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Nature of the Transport Adenosine Triphosphatase-Digitalis Complex: XIV. Inotropy and Cardiac Glycoside Interaction with Na^+,K^+-ATPase of Isolated Cat Papillary Muscles

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SUMMARY

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A mathematical model was developed that allows the use of [^{3}H]ouabain binding to estimate the free and occupied digitalis receptors in crude homogenates from cat right ventricular papillary muscles. Cat papillary muscles were exposed to concentrations of digoxin or ouabain that produced an inotropic effect. The fraction of occupied receptors was estimated using the model. There was a good correlation between the number of receptors occupied and the increase in contractile force achieved. When the inotropic effect was washed out, the number of occupied receptors decreased to control (zero receptors occupied). The results support the concept that the Na^+,K^+-ATPase-membrane system contains the pharmacological receptor for cardiac glycosides. No significant difference in the number of digitalis receptors in right and left ventricle and left atrium was detected. The density of digitalis receptors estimated per unit surface area and the number of receptors per cell in heart is much greater than estimates of receptor capacity for some other drugs.

INTRODUCTION

Cardiac glycosides that produce an inotropic effect also inhibit Na^+,K^+-ATPase isolated from heart in a dose-dependent manner (1). Therefore, it is reasonable to suspect a cause-effect relationship between the interaction of cardiac glycosides with the Na^+,K^+-transport system and positive inotropy. In fact, since the original postulation by Repke (1), evidence has been accumulating to substantiate the hypothesis that Na^+,K^+-ATPase is the receptor for digitalis and that the positive inotropic effect is the result of this receptor occupation (2–11). In spite of this accumulation of evidence, there are some who argue that Na^+,K^+-ATPase is not the pharmacological

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receptor for the inotropic effect of digitalis (12–14).

It is important that studies to elucidate the complex mechanism of cardiac glycoside action include methods that allow simultaneous measurement of inotropy and drug-receptor binding. Toward this end, our experiments utilized right ventricular papillary muscles isolated from cat, a species that is very sensitive to cardiac glycosides. The muscles were exposed to ouabain or digoxin until a chosen level of inotropy was attained. In some experiments, the inotropic effects were washed out. The number of cardiac glycoside receptor sites in these muscles was then measured. It has been suggested (15) that the use of deoxycholate in enzyme extraction procedures causes dissociation of *in vivo* formed ouabain-enzyme complex during isolation. To avoid this possibility, we did not attempt to purify the Na^+,K^+-ATPase but minimally disturbed the cell membranes by preparing homogenates of the papillary muscles. Instead of using Na^+,K^+-ATPase hydrolytic activity (the measurement of which is difficult in these low activity homogenates) to assay the number of active sites, we used [^{3}H]ouabain binding, a technique which is capable of much greater precision. Development of a suitable mathematical model allowed the estimation of the fraction of receptor sites occupied per unit of cell protein. These procedures afforded a small, physiologically stable, controlled muscle with a well defined inotropic state and a quantitative appraisal of the number of receptors occupied during the inotropic states. Included in this study is an assessment of regional variation in the number of cardiac glycoside receptors and an estimation of the number of ouabain molecules that can bind to an average cardiac muscle cell.

MATERIALS AND METHODS

Isolated muscle preparation. Isolated right ventricular papillary muscles (4–8 mg weight) were removed from adult cats and placed in an isometric myograph. The muscle bath contained (mM concentrations): NaCl 117.4, $NaHCO_3$ 25, Na_2HPO_4 1.2, $MgSO_4$ 1.2, KCl 3.6, $CaCl_2$ 2.5 and glucose 11.1. The bath was saturated with 95% O_2–5% CO_2 and maintained at a temperature of 29°. Field stimulation by flat, platinum plate electrodes arranged on both sides of the muscle, delivered square wave pulses of 5 msec duration at approximately 20% above threshold voltage with a stimulation rate of 0.25 Hz. Muscles were equilibrated for a minimum of 2 hr (no deterioration of the muscle occurred) during which the preload was adjusted to load at L_{max}. Ouabain or digoxin (Sigma Chemical Co.) was added to the bath in a concentration range of 10 nM to 10 μM and the contractile force was measured. At an arbitrarily chosen level of inotropy or at peak inotropy for a certain glycoside dose, the muscles were rapidly removed and rinsed in a 4° wash solution. In order to minimize dissociation, this wash solution contained 50 mM Tris-Cl, 100 mM NaCl, and 2.5 mM $MgCl_2$. The muscle was lightly blotted on filter paper and immediately frozen on dry ice. The same procedure was used for muscle samples taken from right atria, left atria, right ventricles and left ventricles with the exception that they were not placed on the myograph. The muscle samples were weighed while frozen and sectioned (if necessary) to weights less than 10 mg.

Ouabain Binding to Homogenates. The [^{3}H]ouabain binding method used is an extension of that used recently by Gelbart and Goldman (16), by Brody (17), and by Ku *et al.* (18). Each muscle was homogenized in the cold by three passes in a Duall Glass homogenizer for 30 sec in 0.4 ml of a medium containing 50 mM Tris-Cl, 100 mM NaCl and 2.5 mM $MgCl_2$. The homogenate was diluted to approximately 10 mg wet weight/ml with the same medium, prior to [^{3}H]ouabain binding and protein assay. Aliquots of the homogenate (0.2 ml) were promptly added to a solution containing Tris-Cl (pH 7.4), NaCl, and $MgCl_2$. After one minute [^{3}H]ouabain (14.4 Ci/mM) was added to initiate binding. The reaction (2.0 ml) contained in final concentration 50 mM Tris-Cl (pH 7.4), 100 mM NaCl, 2.5 mM Na_2ATP, 2.5 mM $MgCl_2$, 10 nM [^{3}H]ouabain and 0.150–0.250 mg of protein. At appropriate times, aliquots (0.4 ml) were removed and filtered through a 0.45 μm Millipore

filter and rinsed three times with 2 ml of cold (4°) water. The filters were dissolved in 10 ml of scintillation fluid (toluene, 10% BBS-3, Fluoralloy, Beckman) and radioactivity was measured in a Beckman LS-200 liquid scintillation spectrometer. The efficiency of this system was 39% so that 12,468 cpm represented one picomole of ouabain. Nonspecific binding was measured in the same reaction medium as above but with the addition of unlabeled ouabain (100 μM final). Protein estimation was done by the method of Lowry *et al.* (19).

RESULTS

In vitro *studies*: *Development of the model.* Using the crude homogenate from papillary muscle, a good separation of specific binding, typically 1600 cpm for 100 μg protein after one hour, and nonspecific binding was obtained. Nonspecific binding is defined as the binding in the presence of 100 μM unlabeled ouabain, typically 90 cpm for 100 μg. All data presented have been corrected for nonspecific binding. The increase above nonspecific levels was ATP dependent (in presence of Mg^{2+} and Na^+). The time course of the binding was dependent upon the amount of protein (and thus the concentration of ouabain receptors) in the binding medium. There was a linear relationship between the concentration of protein and the levels of equilibrium binding. The rate at which equilibrium was obtained, however, was not dependent on the protein concentration. Binding in the presence of magnesium plus inorganic phosphate yielded similar results (data not shown).

Although the ouabain-sensitive Na^+,K^+-ATPase activity in the crude homogenates is low (1 μmole/mg/hr), the total ATPase activity is around 9 μmole/mg/hr. Since ATP was being hydrolyzed during the binding reaction, it was necessary to determine how the concentration of ATP affected the time course of [^{3}H]ouabain binding. The time course of binding (up to 165 min) was examined as described in MATERIALS AND METHODS, except that the initial ATP concentration was varied (2.5, 1.0, 0.5, 0.25, 0.15 and 0.1 mM). At the two lowest concentrations of ATP the binding reached a peak (1.7 and 1.2 picomole/mg) at approximately 50 min and then slowly fell. At concentrations of 0.25 mM and above, there was only a slight effect on the time course of binding. The maximum binding, *Be*, for ATP = 2.5, 1.0, 0.5 and 0.25 mM was 2.92, 2.85, 2.63 and 2.54 picomoles/mg and the effective rate constant, *ke*, was 0.031, 0.033, 0.035 and 0.036/min, respectively.

An initial concentration of ATP of 2.5 mM was therefore sufficient to support [^{3}H]ouabain binding if the enzyme concentration was kept equal or less than 125 μg/ml. Under these conditions the concentration of ATP would fall only to 1.5 mM in one hour and would not be limiting for the experiments described in this paper.

It has been shown (20) that the binding of [^{3}H]ouabain to Na^+,K^+-ATPase is adequately represented by

$$R + ouab^* \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} R \cdot ouab^* \qquad (1)$$

If the concentration of [^{3}H]ouabain, $ouab^*$, is in large excess of the concentration of receptor, R, the approach to equilibrium can be expressed as

$$\ln \frac{[R \cdot ouab^*]\, e - [R \cdot ouab^*]}{[R \cdot ouab^*]_e} = -k_e t \qquad (2)$$

where $[R \cdot ouab^*]$ and $[R \cdot ouab^*]_e$ represent ouabain bound at time t and at equilibrium, respectively. The effective rate constant, k_e, is the sum of the pseudo-first order forward rate constant, $k_1\,[ouab^*]$ and the dissociation rate constant, k_{-1}, for the reverse direction. A plot of equation 2 showed good first-order kinetics (Fig. 1). Five determinations of *ke* yielded a rate constant of 0.029 ± 0.003/min. The dissociation rate constants, k_{-1}, for ouabain and digoxin were determined by a chase method (20). Binding of the tritium-labeled cardiac glycoside was allowed to reach equilibrium. Excess unlabeled cardiac glycoside (final concentration 100 μM) was then added and the loss of radioactivity bound to the homogenate was followed by removing and filtering aliquots of the reaction medium at appropriate times. The dissociation followed first order kinetics (Fig. 2). Digoxin had a slower dissociation rate (0.0013 min^{-1}) than did ouabain (0.0047/min).

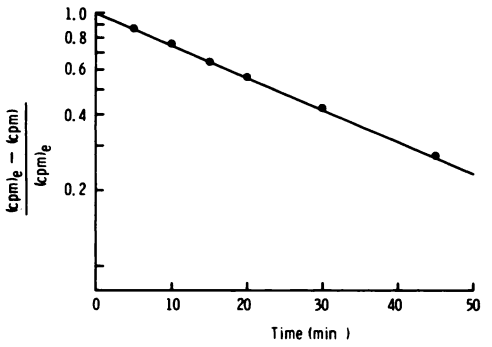

FIG. 1. *First order binding of [^{3}H]ouabain to cat heart homogenate*

The binding was in the presence of MgATPNa as described in METHODS. (CPM) and (CPM)$_e$ are radioactivity expressed as counts per minute bound to the enzyme at time t and at equilibrium, respectively. Protein concentration was 0.11 mg/ml and 1.0 ml aliquots were filtered. At equilibrium (2 hr) 2606 cpm or 1.9 picomoles/mg were bound.

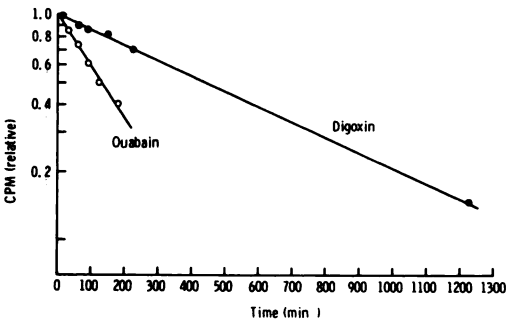

FIG. 2. *Time course of dissociation of [^{3}H]ouabain and [^{3}H]digoxin from cat heart homogenate receptor sites*

Binding of the labeled glycosides was carried out as outlined in Methods. At zero time a 100-fold excess of unlabeled cardiac glycoside was added to the reaction.

To test the hypothesis that occupation of the cardiac glycoside receptor by unlabeled cardiac glycosides would lead to a change in the nature of the [^{3}H]ouabain binding curve, four samples were prepared. A homogenate from cat heart (4.0 mg) was exposed to 0.1 μM unlabeled ouabain in the presence of 50 mM Tris-Cl, 100 mM NaCl, 5 mM $MgCl_2$ and 5 mM Na_2ATP in a volume of 5 ml. After 30 min at 37°, the enzyme suspension was centrifuged at 100,000 × *g* for 60 min. The pellet was resuspended in 10 ml of a cold (4°) wash solution containing 100 mM NaCl, 50 mM Tris-Cl (pH 7.4) and 5 mM $MgCl_2$ and again centrifuged. This pellet was resuspended in 2 ml of the wash solution described above. The Na^+,K^+-ATPase in this homogenate should be saturated with ouabain, i.e., 100% of the cardiac glycoside receptors should be occupied. This sample is labeled sample D. A procedure identical to the above, except that the 0.1 μM unlabeled ouabain was left out of the 37° incubation, was carried out to prepare a control sample (Sample A) in which none of the cardiac glycoside receptors were occupied. Sample B was a mixture of two parts of Sample A and one part of Sample B. Sample C was a mixture of two parts of Sample D and two parts of Sample A. The time course of [^{3}H]ouabain binding to these four samples was followed (Fig. 3). It is clear that occupation of receptors by cardiac glycosides has slowed the apparent rate of [^{3}H]ouabain binding and altered the shape of the binding curve.

Since the characteristics of [^{3}H]ouabain binding to the crude homogenates were qualitatively similar to [^{3}H]ouabain binding to more purified preparations of Na^+,K^+-ATPase (20) and since prior occupation of

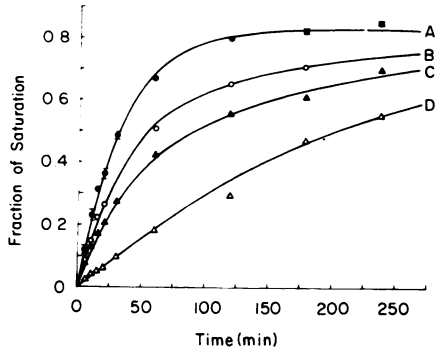

FIG. 3. *Time course of [^{3}H]ouabain binding to cat heart homogenates pretreated with unlabeled cardiac glycoside*

Sample D (△) was pretreated with 0.1 μM unlabeled ouabain in order to saturate the receptors. The drug-receptor complex was then washed free of unbound ouabain as described in the text. Sample A (●) was the control sample which had zero receptors occupied, B (○) was a mixture of 2 parts of A and one part of D; and C (▲) was a mixture of two parts A and two parts D. Enzyme concentration was 75 μg/ml. The lines shown are those predicted by Equation 11 for fraction of saturation ($[R\text{-}ouab^*]/[R_T]$) when $[RD]_o/[R_T]$ equals 0 (A); 0.286 (B); 0.475 (C); 0.871 (D).

cardiac glycoside receptor sites indeed altered the shape of the binding curve (Fig. 3), a kinetic model that would allow the calculation of the fraction of cardiac glycoside receptors occupied in a muscle strip was developed. Addition of a cardiac glycoside, D, to the bathing solution surrounding a papillary muscle produces an increase in the force of contraction of that muscle. If the muscle is then removed from the bath and frozen, interaction of D with its receptor, R, will cease and the muscle will contain a mixture of free receptors, R, and occupied receptors, R·D. Radiolabeled cardiac glycosides, such as [^{3}H]ouabain can then be used to measure the number of free receptors in a homogenate of the muscle. The kinetic description of [^{3}H]ouabain binding to such a mixture is shown below:

$$R \cdot D \xrightarrow{k'} R + D \tag{3}$$

$$R + ouab^* \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} R \cdot ouab^* \tag{4}$$

As soon as the [^{3}H]ouabain reaction is initiated, the free receptor, R, begins to bind [^{3}H]ouabain ($ouab^*$) according to Equation 4. Simultaneously, the occupied receptor, $R \cdot D$, begins to dissociate to yield free receptor which then binds [^{3}H]ouabain. The reverse of equation 3 does not occur to a significant extent since the concentration of unlabelled drug D is low compared to the concentration of [^{3}H]ouabain.

From Equation 4, the change in receptor occupied by radioactive ouabain, $R\text{-}ouab^*$, with respect to time is:

$$d[R \cdot ouab^*]/dt = k_1 [ouab^*][R] - k_{-1} [R \cdot ouab^*] \tag{5}$$

The change in receptors occupied by D, $R \cdot D$, with respect to time is:

$$d[R \cdot D]/dt = -k' [R \cdot D] \tag{6}$$

or

$$[R \cdot D] = [R \cdot D]_o e^{-k't} \tag{7}$$

where $[R \cdot D]_o$ is the concentration of sites occupied by D prior to the initiation of [^{3}H]ouabain binding. The total number of receptors available for binding is:

$$[R_T] = [R] + [R \cdot ouab^*] + [R \cdot D] \tag{8}$$

Substituting Equation 7 into Equation 8 and rearranging, yields:

$$[R] = [R_T] - [R \cdot ouab^*] - [R \cdot D]_o e^{-k't} \tag{9}$$

Substituting Equation 9 into Equation 5 and rearranging, yields:

$$d[R \cdot ouab^*]/dt + (k_1[ouab^*] + k_{-1})[R \cdot ouab^*] = k_1 [ouab^*]([R_T] - [R \cdot D]_o e^{-k't}) \tag{10}$$

Since the concentration of [^{3}H]ouabain is in great excess of the total number of receptors, R_T, the concentration of ouabain can be considered constant over the course of the reaction and Equation 10 is a linear differential equation, the solution of which, setting $[R \cdot ouab^*]$ equal to zero at time equal zero, yields:

$$[R \cdot ouab^*] = a[R_T] - b[R \cdot D]_o \tag{11}$$

where

$$a = (1 - e^{-k_e t})/(1 + K_i/[ouab^*])$$
$$b = (e^{-k't} - e^{-k_e t})/\{1 + (K_i/[ouab^*]) - k'/(k_1 [ouab^*])\}$$
$$k_e = k_1 [ouab^*] + k_{-1}$$
$$K_i = k_{-1}/k_1$$

Equation 11 shows that the concentration of receptors occupied by [^{3}H]ouabain, $[R \cdot ouab^*]$, is a function only of the time of binding, t; concentration of [^{3}H]ouabain, $[ouab^*]$; the rate constants k', k_1, and k_{-1} of equations 3 and 4; the total concentration of receptors, $[R_T]$; and the concentration of receptors, $[R \cdot D]_o$, occupied by the cardiac glycoside, D, prior to initiation of the radioactive binding assay. Since a and b vary only with time, t, Equation 11 is a linear equation dependent on two variables a and b and can be fitted by a bilinear regression to yield the regression coefficients $[R_T]$ and $-[RD]_o$. The relative standard deviation in the derived constants a and b varies from 6–11%. Application of this analysis to the data shown in Fig. 3 yielded for Sample D a value ± S.D. of 87.1 ± 5.6% occupation, for Sample C, 47.5 ± 11.1% occupation, and for Sample B, 28.6 ± 4.6% occupation. These values are in good agreement with the theoretical values of 100%, 50% and 33% respectively.

In vivo *studies: Application of the model.* The time course of [^{3}H]ouabain binding to homogenates of individual papillary mus-

cle, which had developed various degrees of positive inotropy, was measured. Examples of the experimental results are shown in Figure 4. The control muscle had no prior unlabeled ouabain added to the muscle bath; two muscles had force increases of 16 and 38%, respectively, and one muscle developed a contracture. As with the experiments involving ouabain-complexed homogenates, it is evident that when ouabain was added to the muscle bath, the time course of [^{3}H]ouabain binding to the homogenates prepared from these muscles was affected. Application of Equation 11 yielded values for total receptors available and the number of receptors occupied. The fraction ± S.D. of receptors occupied was 0.85 ± 0.11 in the muscle that developed contracture, 0.51 ± 0.16 in the muscle that developed a 38% increase in contractility and 0.26 ± 0.14 in the muscle that developed a 16% increase in contractility. If we assume that the total number of receptors in control papillary muscles are the same as the number of receptors in the cardiac glycoside treated muscles, Equation 11 can be rewritten in another useful form.

$$[RD]_o = (a[R_T] - [R \cdot ouab^*])/b \qquad (12)$$

Using control muscles to calculate $[R_T]$, application of Equation 10 yielded values ± S.D. of receptor occupations of 0.82 ± 0.09, 0.65 ± 0.08 and 0.26 ± 0.05 for the muscles that developed contracture, a 38% increase in force and a 16% increase in force respectively.

A series of muscles were exposed to ouabain or digoxin in order to produce an inotropic effect. When the desired increase in contractility was obtained, the muscles were rapidly removed, blotted and frozen as outlined in Methods. The time course of [^{3}H]ouabain binding to homogenates of these muscles and to control papillary muscles that had not been exposed to a cardiac glycoside was measured. The data from homogenates from the control muscles were used to estimate R_T, the total number of receptors. This value and the data from homogenates of the cardiac glycoside treated muscles allowed the calculation of the number of receptors occupied using equation 12. The results of the analysis are shown in Figure 5 for 9 ouabain-treated and 9 digoxin treated papillary muscles. The increase in contractile force increased as the fraction of receptors occupied increased. Since analysis of the ouabain treated muscles alone and digoxin treated muscles alone yielded regression lines with similar slopes (ouabain 33 and digoxin 34), the data were combined. Six additional muscles were deliberately allowed to de-

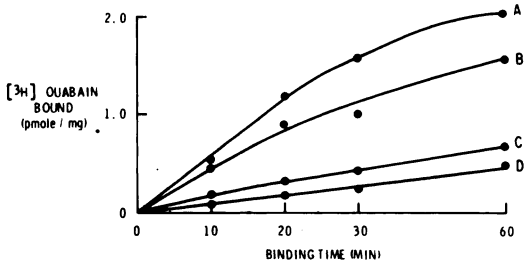

FIG. 4. *[^{3}H]ouabain binding to homogenates of cat right ventricular papillary muscles*

The vertical axis is the amount of [^{3}H]ouabain (pmole) bound to 1 mg of homogenate; horizontal axis is the time from start of [^{3}H]ouabain binding reaction. A) Muscle was not treated with a cardiac glycoside in the bath. B) Muscle was treated with 0.1 μM ouabain and developed a 16% increase in force. C) Muscle was treated with 1 μM ouabain and developed a 38% increase in force. D) Muscle was treated with 1 μM ouabain and developed contracture. Protein concentration was 0.075–0.125 mg/ml.

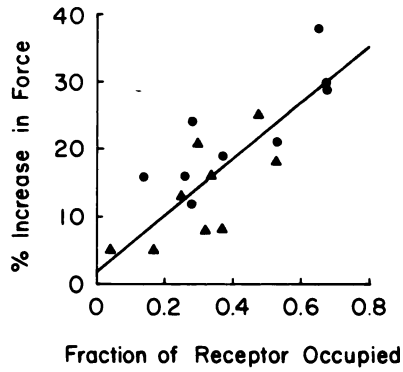

FIG. 5. *Correlation between the increase in contractile force and the fraction of receptors occupied by ouabain, ●; digoxin, ▲*

The least square regression line was $y = 3.70 + 38.64x$. The coefficient of determination was 0.625. See text for experimental details and for calculation of the fraction of receptors occupied.

velop contracture using high concentrations of ouabain (3 muscles) and digoxin (3 muscles). All of the muscles had a high fraction (0.70 ± .09) of their receptors occupied.

Six experiments were designed to determine if "wash out" of the inotropic effect caused a corresponding washout of bound cardiac glycoside and consequently an increase in the number of free receptors (Table 1). The results strongly suggest that occupation of receptors was decreased by the three changes of the Krebs' bicarbonate bath during the times noted in Table 2. For one muscle, which had been exposed to 2 μM ouabain, a 14% increase in force remained after 90 min of washing. This was reflected in the lowered [^{3}H]ouabain binding and thus indicated significant receptor occupation.

Binding of [^{3}H]ouabain was measured in three regions of the heart (Table 2). At each of the three binding times (10, 30, and 60 min), the amount of [^{3}H]ouabain bound was not statistically different in the left atrium compared to the left ventricle. However, at the 10 and 60 min binding times, the atria were different from the right ventricular samples.

DISCUSSION

The method used to assay the number of Na^+,K^+-ATPase sites in small amounts of heart muscle is an extension of the method used first by Ku *et al.* (18) for guinea pig hearts and later by Gelbart and Goldman (16) for dog hearts. The key to the method is the use of [^{3}H]ouabain of high specific radioactivity. Because the "nonspecific" binding is linearly related to the total amount of radioactivity in the reaction tube, it is necessary to use a low molar concentration of the [^{3}H]ouabain. This study and those mentioned above use [^{3}H]ouabain in a concentration of 10 nM with specific radioactivity of 12–14 Ci/

TABLE 1

Effect of drug washout on inotropy and [^{3}H]ouabain binding to homogenates of cat papillary muscles

Final concentration of unlabeled ouabain added to bath	Time from addition of drug to start of washout	Force at start of washout	Time of washout	Force at end of washout	Effect on [^{3}H]-ouabain binding
(M)	*(min)*	*(% change from control)*	*(min)*	*(% change from control)*	*(% change from control)*[b]
4×10^{-7}	90	+9	120	−1	+1
8×10^{-7}	90	+13	120	−2	−2
9×10^{-7}[a]	90	+17	90	+6	−3
2×10^{-6}[a]	90	+21	90	+14	−33
4×10^{-6}	10	+21	120	+3	+4
4×10^{-6}	10	+24	120	+3	−3

[a] Stepwise addition to this concentration.

[b] After washout homogenates were prepared for [^{3}H]ouabain binding. The amount of [^{3}H]ouabain bound was compared to an average of the amount of [^{3}H]ouabain bound to three control muscles from the same right ventricle.

TABLE 2

[^{3}H]Ouabain bound

A comparison of [^{3}H]ouabain binding to right ventricular, left ventricular and left atrial homogenates. The numbers are means ± standard errors and *n* is the number of samples.

Binding time	10 min	30 min	60 min
	(pmole/mg)	*(pmole/mg)*	*(pmole/mg)*
Right ventricle	0.80 ± 0.056 (n = 25)	1.41 ± 0.095 (n = 23)	1.97 ± 0.145 (n = 11)
Left ventricle	0.70 ± 0.063 (n = 9)	1.39 ± 0.179 (n = 9)	not done
Left atrium	0.64 ± 0.044 (n = 15)	1.29 ± 0.114 (n = 15)	1.71 ± 0.102 (n = 6)

mmole. The earlier workers carried out the homogenization of the tissue in sucrose (16, 18). Since the enzyme-cardiac complex formed *in vivo* may be of the unstable type (21), the homogenization of tissue in the present study was carried out in the presence of sodium ions which are known to stabilize this complex.

Both of the earlier studies cited above expressed [^{3}H]ouabain binding in terms of "initial velocity," i.e., the picomoles of [^{3}H]ouabain bound in 3 min (18) or picomoles [^{3}H]ouabain bound per minute, calculated from a rectilinear plot of the first 15 min of the [^{3}H]ouabain binding reaction (16). Analyses of this type are appropriate only if several conditions are met. First, one must assume that none of the drug, D, bound in the isolated muscle bath dissociates during the time course of the [^{3}H]-ouabain binding reaction. This is equivalent to setting $[RD]$ equal to $[RD]_o$ in Equation 8. Second, one must assume that the concentration of free receptor, $[R]$, does not change with time, and that the dissociation of bound [^{3}H]ouabain can be ignored, i.e., $k_{-1}[R \cdot ouab^*]$ is much smaller than $k_1[ouab^*][R]$. If these conditions are met, then Equation 5 reduces to

$$d[R \cdot ouab^*]/dt = k_1[ouab^*][R] \quad (13)$$

which, when integrated, yields

$$[R \cdot ouab^*] = k_1[ouab^*][R]_o \quad (14)$$

Equation (14) indicates that the amount of [^{3}H]ouabain bound per unit time is a function only of k_1, the forward rate constant, the concentration of [^{3}H]ouabain and the concentration of free receptors, $[R]_o$, present at the start of the [^{3}H]ouabain binding reaction. Because the assumptions necessary to reduce Equation 11 to Equation 14 will not in general be valid, especially for insensitive species, the data in this paper have been analyzed using the more general expressions, Equations 11 or 12.

Kuschinsky *et al.* (10) found that at equilibrium the uptake of ouabain by isolated guinea pig atria amounted to about 30 picomoles/g tissue. Under our experimental conditions homogenates from cat atria as well as right and left ventricles bound at equilibrium approximately 200 picomoles/g tissue. Based upon protein, homogenates from cats bound 2.0 picomoles/mg protein. Under conditions similar to ours, Gelbart and Goldman (16) found that homogenates from dog left ventricles bound 4 picomoles/mg protein. In contrast, Ku *et al.* (18) found, also under similar conditions, that homogenates of guinea pig left ventricles bound only 0.3 picomoles/mg protein. These large differences in equilibrium levels of [^{3}H]ouabain binding are probably not caused by differences in the number of ouabain receptors per g of tissue, but rather to differences in the affinity of the receptors for ouabain. Binding at equilibrium (i.e., long periods of time) represents "maximum binding" for the particular conditions employed. It does not necessarily represent saturation of receptors. For example, from our studies the Ki (k_{-1}/k_1) for binding of [^{3}H]ouabain to Na^+,K^+-ATPase from cat hearts at 37° in the presence of Mg^{2+} plus ATP plus Na^+ is about 1.75×10^{-9}. Thus in the presence of 10 μM [^{3}H]ouabain only 85% of the binding sites will be occupied at equilibrium. The half-time for dissociation of ouabain from guinea pig hearts is 2.0 min (22). At a concentration of 0.25 μM ouabain, the half-time for approach to equilibrium of [^{3}H]ouabain binding is approximately 25 sec (22) and at 10 nM is approximately 90 sec (18). Assuming first-order kinetics, ke, the effective rate constant for approach to equilibrium, and k_{-1} can be estimated. Calculation from this data indicates that the Ki for ouabain for Na^+,K^+-ATPase of guinea pig ventricles is in the range of 3.0–6.6×10^{-8}. A Scatchard analysis of [^{3}H]-ouabain binding to homogenates from guinea pig ventricles carried out in our laboratory yielded a Ki of 9×10^{-8} (data not shown). Thus, in the presence of 10 nM ouabain only 10–25% of the binding sites will be occupied at equilibrium. The number of ouabain receptor sites in guinea pig heart homogenates (18) could therefore be as high as 1.2–3.0 picomoles/mg protein, in good agreement with values from the cat (this work) and dog (16). This analysis indicates that care should be exercised in using [^{3}H]ouabain binding to estimate the number of Na^+,K^+-ATPase sites, since the amount bound at equilibrium does not nec-

essarily represent total capacity, particularly when the concentration of drug is low.

In the presence of 10^{-8} [^{3}H]ouabain, homogenates from cat ventricle bound at equilibrium 200 picomoles/g tissue, which we estimate to represent 85% of the receptors present. Phillips *et al.* (23) have suggested that an average left ventricular heart cell from ferrets has the shape of a flattened ribbon-like structure 26.8 μm wide by 8.3 μm high. Such a shape has a surface area per unit of cell volume of 0.32 μm^{-1}, which is in good agreement with the estimation by Page and McCallister that the external sarcolemma of rat left ventricles has 0.30 μm^2 membrane area per μm^3 of cell volume (24). If one assumes 35% extracellular space and a specific gravity of 1.065 for the muscle tissue, the capacity of cat ventricular muscle for ouabain is 7.6×10^{10} molecules per cm^2 of surface area, tenfold higher than other estimates of receptor capacity (25–28) including the 6.0×10^9 molecules/cm^2 estimated by Kuschinsky (10) for the ouabain receptor capacity of guinea pig atrial tissue (Table 3). As noted above, however, values of binding capacity for guinea pig atria are probably underestimated since Na^+,K^+-ATPase of cat heart is more sensitive to ouabain than Na^+,K^+-ATPase of guinea pig heart (see comparison of *Ki*'s above). If the length of a single ventricular cell is assumed to be 100 μm (29) the capacity of cat ventricular muscle is 5.2×10^6 sites per cell. Although our estimates for density of ouabain sites per cell or per square cm are higher than estimates of receptor capacity for other drugs (Table 3), ouabain would cover as a monolayer only 0.08% of the membrane surface, assuming that the biologically active part of ouabain is 10^{-4} cm^2 (10).

The estimate of the capacity/unit surface area of an excitable cardiac cell is 50–400 times larger than that for a red blood cell (Table 3). A similar ratio may be obtained in comparing the Na^+,K^+-ATPase density/membrane area. Assuming that there is one ouabain receptor site per sodium pump site, this suggests the density of pump sites in different cells is dependent upon the function of the cell. Excitable cells may require greater number of cation transport sites.

Regional differences in the ability of heart muscle to bind [^{3}H]ouabain have been reported recently (30) for homogenates of left and right Purkinje fibers and of left and right ventricles. Purkinje fibers bound less [^{3}H]ouabain, suggesting fewer ouabain binding sites and lower Na^+,K^+-ATPase activity. Kubler *et al.* (31) reported that total as well as the ouabain sensitive Na^+,K^+-ATPase activity was lower in conducting tissue than in left ventricle. Conducting tissue was apparently less sensitive to digitalis than left ventricular muscle. Experiments by Palfi *et al.* (32) however, did not reveal any differences in sensitivity. A study (33) in which [^{3}H]digoxin was infused into dogs indicated that the atria and right ventricles contained 44.9% and 78.2% of the amount contained in the left ventricle and septum, respectively. In the present experiments no significant differences were found in the binding of [^{3}H]ouabain to homogenates of atrial muscle when compared to samples of left or right ventricular muscle. It should be pointed out, however, that normalization of different types of muscle tissue by means of the amount of protein in the homogenates may lead to values that reflect only differences in cell protein, and not in number of ouabain sites. It has been suggested that atrial muscle fibers contain more connective tissue components and less contractile protein per cell volume. The actual amount and type of protein probably varies in different regions of the heart. This could contribute to the differences noted between Purkinje fibers and ventricular muscle (30), and the lack of differences between atrial and ventricular fibers found in the present work.

The increase in contractile force of cat papillary muscle caused by ouabain or digoxin shows a high correlation with the increase in occupation of receptors as assayed by [^{3}H]ouabain binding to homogenates from the muscles. These results, consistent with the results of other workers (4–7), suggest that receptor occupation by ouabain is the initial step in the chain of events that leads to the inotropic effect. Since the characteristics of [^{3}H]ouabain binding (e.g., the effects of ligands on the rates of association and dissociation) to these crude ho-

TABLE 3

Comparison of receptor density

Tissue	Drug	Receptor/cm^2	Receptors/cell	Ref
Guinea pig atria	propranolol	3.9×10^9	100,000	25
Guinea pig smooth muscle	atropine	2.0×10^9	16,000	26
Guinea pig atria	ouabain	6.0×10^9	—	10
Red blood cells	ouabain	$0.2–0.9 \times 10^{9a}$	270–1200	27, 28
Cat ventricle	ouabain	76×10^9	5,200,000	[b]
Cat atria	ouabain	46×10^9	1,230,000	[b]

[a] Assuming surface area of 140 μm^2/cell (36).

[b] This work. An atrial cell is assumed to be a cylinder 10 μm by 85 μm with 30% extracellular space (25). Specific gravity of muscle is assumed to be 1.065. Binding at equilibrium is assumed to represent 85% of capacity. Calculations for the ventricle are given in the text.

mogenates are similar in every way to the characteristics of [^{3}H]ouabain binding to purified and semipurified preparations of Na^+,K^+-ATPase, it strongly suggests that Na^+,K^+-ATPase is serving as the pharmacological receptor for the inotropic effect of digitalis. These data are not incontrovertible evidence that there is only one receptor for cardiac glycosides. It has been suggested recently that more than one receptor site may exist in heart (34, 35).

At concentrations of ouabain in excess of 1 μM, some of the cat papillary muscles became arrhythmic and eventually entered a state of contracture in which developed force was zero and resting tension steadily increased. Homogenates from muscles in this state of contracture bound less [^{3}H]-ouabain than muscles that had developed a positive inotropy indicating that more receptor sites were occupied in contracture than in normal or the positive inotropic state. It is clear that occupation of receptor, i.e., Na^+,K^+-ATPase, occurs during both the therapeutic and the toxic state of the muscle. Our studies, however, do not shed any light on whether the toxic effect is a result of increasing inhibition of Na^+,K^+-ATPase, the therapeutic receptor, or whether there are additional sites responsible for the toxic effect, which are occupied only at higher doses.

The hypothesis (12–15) that Na^+,K^+-ATPase is the "toxic receptor" for ouabain and that some other unknown receptor is responsible for the inotropic effect appears to be untenable based on the following argument. If the two effects were due to separate receptors and the toxic receptor were Na^+,K^+-ATPase, then the putative unknown therapeutic receptor would have to have an affinity higher than Na^+,K^+-ATPase since the therapeutic effect occurs *in vivo* at lower concentrations of ouabain. The dissociation constant of Na^+,K^+-ATPase for ouabain is in the range of 1 and 10 nM, depending upon ligand conditions employed and species from which the enzyme is isolated. The affinity of ouabain for the unknown putative therapeutic receptor would have to be higher than this. With an affinity this high, the therapeutic receptor should be easy to detect using [^{3}H]ouabain. In spite of numerous attempts, the only membrane or tissue fraction to which ouabain binds with such a high affinity is the Na^+,K^+-ATPase. It should be emphasized that we did not measure Na^+,K^+-ATPase activity directly via a standard isolation and purification scheme. The direct inhibition of the Na^+,K^+-ATPase enzyme system by ouabain has been well documented. We were interested in determining the rate and amount of ouabain that was bound to the homogenate receptors and, therefore, used a rather mild homogenization procedure and no solubilization by detergents. This was to prevent any greater dissociation of pre-bound unlabeled ouabain and to minimize the disruption of the membrane-bound enzyme system.

The washout experiments for homogenates of cat papillary muscle do not support the concept (12) that the positive inotropic action of cardiac glycosides can be dissociated from the binding of ouabain and subsequent inhibition of Na^+,K^+-ATPase. The cat heart homogenates, which bind cardiac

glycosides with a high affinity, demonstrate that the pre-bound ouabain can be washed from the muscle with a decrease in the observed positive inotropic state.

A high correlation between the positive inotropic effect and binding of ouabain to Na^+,K^+-ATPase has now been observed using guinea pig Langendorff preparations (17, 18), intact dogs (16), and now cat papillary muscles. In hearts from these species, it appears that the Na^+,K^+-ATPase membrane system is serving as the pharmacologic receptor for digitalis.

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