Two Receptor Forms for Ouabain in Sarcolemma-Enriched Preparations from Canine Ventricle

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SUMMARY Some evidence indicates that the inotropic effect of cardiac glycosides occurs at concentrations too low to affect Na⁺,K⁺-ATPase activity. This suggests that some receptor other than Na⁺,K⁺-ATPase mediates the inotropic effect. We studied ouabain binding to sarcolemma-enriched preparations from canine ventricle under conditions known to promote binding to Na⁺,K⁺-ATPase. Profiles for binding and dissociation were characterized by two kinetic components: (1) fast association and slow dissociation; (2) slow association and fast dissociation. Profiles in the absence of supporting ligands were consistent with a single species of receptors with slow association, fast dissociation and minimal effect on Na⁺,K⁺-ATPase activity. Binding supported by magnesium plus inorganic phosphate inhibited Na⁺,K⁺-ATPase activity by 86%. The two binding components were affected differentially by heating at 55°C. It was concluded that the preparation possesses two receptors for ouabain: the Na⁺,K⁺-ATPase and a "new" receptor. The latter may be different chemically from the Na⁺,K⁺-ATPase. The more likely possibility is that the "new" receptor is the Na⁺,K⁺-ATPase in a state characterized by low catalytic activity, low affinity for ouabain, and no requirement of specific ligands for ouabain binding. Further, the data suggest an interdependence between the two forms. This leads to a mechanism which allows an inotropic effect to precede loss of Na⁺,K⁺-ATPase activity even though both result from glycoside binding to Na⁺,K⁺-ATPase. The mechanism involves an equilibrium between inactive and active forms of the Na⁺,K⁺-ATPase such that the inactive form buffers loss of the active form upon exposure to a cardiac glycoside. Circ Res 47: 710-720, 1980

THE Na⁺,K⁺-ATPase, which is thought to catalyze the active transport of sodium and potassium across the plasma membrane of many cells, has a high affinity site for cardiac glycosides that faces the extracellular environment (Dahl and Hokin, 1974; Glynn and Karlish, 1975; Schwartz et al., 1975). Occupation of this site by cardioactive glycosides leads to inhibition of Na⁺,K⁺-ATPase activity, and it has been proposed that inhibition leads to an increase in intracellular sodium which enhances calcium influx or inhibits calcium efflux through sodium-calcium exchange across the cell membrane (Langer, 1972; Reuter, 1974). The ultimate consequence is that more calcium becomes available in the region of the sarcomere upon membrane depolarization such that myocardial contractility is enhanced. Considerable evidence favors this hypothesis (Schwartz et al., 1975; Aker and Brody, 1977), which has been further strengthened by recent studies that are consistent with the existence of a sodium-calcium exchange system in isolated myocardial membrane preparations (Reeves and Sutko, 1979; Pitts, 1979). Conversely, several types of evidence suggest that the inotropic effect of cardiac glycosides is not mediated through the Na⁺,K⁺-ATPase (e.g., see Okita, 1977). In our opinion, the least controvertible of these is that which suggests that increases in myocardial contractility can occur at concentrations of cardiac glycosides which do not affect Na⁺,K⁺-ATPase activity (Rhee et al., 1976; Cohen et al., 1976; Ellis, 1977; Blood and Noble, 1978; Huang et al., 1979). Seemingly, these studies require that the inotropic effect of cardiac glycosides be mediated through some receptor other than the Na⁺,K⁺-ATPase.

To date, there has been no clear demonstration that a receptor exists for cardiac glycosides other than that associated with the Na⁺,K⁺-ATPase. In fact, this is one of the more compelling reasons for believing that the inotropic effect results from glycoside binding to the Na⁺,K⁺-ATPase. There have, however, been reports suggesting the presence of more than one form of the glycoside receptor. For example, [³H]ouabain uptake into guinea pig atrial preparations was characterized by two saturable processes that were differentially affected by potassium (Godfraind and Lesne, 1972; Godfraind and Ghysel-Burton, 1977). Two components of glycoside interaction also have been reported for brain membrane preparations (Tanguchi and Iida, 1972; Erdmann and Schoner, 1973; Hansen, 1976) and for the erythrocyte (Heller and Beck, 1978). The purpose of this study was to address whether or not there is a receptor species for cardiac glycosides in

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myocardial sarcolemma besides the binding site associated with the Na\(^+\),K\(^+\)-ATPase. The recent derivation in this laboratory of an isolation scheme to obtain a sarcolemma-enriched preparation from canine ventricle (Van Alstyn et al., 1979), provided a biological system by which the question could be addressed. In an attempt to maximally retain the characteristics of the in situ sarcolemma, this isolation procedure employs no detergent treatment and the highest salt concentration used is 0.6 m KCl. The fact that glycoside binding to the Na\(^+\),K\(^+\)-ATPase is markedly affected by specific ligands (Schwartz et al., 1975), provided the basis for the probe. The experimental results are consistent with the existence of two receptor forms in the preparation and generated a mechanism to explain how the inotropic effect of cardiac glycosides could precede loss of Na\(^+\),K\(^+\)-ATPase activity even though both result from the binding of glycoside to the Na\(^+\),K\(^+\)-ATPase.

**Methods**

**Sarcolemma-Enriched Preparation**

The procedure employed was that recently described by Van Alstyn et al. (1979). Briefly, canine ventricular tissue was obtained from pentobarbital-anesthetized dogs. After removal of the epicardial and endocardial tissue and fat, the tissue was weighed, minced, and homogenized in a medium containing 10 mM NaHCO\(_3\) and 5 mM NaN\(_3\), pH 7.0, with an Ultra Turrax T-45 Tissumizer (Tekmar Instruments). The homogenate was centrifuged at 8700 \(g\) for 20 minutes, and the pellet was resuspended in the same medium, rehomogenized, and sedimented as described above. The supernatants from the first and second centrifugations were combined and centrifuged at 35,000 \(g\) for 20 minutes. The pellet was suspended in a medium containing 0.6 m KCl and 20 mM Tris-maleate, pH 6.8, and sedimented at 73,400 \(g\) for 20 minutes. The pellet was suspended in 10 mM Tris-Cl, pH 7.4, and layered over a 22% (wt/vol) sucrose solution containing 10 mM Tris-Cl, pH 7.4. Centrifugation at 73,400 \(g\) for 30 minutes yielded a layer on top of the sucrose and a pellet. The former was harvested, washed in 10 mM Tris-Cl, pH 7.4, and resuspended in a small volume of the same medium (protein concentration of about 1 mg protein/ml) and stored at either 2°C or -15°C until use. This fraction was judged to be membranous by electron microscopy.

**Ouabain Binding Assays**

Ouabain binding was carried out as described by Inagaki et al. (1974) at 30° or 37°C in the presence of 10 mM Tris-Cl, pH 7.4, 30-50 μg protein, and other ligands specified for each experiment. The reactions were started by the addition of ouabain containing [\(^3\)H]ouabain (specific radioactivity of 80-120 counts/min per pmol) to yield a final concentration of 1 µM drug in an assay volume of 1 ml. The reactions were terminated by the rapid addition of 1.0 ml of 1 mM unlabeled ouabain. Free drug was separated from bound drug by Millipore filtration (pore diameter of 0.45 µm) and four washes of the filter with 5 ml of ice cold, distilled water. In some cases, ouabain was allowed to react with the membrane preparation under one set of ligand conditions and was then allowed to react under a second set of ligand conditions prior to termination of the assay. Dissociation of bound drug was assessed by allowing binding to occur and subsequently following loss of bound drug in the presence of the initial binding condition plus 1 mM unlabeled ouabain (Lane et al., 1973). Non-specific binding, the amount bound in the presence of unlabeled ouabain, was subtracted from total binding to yield values for specific binding. All assays were carried out in duplicate.

**Assays for Na\(^+\),K\(^+\)-ATPase Activity**

Na\(^+\),K\(^+\)-ATPase activities were assessed at 37°C in the presence of 1 mM Tris-ethylenediaminetetraacetate, 25 mM imidazole, pH 7.4, 5 mM MgCl\(_2\), 100 mM NaCl, 10 mM KCl, and 20-40 μg protein. The reactions were initiated by the addition of Na\(_2\)ATP, pH 7.4, to yield a final concentration of 5 mM ATP in an assay volume of 1 ml and were terminated 6 minutes later by the addition of ice cold, 10% (wt/vol) trichloroacetic acid. Inorganic phosphate produced during the assay was determined by the method of Martin and Doty (1949). Ouabain-inhibitable activity in the presence of sodium and potassium equaled the increase in ATPase activity that was induced by the inclusion of sodium and potassium in the assay.

**Analysis of Data**

A nonlinear least-square, iterative computer program, Simplex (Nelder and Mead, 1965; Lam, 1970), was used in some cases to obtain binding and dissociation rate constants. This routine was stored and executed on a Hewlett-Packard HP2100A computer with 16K memory. Initial estimates for the parameters to be optimized were obtained by graphical techniques.

**Materials**

Disodium adenosine triphosphate and ouabain were obtained from Sigma Chemical Co. Sodium dodecyl sulfate was obtained from Accurate Chemical and Scientific Corp., and [\(^3\)H]ouabain was pur-
chased from New England Nuclear. All other chemicals were of reagent grade.

Results

When large amounts of ouabain are present relative to receptor concentration, ouabain binding to partially purified (Barnett, 1970; Lindenmayer and Schwartz, 1973) or to highly purified (Wallick et al., 1977) Na⁺,K⁺-ATPase preparations occurs via a pseudo-first order process:

\[ R_F = R_T e^{-kt} \]

or

\[ \ln \left( \frac{R_F}{R_T} \right) = -kt \quad (1) \]

where \( R_F \) = free receptor remaining at any time, \( t \), \( R_T \) = total receptor in the assay and \( k \) = the pseudo-first order rate constant. Such a process, by definition, is consistent with binding of the drug to a single species of receptors through one reaction pathway. The first experiment was designed to test the alternate hypotheses that only one receptor or that more than one receptor for ouabain exists in the sarcolemma-enriched preparation. If the first hypothesis were correct, plots of \( \log(R_F/R_T) \) vs. time of exposure to the drug should yield a linear relationship. Conversely, a nonlinear relationship would be consistent with the second hypothesis.

Three binding conditions (30°C) were employed: (1) buffer plus magnesium; (2) buffer, magnesium plus inorganic phosphate; and (3) buffer, magnesium, sodium plus ATP. These conditions promote binding of cardiac glycosides to Na⁺,K⁺-ATPase preparations (Schwartz et al., 1968, 1975). The total number of receptors in the assay, \( R_T \), was determined by allowing the preparation to react with ouabain for extended intervals. Maximal binding was achieved after 30 minutes of exposure at 30°C for each of the three binding conditions used when \( 1 \times 10^{-6} \) M ouabain was included in the assay. As reported previously for a partially purified Na⁺,K⁺-ATPase preparation from brain (Inagaki et al., 1974), assays carried out under these three ligand conditions yielded essentially the same level of maximal binding (see below). Plots of \( \log(R_F/R_T) \) vs. time of exposure of the sarcolemma-enriched preparation to ouabain were clearly nonlinear for each of the three conditions (Fig. 1). These results are consistent with the hypothesis that more than one form of the receptor for ouabain exists in the sarcolemma-enriched preparation.

The second experiment was designed to test further these hypotheses. Lane et al. (1973) showed that radiolabeled ouabain, bound to the Na⁺,K⁺-ATPase, dissociates via a first-order process when chased by an excess of unlabeled drug. Thus, if the drug binds to a single species of receptor in the membrane preparation, the amount of radiolabeled drug should decline in an exponential manner with time after addition of excess unlabeled ouabain (i.e., plots of the logarithm of bound counts/min vs. time should be linear). When ouabain binding to the sarcolemma-enriched preparation was promoted by the three conditions used in the first experiment, plots of the logarithm of bound counts/min vs. time after addition of excess unlabeled ouabain were nonlinear for each condition (Fig. 2). Such profiles,

![Figure 1](http://circres.ahajournals.org/)

**Figure 1** Time course of ouabain binding to the sarcolemma-enriched preparation. The binding reaction was carried out in the presence of 10 mM Tris-Cl, pH 7.4, 1 x 10^{-6} M ouabain with 3HJouabain and: (A) 5 mM MgCl₂; (B) 5 mM MgCl₂ and 5 mM Tris-PO₄, pH 7.4; and (C) 100 mM NaCl, 5 mM MgCl₂, and 5 mM Na₃ATP, pH 7.4. The reactions were started by the rapid addition of 25-50 µg protein (final volume = 1.0 ml). At the times indicated by the data points, 1.0 ml of 2 x 10^{-3} M unlabeled ouabain was added rapidly. Aliquots (1.0 ml) were then filtered through Millipore filters (diameter 0.45 µm). Nonspecific binding, the amount bound in the presence of excess unlabeled ouabain, was subtracted from values obtained to yield the indicated data points. The amount of 3HJouabain bound was used to obtain the percentage of the total receptor concentration that was free (\( R_F \)) at the end of the assay. Total receptor (\( R_T \)) was equated to the maximum binding observed at 30 minutes. Each point is the mean of 3 to 5 experiments. The lines drawn are from a computer fit of the data to the equation, \( R_F = R_T e^{-kt} \) + \( R_0 e^{-kt} \). Values for the best fitting constants are presented in Table 1.
OUABAIN BINDING TO CARDIAC SARCOLEMMA/Wellsmith and Lindenmayer

300
200 CP M
100 CP M
70 CP M
40 CP M

TIME (HOURS)

FIGURE 2 Dissociation of ouabain bound to the sarcolemma-enriched preparation. Ouabain binding reactions were carried out in an Erlenmeyer flask at 37°C in the presence of 10 mM Tris-Cl, pH 7.4, 1 × 10−6 M ouabain with [3H]ouabain, 400–500 μg sarcolemma and: (A) 5 mM MgCl2; (B) 5 mM MgCl2 and 5 mM Tris-PO4, pH 7.4; and (C) 100 mM NaCl, 5 mM MgCl2, and 5 mM Na2ATP, pH 7.4 (final volume = 5.0 ml). The reactions were allowed to proceed for 30 minutes, after which 5.0 ml of 2 × 10−3 M unlabeled ouabain were added (zero time on the abscissa). Aliquots (1.0 ml) were taken and filtered on 0.45-μm Millipore filters at the indicated times. The data shown represent specific binding. The lines represent computer fit of the data to the equation, cpm = R0De−k1t + RbDe−k2t. Values for the best-fitting constants are presented in Table 1.

therefore, are also consistent with the presence of more than one receptor form for ouabain in the sarcolemma-enriched preparation. The profiles for binding (Fig. 1) and for dissociation (Fig. 2) were both well fit by a model with two independent exponential processes (Table 1).

Significant binding to the sarcolemma-enriched preparation was observed in the presence of buffer after exposure to 1 × 10−6 M ouabain for 30 minutes (Fig. 3). Only slow binding to Na+,K+-ATPase preparations occurs under these conditions (e.g., Schwartz et al., 1968). Maximal binding achieved in the presence of buffer alone was approximately 50% of that obtained in the presence of magnesium, magnesium plus inorganic phosphate, or magnesium, sodium plus ATP (Fig. 3). Further, binding assays carried out after prior exposure to 1 or 10 mM Tris-ethylenediaminetetraacetate (EDTA), pH 7.4, yielded values similar to those obtained for control (Fig. 4). The latter results appear to eliminate or at least to minimize the possibility that endogenous magnesium (or calcium) was promoting ouabain interaction with the membrane (Schwartz et al., 1968).

The next two sets of experiments were designed to characteristic further the two binding components. First, ouabain binding to and dissociation from the sarcolemma-enriched preparation were characterized by single first-order processes (Fig. 5) when the reactions were studied in buffer plus drug. The time course for binding corresponded to the slower component in Figure 1, whereas the time course for dissociation was closer to that of the faster component in Figure 2 (Table 1). Further, the time course for binding in the presence of magnesium plus inorganic phosphate was characterized by a single first-order process when the reaction was studied after exposure of the preparation to ouabain in buffer (Fig. 6). Collectively, these results are inconsistent with the contention that a single species of receptors simultaneously reacts with the drug through several reaction pathways. Second, prior experiments showed that both binding components (i.e., that occurring in buffer and that promoted by magnesium plus inorganic phosphate) were eliminated by prolonged heating at 55°C. The time course for loss of the two components, however, differed to a considerable extent. Upon heating at 55°C, the loss of ouabain binding in buffer decreased at a faster rate than the loss of binding promoted by magnesium plus inorganic phosphate (Fig. 7). The difference in heat sensitivity of the two types of binding is consistent with ouabain binding to two membrane components which differ in their physiochemical properties.

It is well documented that potassium reduces the rate of ouabain binding to the Na+,K+-ATPase (Schwartz et al., 1975). Potassium (1 mM) was found to affect the rate constant for ouabain binding to both components in the sarcolemma-enriched preparation (Table 2). The constant for binding in buffer was reduced by 90.3%, whereas the constant for magnesium plus inorganic phosphate-supported binding, after prior exposure to ouabain in buffer (see Fig. 6), was reduced by 57.6%.

The next set of experiments was designed to test whether the binding that occurred in buffer represented binding to and caused inhibition of the Na+,K+-ATPase. Sarcolemma-enriched preparations were exposed to ouabain in the absence and presence of magnesium plus inorganic phosphate for 30 minutes at 37°C. The samples were then centrifuged at 2°C and resuspended in 10 mM Tris-Cl,
TABLE 1  Compartmental Analysis of the Binding Profiles (Fig. 1) and Dissociation Profiles (Fig. 2)

<table>
<thead>
<tr>
<th>Binding condition</th>
<th>Binding profiles (Fig. 1)*</th>
<th>Dissociation profiles (Fig. 2)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_1$</td>
<td>$R_0/R_1$</td>
</tr>
<tr>
<td>Buffer†</td>
<td></td>
<td>±0.034</td>
</tr>
<tr>
<td>Buffer plus magnesium</td>
<td>1.26</td>
<td>±0.21</td>
</tr>
<tr>
<td></td>
<td>±0.27</td>
<td>±0.06</td>
</tr>
<tr>
<td></td>
<td>0.17</td>
<td>±0.06</td>
</tr>
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| pH 7.4, to remove unbound drug, and were maintained on ice prior to assay for Na⁺,K⁺-ATPase activity to minimize loss of bound drug. Separate studies, using $[^{3}H]$ouabain, showed that ouabain remained bound throughout these steps (data not shown). Assays of the preparations, previously exposed to magnesium plus inorganic phosphate, yielded a Na⁺,K⁺-ATPase activity of 17 pmol phosphate released per mg protein per hour. Inclusion of ouabain in the pretreatment caused a reduction in activity of 86%. Conversely, pretreatment with ouabain in buffer reduced activity by only 13% (Fig. 8). If two forms of the Na⁺,K⁺-ATPase exist in the preparation (i.e., reflecting the two binding components), the form binding ouabain in buffer alone must have considerably less catalytic activity than the form to which binding is promoted by magnesium plus inorganic phosphate. It is also conceivable that the former has no catalytic activity or, in fact, is not a form of the Na⁺,K⁺-ATPase, since slow binding to partially or highly purified Na⁺,K⁺-ATPase preparations can occur in buffer alone (Wallick et al., 1977), and some carry-over of free ouabain into the assay for Na⁺,K⁺-ATPase activity was detected by use of $[^{3}H]$ouabain (i.e., an amount which equated to a final concentration of about 1 X $10^{-8}$ M in the assays). The latter, of course, could have reacted with the catalytically active form of the Na⁺,K⁺-ATPase during the assay for Na⁺,K⁺-ATPase activity.

**Figure 3** Maximal ouabain binding to the sarcolemma-enriched preparation in various media. Aliquots (45-50 µg) of the sarcolemma preparation were exposed to 1 X $10^{-6}$ M ouabain with $[^{3}H]$ouabain for 30 minutes at 30°C in the presence of 10 mM Tris-Cl, pH 7.4, and: (A) no other ligands; (B) 5 mM MgCl₂; (C) 5 mM MgCl₂ and 5 mM Tris-PO₄, pH 7.4; and (D) 5 mM MgCl₂, 100 mM NaCl, and 5 mM Na₂ATP, pH 7.4, in a final volume of 1.0 ml. Each value, corrected for nonspecific binding, represents the mean ± SEM (n = 8).

**Figure 4** Ouabain binding to the sarcolemma-enriched preparation following treatment with ethylenediaminetetraacetate (EDTA). Aliquots of the preparation were incubated for 15 minutes at 0°C in 10 mM Tris-Cl, pH 7.4, in either 0, 1, or 10 mM Tris-EDTA, pH 7.4. The suspensions were then centrifuged at 160,000 g for 20 minutes at 2°C, resuspended in 10 mM Tris-Cl, pH 7.4, and exposed for 30 minutes to 2 X $10^{-8}$ M ouabain with $[^{3}H]$ouabain and 10 mM Tris-Cl, pH 7.4, in the absence and presence of 0.5 mM MgCl₂ and 0.5 mM Tris-PO₄ at 37°C (final volume = 1 ml). Each value, corrected for nonspecific binding, represents the mean ± SEM (n = 4).
It is well known that the lipid environment around the Na\textsuperscript{+},K\textsuperscript{+}-ATPase affects the catalytic activity of this system (Dahl and Hokin, 1974; Schwartz et al., 1975). Thus, if two forms of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase were responsible for the binding components, it seemed reasonable that the two forms could reflect segregation of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase into different lipid domains in the membrane. The next experiment was designed to explore this and other possibilities by use of the detergent, sodium dodecyl sulfate. The rationale behind the experiment was that the detergent, by modifying protein-lipid interactions, might selectively solubilize or destroy one of the two binding components. Alternatively, the detergent might convert one of the two into the other if, in fact, both reflect binding to the Na\textsuperscript{+},K\textsuperscript{+}-ATPase. The results were consistent with the latter possibility in the sense that binding in buffer alone decreased by 32% and 54% after exposure to 0.2 and 0.3 mg detergent/ml, respectively, whereas binding dependent upon magnesium plus inorganic phosphate increased by 43% and 62% (Fig. 9). Pretreatment with 0.3 mg detergent/ml increased Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity by 3.1-fold (legend of Fig. 9). This is disproportionate to the increase in number of sites requiring magnesium plus inorganic phosphate for ouabain binding and may be due to one or more of the following: (1) conversion of inactive Na\textsuperscript{+},K\textsuperscript{+}-ATPase to an active form, (2) disruption of intact vesicles (Besch et al., 1976), and (3) modification of lipid or other types of constraints on Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity.

**Discussion**

The results of this study clearly support the existence of two receptor species for the cardiac glycoside, ouabain, in a sarcolemma-enriched preparation from canine ventricle. Unlike binding to partially or to highly purified Na\textsuperscript{+},K\textsuperscript{+}-ATPase preparations, substantial binding was observed in buffer alone. The association of ouabain with this receptor occurred through a single first-order process. If one assumes that this process was in fact, pseudo-first order (i.e., reflecting a second-order binding process with free drug in considerable excess of binding sites), the second-order rate constant for association was 570/M per sec (30°C). Dissociation of drug bound to the receptor was characterized by a first-order rate constant of 1.08 × 10\textsuperscript{-4}/sec (37°C). Conditions which allowed the interaction of ouabain with this receptor were associated with a relatively small decrease in Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity.
little effect on the above rate constants but did generate a second component of binding. The second-order rate constant for the additional component ranged from 21,000 to 22,800/M per sec (30°C) for the forward reaction. These values are similar to the value of 22,000/M per sec (30°C) reported for a partially purified Na+,K+-ATPase preparation from bovine brain (Lindenmayer and Schwartz, 1973). The rate constant for dissociation of drug bound to this receptor ranged from $1.83 \times 10^{-5}$ to $2.17 \times 10^{-5}$/sec (37°C), which is within an order of magnitude of that reported for a highly purified Na+,K+-ATPase preparation from the outer medulla of canine kidney (viz., $7.7 \times 10^{-5}$/sec at 37°C; Lane et al., 1973). Ouabain binding to this receptor caused a large decrease in Na+,K+-ATPase activity. We have concluded, therefore, that binding to the second component (i.e., the one that requires specific ligands and is characterized by fast association and slow dissociation) reflects the interaction of ouabain with the Na+,K+-ATPase. Binding to the other component (i.e., the one that does not require specific ligands and is characterized by slow association and fast dissociation) reflects ouabain binding to a "new" receptor.

If the two binding forms are independent, the characteristics of the "new" receptor are not those required to elicit an inotropic effect prior to inhibition of Na+,K+-ATPase activity (see above). For technical reasons, ouabain association and dissociation were studied at 30 and 37°C, respectively, but one can calculate a lower limit for the equilibrium constant, $K_{eq}$, by assuming that the rate constants for association at 30°C apply to 37°C. The equilibrium constant is defined as the second-order rate constant for association divided by the first-order rate constant for dissociation. For the case where binding was studied in the presence of magnesium, sodium plus ATP (Table 1), $K_{eq}$ equals $1.21 \times 10^9$/M for the Na+,K'-ATPase and $5.15 \times 10^6$/M for the

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### Table 2  Effect of Potassium on the Two Binding Components

<table>
<thead>
<tr>
<th>KCl (mM)</th>
<th>Buffer</th>
<th>Magnesium + phosphate dependent*</th>
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<tbody>
<tr>
<td>0</td>
<td>2470 ± 1050</td>
<td>3600 ± 1300</td>
</tr>
<tr>
<td>1</td>
<td>240 ± 80</td>
<td>1610 ± 320</td>
</tr>
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</table>

* After prior exposure to ouabain in buffer alone, as described in the legend of Figure 6.
Further, the inactive and active forms of the Na\(^+,K\(^+\)-ATPase may exist in equilibrium.

Experiments using 90° light scatter to measure vesicular response to osmotic interventions indicated that a fraction of the vesicles in the sarcolemma preparation are impermeable (D.W. Schuil and C. E. Lindenmayer, unpublished observations), which raises the possibility that accessibility of ouabain to a receptor species could be limited. However, the evidence reported in this study is not consistent with the hypothesis. First, binding occurs fairly rapidly and to an appreciable extent in the presence of buffer alone. Only slow binding occurs to partially or highly purified Na\(^+,K\(^+\)-ATPase preparations under this condition (e.g., Schwartz et al., 1968; Wallick et al., 1977). Further, if buffer alone does support binding to an access-limited but otherwise active form of the Na\(^+,K\(^+\)-ATPase, it should support binding to the access-unlimited, active form of the enzyme. Thus, binding in buffer alone should occur through a two-component process and reach the same level of maximal binding that is observed for the other ligand conditions employed (e.g., magnesium plus inorganic phosphate), and such binding should result in inhibition of Na\(^+,K\(^+\)-ATPase activity. None of these criteria was met by the experimental results (Fig. 3, 5 and 8). Second, if accessibility accounts for the difference between the two sites, the ratio of the sites, R\(_a\)/R\(_n\), should be identical regardless of the conditions used to promote binding, and the ratio determined from dissociation profiles, R\(_D\)/R\(_D\), should agree with those determined from the binding profiles, R\(_a\)/R\(_n\). Again, these conditions were not satisfied by the results (Table 1). Third, the binding rates to each component are differentially affected by pretreatment of the sarcolemma preparation with heat (Fig. 7). While possible, it seems unlikely that the susceptibility of the Na\(^+,K\(^+\)-ATPase to heat-denaturation would be significantly related to the permeability of the vesicles to ligands.

The studies reported herein do not eliminate the possibility that the "new" receptor is chemically different from the Na\(^+,K\(^+\)-ATPase. Overall, however, the evidence seems to be most consistent with the third hypothesis. First, pretreatment with sodium dodecyl sulfate may have converted an inactive form of the Na\(^+,K\(^+\)-ATPase into one with high catalytic activity and with a requirement of specific ligands for ouabain binding. The studies reported herein potentially could be explained by one or more of the following hypotheses: (1) The "new" receptor reflects binding to the active form of the Na\(^+,K\(^+\)-ATPase, but access to the receptor site is modified (e.g., presence of intact inside out vesicles in the preparation with receptor region located on the intravesicular surface). (2) The "new" receptor is chemically different from the Na\(^+,K\(^+\)-ATPase. (3) The "new" receptor is actually the Na\(^+,K\(^+\)-ATPase in a physiochemical state that has little if any catalytic activity and no requirement of specific ligands for ouabain binding.
ponents was carried out simultaneously (Table 1). On the other hand, the rate constant for binding in the presence of magnesium plus inorganic phosphate was only $3.200\,\text{M}^{-1}\,\text{sec}^{-1}$ when binding was carried out after exposure of the preparation to ouabain in buffer alone (Fig. 6). Thus, prior binding to one receptor form in the isolated membrane preparation was associated with substantial reduction in the rate of subsequent binding to the other form. This also suggests that ouabain binding to the two forms may be interdependent.

A number of studies have suggested that glycoside binding causes an increase in a pool of calcium associated with the myocardial cell surface (Nayler, 1973; Carrier et al., 1974; Lullman and Peters, 1974; Lullman et al., 1975; Gervais et al., 1975; Proppe, 1976; Lullman and Peters, 1976; Gervais et al., 1977; Bailey, 1977; Sheridan, 1978). This pool, presumably released into the cell upon membrane depolarization, does not require in a conceptual sense that internal sodium be increased through net loss of Na⁺,K⁺-ATPase activity. Accordingly, the inotropic response to a cardiac glycoside may be related to the number of Na⁺,K⁺-ATPase units complexed with drug but unrelated to the number of Na⁺,K⁺-ATPase units inhibited by the drug. This paradox can be resolved by an interdependence between catalytically inactive and active forms of the Na⁺,K⁺-ATPase where the inactive form buffers the loss of the active form upon exposure to the drug.

The following scheme shows how such an interdependence could lead to an inotropic effect without significant inhibition of sodium efflux:

\[ \text{Sodium Efflux} \]

- \( R_i \) and \( R_2 \) represent the active and inactive forms of the Na⁺,K⁺-ATPase, respectively, and \( R_iD \) and \( R_2D \) represent complexes of the two forms with a cardiac glycoside, \( D \). Sodium efflux would derive from \( R_i \), while the inotropic effect would derive from both \( R_iD \) and \( R_2D \). We have considered three cases for a hypothetical cell in terms of the number of units of \( R_i \) and \( R_2 \) present prior to addition of the drug.

### Table 3: Equilibrium Relationships between \( R_i \), Bound Drug, and Cardiac Glycoside Concentrations as defined by Equations 2 and 3

<table>
<thead>
<tr>
<th>Cardiac glycoside (nM)</th>
<th>( R_i )</th>
<th>( R_D )</th>
<th>( R_i )</th>
<th>( R_D + R_D )</th>
<th>( R_j )</th>
<th>( R_D + R_D )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.01</td>
<td>99.0</td>
<td>1.0</td>
<td>99.5</td>
<td>1.0</td>
<td>99.9</td>
<td>1.0</td>
</tr>
<tr>
<td>0.03</td>
<td>97.1</td>
<td>2.9</td>
<td>98.5</td>
<td>3.0</td>
<td>99.7</td>
<td>3.0</td>
</tr>
<tr>
<td>0.1</td>
<td>90.9</td>
<td>9.1</td>
<td>95.2</td>
<td>9.5</td>
<td>99.1</td>
<td>10.0</td>
</tr>
<tr>
<td>0.3</td>
<td>76.9</td>
<td>23.1</td>
<td>86.9</td>
<td>26.1</td>
<td>97.3</td>
<td>29.5</td>
</tr>
<tr>
<td>1.0</td>
<td>50.0</td>
<td>50.0</td>
<td>66.6</td>
<td>66.7</td>
<td>91.6</td>
<td>92.5</td>
</tr>
</tbody>
</table>

*Ratio in the absence of drug.
OUABAIN BINDING TO CARDIAC SARCOLEmma/Wellsmith and Lindenmayer

For \( R_2/R_1 = 10 \) in the absence of drug, \( 1 \times 10^{-7} \) m drug gives almost as many units of \( R_1D \) as \( R_2D \) as was originally present as \( R_1 \) (i.e., 100 units) with a decrease in \( R_2 \) to only 91.6 units. Thus, it is entirely feasible that an inotropic effect could be elicited by cardiac glycosides prior to any notable loss of Na\(^{+}\),K\(^{-}\)-ATPase activity when inactive and active forms of the Na\(^{+}\),K\(^{-}\)-ATPase exist in equilibrium.

In addition, the scheme also takes into account two other important aspects: (1) It allows the ratio of the two components for binding profiles to be ligand dependent and the ratio of the two components for dissociation profiles to be ligand independent (Table 1), and (2) the scheme accounts for the fact that loss of the inotropic effect in dogs upon washout of ouabain has a half-time that is much less than 9 to 10 hours (i.e., \( k_a \), in Table 1). Upon washout of free drug, the disappearance of bound drug (and the inotropic effect) would take place as follows:

\[
\begin{align*}
\text{(fast)} & \quad k_3 \quad k_{-3} \\
R_2D & \quad k_4 \quad k_{-4} \\
R_1D & \quad (slo) \\
\end{align*}
\]

Kinetically, 64% to 70% of drug bound to the isolated membrane preparation was observed to disappear through a process with half-time of 1.3 to 1.4 hours at \( 37^\circ \text{C} \) (dissociation profiles; Table 1). If equilibrium between \( R_1D \) and \( R_2D \) is reached prior to removal of free drug, the ratio of the fast and slow dissociation components could be independent of the relative contributions of the pathways through which binding occurred.

Several limitations must be placed on the use of the rate constants presented in this study. First, the data analysis summarized in Table 1 is based on an independent two-site model rather than the independent site model presented above. Therefore, instead of a direct correspondence, for example, of \( k_2 \) to \( k_a \) and of \( k_{-2} \) to \( k_{-a} \), the rate constants \( k_a \) and \( k_{-a} \) as well as \( k_b \) and \( k_{-b} \) are complex functions of the individual rate constants shown in the scheme (Weston and Schwarz, 1972). Second, caution must be exercised in extrapolating values for the rate constants determined in vitro to those for the positive inotropic response and inhibition of sodium efflux in intact myocardial preparations. The rate constants will be modified by the presence of potassium and factors unique to in situ conditions. The major point to be considered is that the proposed reaction scheme, generated by results from the in vitro study, may be applicable to the in situ case.

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