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EFFECTS OF BASIC DRUGS ON THE HEPATIC TRANSPORT OF CARDIAC GLYCOSIDES IN RATS*

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Abstract—The effects of some basic and acidic drugs on the hepatic uptake of digoxin and ouabain were studied in isolated rat hepatocytes. Digoxin accumulated against a concentration gradient, and its initial uptake was energy- and temperature-dependent. Digoxin competitively inhibited the uptake of ouabain (K_i = 1.3 μM), which was reported to be transported by a carrier-mediated active transport system. All basic drugs tested (verapamil, dipyridamole, amiodarone, nifedipine, diltiazem, ajmaline, chlorpromazine, imipramine, disopyramide, quinidine, procainamide, propranolol and lidocaine: 50 μM) except for procainamide, propranolol and lidocaine significantly ($P < 0.05$) reduced the uptake of digoxin, whereas acidic drugs (salicylic acid and phenytoin) had no effect. The same inhibitory effects were observed for ouabain uptake, whereas the uptake of alanine was not changed by these drugs. Quinidine inhibited the uptake of ouabain in a noncompetitive manner (K_i = 88 μM). These basic drugs had no effect on the permeability of the cells assessed by the trypan blue exclusion test and succinate-stimulated oxygen consumption. But carbonylcyanide-*m*-chlorophenyl hydrazone-stimulated oxygen consumption decreased in the presence of some basic drugs and correlated with their inhibitory effects on digoxin uptake. Therefore, one of the mechanisms of the inhibitory effects of these drugs on digoxin uptake was the inhibition of oxidative phosphorylation. These basic drugs had no effect on the microtubular system, which was assessed by the measurement of tubulin polymerization and colchicine binding to tubulin. The results of our study suggested that many basic drugs have a potential to inhibit the hepatocellular uptake of cardiac glycosides.

Quinidine, when administered with digoxin, causes the occurrence of severe side-effects due to the elevation of the serum concentration of digoxin [1–8]. This pharmacokinetic drug interaction was explained by quinidine-induced decreases in both tissue distribution and total body clearance of digoxin [4, 5, 8]. Previously, we reported that the mechanism by which quinidine decreases the tissue distribution of digoxin to the heart and muscle of guinea pig is due to inhibition of tissue binding [9]. However, we failed to explain the decrease in hepatic distribution of digoxin by inhibition of tissue binding. These findings suggested that the mechanism of quinidine-induced decrease in hepatic transport of digoxin was due to inhibition of the hepatic uptake process. The quinidine-induced decrease in total body clearance of digoxin was mainly attributed to the decrease in the renal clearance, which was caused by the inhibition of renal tubular secretion. In humans [10], rats [11] and guinea pigs [8], however, it was reported that hepatic clearance contributes significantly to total body clearance of digoxin and that quinidine reduces the hepatic clearance [8]. But the mechanism of quinidine-induced decrease in the hepatic clearance has not been elucidated yet. Kupferberg and Schanker [12] found that ouabain, another cardiac glycoside, accumulates in rat liver slices. Eaton and Klaassen [13] demonstrated an active carrier-mediated transport process for ouabain in isolated rat hepatocytes and found that quinine, another cinchona alkaloid, inhibits this transport system. One of the possible mechanisms of quinidine-induced decrease in the hepatic clearance of digoxin is inhibition of the hepatic uptake of digoxin. Recently, many other basic drugs such as verapamil [14], amiodarone [15] and nifedipine [16] were reported to increase the serum concentration of digoxin in patients who had received this drug. However, the mechanism of these drug interactions is also unknown. The purpose of the present study was to determine the effects of quinidine and other basic drugs on the hepatic transport of digoxin and ouabain in isolated rat hepatocytes.

MATERIALS AND METHODS

Materials. [^{3}H]Digoxin (12α-labeled, 14.0 Ci/mmol), [^{3}H]ouabain (generally labeled, 19.5 Ci/mmol), [^{3}H]colchicine (ring C, methoxy labeled, 27.5 Ci/mmol) ^3H_2O (5 mCi/ml) and [^{14}C]inuline (carboxyl labeled, 2.4 mCi/g) were obtained from New England Nuclear, Boston, MA. [^{3}H]L-Alanine (2,3-labeled, sp. act. 59 Ci/mmol) was obtained from RCC Amersham, Buckinghamshire, England. Nonradioactive digoxin and ouabain were obtained from the Sigma Chemical Co., St. Louis, MO. Quinidine, phenytoin and phthalic acid diisobutyl ester (d = 1.041) was obtained from the Tokyo Kasei Kogyo Co., Tokyo, Japan. Collagenase was obtained from

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the Wako Pure Chemical Industries Co., Osaka, Japan. Verapamil HCl was a gift from the Eisai Pharm. Co., Tokyo, Japan. Nifedipine was a gift from the Bayer Yakuhin. Co., Shiga, Japan. Diltiazem HCl was a gift from the Tanabe Pharm. Co., Osaka, Japan. All other chemicals were commercial products and of 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 [17] as modified by Baur *et al.* [18]. 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 $MgCl_2 \cdot 6H_2O$, 0.205 g $MgSO_4 \cdot 7H_2O$, 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 7.40). The viability of isolated hepatocytes was tested by the trypan blue exclusion test, and the preparation was considered suitable by the criterion that more than 90% of the cells excluded the dye.

Uptake experiments. Isolated hepatocytes (final concentration of 2 mg protein/ml of incubation buffer) were preincubated with a 50 μM concentration of test compounds at 37° for 2 min. For determination of alanine uptake, hepatocytes were preincubated with 5 mM aminooxyacetic acid for 5 min to avoid the metabolism of alanine in the cells [19]. Uptake was initiated by the addition of radiolabeled digoxin, ouabain and alanine 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 the upper and lower sides was determined in a Packard Tri-carb counter (model 3255, Packard Instruments Corp., Downers Grove, IL) in 10 ml of liquid scintillation fluid (0.1 g POPOP*, 4.0 g of DPO, and 500 ml of Triton X-100/liter of toluene) after neutralization with 2 M HCl. The aqueous cellular volume and the volume of adherent fluid were corrected by measuring the uptake of ^3H_2O and [^{14}C]inulin respectively.

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

Viability test. To determine the effects of these test drugs on the viability of hepatocytes, we checked the viability by two tests according to Eaton and Klaassen [13] in addition to the trypan blue exclusion test. The respiratory capabilities of the cells were measured with a dissolved oxygen monitor (Yellow Springs Instruments, Yellow Springs, OH). After 5 min of preincubation at 37° with the test drugs, initial rate was determined. Then a final concentration of 1 mM succinate or 2 μM carbonylcyanide-*m*-chlorophenyl hydrazone (CCP) was added, and the oxygen utilization rate was measured.

Colchicine binding assay. The colchicine binding assay was performed by the method of Wierzba *et al.* [20]. Male Wistar rats were killed by cervical dislocation and then exsanguinated. Brain was removed and placed in ice-cold buffer composed of 100 mM MES [2(*n*-morpholino)ethanesulfonic acid], 1 mM EGTA (ethylene-glycolbis-*N*,*N*-tetra-acetic acid), 1 mM $MgSO_4$, 0.02% NaN_3 in 0.01 M Na_2HPO_4/NaH_2PO_4 buffer, pH 6.75. The tissue was blotted, weighed, and homogenized at 0–4° with 2 vol. of the above buffer [33% (w/v) homogenate] using a hand-driven Teflon pestle and glass homogenizer. The homogenates were kept at 0° for 30 min to allow for the dissociation of microtubular tubulin to the free form of tubulin, and then were centrifuged at 100,000 *g* for 40 min at 4°. The resulting supernatant fractions were assayed for colchicine binding capacity by the charcoal method on the day of preparation.

Tracer [^{3}H]colchicine was added to an aliquot of 100,000 *g* supernatant fraction to the final radioactivity of 0.2 μCi/50 μl of supernatant. Then, 50-μl samples of the supernatant were transferred to microcentrifuge tubes. Blanks contained 50 μl of the buffer in place of the supernatant. Standard was identical to blank except that distilled water (400 μl) was used in place of the activated charcoal suspension. The samples were incubated for 60 min at 37° (the blank sample was kept on ice). The reaction was stopped by addition of 400 μl of 0.25% distilled-water-activated charcoal suspension, and vigorous mixing. Ten minutes later, the samples were centrifuged in a Beckman microfuge B (Beckman Instruments) for 4 min. One hundred microliters of the supernatant fraction was counted in 10 ml of scintillation fluid described above.

Turbidometric assay of microtubule polymerization. Microtubule polymerization was performed by the usual method [21]. Polymerization of microtubule protein beginning with soluble protein (initiation conditions) was monitored by light scattering at 350 nm in a Hitachi 557 spectrophotometer using a water-jacketed chamber. The turbidity was directly proportional to the mass of microtubules formed and was insensitive to the lengths of the microtubules. Assembly was carried out at 37° in buffer composed of 100 mM MES, 1 mM EGTA, 1 mM $MgSO_4$, 0.02% NaN_3 and 100 μM GTP in 0.01 M Na_2HPO_4/NaH_2PO_4 buffer, pH 6.75, at approximately 2 mg/ml of total microtubule protein in the presence or absence of 100 μM verapamil.

Statistical analysis. Statistical significance was analyzed according to the two-tailed Student's *t*-test and Dunnett's test.

RESULTS

General kinetics of digoxin uptake. The time course of digoxin uptake into the isolated hepatocytes is shown in Fig. 1. The uptake process was almost linear within the first minute and reached a plateau at 3 min. The cell to medium concentration ratio (C/M ratio) of digoxin at 3 min between 50 and 60. The initial uptake rate of digoxin, calculated from the slope of each line, was decreased significantly in the presence of 50 μM quinidine. The Dixon plot for inhibition (Fig. 2) shows a common point of

* Abbreviations: POPOP, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene; and DPO, diphenyloxazole.

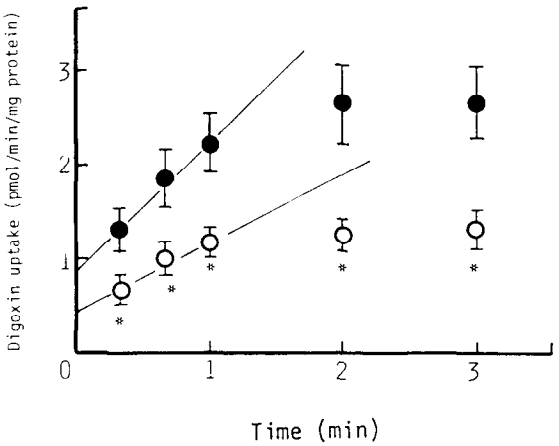

Fig. 1. Effect of quinidine on the hepatic uptake of digoxin by isolated rat hepatocytes. The freshly isolated hepatocytes (final concentration of 2 mg cellular protein/ml) were preincubated for 2 min at 37° in Hanks' buffer in the absence (●) or presence (○) of 50 μM quinidine. Then radiolabeled digoxin was added at a final concentration of 20 nM. Uptake was measured by sampling 200 μl of the suspension at the times indicated and by separating cells with the centrifugal filtration method as described in Materials and Methods. Radioactivity was determined in the cells and in the supernatant fraction. Each point represents the mean ± SE of three experiments. An asterisk represents a statistically significant difference from the control value ($P < 0.05$).

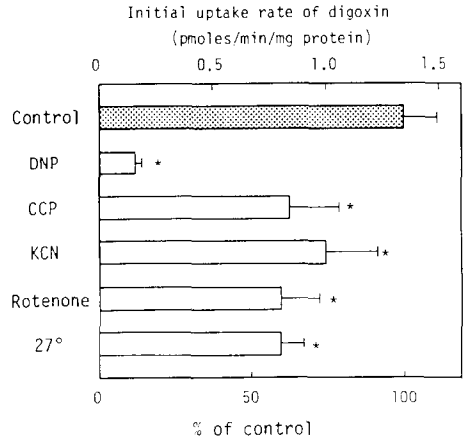

Fig. 3. Effects of metabolic inhibitors and incubation temperature on the initial uptake rate of digoxin. Substrate concentration was 20 nM, and the inhibitor concentrations were: 2,4-dinitrophenol (DNP), 1 mM; CCP, 2 μM; rotenone, 10 μM; and KCN, 1 mM. Each point represents the mean and SE of three experiments. An asterisk represents a statistically significant difference from the control value ($P < 0.05$).

intersection above the x-axis. This indicates that ouabain uptake was inhibited in a competitive manner with an apparent K_i value of 1.3 μM digoxin.

Effects of metabolic inhibitors. To determine whether digoxin transport is dependent on metabolic energy, 20 nM digoxin was added to cells preincubated for 2 min with four different metabolic inhibitors (Fig. 3). Cyanide ion (1 mM), which blocks electron transport at the final step, inhibited the initial uptake rate of digoxin by 30%, while rotenone (10 μM), which blocks electron transport near the initial step, inhibited the initial uptake rate by 40%. The oxidative phosphorylation uncouplers 2,4-dinitrophenol (1 mM) and CCP (2 μM) reached the uptake rate by 90 and 40% respectively. In addition to these chemical inhibitors, reduction of the incubation temperature from 37° to 27° decreased the initial uptake rate by 40%. These inhibitory effects of metabolic inhibitors were smaller than those for ouabain reported by Eaton and Klaassen [13], which was accounted for by the shorter preincubation time (2 min) compared with their experimental conditions (10 min).

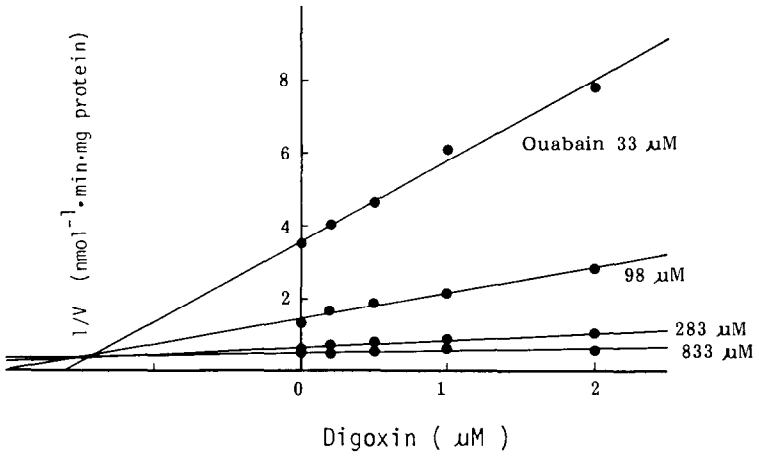

Fig. 2. Effect of digoxin on ouabain uptake. Hepatocytes (2 mg protein/ml) were preincubated for 2 min at 37° in the Hanks' buffer. Then unlabeled digoxin and [^{3}H]ouabain were added simultaneously, and the initial uptake rate of ouabain was determined in the presence of the following concentrations of digoxin: 0, 0.2, 0.5, 1.0 and 2.0 μM. Ouabain concentrations were 33, 98, 283 and 833 μM.

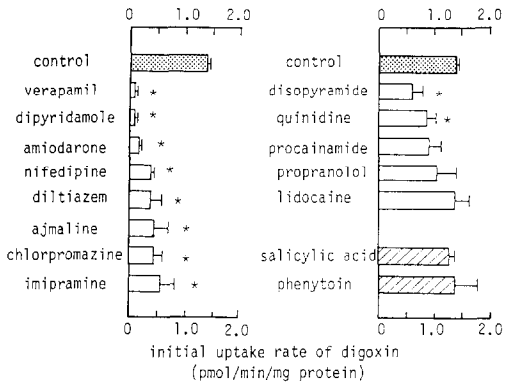

Fig. 4. Inhibitory effects of thirteen basic drugs (open bars) and two acidic drugs (shaded bars) on the initial uptake rate of digoxin. Isolated hepatocytes were preincubated for 2 min at 37° in Hanks' buffer containing basic or acidic drugs. All drugs except amiodarone (3 μM) were added to a final concentration of 50 μM. Substrate concentration was 20 nM. Each point represents the mean and SE of three separate experiments. An asterisk represents a statistically significant difference from the control value ($P < 0.05$).

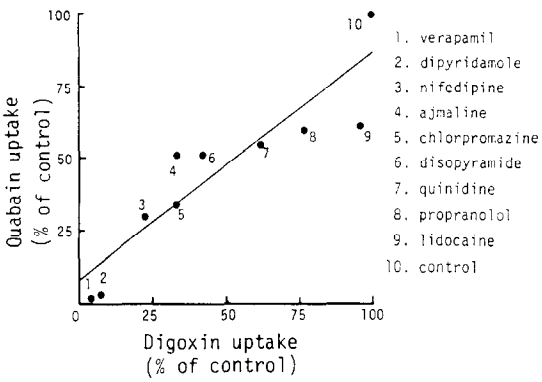

Fig. 5. Correlation between the inhibitory effects of basic drugs on the initial uptake rates of digoxin and ouabain. Digoxin uptake data were obtained from Fig. 4. Ouabain uptake was determined as follows: hepatocytes were preincubated for 2 min with Hanks' buffer containing various concentrations of basic drugs. Ouabain concentration was 1.0 μM. Each point represents the mean of three separate experiments in the presence of one basic drug (50 μM). The correlation was significant according to Student's t-test ($r = 0.902$, $P < 0.05$).

Effects of different compounds on digoxin uptake. The initial uptake of digoxin was determined in the presence of different basic or acidic drugs. As shown in Fig. 4, a 50 μM concentration of basic drugs had a quite varied effect on digoxin uptake. All of these basic drugs except procainamide, propranolol and lidocaine significantly decreased the uptake of digoxin. Verapamil, amiodarone, nifedipine, diltiazem and quinidine, which were reported to increase the serum concentration of digoxin when they were administered with digoxin, decreased the initial uptake rate of digoxin to a great extent, whereas the same concentration of two organic acids, phenytoin and salicylate, had no effect on this transport system. The same inhibitory effects of basic drugs were observed for ouabain transport, and the correlation between the inhibitory effect of basic drugs on the initial uptake of digoxin and ouabain was significant ($r = 0.902$, $P < 0.05$, Fig. 5). Figure 6 shows that quinidine inhibited the uptake of ouabain in a noncompetitive manner ($K_i = 88$ μM). As shown in Fig. 7, dose-dependency of the inhibitory effects on the initial uptake rate of ouabain was observed in four basic drugs studied, i.e. quinidine, chlorpromazine, dipyridamole and verapamil, although the type of inhibition was not determined except for quinidine.

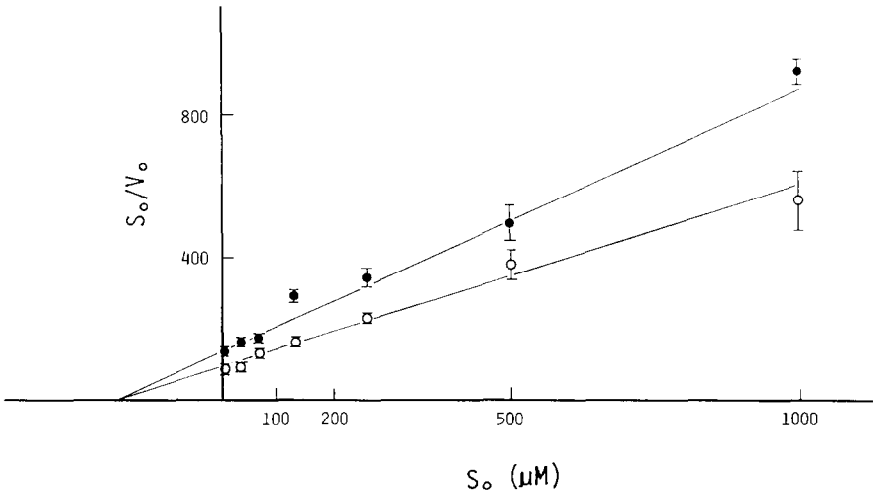

Fig. 6. Effect of quinidine on the initial uptake rate of ouabain. Hepatocytes were preincubated with Hanks' buffer in the absence (●) or presence (○) of 50 μM quinidine. Each point represents the mean and SE of three separate experiments. Single reciprocal linear transformation produced a straight line from which the K_m and V_{max} values were determined.

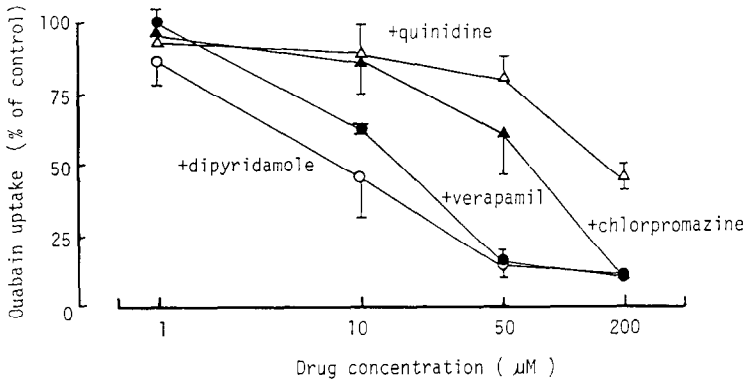

Fig. 7. Dose-dependency of the inhibitory effects of basic drugs on the initial uptake rate of ouabain. Substrate concentration was 1 μM. Hepatocytes were preincubated for 2 min with Hanks' buffer containing various concentrations of quinidine (△), chlorpromazine (▲), verapamil (●), or dipyridamole (○). Each point represents the mean and SE of three separate experiments.

Effects of basic drugs on alanine uptake. The initial uptake rate of alanine, which is reported to be transported by a sodium-dependent carrier-mediated active transport system [22], was determined in the presence of three basic drugs, which had varied effects on the uptake of cardiac glycosides. As shown in Fig. 8, only verapamil decreased the alanine uptake to some extent, but it was not significant. And there was no correlation between the inhibitory effects of these drugs on the alanine uptake and the digoxin uptake.

Effects of basic drugs on the viability of hepatocytes. To determine the effects of these basic drugs on the plasma membrane permeability of hepatocytes, we studied succinate stimulation of oxygen consumption and the trypan blue exclusion test. At this concentration (50 μM), these basic drugs did not change the membrane permeability assessed by

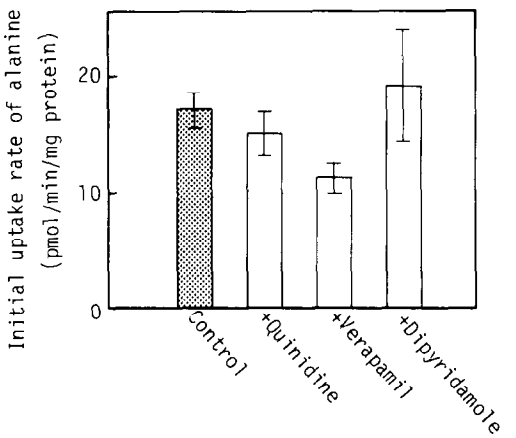

Fig. 8. Effects of basic drugs on the initial uptake rate of alanine. Substrate concentration was 1 μM. To avoid the metabolism of alanine in the cells, hepatocytes were preincubated with 5 mM aminooxyacetic acid for 5 min. Basic drugs were added to a final concentration of 50 μM to the incubation mixture 2 min before addition of the substrate. Each point represents the mean and SE of three separate experiments.

succinate stimulation of oxygen consumption, whereas the correlation between the effects of these drugs on digoxin transport and the trypan blue exclusion test was close to being statistically significantly ($r = -0.42$; $P = 0.05$). However, each value of the percent of trypan blue stained cells was not significantly different from the control value (Fig. 9).

To determine the effects of basic drugs on the mitochondrial respiratory function, we determined the endogenous oxygen consumption and the response of cells to the oxidative phosphorylation uncoupler, CCP. The CCP-stimulated oxygen consumption decreased in the presence of some basic drugs and significantly ($P < 0.05$) correlated with the inhibitory effect on the digoxin uptake (Fig. 10).

Effects of basic drugs on the microtubular system. The initial uptake rate of digoxin was reduced markedly (from 1.0 to 0.2 pmol/min/mg protein) after 15 min of preincubation with colchicine (50 μM), which is known to be a specific inhibitor of tubulin polymerization.

To determine the effects of these basic drugs on the microtubular system, we studied the tubulin assembly in the presence or absence of 100 μM verapamil, which dramatically inhibited the transport of cardiac glycosides. However, verapamil did not change the assembly of tubulin. We also determined the effects of basic drugs on the colchicine binding to tubulin, but the binding was not changed (data not shown).

DISCUSSION

In the present study we showed that many basic drugs inhibited the initial uptake rate of digoxin and ouabain by isolated rat hepatocytes. Eaton and Klaassen [13] previously reported that some acidic compounds, such as indocyanine green, iopanic acid, rose bengal and sulfobromophtalein, and steroidal compounds, dramatically inhibited the uptake of ouabain. However, in the case of basic drugs only quinine, a dextroisomer of quinidine, had a great effect on ouabain uptake.

Recently, many basic drugs, such as quinidine [1–

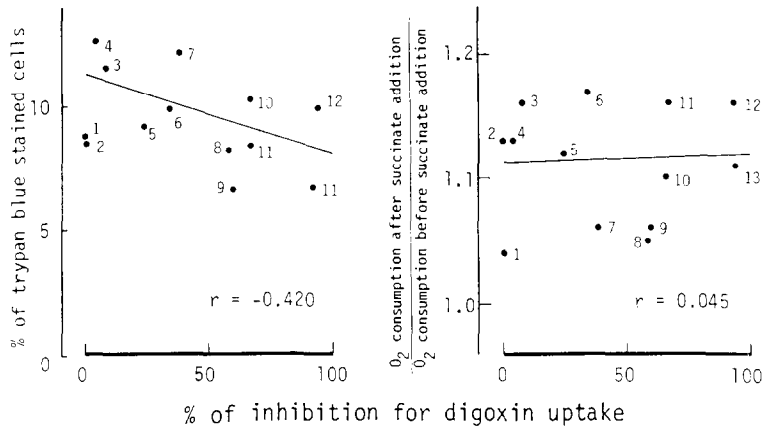

Fig. 9. Correlation between the effects of acidic and basic drugs on the digoxin uptake and the viability (permeability of the membrane) of the isolated rat hepatocytes. Left panel: the permeability of cells assessed by the trypan blue exclusion test (percent of trypan blue stained cells *vs* percent of inhibition for digoxin uptake). Right panel: the permeability of cells assessed by succinate-stimulated oxygen consumption monitored by using the dissolved oxygen monitor as described in Materials and Methods (the ratio of O_2 consumption vs percent of inhibition for digoxin uptake). The correlation was not statistically significant (P = 0.05), according to Student's *t*-test. Each point represents the mean of three separate experiments in the presence of one basic drug (50 μM). Key: (1) control, (2) phenytoin, (3) salicylic acid, (4) propranolol, (5) lidocaine, (6) procainamide, (7) quinidine, (8) disopyramide, (9) imipramine, (10) chlorpromazine, (11) ajmaline, (12) dipyridamole, and (13) verapamil.

8], verapamil [14], amiodarone [15], and nifedipine [16], have been reported to increase the serum digoxin concentration and serious side-effects in patients. One possible mechanism for the interaction between these drugs is a decrease in hepatic clearance of digoxin. In this study we showed that these basic drugs greatly decreased the hepatic uptake of digoxin. Therefore, these basic drugs may lower the hepatic clearance, and thus increase serum concentration of digoxin. However, the concentration of basic drug used in this study was rather high compared with the therapeutic concentration range. For example, the K_i value of quinidine (88 μM) for ouabain uptake (Fig. 6) was approximately twenty times higher than the therapeutic serum unbound concentration (2–5 μM). However, to predict drug interactions in humans, the interspecies difference between rats and humans must be considered. Furthermore, it is possible that these basic drugs may change the renal clearance of digoxin as well as the hepatic clearance.

The specific biochemical mechanism of the inhibitory effects of these drugs on the cellular uptake of cardiac glycosides remains uncertain. The correlation between inhibitory effects and decreased CCP-stimulated oxygen consumption suggests that these basic drugs may interfere with energy delivery to the transport system (Fig. 10). Tsao *et al.* [23] reported that chlorpromazine decreases CCP-stimulated oxygen consumption in isolated rat hepatocytes and discussed that such changes were related to alterations in oxidative phosphorylation. However, these drugs fail to decrease the uptake of alanine, which is also known to be transported into liver by an energy-dependent and a carrier-mediated process [21].

Another possibility is that these basic drugs may change the membrane potential of liver cells. It is thought that the transport of neutral substances such as digoxin and ouabain is not affected by the membrane potential. But if they are co-transported with some ions, the membrane potential could change transport rates. Yachi *et al.* [24] reported that the

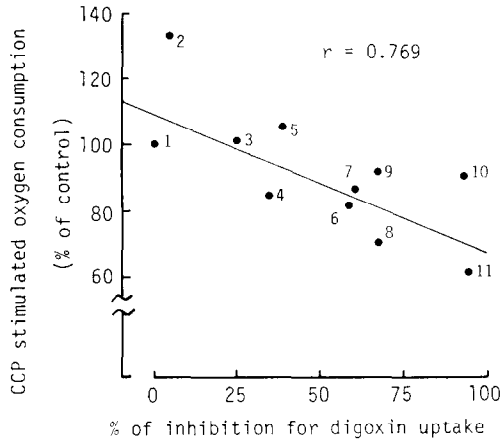

Fig. 10. Correlation between the inhibitory effects of basic drugs on the digoxin uptake (percent of inhibition) and the CCP-stimulated oxygen consumption (percent of control). CCP-stimulated oxygen consumption was monitored by using the monitor of a dissolved oxygen as described in Materials and Methods. Each point represents the mean for three separate experiments in the presence of one basic drug (50 μM). The correlation was significant according to Student's *t*-test (r = 0.769, P < 0.05). Key: (1) control, (2) lidocaine, (3) propranolol, (4) procainamide, (5) quinidine, (6) disopyramide, (7) imipramine, (8) chlorpromazine, (9) ajmaline, (10) dipyridamole, and (11) verapamil.

transport of ouabain is stimulated by the inside negative diffusion potential using isolated rat sinusoidal plasma membrane vesicles. The hepatic uptake of taurocholate, which was reported to inhibit the hepatocellular uptake of ouabain in a competitive manner [25, 26], is also stimulated by the inside negative membrane potential [27].

Colchicine, which is a specific inhibitor of tubulin polymerization, also dramatically decreased the uptake of ouabain (data not shown). Reichen *et al.* [28] reported that the cellular uptake of taurocholate is reduced by 15-min preincubation with colchicine without changing the cell viability assessed by the determination of the membrane potential. They discussed that the cytoskeleton might participate in the hepatocellular transport of taurocholate. However, we did not observe any effect of verapamil, which had a strong inhibitory effect on digoxin uptake into hepatocytes, on polymerization of tubulin (data not shown). These results indicated that microtubules may not be involved in the inhibitory effects of these basic drugs on the hepatocellular uptake of cardiac glycosides.

In summary, the present investigation demonstrated that the hepatocellular transport of digoxin was a carrier-mediated and active process, and many basic drugs have a potential to inhibit this process. These inhibitory effects correlated with uncoupling of oxidative phosphorylation. However, the uptake of alanine, another active transport process, was not changed by these basic drugs. Therefore, the mechanism by which basic drugs inhibited the transport of cardiac glycosides remains to be elucidated.

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