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KINETIC EVALUATION OF CARRIER-MEDIATED TRANSPORT OF OUABAIN AND TAUROCHOLIC ACID IN ISOLATED RAT HEPATOCYTES

EVIDENCE FOR INDEPENDENT TRANSPORT SYSTEMS*

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Abstract—Ouabain and taurocholic acid (Tch) share in common the cyclopentanophenanthrene (steroid) nucleus, and both are concentrated in the liver by hepatic sinusoidal carrier-mediated transport processes. Multiple transport systems for Tch uptake have been implicated, and Tch is an effective inhibitor of ouabain uptake. To determine if the ouabain transport system is related to transport of Tch, kinetic studies were conducted to examine the nature of cross-inhibition of ouabain and Tch. Tch was found to inhibit ouabain uptake in a competitive manner, with a K_i approximately ten times less than the K_m for ouabain. However, ouabain failed to inhibit total Tch uptake in a competitive manner when added to the system at the same time as the Tch substrate. Preincubation of cells with ouabain resulted in non-competitive inhibition of sodium-dependent Tch uptake but had no effect on sodium-independent Tch uptake. Ouabain had a weak competitive inhibitory effect on sodium-independent transport of Tch, but the K_i was approximately ten times greater than the K_m for ouabain. These results demonstrate that the ouabain transport system is distinct from both the sodium-dependent and sodium-independent transport systems for Tch. Tch apparently binds competitively to the ouabain transport system but is not effectively transported across the cell membrane by this system.

The cardiac glycoside ouabain undergoes rapid hepatic extraction and biliary excretion in the rat [1], apparently as a result of active, carrier-mediated transport processes present at both the sinusoidal and canalicular membranes [1, 2]. Based on studies with liver slices, Kupferberg [3] suggested that a carrier selective for steroidal compounds was responsible for the hepatic uptake of ouabain, bile acids and steroid hormones. More recent work with isolated hepatocytes has demonstrated that the sinusoidal uptake process for ouabain is an active, sodium-independent, carrier-mediated process [2], whereas bile acid uptake occurs by at least two transport systems, one sodium dependent and one sodium independent [4, 5]. Cholate and taurocholate (Tch) were proposed to share the same sodium-independent transport system but to have different sodium-dependent systems [5].

Both cholate and Tch are potent inhibitors of ouabain uptake, although the nature of inhibition has not been determined [2]. When hepatocytes are preincubated with ouabain, it is an effective inhibitor of Tch uptake, presumably because of a non-competitive inhibitory effect on (Na^+-K^+)ATPase [2, 4, 5]. However, it is possible that ouabain is also a competitive inhibitor of bile acid uptake if a common carrier is shared. As ouabain uptake occurs via a sodium-independent system [2], the purpose of this investigation was to determine if the sodium-independent bile acid transport system and the ouabain transport system share a common carrier protein.

MATERIALS AND METHODS

[^{3}H]Ouabain (20 Ci/mmole, >98.5% radiochemical purity by TLC) and [^{3}H]-taurocholic acid (6 Ci/mmole, > 98.5% radiochemical purity by TLC) were obtained from the New England Nuclear Corp. (Freehold, NJ). Ouabain, taurocholic acid and other chemical reagents were obtained from the Sigma Chemical Co. (St. Louis, MO). Collagenase (class II, 120–180 units/mg) was obtained from the Cooper Biomedical Corp. (Malverne, PA). Silicone oil was obtained from the S.W.S. Silicone Corp. (Adrian, MI).

Preparation of isolated hepatocytes. Isolated hepatocytes were prepared by collagenase perfusion as described previously [2]. Adult male, Sprague–Dawley rats, 250–300 g, were used as liver donors. Animals were maintained in a controlled environment with a 12-hr light cycle, and were provided water *ad lib.* Food was withheld for 12 hr prior to hepatocyte preparation. Hepatocytes were prepared between 8:30 and 9:30 in the morning, and were utilized within 2 hr. Viability was assessed by trypan blue exclusion, and only hepatocytes with 90% or better viability were utilized for kinetic experiments.

Determination of initial velocities of uptake. Isolated hepatocytes were suspended in a Tris incubation buffer [2] at a final concentration of 2.0 to

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2.5 mg protein/ml. After temperature equilibration for 10 min, uptake experiments were initiated by addition of radiolabeled substrate. Uptake kinetics for [^{3}H]-ouabain were determined at substrate concentrations of 25, 50, 100, 150, 300, 500 and 750 μM. Duplicate 200-μl aliquots of cell suspension were removed at 1, 2, 3 and 4 min and added to 400-μl microcentrifuge tubes previously layered with 50 μl of 3 M KOH and 100 μl of silicone oil ($\delta = 1.015$, obtained by mixing Silicone oil AR200 and AR20 in a ratio of 1:1). In inhibition experiments, Tch was added at the same time as the radiolabeled substrate. At the appropriate time, the tubes were centrifuged at 10.000 g for 15 sec in a Beckman microfuge B. After standing overnight, the tubes were cut at the oil:KOH interface, and the radioactivity in the pellet fraction was determined by scintillation counting after neutralization with HCl. An aliquot of the incubation mixture was also obtained to determine total specific activity for each substrate concentration.

Uptake of [^{3}H]-Tch was determined in a similar manner at substrate concentrations of 5, 15, 30, 50, 100 and 200 μM. Because of the more rapid uptake and shorter period of linearity, initial velocities of uptake for Tch were determined in duplicate samples taken at 20, 40 and 60 sec. In experiments to delineate if ouabain competitively inhibits Tch uptake, except where noted, ouabain was added at exactly the same time as the [^{3}H]-Tch substrate. Uptake of both substrates was linear during the time intervals chosen, and initial velocities of uptake were determined from the slopes of the least-squares regression lines of uptake (nmoles/mg) versus time for each substrate concentration. As adherent fluid (extracellular incubation medium passing through the oil with the hepatocytes) is constant within an experiment and thus affects only the intercept of the regression line (e.g. has no effect on the initial velocity of uptake), corrections for adherent fluid were not made [2].

In virtually all cases, correlation coefficients greater than 0.990 were obtained, indicating the linearity of this technique during the time points sampled. In experiments examining sodium-independent uptake processes, the final wash of the cell preparation was conducted with incubation buffer containing an equimolar amount of choline chloride in place of sodium chloride. All other sodium salts were replaced with potassium salts [2]. The incubation medium and all solutions added to it were made with this same sodium-free incubation buffer. Because of the residual sodium present within the hepatocyte fraction even after washing with sodium-free buffer, the system is not totally free of sodium, but is reduced to less than 1 mM (unpublished observation). Replacement of sodium with choline had no apparent effect on cell viability after incubation for 30 min. Protein content was determined by the method of Lowry *et al.* [6].

Determination of K_m, V_{max} *and* K_i *from initial velocity data.* For both ouabain and Tch, no significant non-saturable uptake was noted. Thus, determination of kinetic parameters was accomplished directly from single-reciprocal linear transformation (S_0/v_0 vs S_0,: Hanes-Woolf plot) of the initial velocity of uptake data. Values were also calculated by double-reciprocal linear transformations (Lineweaver–Burk plot), and generally varied less than 15% from those obtained by single-reciprocal transformation. However, because of the extensive weighting of low substrate concentrations by double-reciprocal transformation, occasional K_m and V_{max} values were skewed greatly by relatively small variations in the lowest concentration time point. For this reason, all K_m, K_p and V_{max} values reported were determined from single-reciprocal transformations. Graphical presentation of inhibitor data is presented as double-reciprocal transformation because of convention and the ease of visual interpretation of this plot. K_i values were determined algebraically from inhibitory data by the rearrangement of the Michaelis–Menten equation describing competitive inhibition as follows:

$$K_i = \frac{i}{(K_p/K_m) - 1},$$

where K_m is the apparent Michaelis constant in the absence of inhibitor, and K_p is the apparent Michaelis constant in the presence of inhibitor at i concentration.

Statistical evaluation of the data. All experiments were conducted with at least four separate liver cell preparations. K_m and V_{max} values in the presence of inhibitor were analyzed by a one-way analysis of variance, and individual treatments were compared for statistical differences from the control values by the Dunnett's test for multiple comparisons [7]. The level of significance was set at $P > 0.05$.

RESULTS

Inhibition of ouabain transport by Tch. Uptake of ouabain was completely saturable at high substrate concentration, and was reduced in a dose-related fashion by the presence of Tch (Fig. 1A). Tch increased the K_m but had no effect on the V_{max} for ouabain uptake, indicative of competitive inhibition (Fig. 1B, Table 1). The K_m and V_{max} values for ouabain in the absence of inhibitors were 127 ± 13 μM and 1.68 ± 0.11 nmoles/min/mg respectively. These values are consistent with previous reports of kinetic parameters for this transport system [2, 8, 9]. The K_i for Tch inhibition of ouabain transport was 10.4 ± 1.0 μM when averaged over three inhibitor concentrations (Table 1). This value was approximately ten times less than the K_m for ouabain, and slightly less than the K_m of total Tch transport (Table 2), indicating that Tch is a very effective competitor for the ouabain transport site.

Inhibition of uptake of taurocholate by ouabain. Tch transport was completely saturable at high substrate concentrations and was not affected by ouabain when added at the same time as the substrate (Fig. 2). The K_m and V_{max} values for Tch in the absence of inhibitor were 19.3 ± 1.6 μM and 2.4 ± 0.1 nmoles/min/mg respectively (Table 2). These values are similar to previously reported kinetic parameters for Tch transport in isolated rat hepatocytes [4, 5, 9], and represent the apparent kinetic parameters for Tch uptake with both sodium-

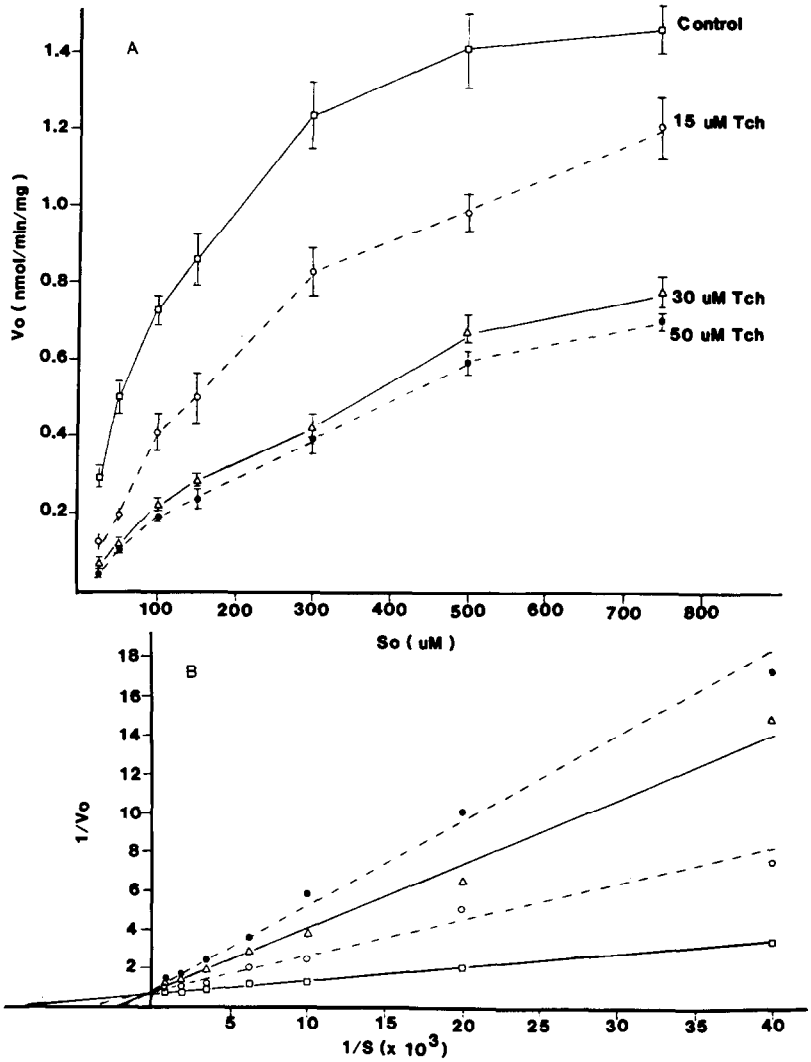

Fig. 1. Concentration-dependent inhibition of ouabain uptake by Tch. (A) Initial velocity of uptake (v_0) of ouabain vs substrate concentration at different Tch concentrations. V_0 was determined from least-squares regression of uptake vs time plot at each S_0. (B) Double-reciprocal linear transformation demonstrating a common y-intercept, indicative of competitive inhibition. Each value represents the mean ± S.E.M. of five cell preparations.

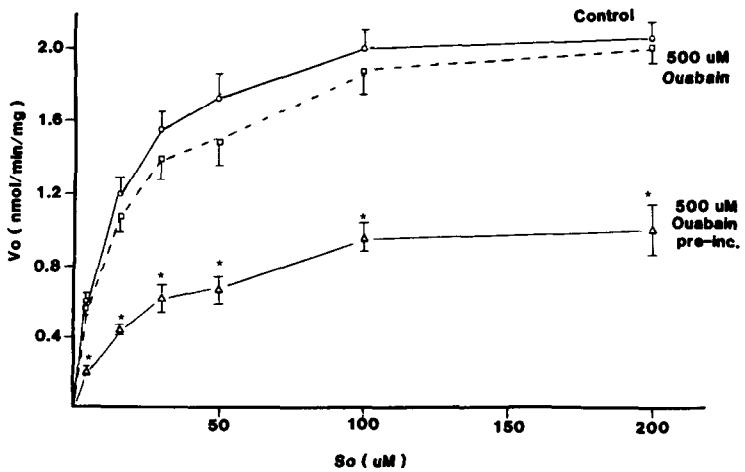

Fig. 2. Effects of ouabain on Tch uptake. Ouabain (500 μM) was added to the cells immediately at the time of Tch addition (□—□) or 15 min prior to addition of Tch substrate (△—△). Each value represents the mean of five to eight cell preparations. An asterisk (*) indicates significance at $P < 0.05$.

Table 1. Kinetic parameters for ouabain transport in the presence of various concentrations of Tch

Tch concn (μM)	K_m (μM)	V_{max} (nmoles/min/mg)	K_i (μM)
0	127 ± 13	1.68 ± 0.11	
15	361 ± 53	1.73 ± 0.12	8.3
30	492 ± 77*	1.34 ± 0.10	11.6
50	694 ± 127*	1.45 ± 0.15	11.2

Kinetic parameters were determined from single-reciprocal linear transformation (S_0/v_0 vs S_0). Each value represents the mean ± S.E.M. of four to six different cell preparations.

* Significantly different from control, $P < 0.05$.

dependent and sodium-independent transport systems operational. Although addition of ouabain at the same time as the substrate had no effect on Tch transport, preincubation of cells with 500 μM ouabain for 15 min reduced the uptake of Tch at all substrate concentrations (Fig. 2). Reciprocal transformation of these data indicated that preincubation of cells with ouabain reduced the V_{max} for Tch transport without significantly affecting the K_m, consistent with a non-competitive type of inhibition (Table 2).

Effects of ouabain on sodium-independent Tch uptake. The V_{max} for sodium-independent Tch uptake was 0.50 ± 0.06 nmoles/mg/min (Table 3), which accounts for approximately 20% of the total Tch uptake in the presence of sodium (Table 2). The K_m for sodium-independent Tch uptake was slightly greater than the K_m of the combined transport systems (Tables 2 and 3). When 500 μM ouabain was added to the cells at the same time as the Tch substrate, the K_m was increased slightly but significantly, whereas no significant effect on V_{max} was observed, suggestive of weak competitive inhibition (Table 3). The K_i for ouabain inhibition of sodium-independent Tch uptake was 1140 μM, or about ten times higher than the K_m for ouabain, and forty times greater than the K_m for sodium-independent Tch uptake. In contrast to its marked effects on sodium-dependent Tch uptake, preincubation of cells with ouabain for 15 min had no significant effect on sodium-independent Tch transport (Table 3). The kinetic parameters for ouabain uptake determined in the absence of sodium were not significantly different from those determined in a sodium-sufficient medium (data not shown), consistent with previous studies demonstrating that ouabain transport occurs by a sodium-independent transport system [2].

DISCUSSION

Kupferberg [3] first suggested that the hepatocyte membrane possessed a carrier-mediated transport system selective for exogenous as well as endogenous chemicals with a steroidal nucleus. This transport system has been referred to extensively in the literature as the "neutral" transport system, although the specificity of this system has not been well defined. As bile acids contain the steroid nucleus, it was initially thought that ouabain and bile acids might share the same transport system. However, Klaassen [10] demonstrated that newborn rats lack the ability to transport ouabain from plasma into liver, yet maintain normal bile acid transport capabilities, demonstrating that the two systems are not identical. Eaton and Klaassen [8] provided further evidence that bile acid and ouabain transport are at least partially different because the microsomal enzyme inducer pregnenolone-16-α-carbonitrile (PCN) produces a 2-fold increase in the V_{max} for ouabain transport yet has no effect on Tch uptake. Furthermore, Tch uptake is strongly sodium dependent, whereas ouabain uptake is sodium independent. However,

Table 2. Effects of ouabain on total Tch transport

Ouabain concn. (μM)	Preincubation (min)	K_m (μM)	V_{max} (nmoles/min/mg)
0		19.3 ± 1.6	2.4 ± 0.1
500	0	18.7 ± 4.2	2.0 ± 0.1
500	15	27.1 ± 6.0	1.2 ± 0.2*

Kinetic parameters were determined as described in Table 1. Each value represents the mean ± S.E.M. of five to eight cell preparations.

* Significantly different from the control, $P < 0.05$.

Table 3. Effects of ouabain on sodium-independent transport of Tch

Ouabain concn. (μM)	Preincubation (min)	K_m (μM)	V_{max} (nmoles/min/mg)
0		27.8 ± 2.2	0.50 ± 0.06
500	0	40.9 ± 2.7*	0.41 ± 0.06
500	15	32.2 ± 4.8	0.61 ± 0.02

Kinetic parameters were determined as described in Table 1. Each value represents the mean ± S.E.M. of four to seven cell preparations.

* Significantly different from the control, $P < 0.05$.

Tch has been found to inhibit ouabain uptake without affecting the transport of the organic cation procaineamide ethobromide (PAEB), suggesting that some specific interaction at the ouabain transport protein is occurring [2, 11].

The data provided in this study demonstrate competitive inhibition by Tch of ouabain transport. Previous studies have suggested that bile acid uptake occurs by multiple transport systems [4, 5, 12, 13], and specifically that Tch uptake occurs, in part, by a sodium-independent transport system. Ouabain inhibits the uptake of Tch after preincubation of isolated hepatocytes with ouabain, presumably as a result of inhibition of (Na^+-K^+)ATPase [5]. We found that ouabain did not competitively inhibit total Tch uptake even at concentrations twenty-five times greater than the K_m for Tch uptake but did inhibit Tch uptake in a non-competitive manner when hepatocytes were preincubated for 15 min with ouabain. However, ouabain did slightly competitively inhibit the uptake of sodium-independent Tch transport, suggesting that there was some interaction of ouabain with this carrier system. However, the K_i for ouabain inhibition of sodium-independent Tch was 10-fold greater than the K_m for ouabain transport and nearly forty times greater than the K_m for sodium-independent Tch uptake. Furthermore, the V_{max} for sodium-independent Tch transport was approximately one-third that of ouabain. Thus, the competitive interaction of ouabain at the sodium-independent transport site for Tch was very weak, and thus the two systems were not the same. As ouabain failed to significantly affect transport of Tch into isolated hepatocytes, the competitive inhibition of ouabain transport by Tch was apparently a result of a strong competitive interaction of Tch at the ouabain transport site, without significant translocation of Tch across the membrane by the ouabain carrier.

These results, taken with data from previous studies, demonstrate mediations of ouabain transport by a carrier distinct from both sodium-dependent and sodium-independent transport systems for Tch. Whether a physiological ligand exists for the ouabain transport system is uncertain. As other reports have demonstrated that endogenous steroid hormones such as estrogens [14], testosterone [14] and cortisol [15] are taken into the liver in part by carrier-mediated processes, it is possible that ouabain transport occurs by a physiological transport system for one or more of these steroid hormones. We have shown previously that a variety of steroid hormones do inhibit the uptake of ouabain [2], although the mode of inhibition remains to be elucidated.

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