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CONCENTRATIVE UPTAKE OF DIGOXIN BY SLICES OF CHICKEN RENAL CORTEX

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Abstract—The purpose of this investigation was to define, under controlled *in vitro* conditions, the processes contributing to the uptake and accumulation of [^{3}H]digoxin by incubated slices of chicken renal cortex. Progressive uptake was evident in time–course experiments with the slice-to-medium concentration ratio (S/M) reaching 5.25 after 120 min. No metabolism was evident. Increasing the ratio of unlabeled to labeled digoxin resulted in a concentration-dependent decrease in relative uptake of the label, suggesting saturability. Incubation under conditions of metabolic inhibition reduced digoxin S/M by about 50%, indicating that both energy-requiring and passive mechanisms contribute to the overall accumulation process. The structural nature of the uptake process was explored by incubating digoxin in the presence of potential inhibitors of transport. The organic cation quinine and the non-glycosidic steroids digoxigenin and spironolactone were without effect even at greater than 1000-fold excess compared to digoxin. Similarly, the sugar digitoxose had no inhibitory activity on digoxin accumulation by the slices. On the other hand, the glycosides digitoxin, digoxigenin-bis-digitoxoside and digoxigenin-mono-digitoxoside inhibited dogoxin uptake in a concentration-dependent manner. These results indicate a structural preference for an intact glycoside rather than for either the steroidal or sugar portion of the molecule alone. An inhibitory effect of ouabain and a stimulatory effect of reduced medium potassium concentration suggest a possible role for Na^+,K^+-ATPase in the uptake of digoxin by the renal cortex.

Despite a narrow therapeutic index, digoxin is the most widely used cardiac glycoside for treatment of congestive heart failure. Because digoxin is largely eliminated in unchanged form in the urine, the kidney plays a central role in determining its disposition, a fact of particular importance in the elderly population in which the incidence of toxicity may exceed 20%. Renal excretion of digoxin in humans involves glomerular filtration, tubular reabsorption and tubular secretion. Of the tubular processes, reabsorption appears to be passive but some evidence indicates an active secretory component [1, 2]. The nature of this latter process is unclear since current definitions of renal tubular secretory processes do not include a process capable of transporting an uncharged glycosidic steroid such as digoxin. Steiness [1] has spectulated the existence of a steroid-specific active transport process, a hypothesis supported by the inhibitory effect of the non-glycosidic steroid spironolactone on digoxin renal clearance. O'Brien *et al.* [3], on the other hand, have proposed that binding of digoxin to Na^+,K^+-ATPase is involved in the renal tubular transport of this glycoside. The purpose of the present study was to examine the process of accumulation of digoxin by incubated renal cortex slices. The experimental model was the chicken kidney because of the close similarities of digoxin disposition and excretion kinetics *in vivo* in this species of those of humans [4], and because of preliminary evidence for the participation of an energy-dependent component in digoxin uptake by avian (but not mature rat) renal cortex [5]. This generally supported earlier findings of Orloff and Burg [6] that the cardiac agent, strophanthidin, was cleared at a rate in excess of the glomerular filtration rate in the chicken, suggesting secretion.

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MATERIALS AND METHODS

Preparation and incubation of slices. The animals used in the study were mature Rhode Island Red laying hens of about 2.5 kg body weight. Food but not water was withheld on the day before an experiment. Following sacrifice by CO_2 administration, both kidneys were rapidly removed and placed in ice-cold Ringer's solution (see composition below). Thin (0.3–0.5 mm) slices of cortex were prepared from the cranial and caudal lobes by use of a Stadie–Riggs microtome as described previously [7]. Slices were individually incubated under O_2/CO_2 (95/5) and 25° in a Blue M metabolic shaker bath fitted with an atmosphere hood (Blue M Electric, Blue Island, IL). Incubation vessels were 30-mL beakers containing 5 mL of bicarbonate-buffered Ringer's solution (pH 7.4) of the following composition: NaCl, 125 mM; KCl, 5 mM; $NaHCO_3$, 25 mM; NaH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; and $CaCl_2$, 1 mM. In some experiments a potential inhibitor of transport was added to the basic Ringer's solution. Anaerobic conditions were produced by de-gassing the incubation medium under vacuum and vigorously bubbling the solution with N_2/CO_2 (95/5) for 15 min. The incubation in such experiments was conducted under a N_2/CO_2 atmosphere. Potassium concentration was also changed in some experiments with an equiosmolar amount of sodium chloride added or sub-

tracted to maintain osmotic balance. After a 20-min preincubation in the presence or absence of an inhibitor, sufficient [^{3}H]digoxin was added to yield an initial medium concentration of 3.2 nM and the timed incubation begun. This concentration lies within the therapeutic range for digoxin. At the end of the incubation, slices were rapidly separated from medium by filtration through gauze, blotted, and placed in tared vials containing 3 mL of 0.5 N NaOH. After reweighing the vials to obtain the tissue wet weight, slices were solubilized in the NaOH by incubating for 90 min at 70° with occasional shaking.

Analysis. Radioactivity in slice solutions (acidified to prevent chemiluminescence) and media was quantified by quench-corrected liquid scintillation spectroscopy using 3a70B mixture (RPI, Arlington Heights, IL) and a Beckman LS-7000 counter. Uptake was expressed as a slice-to-medium concentration ratio (S/M) where S = nanomoles per gram wet weight and M = nanomoles per milliliter medium. Accumulation was defined as S/M > 1. Statistical analysis of the uptake data was by one-way-analysis of variance or Student's *t*-test, with $P < 0.05$ considered significant.

The identity of the tritium label in slices and medium was determined by HPLC as described by Gault *et al.* [8]. Retention times (min) were: digoxin, 11.2; digoxigenin-bis-digitoxide, 6.63; digoxigenin-mono-digitoxide, 4.35; and digoxigenin, 3.87. Rates of tissue oxygen consumption were determined at 37° with a YSI model 53 oxygen monitor using a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH).

Chemicals. Digoxin [12-α-^{3}H(N)] (15.4 Ci/mmol), tetraethylammonium [1-^{14}C] (4.8 mCi/mmol) and *p*-aminohippuric acid [*glycyl*-^{14}C] (57.6 mCi/mmol) were obtained from DuPont NEN Research Products (Wilmington, DE). The 12-α form of labeled digoxin was used because of its stability in aqueous solution [9, 10]. Unlabeled digoxin metabolites were gifts from the Burroughs Wellcome Co. (Research Triangle Park, NC). All other chemicals were of reagent grade or better and were purchased from major commercial sources.

RESULTS

Quantification of uptake. The results of time-dependent uptake experiments are presented in Fig. 1. Slices incubated in the presence of 3.2 nM digoxin took up the drug at a progressive rate with accumulation (S/M > 1) evident within 10 min of incubation. At the conclusion of the 120-min incubation period, the mean tissue digoxin level was 13.1 μg/g, corresponding to a S/M (± SD) of 5.25 ± 1.80. Uptake was linear ($r = 0.991$, $P < 0.05$) during the first 30 min with the approximate rate of uptake (estimated from the slope) being 0.11 nmol/g wet wt/min. After 30 min, the uptake was also linear ($r = 0.979$, $P < 0.05$) with an approximate rate of 0.017 nmol/g wet wt/min. No steady state was achieved over the 2-hr time course. HPLC analysis of slice extracts and media incubated for 120 min with labeled digoxin revealed the presence of a single labeled compound whose retention time corresponded to authentic digoxin. Saturability of the uptake process was assessed by comparing the uptake of [^{3}H]digoxin by slices incubated in the absence (control) or presence of comparatively high concentrations of unlabeled digoxin. The S/M for [^{3}H]digoxin was not affected by 0.5 μM unlabeled digoxin (99.4% of control) but was reduced significantly ($P < 0.05$) in the presence of unlabeled digoxin at concentrations of 5 μM (59.6% of control) and 50 μM (40.7% of control). These results are summarized in Fig. 2.

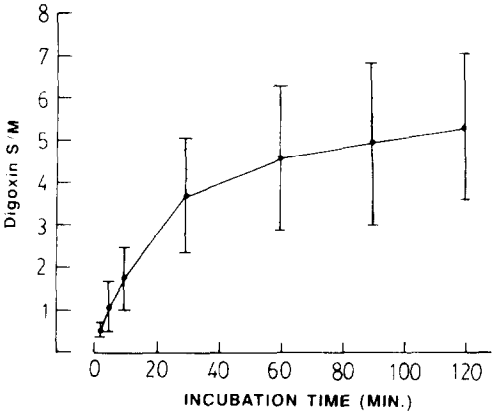

Fig. 1. Time course of [^{3}H]digoxin uptake by chicken renal cortex slices. Each point is the mean ± SD of at least 15 slices individually incubated in bicarbonate-buffered Ringer's medium at 25° in the presence of 3.2 nM digoxin. S/M = slice-to-medium concentration ratio with S = nmol/g wet wt and M = nmol/mL medium.

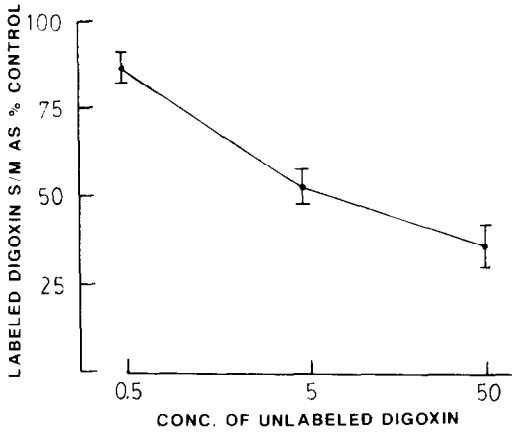

Fig. 2. Effect of unlabeled digoxin on uptake of [^{3}H]digoxin by renal cortex. Each value is the mean ± SD from 9 to 12 slices. Slices were incubated individually with 3.2 nM [^{3}H]digoxin alone (control) or in the presence of 0.5, 5 or 50 μM unlabeled digoxin for 120 min. Control S/M for digoxin in these experiments was 4.78.

Effect of metabolic inhibition on digoxin uptake. To determine whether the accumulation of digoxin involved an energy-dependent process, the time course of digoxin uptake was determined under anaerobic conditions (N_2/CO_2 atmosphere) or in the presence of 10 mM NaCN. Parallel incubations of slices with digoxin alone under an atmosphere of O_2/CO_2 served as control. The results are presented in

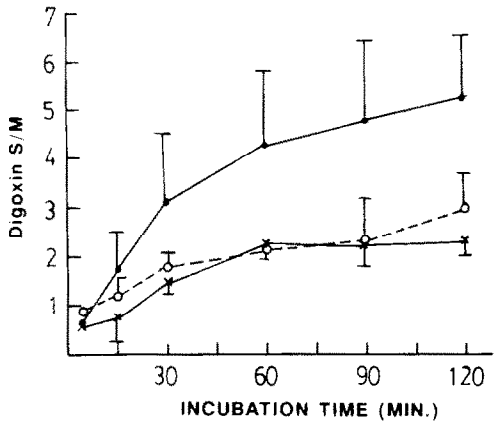

Fig. 3. Effect of metabolic inhibition on [^{3}H]digoxin uptake by renal cortex. Each value is the mean ± SD from 9 to 15 individually incubated slices. [^{3}H]Digoxin concentration was 3.2 nM in all experiments. Key: (●) digoxin alone + O_2 atmosphere, (○) digoxin alone + N_2 atmosphere, and (×) digoxin + 10 mM NaCN + oxygen atmosphere.

Fig. 3, and demonstrate that the slices accumulated digoxin in the presence of metabolic inhibition but S/M values were only about 50% (range 44 to 56%) of control values at all times points beyond 15 min. These represent significant ($P < 0.05$) differences from control values.

Effects of probenecid and quinine on digoxin uptake. We examined the ability of the organic anion transport blocker probenecid and the corresponding inhibitor of the cation system quinine to inhibit digoxin accumulation by the slices. The concentrations of the inhibitors were sufficient to selectively inhibit uptake of either the anion *p*-aminohippurate or the cation tetraethylammonium by at least 75% without affecting that of the oppositely charged reference. The results, presented in Fig. 4, demonstrate that neither inhibitor significantly altered digoxin uptake.

Effects of structural analogues on digoxin uptake. If digoxin uptake by renal cortical slices involves specific carriers, structurally related compounds should inhibit the process. To determine this, slices were preincubated for 20 min in the presence of a potential inhibitory analogue, after which digoxin was added, the incubation continued for an additional 120 min, and a S/M value for digoxin calculated. The effects of four analogues were examined: digitoxin, digoxigenin-bis-digitoxoside (BIS), digoxigenin-mono-digitoxoside (MONO), and digoxigenin. The latter three differ from digoxin in the number of digitoxose residues associated with the steroid portion of the molecule. Digitoxin differs in that it has a hydrogen atom rather than a hydroxyl group at the C-12 position of the steroid nucleus. The results (Fig. 5) demonstrate a significant ($P < 0.05$) dose-related reduction of digoxin S/M for slices incubated in the presence of digitoxin (three sugars), BIS (two sugars) and MONO (one sugar) but no such effect ($P > 0.05$) in the presence of digoxigenin (no sugars). Although there was not a statistical difference in potencies among the three inhibitory analogues, a trend was evident by which inhibitory potency was related to the number of sugars present (i.e. digitoxin > BIS > MONO > digoxigenin). None of the analogues caused a statistically significant reduction of tissue oxygen utilization rate after 2 hr of incubation when compared to the control value of 0.0159 ± 0.005 μL O_2/mg wet wt/min. This suggests that inhibition of digoxin S/M by digitoxin, BIS and MONO could not be attributed to generalized cellular toxicity.

Effect of digitoxose on digoxin uptake. The results of Fig. 5 can be interpreted to indicate that digoxin uptake is via a sugar transport process. Accordingly, we tested the ability of digitoxose to interfere with the ability of the renal cortex to accumulate digoxin. Digoxin (3.2 nM) uptake in the presence of 5 mM digitoxose (S/M 7.0 ± 2.01) was not significantly different from controls (S/M 7.30 ± 0.95).

Effect of ouabain on digoxin uptake. The effect of the glycoside ouabain (50 μM) on the tissue accumulation of 3.2 nM digoxin was examined. This concentration of ouabain is sufficient to cause functional inhibition of renal Na^+,K^+-ATPase [11] including that in chicken renal cortex slices [12]. Ouabain decreased the absolute tissue uptake of digoxin from a mean control level of 9.1 ± 1.1 ng/g wet wt to 5.4 ± 0.4 ng/g wet wt (N = 3 chickens).

Effect of spironolactone on digoxin uptake. The ability of renal cortical slices to accumulate digoxin (3.2 nM) in the presence of 50 μM spironolactone was compared to control slices incubated with digoxin alone (N = 4 chickens). Spironolactone had no inhibitory effect. Mean control S/M digoxin in these experiments was 4.78 ± 0.41, while that in the presence of spironolactone was 4.49 ± 0.54, a statistically insignificant difference.

Effect of medium potassium concentration on digoxin uptake. To examine the influence of medium potassium concentration on digoxin accumulation, slices were incubated for 120 min with 3.2 nM digoxin in medium containing either 0 mM potassium, 5 mM potassium (control) or 15 mM potassium. Osmolarity was maintained by adjusting sodium levels. The results are presented in Fig. 6. The effects of these treatments on uptake of the reference organic anion

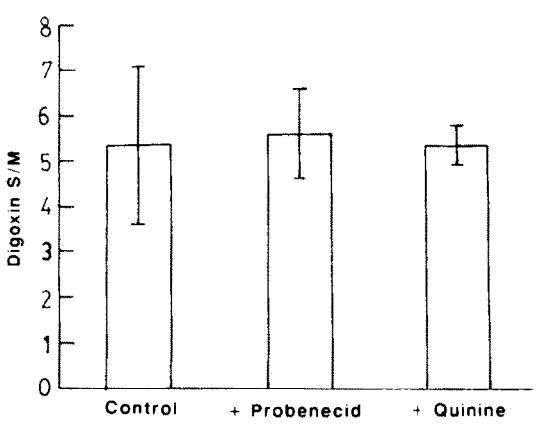

Fig. 4. Effect of organic ion transport inhibitors on [^{3}H]digoxin uptake by renal cortex. Each value is the mean ± SD from 15 individually incubated slices. Concentrations: [^{3}H]digoxin, 3.2 nM; probenecid, 0.25 mM; quinine, 0.25 mM. Incubation time was 120 min.

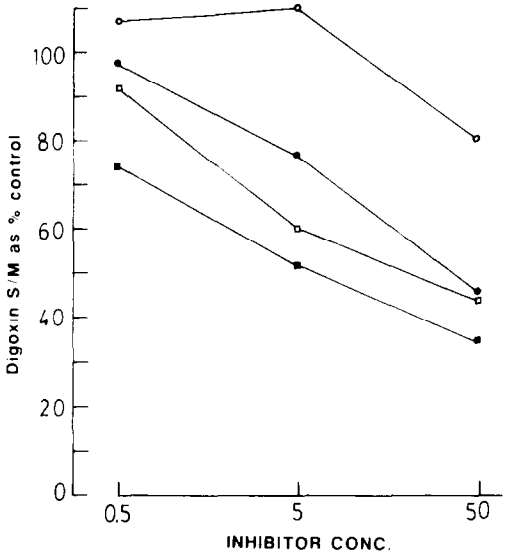

Fig. 5. Effects of structural analogues on [^{3}H]digoxin uptake by renal cortex. Each point is the mean of at least 12 individually incubated slices. Control S/M for [^{3}H]digoxin in these experiments was 3.72. Standard deviations are omitted for clarity but all were within 15% of the mean. The digoxin concentration was 3.2 nM in all experiments. Inhibitor concentrations are expressed as micromolar. Inhibitor key: (○) digoxigenin, (●) digoxigenin-mono-digitoxoside, (□) digoxigenin-bis-digitoxoside, and (■) digitoxin.

[^{14}C]*p*-aminohippuric acid (PAH, 0.01 mM) are also presented. The pattern of S/M digoxin varying inversely with medium potassium concentration is clearly evident. The accumulation of [^{14}C]PAH, on the other hand, was directly proportional to the medium potassium concentration, a finding that parallels the data of Dantzler [12] in a study of organic anion transport by chicken renal cortex slices.

DISCUSSION

This study has examined the uptake and accumulation of digoxin by avian renal cortex under controlled *in vitro* conditions. The results indicate that digoxin uptake involves both passive and energy-dependent mechanisms.

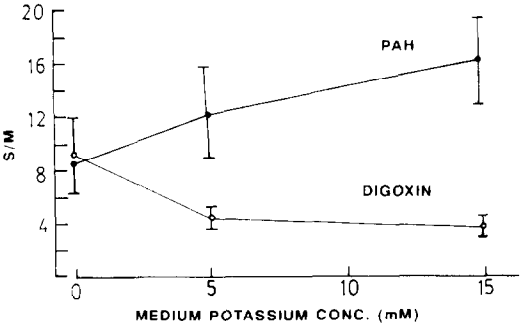

Fig. 6. Effect of incubation medium potassium concentration on uptake of digoxin and *p*-aminohippuric acid (PAH) by renal cortex. Each value is the mean ± SD of 18 individually incubated slices. Incubation time was 120 min. Concentrations: digoxin, 3.2 nM; and PAH, 10 μM.

The chicken renal cortex is capable of accumulating digoxin against at least a 5:1 concentration gradient. The results of the metabolic inhibitor experiments (Fig. 3) indicate that about half of the uptake can be accounted for by passive processes. Simple passive diffusion alone, however, is insufficient to account for the observed tissue accumulation (S/M > 1) under these anaerobic conditions. In view of the large volume of distribution of digoxin *in vivo* relative to that of ouabain, a glycoside with high specificity for Na^+,K^+-ATPase alone, it seems likely that the non-energy-dependent component in our experiments involved passive binding to tissue components.

As to the nature of the energy-dependent, saturable component of digoxin binding, one might first consider that the two most clearly identified renal secretory transport processes for organic molecules are the active carrier-mediated systems for organic anions and organic cations. Neither, however, seemed to play a role in digoxin accumulation since both anionic probenecid and cationic quinine were without inhibitory effect. Although this may seem quite reasonable in view of the fact that digoxin is a non-electrolyte, Kaplinsky *et al.* [13] have reported a small but significant reduction in accumulation of digoxin by rat renal cortex slices incubated in the presence of the cation quinidine, an isomer of quinine. This is probably attributable to displacement of digoxin from tissue sites unrelated to specific Na^+,K^+-ATPase sites [14]. This effect was stereospecific since quinine had no such action on digoxin disposition. Since quinine is widely applied as the definitive inhibitor of renal organic cation transport, our results indicate that this process plays no role in digoxin accumulation by chicken renal cortex slices.

An examination of the digoxin molecule suggests that at least three possible structural features may be essential for transport by renal tubular cells: the steroid ring system, the sugar portion, or the intact glycoside (steroid + sugars). If the steroid portion of the molecule was alone the essential feature, the aglycone digoxigenin should have been a potent competitor for digoxin uptake. This was not observed even at a greater than 10,000-fold molar excess. Steiness [1] noted an inhibitory effect of the spironolactone on digoxin renal clearance in heart patients and suggested an interaction of these drugs via a shared renal steroid transport process. The lack of inhibitory effect in our experiments with spironolactone suggests that in the chicken this is not the case and is consistent with the conclusion that the steroid structure alone is not sufficient to account for the observed uptake of digoxin. Though the present results seem to be in conflict with the inhibitory effect of spironolactone on digoxin excretion *in vivo*, it should be pointed out that the renal slice technique reflects primarily events associated with only the basolateral membrane and possibly interaction of substrates with intracellular components, tubular lumina being largely collapsed and/or filled with debris [15]. Given this and the complex pharmacodynamic profile of orally administered spironolactone including the presence of twenty or more

metabolites [16], it becomes clear that mechanistic comparison between *in vivo* observations of Steiness and our simpler *in vitro* model system results is difficult. Our results do suggest, however, that spironolactone did not interfere directly with the interaction of digoxin with the basolateral membrane. Neither does the sugar portion appear to be the sole essential molecular moiety since digitoxose was also without inhibitory effect on digoxin uptake. On the other hand, the three intact glycosidic analogues showed a clear dose-dependent inhibitory ability, supporting the concept of a structure-specific glycoside transport process. Dutta and Marks [17] have proposed that cardiac glycosides may be physically transported into cardiac muscle cells via a "transporting" Na^+,K^+-ATPase. There is, however, no convincing evidence to date for a role of renal ATPase in the tubular secretion of digoxin. Although our present experiments were not designed to definitively address the important question of a contribution of direct ATPase binding to slice accumulation of digoxin, the inhibitory action of ouabain and the stimulatory effect of potassium-free incubation medium are provocative and suggest that this merits investigation by more direct methods.

Some perspective may be gained by considering studies of cardiac glycoside uptake in other tissues. Some differences as well as similarities with our current results in the kidney are notable. Ouabain uptake by liver cells has been studied extensively and been found to occur by a saturable, energy-dependent transport process [18]. In contrast to digoxin uptake by chicken kidney slices, however, ouabain transport in isolated hepatocytes is competitively inhibited by non-glycosidic steroids such as the bile acid taurocholate, diethylstilbesterol and spironolactone [19, 20]. Digitoxin and digoxin were found to be potent inhibitors of ouabain transport and so presumably share a common mechanism [21]. Similar carrier-mediated transport processes for cardiac glycosides have been identified in brain microvessels/choroid plexus [22] and intestinal mucosa [23]. There is not enough compatible experimental data at present to reconcile the glycoside transport processes in various tissues into a general, perhaps common, glycosidic steroid transport system, but the close similarities, as well as some differences, in the uptake mechanisms of various tissues for these drugs make this an idea worthy of further research.

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