Organic Anion Transporter oatp2-Mediated Interaction between Digoxin and Amiodarone in the Rat Liver

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Purpose. The interaction between amiodarone and digoxin has been known to increase serum concentrations of digoxin in humans and rats. In this study, we assessed the molecular mechanism(s) of that drug interaction, focusing on digoxin transport mediated by P-glycoprotein (Pgp) and by rat liver organic anion transporter (oatp2).
Methods. Digoxin transport by Pgp and oatp2 was assessed using Pgp-overexpressing transfectant LLC-GA5-COL150 monolayers and oatp2-expressing *Xenopus* oocytes, respectively. The digoxin uptake into the isolated rat hepatocytes was also examined.
Results. Amiodarone (10 μM) inhibited slightly the transcellular transport of digoxin in LLC-GA5-COL150 monolayers, whereas itraconazole (10 μM), a potent Pgp inhibitor, markedly blocked the transport. The digoxin uptake by the isolated rat hepatocytes and by the oatp2-expressing *Xenopus* oocytes was decreased markedly in the presence of amiodarone but not in the presence of itraconazole. In addition, amiodarone inhibited the oatp2-mediated digoxin uptake in a competitive manner with an apparent inhibition constant value of 1.8 μM.
Conclusion. These findings suggest that rat oatp2 rather than Pgp may be one of the interaction sites for digoxin and amiodarone in the liver.

KEY WORDS: amiodarone; digoxin; hepatocyte; interaction; organic anion transporter; P-glycoprotein.

INTRODUCTION

Digoxin, a cardiac glycoside commonly used to treat congestive heart failure and tachycardia, should be administered with caution because it has a narrow therapeutic concentration range and interacts with several drugs when administered concomitantly. Digoxin is eliminated mainly in urine by renal glomerular filtration and tubular secretion and partially by other pathways, including biliary secretion (1). The pharmacokinetic interactions between digoxin and other drugs would occur in the liver as well as the kidney.

Amiodarone, a benzofuran derivative, prolongs action potential duration time and the refractory period in all cardiac tissues and is classified as a class three antiarrhythmic drug (2). Uncontrolled retrospective studies have suggested that amiodarone therapy prolonged survival periods of patients with congestive heart failure or arrhythmia (3). It has been reported that amiodarone interacts with digoxin and causes an elevation of serum digoxin levels in humans (4–8). Lambert *et al.* (9) have shown that amiodarone increased the blood concentration of digoxin in rats by inhibiting its uptake into hepatocytes; however, the molecular mechanism has not been revealed.

The *MDR1* gene product P-glycoprotein (Pgp) has been suggested as a potential site of interaction between digoxin and other drugs, such as quinidine, verapamil, itraconazole, or clarithromycin when administered concomitantly (10–14). However, the molecular mechanism(s) of the interactions between digoxin and these drugs cannot be explained fully based only on the interaction with Pgp. Recently, an organic anion transporting polypeptide (oatp2) was cloned from the rat liver (15,16). Noé *et al.* (15) have reported that the rat oatp2 was localized in the basolateral membranes of the brain endothelial cells and liver and that cardiac glycoside as well as taurocholic acid was transported by oatp2-expressing *Xenopus* oocytes. Although some species-specific characteristics could be found between rats and humans, these findings encouraged us to examine whether the interaction between amiodarone and digoxin was part of the process of hepatic uptake via oatp2 and/or renal tubular secretion via Pgp. In this study, we analyzed the effect of amiodarone on the digoxin transport by oatp2 and Pgp, comparing it with the effect of itraconazole, a potent inhibitor of Pgp-mediated digoxin transport (13,17). Our data indicated that the major target transporter of digoxin-amiodarone interaction was the hepatic oatp2.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]Digoxin (555 GBq/mmol) and [methoxy-^{14}C]inulin (81.77 MBq/g) were purchased from Du Pont-New England Nuclear Research Products (Boston, Massachusetts). Itraconazole was kindly supplied by Janssen Pharmaceutica N. V. (Beerse, Belgium). Amiodarone was a gift from Novartis Pharma KK, Co. Ltd. (Tokyo, Japan). Unlabeled digoxin was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals used were of the highest purity available.

Cell Culture and Transport Study in Pgp-Expressing LLC-PK$_1$ Monolayers

The transcellular transport of [^{3}H]digoxin (100 nM, 37 kBq/mL) by LLC-PK$_1$ cells expressing human Pgp, designated LLC-GA5-COL150, was measured using monolayer cultures grown in a Transwell chamber (3-μm pores, 4.71 cm^2 growth area; Costar, Cambridge, Massachusetts) as described previously (12,13). The composition of the incubation medium was as follows (in mM): 145 NaCl, 3 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 D-glucose, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4). For the transport experiments, cell monolayers grown in Transwell chambers were incubated with 2 mL of incubation medium containing [^{3}H]digoxin and [^{14}C]inulin (10 μg/mL, 3.7 kBq/mL) for specified periods at 37°C. After the incubation, aliquots (100

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μL) of the incubation medium on the other side were taken at specified time points, and the radioactivity of [^{3}H]digoxin and [^{14}C]inulin was measured. For the accumulation study, the medium was removed by aspiration at the end of the incubation period, and the monolayers were rapidly washed twice with 2 mL of ice-cold incubation medium on each side. The filters with monolayers were detached from the chambers, the cells on the filters were solubilized with 0.5 mL of 1 N NaOH, and the radioactivity in aliquots (200 μL) was measured. The radioactivity in the medium collected and in the solubilized cell monolayers was determined in 5 mL of ACS II (Amersham Pharmacia Biotech, Uppsala, Sweden). The protein contents of the cell monolayers solubilized in 1 N NaOH were determined by the method of Bradford (18) using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, California) with bovine γ-globulin as a standard.

Isolation of Hepatocytes and Transport Study

Hepatocytes from the male Wistar rats (230–260 g) were isolated after perfusion of the liver with 0.08% collagenase in accordance with the method of Seglen (19). The viability of the isolated hepatocytes was shown to be between 90 and 95% by trypan blue exclusion test and between 90 and 92% by lactate dehydrogenase latency test.

The uptake of [^{3}H]digoxin (60 nM, 37 kBq/mL) was measured by a rapid filtration technique (20). Usually, the hepatocytes resuspended in cell suspension buffer (0.1% D-Glucose, 10 mM HEPES in Krebs-Henseleit Bicarbonate Buffer Solution, pH 7.4), were preincubated for 10 min at 37°C before initiation of the uptake study. The reaction was initiated rapidly by adding 200 μL of the cell suspension buffer containing substrate to 20 μL of cell suspension (6 mg of cell protein/mL) at 37°C. At specified times after the incubation of digoxin and hepatocytes was started, the uptake reaction was stopped by diluting with 2 mL of ice-cold cell suspension buffer containing 10 μM of unlabeled digoxin (stop solution), and the tube contents were immediately poured onto Millipore filters (HAWP, 0.45 μm, 2.5 cm diameter) and washed twice with 5 mL of ice-cold stop solution. Radioactivity trapped on the filters was determined in 5 mL of ACSII (Amersham Pharmacia Biotech) by liquid scintillation counting.

Functional Expression in *Xenopus* Oocytes

Five nanograms of capped cRNA transcribed *in vitro* were injected into *Xenopus* oocytes. Injected oocytes were maintained in modified Barth's medium at 18°C for 2 days. In general, the functional expression of oatp2 was analyzed by measuring the uptake of [^{3}H]digoxin (25 nM, 37 kBq/mL) in groups of oocytes injected with 50 nL of water or cRNA as described (16). Oocytes were incubated for the specified times at 25°C in an uptake buffer (pH 7.4) (in mM: 100 NaCl, 2 KCl, 1 $MgCl_2$, 10 HEPES) containing 25 nM [^{3}H]digoxin (37 kBq/mL). After the incubation, the oocytes were washed three times in 1.5 mL of ice-cold uptake buffer (pH 7.4) and solubilized in 10% sodium dodecyl sulfate solution. The radioactivity was determined in 5 mL of ACSII (Amersham Pharmacia Biotech) by liquid scintillation counting.

Statistical Analysis

Statistical significance of differences between mean values was calculated using the paired *t* test for the data of patients and the non-paired *t* test for the data of transport experiments. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Inhibitory Effects of Itraconazole and Amiodarone on Digoxin Transport via Pgp in LLC-GA5-COL150 Monolayers

We examined the effect of amiodarone on digoxin transport via Pgp using LLC-GA5-COL150 monolayers comparing it with that of itraconazole. In the presence of 10 μM itraconazole, the digoxin transport from basolateral-to-apical chamber was decreased by 50%, whereas it was significantly increased by approximately three-fold in the opposite direction at 15, 30, and 60 min (Fig. 1A). A slight but significant inhibitory effect of 10 μM amiodarone on the digoxin transport was observed in the transfectant. The effect of itraconazole was more potent than that of amiodarone (Fig. 1A). In addition, the cellular accumulation of digoxin from both basolateral and apical sides was increased in the presence of itraconazole and amiodarone at a concentration of 10 μM (Fig. 1B). These results suggested that itraconazole had a more potent effect on Pgp activity than amiodarone.

Inhibitory Effects of Itraconazole and Amiodarone on Digoxin Uptake by Isolated Hepatocytes

To examine whether amiodarone and itraconazole had inhibitory effects on the hepatic distribution of digoxin, we performed [^{3}H]digoxin uptake study using the isolated hepatocytes. Ten micromolar unlabeled digoxin and amiodarone markedly inhibited the uptake of [^{3}H]digoxin by the isolated hepatocytes, but 10 μM itraconazole had no inhibitory effect (Fig. 2A). In addition, the [^{3}H]digoxin uptake into the isolated hepatocytes was inhibited by unlabeled digoxin and amiodarone in a dose-dependent manner with IC_{50} values of 0.5 μM and 1 μM, respectively (Fig. 2B).

Inhibitory Effects of Itraconazole and Amiodarone on Digoxin Transport via oatp2 Expressed in *Xenopus* Oocytes.

To clarify whether oatp2 was a major site of interaction between digoxin and amiodarone, we performed experiments on oatp2-mediated uptake of digoxin using the *Xenopus* oocyte expression system and the average therapeutic concentration of amiodarone (21). The oatp2-mediated uptake of [^{3}H]digoxin was decreased markedly in the presence of 10 μM unlabeled digoxin and amiodarone but not 10 μM itraconazole (Fig. 3). In addition, the oatp2-mediated [^{3}H]digoxin uptake was inhibited by amiodarone in a dose-dependent manner (Fig. 4A). Furthermore, Dixon plot analysis demonstrated that amiodarone inhibited digoxin uptake in a competitive manner with an apparent inhibition constant (K_i) of 1.8 μM (Fig. 4B).

When the concentration-dependent uptake of digoxin was assessed in the presence or absence of 2 μM amiodarone, the apparent Km value of digoxin was significantly increased, but the V_{max} value was not changed significantly (Table I).

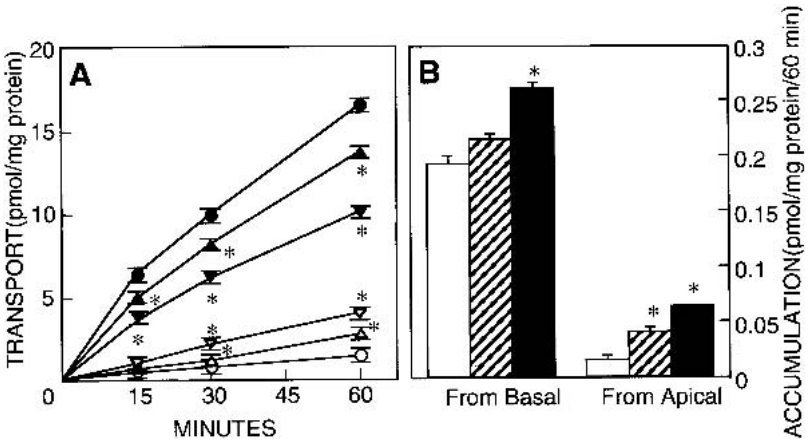

Fig. 1. Effects of amiodarone or itraconazole on the transcellular transport (A) and intracellular accumulation (B) of digoxin using LLC-GA5-COL150 monolayers. A, Intracellular radioactivities were determined after incubation with 100 nM [^{3}H]digoxin, which was added to either the basolateral (●, ▲, ▼) or the apical (○, △, ▽) side in the absence (○, ●) or presence of 10 μM amiodarone (△, ▲) or itraconazole (▽, ▼) at 37°C. Both inhibitors were added to the medium on both sides of the cell monolayers during preincubation and incubation periods. Radioactivity on the opposite side was measured after presence of amiodarone (hatched columns) or itraconazole (solid columns) 60 min after the transport experiments. Each column represents the mean ± SE of three monolayers. *$P < 0.05$, significantly different from control.

DISCUSSION

In the present study, 10 μM amiodarone had a potent inhibitory effect on the digoxin uptake by oatp2-expressing oocytes (Fig. 3) as well as the isolated rat hepatocytes (Fig. 2). However, 10 μM amiodarone had only a slight though significant inhibitory effect on Pgp-mediated digoxin transport (Fig. 1). Robinson *et al.* (22) reported that the high levels of serum digoxin concentration in 10 patients after coadministration with amiodarone did not appear to be due to a change in the renal clearance of digoxin. Nademanee *et al.* (5) demonstrated that the total body clearance and nonrenal clearance of digoxin in combination with amiodarone were reduced significantly by 29% and 33%, respectively, but the renal clearance was reduced only by 22%, which was not statistically significant. In the rats fed with 0.1% amiodarone for more than 10 days, the biliary clearance of digoxin was reduced significantly by 55%, but the renal clearance was not significantly changed (23). Using the isolated-perfused liver system, amiodarone was found to increase the serum digoxin concentration by inhibiting the uptake of digoxin into the rat liver cells (9). Therefore, amiodarone increased serum digoxin concentration in human as well as rats by decreasing the hepatic distribution of digoxin and not by urinary secretion.

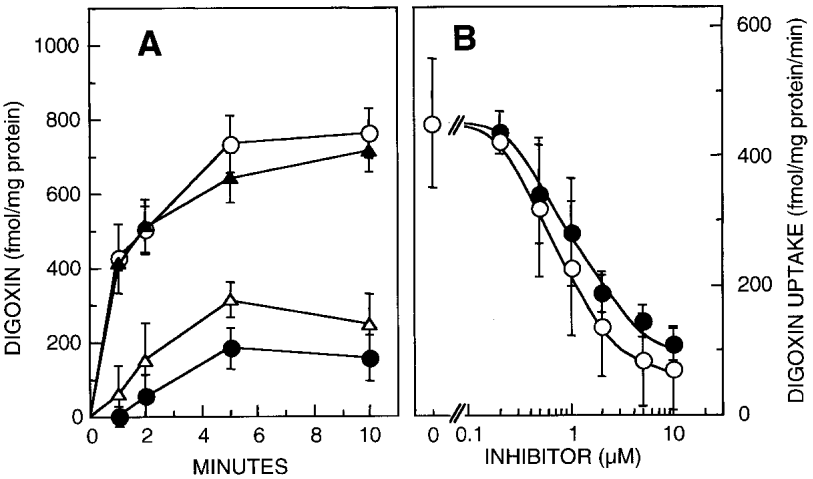

Fig. 2. Effects of amiodarone or itraconazole on digoxin uptake by isolated rat hepatocytes. A, Uptake of 60 nM [^{3}H]digoxin by isolated rat hepatocytes was assayed for specified periods at 37°C in the absence (○)or presence of 10 μM unlabeled digoxin (●), amiodarone (△), or itraconazole (▲). B, Uptake of 60 nM [^{3}H]digoxin by isolated rat hepatocytes in the presence of various concentrations (0, 0.2, 0.5, 1, 2, 5, and 10 μM) of digoxin (○), or amiodarone (●) were measured for one minute at 37°C. Each point represents the mean ± SE of three experiments.

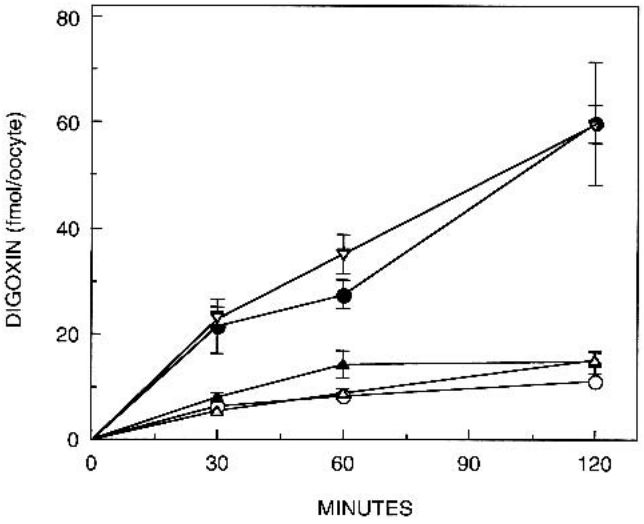

Fig. 3. Effects of amiodarone or itraconazole on digoxin uptake by *Xenopus* oocytes expressing rat oatp2. Uptake of 25 nM [^{3}H]digoxin by water (○) or rat oatp2 cRNA-injected oocytes was measured for specified periods at 25°C in the absence (●) or presence of 10 μM unlabeled digoxin (△), amiodarone (▲), or itraconazole (▽). Each point represents the mean ± SE of four to eight oocytes.

These findings and the present results suggest that the inhibitory effect of amiodarone on the hepatic uptake of digoxin by oatp2 is greater than that on the tubular and/or biliary secretion of digoxin via Pgp. Recently, the human liver specific transporter LST-2 (or also called OATP8), whose amino acid sequence showed only 45% identity with that of the rat oatp2, was cloned and shown to transport digoxin (24,25). Although both transporters were expressed predominantly in the liver and mediated basolateral uptake of digoxin, the rat oatp2 probably does not represent an orthologous gene product of LST-2. The inhibition constants of various drugs including amiodarone on the transport of digoxin via oatp2 and LST-2 should be determined in the future to clarify whether the rat oatp2 is a functional counterpart for the human LST-2 with the respect to hepatic uptake of digoxin.

Table I. Apparent Kinetic Parameters for Digoxin Uptake by *Xenopus* Oocytes Expressing Rat oatp2

	K_m (μM)	V_{max} (fmol/oocyte/30 min)
Digoxin	0.38 ± 0.06	320.6 ± 61.6
Digoxin with amiodarone	2.16 ± 0.67[a]	524.5 ± 95.6

Note: [^{3}H]Digoxin uptake at various concentrations (0.05–2 μM) was measured for 30 min at 25°C in the absence and presence of 2 μM amiodarone. Each concentration was examined in 3 to 4 oocytes. The K_m and V_{max} values were calculated from plots based on the Michaelis–Menten equation using nonlinear least square regression analysis. Each value represents the mean ± SE of three experiments.
[a] $P < 0.05$, significantly different from digoxin alone.

Holt *et al.* (21) determined the relationship between dose and plasma concentration of amiodarone in 170 patients receiving amiodarone maintenance therapy at 200, 400, or 600 mg daily for 1 month or longer. Most of the patients (n = 101) were administered 200 mg/day of amiodarone. The average plasma concentration was 1.64 μM. In addition, 400 or 600 mg/day of amiodarone was administered to 51 or 18 patients with average plasma concentrations of 2.99 or 5.36 μM, respectively. In the rats, the repeated oral administration of amiodarone at 66 mg/kg/day for 18 days resulted its serum

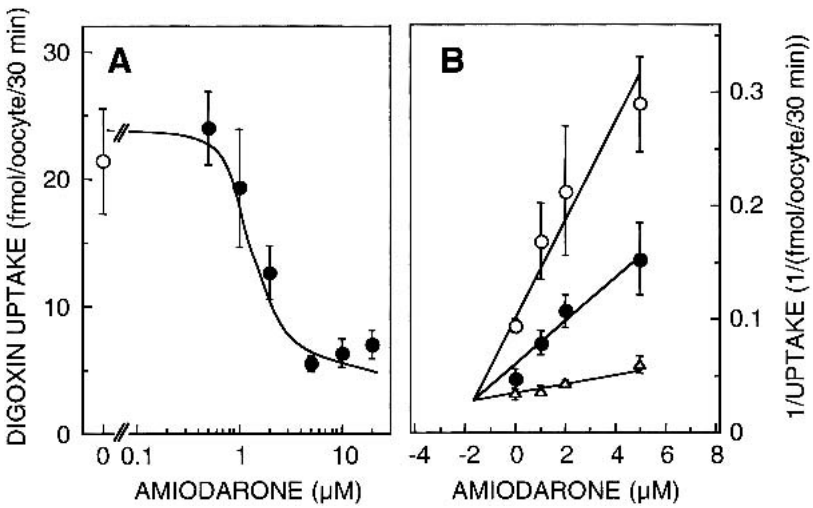

Fig. 4. Concentration-dependent inhibition by amiodarone (A) and Dixon plots for inhibitory effect of amiodarone (B) on [^{3}H]digoxin uptake by *Xenopus* oocytes expressing rat oatp2. A, Uptake of 25 nM [^{3}H]digoxin by rat oatp2 cRNA-injected oocytes in the absence (○) or presence of various concentrations of amiodarone (0.5, 1, 2, 5, 10, and 20 μM) (●) was measured for 30 min at 25°C. B, Digoxin uptake at each concentration of 25 (○), 50 (●), and 100 (△) nM in the presence of 1, 2, and 5 μM of amiodarone was measured for 30 min at 25°C. The oatp2-mediated uptake of digoxin was calculated by subtracting the uptake value of digoxin into water-injected oocytes from that into oatp2 cRNA injected oocytes. Data are expressed as 1/uptake (fmol/oocyte/30 min). Each point represents the mean ± SE of four to eight oocytes.

concentration of 1.55 μM and caused an elevation of serum digoxin levels three-fold (26). In the present study, the nonlinear least regression analysis on Dixon plots (Fig. 4B) and Michaelis–Menten equation (Table I) revealed that amiodarone competitively inhibited the oatp2-mediated uptake of digoxin with an apparent K_i value of 1.8 μM. In addition, the IC_{50} value of amiodarone on the digoxin uptake by the isolated rat hepatocytes was 1 μM (Fig. 2B). Because the fraction of amiodarone bound to human serum protein accounted for 95.6% (27), the enhancement of the serum concentration of digoxin during concomitant administration with amiodarone could not be fully explained by the competitive inhibition of amiodarone on the oatp2-mediated digoxin transport. To our knowledge, this is the first report identifying the target transporter involved in the drug interaction between digoxin and amiodarone in the liver. Considering the protein binding of amiodarone, the Ki value of amiodarone on the digoxin uptake via oatp2 in rat serum should be clarified in the future.

We found digoxin-itraconazole interactions in three patients in our hospital, whose total body clearance of digoxin was significantly decreased to 50.5 ± 8.8% (mean ± SD of three patients, $P = 0.047$) by the administration of itraconazole (28). Woodland *et al.* (14) demonstrated that itraconazole and ketoconazole inhibited Pgp-mediated digoxin transport using cultured MDCK monolayers. In this study, the Pgp-mediated digoxin transport in the transfectant was depressed markedly in the presence of 10 μM itraconazole. However, 10 μM itraconazole had no inhibitory effect on the digoxin uptake by either isolated hepatocytes (Fig. 2A) or oatp2-expressing oocytes (Fig. 3). These results indicated that Pgp in the renal brush-border membranes and/or bile canalicular membranes could be responsible for the interaction between digoxin and itraconazole.

In conclusion, the present results suggest that an amiodarone serum concentration of approximately 2 μM inhibits predominantly the oatp2-mediated uptake of digoxin into the liver in a competitive manner. In contrast, itraconazole appears to inhibit primarily the Pgp-mediated excretion of digoxin. The present results may provide useful information for examining the molecular mechanism of the drug interaction between digoxin and amiodarone in humans. In addition, interactions between digoxin and other drugs in rats could be evaluated *in vitro* by using expression systems for oatp2 and Pgp.

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