CORRELATION OF THE SUBCELLULAR DISTRIBUTION OF DIGOXIN WITH THE POSITIVE INOTROPIC EFFECT

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ABSTRACT


We have investigated the effect of chlorpromazine and aldosterone on the positive inotropic effect and the subcellular distribution of 3H-digoxin in isolated, electrically driven guinea-pig hearts. Both drugs decreased the positive inotropic effect of 3H-digoxin and the uptake of the drug by the microsomal fraction, whereas the total tissue uptake and the tissue/medium ratio of the radioactivity did not differ significantly from control. Because all measurements were made in the same experiments, we were able to show a significant correlation between the positive inotropic effect and the content of 3H-digoxin in a crude microsomal fraction, but not in the mitochondrial or nuclear fractions. A significant fraction of the bound drug was released into the supernatant when the microsomal pellet was shaken in Krebs' solution and recentrifuged. There was a greater correlation of the inotropic effect with the loosely bound drug than with the total microsomal binding; there was no correlation between contractility and the drug remaining in the microsomal pellet. Gel filtration of the supernatant showed that the drug was not bound to a macromolecule. We suggest that the digoxin taken up by the cell and bound loosely to a constituent of the microsomal fraction is responsible for the positive inotropic effect.

As a result of their study of the kinetics of uptake, exchange and release of several cardiac glycosides and on the time required for the development of the positive inotropic effect, Kuschinsky et al. (1968a,b) and Kuschinsky and Van Zwieten (1969) concluded that total tissue uptake was not related to the increase in contractility. However, considerable evidence has accumulated to suggest that the positive inotropic effect of digitalis glycosides may be related to their uptake by the microsomal fraction of the heart. Dutta et al. (1968b) showed that 3H-digoxin uptake and the positive inotropic effect of digoxin are correlated. Therefore, this study was designed to determine if there is a correlation between the uptake of digoxin and the positive inotropic effect.
digoxin is concentrated in a crude microsomal fraction to a greater extent than in the other subcellular fractions of guinea-pig hearts, and that microsomal uptake of various glycosides and aglycones was consistent with their pharmacological activities. They suggested that this fraction contains the digitalis receptor. They have also reported that both the total tissue uptake of \(^3\)H-ouabain and its uptake by the microsomal fraction were related to the concentrations of Na\(^+\) and K\(^+\) in the perfusion medium in a manner consistent with the known effects of these ions on the positive inotropic effect of the drug (Dutta et al., 1968a; Dutta and Marks, 1969). They have proposed that a carrier located in the cardiac cell membrane binds and translocates glycosides from the outside to the inside of the cell, where it becomes accessible for further binding and interaction with the receptors (Dutta et al., 1968a). Their studies and those of Kuschinsky et al. (1968a), and Kuschinsky and Van Zwieten (1969) suffered from the fact that tissue uptake or distribution of the glycoside was not measured in the same preparations in which the pharmacological effect was determined. Taking this additional precaution, we found (Kim et al., 1970; Kim and Dresel, 1970) that the action of insulin to increase the positive inotropic effect of \(^3\)H-digoxin in perfused guinea-pig hearts was directly related to the increase in the uptake of the glycoside by the microsomal fraction, but was not related to the total content of digoxin of the hearts, to the tissue/medium ratio, or to the binding to the crude nuclear and mitochondrial fractions. Our results were in agreement with those of Kuschinsky et al. (1968a,b) and Kuschinsky and Van Zwieten (1969) and showed that the total uptake of a given compound by the heart under constant conditions was not necessarily an index of drug binding to a specific receptor. We now suggest that the total uptake by the microsomal fraction may also not be the most appropriate index of the portion of bound digoxin which may be responsible for the pharmacological effect.

The objective of the present work was to interfere with the positive inotropic effect of digoxin or with its binding to various subcellular fractions by agents which, unlike the changes in ionic concentrations examined by Dutta and Marks (1969), would not cause major changes in total tissue uptake of digoxin, but which might affect the cellular distribution of the glycoside.

Lefer and Sayers (1965) and Tanz (1962) reported that aldosterone exerted a small but significant positive inotropic effect in isolated cardiac tissue. When aldosterone and ouabain were administered together, aldosterone antagonized the positive inotropic effect of ouabain in cat papillary muscle (Lefer and Sayers, 1965; Lefer, 1966). Fujino et al. (1969) demonstrated an antiouabain action of aldosterone in frog ventricle strips. In addition, Read et al. (1964) reported that ouabain decreased the accumulation of aldosterone by the heart and suggested that the two compounds competed for a binding site on the cell membrane. On the other hand, Levy (1969) has found that aldosterone did not antagonize the positive inotropic effect of ouabain on rabbit atria under the conditions caused by Lefer (1966).

Chlorpromazine has been shown to be bound "nonspecifically" to liver microsomes (Dingell et al., 1961), and Idinpasin-Heikkila et al. (1968) have shown autoradiographically that the drug was concentrated by the heart. Although high concentrations of chlorpromazine have a cardiac depressant effect in the isolated perfused rabbit heart (Melville, 1954), we know of no report concerning the interference of chlorpromazine with the positive inotropic effect of digitalis, and we hoped that low concentrations of chlorpromazine might affect the nonspecific binding of digoxin without altering the pharmacological effect.

**Methods**

**Perfusion procedure.** Guinea pigs of both sexes weighing approximately 300 g were killed by a blow on the head and their hearts were rapidly removed. Immediately after extraneous tissues were removed, the hearts were perfused through the aorta at a constant pressure of 60 mm Hg. The composition of the modified Krebs-Henseleit solution was: NaCl, 118 mM; KCl, 4.7 mM; CaCl\(_2\), 2.5 mM; NaH\(_2\)PO\(_4\), H\(_2\)O, 1.18 mM; MgSO\(_4\)·7H\(_2\)O, 1.18 mM; NaHCO\(_3\), 26.2 mM; and glucose, 11 mM (Krebs and Henseleit, 1932). The perfusion medium was equilibrated with 95% O\(_2\) and 5% CO\(_2\) and maintained at 30°C. Isometric contractile force was measured with a Grass FT-03 force-displacement transducer attached by a stainless-steel clip and prestretched nylon monofilament line to the apex of the heart. The force of contraction was recorded on a Grass model 5D polygraph. The clip on the apex served as an indifferent electrode and the hearts were stimulated at twice threshold voltage at a rate of 210/min by a second stainless-steel
contractility and \( ^{3}H \)-digoxin distribution

with an equal amount of unlabeled digoxin. Aldosterone (2 \( \times \) \( 10^{-7} \) g/ml, Sigma Chemical Company, St. Louis, Mo.) or chlorpromazine hydrochloride (2 \( \times \) \( 10^{-7} \) g/ml, May and Baker, Ltd., Dagenham, England) was added to the perfusate 30 minutes before the exposure to digoxin and remained in the perfusate throughout the rest of the experiment. At the end of 30 minutes of exposure to digoxin the hearts were washed free of all drugs with Krebs-Henseleit solution for four minutes. This period of washing is shorter than that used by Dutta et al. (1968b). Figure 1 shows the time course of removal of \(^{14}C\)-inulin (New England Nuclear) from the extracellular space of guinea-pig hearts which had been perfused with inulin for 30 minutes; approximately 95% of the marker was removed after four minutes of washout with inulin-free medium. We therefore assume that most of the digoxin which occupies the inulin space is also removed by this procedure.

Preparation of subcellular fractions. The hearts were removed from the cannula and blotted on filter paper to remove excess moisture. A sample of ventricular muscle weighing approximately 200 mg was homogenized in a glass homogenizer, extracted for three hours with 2 ml of dioxane and the extract was counted in a liquid scintillation counter. The remainder of the ventricle was fractionated by the method of Dutta et al. (1968b). Briefly, the procedure was as follows. Approximately 800 mg of the ventricle were minced in an ice bath and homogenized in 10 volumes of cold (4\(^{\circ}\)C) 0.33 M sucrose solution containing 0.001 M ethylenediamine tetraacetic acid (EDTA) by seven gentle strokes of a glass Potter-Elvehjem homogenizer fitted with a motor-driven Teflon pestle. The homogenate was spun in an International Preparative Ultracentrifuge (International Equipment Company, Needham Heights, Mass.), model 60, at 166,000 \( \times g \) for 60 minutes at 4\(^{\circ}\)C to sediment all particulate matter. The pellet was resuspended in 10 volumes of cold (4\(^{\circ}\)C) sucrose-EDTA solution and centrifuged in a Sorvall refrigerated centrifuge at 700 \( \times g \) for 10 minutes to remove nuclei, myoffibrils and other cell debris. The sediment was called the nuclear fraction and the supernatant was centrifuged in the same centrifuge at 12,000 \( \times g \) for 15 minutes at 4\(^{\circ}\)C. The resulting pellet was the mitochondrial fraction.

\[\text{Fig. 1. Time course of } ^{14}C\text{-inulin clearance in guinea-pig hearts.} \]

The supernatant was centrifuged in the ultracentrifuge at 166,000 \( \times g \) for 60 minutes at 4\(^{\circ}\)C. The pellet represented the microsomal fraction. Dutta et al. (1968b) have shown by electron microscopy that this fraction consists of vesicles and membrane fragments. Each of the pellets was suspended in a known amount of distilled water or Krebs' solution and the \(^{3}H\)-digoxin content of each subcellular fraction and perfusion medium was determined in a liquid scintillation spectrometer. The scintillation mixture contained 5.3 g/l of 2,5-diphenyloxazole (PPO) and 66.7 mg/l of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) dissolved in a toluene-Triton X-100 2:1 (v/v) scintillation mixture (Patterson, 1965). The measured \(^{3}H\)-digoxin was assumed to represent unchanged digoxin, since various studies (Lage and Spratt, 1965; Kuschinsky et al., 1967) have demonstrated that guinea-pig myocardium does not metabolize digoxin in vitro. In order to normalize the digoxin concentration for the purposes of comparison, the protein concentrations in aliquots of each fraction were measured by the method of Lowry et al. (1951), modified by the addition of 5% deoxycholate to each sample to solubilize the protein. Bovine serum albumin was used as the standard.

Determination of loosely bound digoxin. To determine loosely bound digoxin, the microsomal fraction was resuspended by shaking for 5 minutes with a Vortex Mixer in 2 ml of Krebs-Henseleit solution. The suspension was allowed to stand in the cold (4\(^{\circ}\)C) for 40 minutes. Aliquots were taken for the determination of protein and total microsomal digoxin concentrations (as above) and the
remainder was centrifuged in the ultracentrifuge at 166,000 × g for 60 minutes at 4°C. The radioactivity in the supernatant was called the loosely bound digoxin.

Gel filtration. A 1-ml aliquot of the supernatant containing loosely bound digoxin was placed on a 1.5 × 22 cm column containing 20 g of Sephadex G-15 (Pharmacia Fine Chemicals, Uppsala, Sweden) prepared according to the manufacturer's recommendation and was eluted with 0.9% NaCl solution. Flow through the column was 30 ml/hr. Protein concentration and radioactivity were determined for each milliliter of the eluate.

Drugs. Samples of the 3H-digoxin were tested for purity by thin-layer chromatography on Eastman Silica Gel G chromatoplates. The plates were prepared according to the manufacturer's recommendation and was eluted with 0.9% NaCl solution. Flow through the column was 30 ml/hr. Protein concentration and radioactivity were determined for each milliliter of the eluate.

The effect of aldosterone and chlorpromazine on the positive inotropic effect of 3H-digoxin. The effects of aldosterone and chlorpromazine on the positive inotropic effect of 3H-digoxin are shown in Table 1. Contractile force of untreated hearts did not change significantly during these experiments. Digoxin (2.6 × 10^{-7} M) alone caused an increase in contractile force of 47.9 ± 2.4% (mean ± S.E.) after 30 minutes of perfusion. This effect was not increased by longer exposure to the drug. Perfusion with aldosterone (5.5 × 10^{-7} M) for 30 minutes caused cardiac contractile force to increase approximately 7% (P > .05). The positive inotropic response to digoxin was reduced to 17.7% when aldosterone was administered 30 minutes before and throughout the exposure to digoxin; this represents a 63% reduction in the normal response to digoxin (P < .05). This is consistent with the observation of Lefer and Sayers (1965) that aldosterone antagonizes the effects of ouabain on contractility in the isolated papillary muscle. Addition of 95% ethanol in amounts corresponding to those added with aldosterone did not affect contractility or the response to digoxin.

Perfusion of chlorpromazine (6.3 × 10^{-7} M) for 30 minutes caused a 4% increase (P > .05) in contractile force. However, contractile force decreased to 18% below control during the next

<table>
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<tr>
<th>Treatment</th>
<th>Force (Increase)</th>
<th>Digoxin Uptake</th>
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<tr>
<td></td>
<td></td>
<td>Total tissue</td>
</tr>
<tr>
<td>Digoxin, 2.6 × 10^{-7} M (4)*</td>
<td>47.9 ± 2.3%</td>
<td>445.73 ± 30.4</td>
</tr>
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<td>Digoxin plus chlorpromazine 6.3 × 10^{-7} M (4)</td>
<td>32.4 ± 1.5%</td>
<td>410.25 ± 8.85</td>
</tr>
<tr>
<td>Digoxin plus aldosterone 5.5 × 10^{-7} M (4)</td>
<td>17.7 ± 2.0%</td>
<td>426.69 ± 21.06</td>
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* Number in parentheses indicates the number of experiments.

* Mean ± S.E.

* Significant reduction from values measured in hearts treated with digoxin alone (P < .05).

Table 1

The effect of aldosterone and chlorpromazine on the positive inotropic effect and the uptake of H-digoxin in the guinea-pig myocardium.
30 minutes (three experiments). The positive inotropic effect of digitoxin was reduced significantly (P < .05) when chlorpromazine was given 30 minutes before and throughout exposure to digitoxin, but not to the extent seen after aldosterone pretreatment (table 1).

Effect of aldosterone and chlorpromazine on the uptake and distribution of 3H-digitoxin. Table 1 shows that the total tissue uptake of digitoxin by the control hearts was 445.7 ± 30.4 ng/g of tissue and the tissue/medium radioactivity ratio was 2.23 ± 0.17. Neither of these measurements was affected significantly by aldosterone or chlorpromazine. The two drugs decreased the concentration of 3H-digitoxin in the nuclear and mitochondrial fractions from that measured in hearts treated with digitoxin alone, but these changes were not statistically significant (table 1). The supernatant/pellet ratio was increased from 0.35 ± 0.02 in control hearts to 0.37 ± 0.02 and 0.40 ± 0.01 in chlorpromazine- and aldosterone-treated hearts, respectively; these changes were also not statistically significant.

In four control hearts, the concentration of 3H-digitoxin in the microsomal fraction (6.12 ± 0.23 ng/mg of protein) was at least twice the concentration measured in the mitochondrial and nuclear fractions. Both aldosterone and chlorpromazine significantly decreased the concentration of digitoxin in the microsomal fraction to 4.34 ± 0.23 and 4.60 ± 0.22 ng/mg of protein, respectively (P < .05; table 1). The difference in microsomal uptake of 3H-digitoxin between the two pretreated groups of hearts was not significant statistically (P > .05; table 1). The difference in the inotropic effects was significant. Despite this apparent dissociation of the inotropic effect from total microsomal uptake as concluded from the data in table 1, we were able to show that a relationship exists between the two parameters. Figure 2 shows this relationship measured in 12 experiments. The two measurements were correlated significantly with a coefficient of correlation $r = 0.73$ (P < .05). When the regression line was extrapolated to the digitoxin concentration where no positive inotropic effect would be expected to occur, the intercept indicated that 2.0 ng of digitoxin per mg of protein must be bound before there would be any evidence of a positive inotropic effect. There was no significant correlation between the positive inotropic effects and the binding to the nuclear or mitochondrial fractions.

Loosely bound digitoxin. We attempted, as part of a series of experiments reported previously (Kim et al., 1970), to determine the binding of digitoxin to various subfractions of the microsomes by recentrifugation of the microsomal fraction on a continuous sucrose density gradient. These experiments were unsuccessful because a major portion of the radioactivity remained at the top of the gradient, indicating that the radioactivity had become dissociated from the particulate fraction as a result of the resuspension. We therefore resuspended the microsomal pellets obtained in the present series of experiments in Krebs-Henseleit solution under standardized conditions (cf. “Methods”). Aliquots of each microsomal resuspension were analyzed for protein concentration and radioactivity to yield the data on total microsomal uptake shown in table 1. The remainder of the suspension was centrifuged at 166,000 × g for 60 minutes. We have called the digitoxin in the resultant supernatant “loosely bound.” It contains digitoxin which has been removed from the microsomes by the resuspension procedure in Krebs-Henseleit solution. As shown in table 1, the mean amount of loosely bound digitoxin in the control hearts was 1.96 ± 0.06 ng/mg of microsomal protein. Aldosterone reduced this to 1.17 ± 0.08
ng/mg of microsomal protein (P < .05). The mean quantity of loosely bound digoxin in chlorpromazine-treated hearts did not differ significantly from that in the controls. The ratio of loosely bound to total microsomal digoxin was 0.32 for the control hearts. This ratio was reduced by aldosterone but was increased by chlorpromazine.

We then correlated the positive inotropic effect with the content of the two subfractions of the total microsomal uptake, i.e., the loosely bound fraction and the digoxin which remained bound to the microsomes after resuspension. Figure 3 shows that loosely bound digoxin is clearly correlated with the positive inotropic effect. The coefficient of correlation, $r = 0.84$, is considerably larger than that associated with the correlation of total microsomal uptake shown in figure 2, but this difference did not reach statistical significance. However, the amount of digoxin which remained bound after the resuspension procedure was not correlated significantly with the inotropic effect. The coefficient of correlation, $r = 0.37$, was not significantly different from zero (P > .05) and differed significantly from $r = 0.84$ (P < .05), the value for the data in figure 3. When the regression line was extrapolated to the digoxin concentration where no positive inotropic effect would be expected to occur, the intercept indicated that there must be 0.44 ng/mg of protein of the loosely bound digoxin before there would be evidence of a positive inotropic effect.

Loosely bound digoxin from a separate series of experiments was used to determine whether the radioactivity removed from the particulate fraction was free or remained associated with a protein component. The elution pattern obtained from one such experiment is shown in figure 4. Retention of the radioactivity was the same as

**Fig. 3.** Correlation between the loosely bound digoxin released from the microsomal fraction during resuspension and the changes in cardiac contractile force after digoxin perfusion. Each dot represents a heart perfused for 30 minutes with $^3$H-digoxin alone or in the presence of aldosterone (5.5 $\times$ 10$^{-7}$ M) or chlorpromazine (6.3 $\times$ 10$^{-7}$ M).

**Fig. 4.** Gel filtration elution diagram of loosely bound digoxin fraction. (Sephadex G-15 column, 1.5 $\times$ 22 cm, was eluted with 0.9% sodium chloride.) The right scale of ordinates indicates the radioactivity of the eluate; the left indicates the optical density at 280 m$\mu$; the abscissa are the elution volume in milliliters. $\cdots \cdots$, protein content of the eluate (optical density); $\cdots \cdots$, $^3$H-digoxin content of the eluate in counts per minute per milliliter.
for an authentic sample of free \(^{3}H\)-digoxin. The protein peak corresponds to the elution volume of serum albumin. We conclude that free digoxin is removed from the microsomes during resuspension.

**Discussion**

The results of the present experiments are in general agreement with the conclusions of Dutta *et al.* (1968a,b) and of Gerber and co-workers (1968), which indicated that the crude microsomal fraction contained sites of uptake for digitalis glycosides that can best account for the positive inotropic effect. We have observed again that the total tissue uptake of a glycoside cannot be correlated with the pharmacological effect and that the uptake by nuclear and mitochondrial fractions is also not related to effectiveness (Kuschinsky *et al.*, 1967; Kim *et al.*, 1970; Roth-Schechter *et al.*, 1970; Dutta and Marks, 1969). Kuschinsky hypothesized that the cellular uptake at different concentrations of digoxin may be the sum of two different components. The linear component was not saturable and possibly represents the passive movement of digoxin across the cell membrane. The other component, which was saturable, might represent the accumulation of digoxin at the active site (Kuschinsky *et al.*, 1967). The availability of the saturable sites was reduced either in the presence of another glycoside or after incubation with metabolic inhibitors (Godfraind and Lesne, 1970). It has also been demonstrated that in the guinea-pig heart the microsomal uptake of digoxin is inhibited by dinitrophenol (Dutta *et al.*, 1969) and that binding is adenosine triphosphate-dependent in fragments of sarcoplasmic reticulum of the bovine heart (Dutta *et al.*, 1968a). We have shown a significant correlation between the positive inotropic effect and the uptake of digoxin by the crude microsomal fraction. Re-suspension of the microsomal pellet in Krebs-Henseleit solution permitted the total microsomal digoxin to be separated into two subfractions: one that was removed from the microsomes, and another that remained bound to them after this procedure. The correlation between loosely bound digoxin and the positive inotropic effect appeared to be better than that of the total microsomal uptake. The more tightly bound drug was no longer correlated with the positive inotropic effect. These results strongly suggest that it is the digoxin bound loosely to the microsomes that may be causal to the positive inotropic effect whereas that which remained associated with the particulate was not. It should be emphasized that this represents a further fractionation of the bound drug, not of the microsomes, and that no evidence is available at present concerning the constituent of the microsomal fraction which contains the loosely bound drug.

The correlations which we have shown are based on the use of drugs which decrease the positive inotropic effect of digoxin. This action is known for aldosterone (Lefer, 1965). We recognize that the effect of chlorpromazine may be partly due to a physiological antagonism despite the fact that total microsomal binding of digoxin was reduced significantly. Neither drug changed the total uptake of digoxin by the tissue, so that differences in their effect on the intracellular distribution may in fact be causal to their differing antidigitalis actions. It is thus of considerable interest that the two drugs affect differently the ratio of loosely bound to more tightly bound digoxin. Aldosterone, on the one hand, decreased total microsomal uptake and decreased the quantity of loosely bound digoxin to an even greater extent. This effect on digoxin binding was accompanied by a considerable decrease in the positive inotropic effect. Chlorpromazine, on the other hand, which is known to be bound nonspecifically to liver microsomes and to be concentrated by cardiac tissue (Idänpään-Heikkila *et al.*, 1968), decreased the total microsomal uptake of digoxin as much as did aldosterone. However, chlorpromazine interfered less than aldosterone with the uptake of digoxin by the labile binding sites. Correspondingly, the positive inotropic effect of digoxin was decreased only slightly, although significantly, in the chlorpromazine-treated hearts.

Extrapolation of the regression lines (figs. 2 and 3) to zero effect indicates that there may be considerable binding of digoxin before a positive inotropic effect could be observed under the conditions of our experiments. That is, there is probably a threshold concentration of bound digoxin below which no pharmacological effect occurs. We perfused two preparations with drug-free medium until contractility returned to control levels and found that the quantities of digoxin (total and loosely bound) in the micro-
somal fraction closely approximated those predicted by the extrapolation.

The fact that the loosely bound doxigoxin was not released by the prior suspensions in sucrose-EDTA solutions required for the preparation of the microsomal fraction indicates that there are profound differences in the binding capacities of microsomes suspended in saline solutions and sucrose solutions. These may be due to changes in ionic strength, or one or more specific constituents of Krebs-Henseleit solution may be responsible.

Conclusions

Of the intracellularly bound fractions of digitoxin, the one best correlated with the positive inotropic effect of digitoxin is a loosely bound component of the microsomal fraction which can be removed from the microsomes by mechanical agitation in Krebs-Henseleit solution. Changes in the quantity of digitoxin bound loosely to the microsomes are correlated directly with time positive inotropic effect in the presence of aldosterone or chlorpromazine. The results suggest that the digitoxin responsible for the positive inotropic response is a labile fraction of the total digitoxin bound to the microsomes.

References


