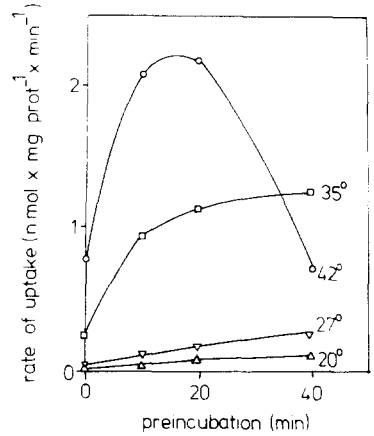

Fig. 3. Effect of preincubation at various temperatures. Isolated liver cells (1 mg cell protein) were taken from the ice-cold stock suspension and preincubated in 1 ml Leibowitz medium for various lengths of time at the following temperatures: 42 (○), 35 (□), 27 (▽) and 20°C (△). Then [^{14}C]taurocholate was added to the final concentration of 10 μM. Initial rates of uptake were measured at the temperatures of preincubation by assessing uptake at 15, 30, 45 and 60 s after addition of the bile acid.

cholate uptake has been shown to be sodium-dependent [1,23].

To test whether alterations of membrane fluidity are involved, we used an indirect approach. Hepatocytes were preincubated at four different temperatures above and below the formerly described temperature break in the Arrhenius plot (29°C), which was interpreted as phase transition point of the cell membrane [1]. It turned out that although uptake was slower at lower temperatures the relative effect of preincubation was similar both above and below that critical temperature (Fig. 3; the downward bend of the curve at 42°C may be due to energy depletion and is not considered further). Thus, it is unlikely that phase transitions contribute much to the effect of preincubation.

Alternatively, the effect of preincubation may be related to the well-known stimulatory effect of Na^+ [1,23,24] on taurocholate influx. During preincubation the transmembrane Na^+- and K^+-gradients increase in the same fashion as taurocholate uptake [16,19]. Both processes reach their steady state within 20–30 min at 37°C, and are protracted at lower temperatures. If the effect of preincubation was a consequence of the increasing cation gradient, then the effect should disappear when the gradients are experimentally suppressed. We tested this hypothesis by performing preincubation in a standard medium where Na^+ was replaced by an equimolar amount of K^+. The results are included in Fig. 1, the effect of preincubation on taurocholate uptake was abolished. This indicates an interdependence between cation distribution and effect of preincubation. Recent studies on uptake of ouabain yielded complementary results [25]. Transport of this uncharged steroid resembles that of taurocholate in most respects. But it differs in two ways: It is independent of both Na^+ and preincubation suggesting a coupling of the two phenomena.

Taking all these results into account, there is now strong evidence that preincubation affects the uptake of taurocholate via alteration of the transmembrane cation distribution.

Independent of the yet unknown mechanism of the coupling between cation distribution and transport (possible mechanisms are discussed by Kimmich, using isolated intestinal cells [26]) the present results have consequences for the practical application of isolated liver cells in transport studies: Firstly, the optimal length of preincubation depends on the experimental temperature; once the length is fixed, it

should be maintained accurately. Secondly, the length of preincubation is an important parameter for assessing Na^+-dependence of transport; in the absence of preincubation, taurocholate uptake is only slightly Na^+-dependent (Fig. 1). Finally, it appears possible that variations in the length of preincubation will provide a tool for studying the coupling between cation gradients and transport of taurocholate or other substrates.

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