Correlation Between the Inhibitory Effects of Basic Drugs on the Uptake of Cardiac Glycosides and Taurocholate by Isolated Rat Hepatocytes

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The role of the multispecific bile acid transporter for cardiac glycoside uptake is still controversial. This study was designed to examine the inhibitory effects of basic drugs (verapamil, dipyridamole, nifedipine, chlorpromazine, disopyramide, quinidine, propranolol, and lidocaine) on taurocholate uptake by isolated rat hepatocytes and to compare these effects with inhibition of ouabain uptake. Sodium-dependent taurocholate uptake was significantly reduced, to 50–70% of the control value, by 50 μM verapamil, dipyridamole, and nifedipine. Sodium-independent taurocholate uptake was more extensively inhibited, to 20–40%, by these basic drugs. The inhibition of ouabain uptake correlated better with sodium-independent taurocholate uptake ($\gamma = 0.918$) than with sodium-dependent taurocholate uptake ($\gamma = 0.714$). Taurocholate competitively inhibited ouabain uptake in the absence of sodium. These results indicate that the cardiac glycoside transport system is similar to the sodium-independent taurocholate transport system.

KEY WORDS: taurocholate; ouabain; verapamil; multispecific bile acid transporter; hepatic uptake.

INTRODUCTION

Recently some basic drugs such as quinidine (1–7), verapamil (8), amiodarone (9), and nifedipine (10) were reported to increase the serum digoxin concentration in patients and, in consequence, causing the occurrence of severe side effects. One of the mechanisms of the drug interaction is the decreased hepatic clearance of digoxin (3,4,7,11). In our previous study with isolated rat hepatocytes (12) we showed that many basic drugs, such as verapamil, diltiazem, dipyridamole, nifedipine, ajmaline, chlorpromazine, imipramine, disopyramide, and quinidine reduced hepatic uptake of digoxin. Further, digoxin was taken up by the same uptake process as ouabain, another cardiac glycoside (12).

The cardiac glycosides such as ouabain and digoxin undergo extensive hepatic extraction in the rat (13). Studies with isolated hepatocytes have demonstrated that the sinusoidal uptake process for the cardiac glycosides is an active, sodium-independent, carrier-mediated process (14), while bile acid uptake occurs by at least two transport systems, one sodium dependent and the other sodium independent (15,16). Recently, the role of the bile acid transporter for the hepatic clearance of ouabain has been discussed (17). In hepatocytes, ouabain uptake is positively correlated with bile acid uptake (18). Cardiac glycosides and bile acids are mutual inhibitors of their hepatocellular uptake (18–20). Furthermore, a photoreactive ouabain derivative was found to bind to the same protein in the plasma membrane as bile acid analogues (17). Therefore, it may be assumed that hepatic uptake of ouabain is mediated by carrier protein of the bile acid transport system, which has recently been called a multispecific transporter (21). However, other experimental evidence suggested independent transport for bile acids and for ouabain (22). Recently, Eaton and Richards (20) have reevaluated in detail the carrier-mediated transport of ouabain and taurocholate and proposed kinetic evidence for independent transport systems.

The relation between the transport systems for taurocholate (sodium dependent and independent) and cardiac glycosides is thus controversial. In the present study, we attempted to examine the inhibitory effects of many basic drugs on sodium-dependent and independent hepatic uptake of taurocholate and to gain some insight into the mutual relation between the transport systems for bile acids and cardiac glycosides.

MATERIALS AND METHODS

Materials

^{3}H-Taurocholate (6.8 Ci/mmol), ^{3}H-ouabain (19.5 Ci/mmol), ^3H_2O (5 mCi/ml), and ^{14}C-inulin (carboxy labeled, 2.4 mCi/g) were obtained from New England Nuclear Co. (Boston, MA). Nonradioactive taurocholate and ouabain were obtained from Sigma Chemical Co. (St. Louis, MO). Quinidine and phthalic acid diisobutyl ester ($d = 1.041$) were obtained from Tokyo Kasei Kogyo Co. (Tokyo). Collagenase was obtained from Wako Pure Chemical Industries Co. (Osaka, Japan). Verapamil HCl was a generous gift from Eisai Pharmaceutical Co. (Tokyo). Nifedipine was a generous gift from Bayer Yakuhin Co. (Shiga, Japan). All other chemicals were commercial products and analytical grade.

Isolation of Hepatocytes

Male Wistar rats weighing 180–200 g were used as liver donors. Hepatocytes were prepared by the procedure of Berry and Friend (23) as modified by Baur *et al.* (24). 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 g NaH_2PO_4, 0.151 g Na_2HPO_4, 0.35 g $NaHCO_3$, and 0.9 g glucose per liter of water, oxygenated with 95% O_2–5% CO_2, pH 7.4). The viability of isolated hepatocytes was tested by the trypan blue exclusion test and the preparation was considered suitable

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by the criterion that more than 90% of the cells excluded the dye.

Uptake Experiments

In all experiments except for studies to examine the type of inhibition, isolated hepatocytes (final concentration, 2 mg protein/ml of incubation buffer) were preincubated with a 50 μM concentration of the test compounds (inhibitors) at 37°C for 2 min. Uptake was initiated by the addition of radiolabeled taurocholate or ouabain to the cell suspension. Aliquots of the cell suspension were withdrawn at appropriate intervals. Cells were separated from the incubation medium by rapid centrifugation in a Beckman Microfuge B (Beckman Instruments, Fullerton, CA) through the layer of phthalic acid diisobutyl ester and were further dissolved in 3 M KOH. After standing overnight, the tubes were cut at the oil layer. The radioactivity of upper and lower sides was determined. The aqueous cellular volume and the volume of adherent fluid were corrected by measuring the uptake of ^3H_2O and ^{14}C-inulin, respectively.

The uptake of taurocholate in the absence or presence of sodium was linear within the first 1 min. The uptake of ouabain was slower and linear within the first 5 min. Initial uptake rates were determined over 1 min at 15-sec intervals for taurocholate and over 5 min at 1-min intervals for ouabain.

Protein concentration was determined by a Bio-Rad protein assay kit with bovine serum albumin as a standard.

Determination of Sodium-Dependent and -Independent Taurocholate Uptake

Taurocholate was reported to be transported into hepatocytes by a sodium-dependent and a sodium-independent uptake process (16,17). In experiments examining sodium-independent uptake process, the final wash of the cell preparation was conducted with incubation buffer containing an equimolar of lithium chloride instead of sodium chloride. All other sodium salts were replaced with potassium salts according to Anwer *et al.* (16). Sodium-dependent transport rates were calculated by subtraction of values measured in the absence of Na^+ from those obtained in the presence of Na^+ (the total uptake of taurocholate). Sodium-independent transport rates were obtained from the uptake rates in sodium free buffer.

Kinetic Analysis

The relation between the initial uptake rate (V_o) and the concentration of ligand (C) was analyzed according to the Michaelis–Menten equation:

$$V_o = \frac{V_{max} \cdot C}{K_m + C} + P_{diff} \cdot C$$

In this equation, V_{max} and K_m are maximum uptake rate and Michaelis constant, respectively, and P_{diff} is a constant representing nonsaturable uptake. In the case of ouabain uptake, this nonsaturable component was negligible. The analysis was performed by the nonlinear iterative least-squares method (25).

Viability Test

To determine the effect of these basic drugs on the viability of hepatocytes, we checked the trypan blue exclusion and the oxygen utilization of the cells after 5 min of incubation with the basic drugs according to Eaton and Klaassen (14). However, these basic drugs had no significant effect on the viability of hepatocytes assessed by these tests.

Statistical Analysis

Statistical significance was analyzed according to the two-tailed Student's t test and Dunnett's test. Values are expressed as mean ± SE, with $P < 0.05$ considered significant.

RESULTS

Effects of Different Compounds on Taurocholate Uptake

Sodium-dependent and -independent uptake rates of taurocholate were determined in the presence of 50 μM of different organic bases (secondary and tertiary amines). The sodium-dependent uptake contributed about 80% or more of the total uptake. As shown in Table I, organic bases had varied effects on taurocholate uptake. Sodium-dependent taurocholate uptake was significantly reduced, to 50–70% of the control value, by verapamil, dipyridamole, and nifedipine. Sodium-independent taurocholate uptake was more extensively inhibited by these drugs, that is, the rate was reduced to 20–40% of the control values. Especially verapamil, dipyridamole, and nifedipine, which were reported to decrease the uptake rate of digoxin and ouabain (12), also decreased taurocholate uptake to a great extent.

In order to examine the effect of quaternary amine, taurocholate uptake was studied in the presence of 50 μM tubocurarine. However, tubocurarine had no effect on both sodium-dependent and -independent taurocholate uptake.

Table I. Inhibitory Effects of Eight Basic Drugs on the Sodium-Dependent and -Independent Uptake of Taurocholate[a]

Inhibitors	Initial uptake rate of taurocholate[b]	
	Sodium dependent	Sodium independent
None	19.5 ± 0.5 (100)[c]	3.53 ± 0.28 (100)
Verapamil	10.8 ± 1.5* (55)	0.73 ± 0.15* (20)
Dipyridamole	13.7 ± 2.4* (70)	0.71 ± 0.28* (20)
Nifedipine	10.5 ± 2.5* (54)	1.40 ± 0.28* (40)
Chlorpromazine	19.1 ± 2.1 (98)	2.32 ± 0.13 (66)
Disopyramide	21.0 ± 1.7 (108)	2.81 ± 0.25 (80)
Propranolol	16.4 ± 1.7 (85)	2.55 ± 0.25 (73)
Lidocaine	17.8 ± 1.5 (91)	2.51 ± 0.26 (71)
Quinidine	19.5 ± 1.0 (100)	2.01 ± 0.41* (56)

[a] Isolated hepatocytes were preincubated for 2 min at 37°C in the Hanks' buffer containing 50 μM of basic drugs. Substrate concentration was 0.1 μM. Each point represents the mean ± SE for three separate experiments.
[b] Expressed as pmol/min/mg protein ± SE.
[c] Percentage in parentheses.
* Statistical difference in comparison to control values ($P < 0.05$).

The nature of the inhibition of verapamil (50 μ*M*) for sodium-independent taurocholate uptake was examined by determining the initial rates of taurocholate uptake over a wide concentration range (1–200 μ*M*). Eadie–Hofstee plots of these kinetic data are shown in Fig. 1. Values for K_m (22.9 ± 2.6 μ*M*) and V_{max} (1.04 ± 0.10 nmol/mg protein/min) for sodium-independent uptake in the absence of verapamil are comparable with previously reported kinetic parameters (16,20). Kinetic analysis indicates that verapamil noncompetitively inhibits sodium-independent taurocholate uptake, that is, the V_{max} (0.35 ± 0.11) was reduced to 30% of control values, whereas no significant change in the K_m value (21.5 ± 8.5) was observed. The inhibition constant, K_i, of verapamil for sodium-independent taurocholate uptake was 25.4 μ*M*.

Correlation Between the Inhibitory Effects of the Basic Drugs on the Uptake Rate of Taurocholate and Ouabain

We compared the initial rate of sodium-dependent or -independent taurocholate uptake and ouabain uptake in the presence of eight basic drugs (Fig. 2). The initial uptake data for ouabain were obtained in our previous report (12). Ouabain uptake (x) was correlated better with sodium-independent taurocholate uptake (y) (correlation coefficient = 0.918), and the regression line passed through a point close to the origin. Ouabain uptake also correlated with sodium-dependent taurocholate uptake (correlation coefficient = 0.714), but the regression line showed a large y intercept value (approximately 60% of the control value).

Effect of Taurocholate on Ouabain Uptake

The inhibition of ouabain uptake by taurocholate was examined in the absence of sodium. The Dixon plot of these kinetic data (Fig. 3) had a common point of intersection above the X axis, indicating that inhibition is competitive. Values for K_m (258 ± 23 μ*M*) and V_{max} (1.55 ± 0.06 nmol/mg protein/min) for ouabain uptake are comparable with previously reported kinetic parameters (14,19,20). The K_i value (28.0 μ*M*) of taurocholate inhibition for ouabain uptake is also comparable with the K_m value (22.9 μ*M*) for sodium-independent taurocholate uptake obtained in the present study (Fig. 1).

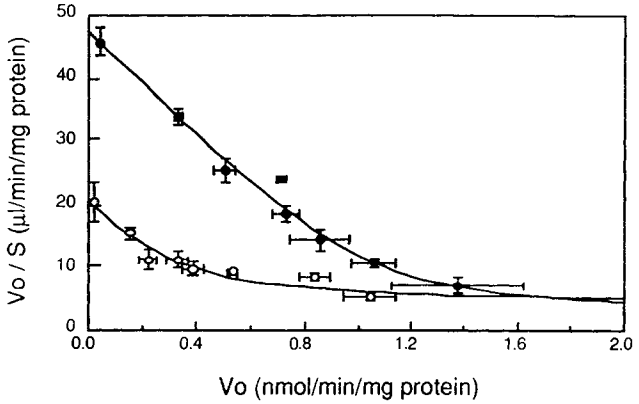

Fig. 1. Eadie–Hofstee plots for the sodium-independent taurocholate uptake in the absence (●) and presence (○) of 50 μ*M* verapamil. The freshly isolated hepatocytes (final concentration of 2 mg cellular protein/ml) were preincubated for 3 min at 37°C in Hanks' buffer. In the absence of verapamil K_m is 22.9 ± 2.6 μ*M*, V_{max} is 1.04 ± 0.10 nmol/mg protein/min, and P_{diff} is 2.2 ± 0.6 μl/mg protein/min. In the presence of verapamil K_m is 21.5 ± 8.5 μ*M*, V_{max} is 0.35 ± 0.11 nmol/mg protein/min, and P_{diff} is 4.1 ± 0.9 μl/mg protein/min. The inhibition constant, K_i, of verapamil for taurocholate uptake is 25.4 μ*M*. Each point represents the mean ± SE for triplicate analysis of two separate experiments.

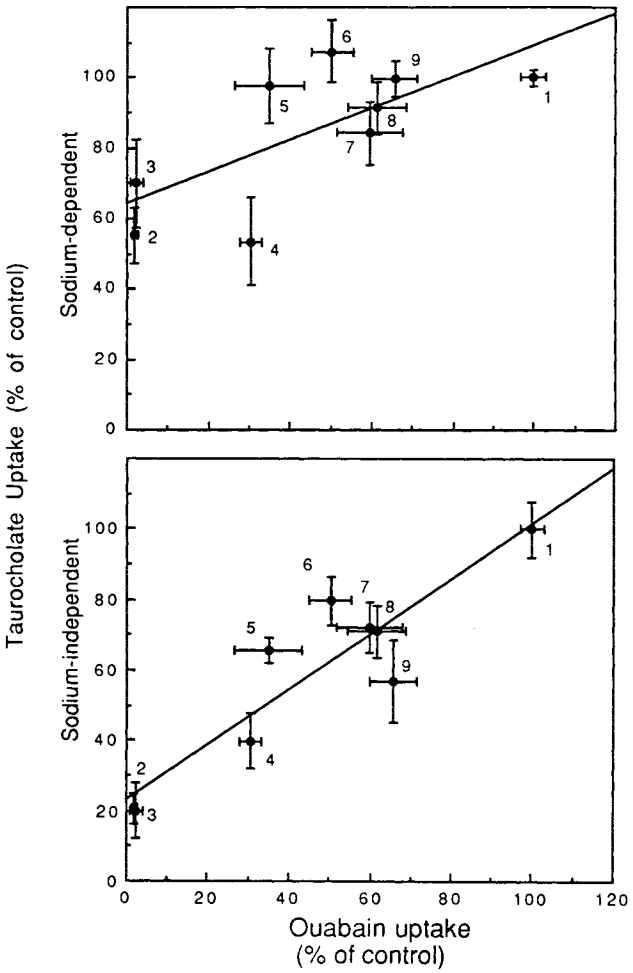

Fig. 2. Correlation between the inhibitory effects of basic drugs (50 μ*M*) on ouabain uptake and sodium-dependent (top) or -independent (bottom) uptake of taurocholate. Ouabain uptake data were obtained from our previous report (12). Taurocholate uptake data were obtained from Table I. Correlation coefficients for sodium-dependent and -independent uptake of taurocholate are 0.714 ($P < 0.05$) and 0.918 ($P < 0.05$), respectively. (1) Control; (2) verapamil; (3) dipyridamole; (4) nifedipine; (5) chlorpromazine; (6) disopyramide; (7) propranolol; (8) lidocaine; (9) quinidine.

DISCUSSION

We previously showed that digoxin was transported into the isolated rat hepatocytes by the same transport carrier as ouabain, and many basic drugs such as verapamil, dipyridamole, nifedipine, chlorpromazine, disopyramide, quinidine, and lidocaine inhibited this uptake system (12). Here we report the inhibitory effects of these basic drugs on the

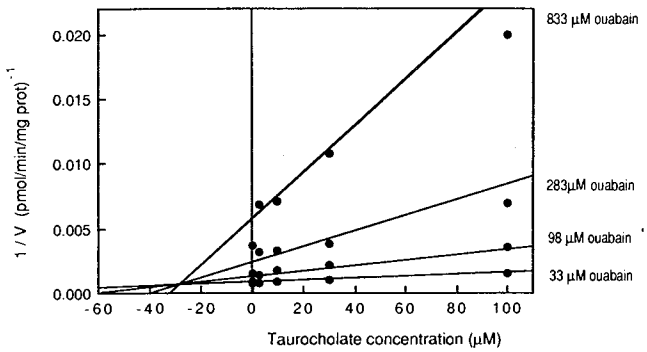

Fig. 3. Dixon plot to examine the inhibition type of ouabain uptake by taurocholate in sodium free buffer. The freshly isolated hepatocytes (final concentration of 2 mg cellular protein/ml) were preincubated for 2 min at 37°C in Hanks buffer. Final taurocholate concentrations of 0, 3, 10, 30, and 100 μ*M* and final ouabain concentrations of 33, 98, 283, and 833 μ*M* were used. In these experiments a K_m of 258 ± 23 μ*M* and a V_{max} of 1.55 ± 0.06 nmol/mg protein/min were obtained for ouabain uptake. The inhibition constant, K_i, of taurocholate is 28.0 μ*M*. Each point represents the mean from two separate experiments.

hepatocellular sodium-dependent and -independent uptake of taurocholate. Three of these drugs, quinidine (1–7), verapamil (8), and nifedipine (10), were reported to increase the serum digoxin concentration in patients, thereby causing severe side effects. One of the mechanisms of the drug interaction is the reduced hepatic clearance of digoxin (3,4,7,11). Therefore it is possible that these basic drugs decreased the hepatic clearance of taurocholate *in vivo*.

The specific biochemical mechanisms for the inhibitory effects of these drugs on the uptake of cardiac glycosides and taurocholate remain unresolved. One of the possible mechanisms is the competition for the same transport carrier site. Recently Meijer *et al.* (26) identified at least two separate carrier systems for hepatic uptake of organic cations through kinetic and photoaffinity labeling studies. One is the carrier for monovalent organic cations such as triethylmethylammonium, and the other is the carrier for bivalent organic cations such as tubocurarine and vecuronium. It was reported (26) that uptake of vecuronium was inhibited by taurocholate, ouabain, quinidine, and verapamil. They discussed that the uptake system for vecuronium might be shared with bile salts and cardiac glycosides. Therefore it is possible that some basic drugs such as quinidine and verapamil competitively inhibited the uptake of taurocholate and cardiac glycosides. However, present analysis clarifies that verapamil noncompetitively inhibited sodium-independent taurocholate uptake (Fig. 1).

We showed the correlation between the inhibitory effect of these basic drugs on sodium-dependent or -independent taurocholate uptake and ouabain uptake (Fig. 2). These results suggested two hypotheses: first, that taurocholate and cardiac glycosides share a common carrier protein and, second, that the carrier proteins are different from each other but are affected by these drugs in a similar way. Therefore, the type of inhibition of ouabain uptake by taurocholate was examined in the absence of sodium; taurocholate competitively inhibited the hepatocellular uptake of ouabain (Fig. 3). Other investigators (18–20) demonstrated that taurocholate competitively inhibited ouabain uptake also in the presence of sodium. Petzinger *et al.* (18) showed that inhibitors of taurocholate uptake such as cevadine, probenecid, DIDS, and furosemide decreased ouabain uptake in a similar way. Therefore, it may be assumed that hepatic uptake of ouabain is mediated by carrier proteins for taurocholate transport, which was recently designated as a multispecific transporter (21).

However, other experimental evidence suggested independent transport for bile acids and for ouabain. Klaassen (22) showed that newborn rats lack the ability to transport ouabain from plasma into liver yet maintain normal bile acid transport capabilities. Eaton and Richards (20) proposed that taurocholate binds competitively to the ouabain transport system but is not effectively transported across the cell membrane by this system. Zimmerli (27) showed that cardiac glycosides (digitoxin and ouabain) did not affect sodium-dependent taurocholate uptake in basolateral rat liver plasma membrane vesicles. These results suggest that the sodium-dependent taurocholate uptake system is different from the ouabain uptake system.

However, little is known about the relation between sodium-independent taurocholate uptake and ouabain uptake. A photoreactive ouabain derivative was found to bind to the same proteins in the plasma membrane as bile acid analogues (17). In the present study ouabain uptake was correlated better with sodium-independent taurocholate uptake than with sodium-dependent taurocholate uptake, and in the former case, the regression line passed through a point close to the origin (Fig. 2). Further, we showed that taurocholate competitively inhibited the hepatocellular uptake of ouabain in the absence of sodium (Fig. 3). These results may suggest that the transport system for cardiac glycosides is similar to the sodium-independent taurocholate transport system.

In conclusion, many basic drugs, which were previously reported to inhibit ouabain uptake, also inhibited sodium-dependent and -independent taurocholate uptake by isolated rat hepatocytes. Significant correlations between ouabain uptake and sodium-dependent or -independent taurocholate uptake were observed, but the correlation was better with sodium-independent taurocholate uptake. In addition, taurocholate competitively inhibited ouabain uptake in the absence of sodium. These results may suggest that the transport system for cardiac glycosides is similar to the sodium-independent taurocholate transport system.

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