A Possible Molecular Mechanism of the Action of Digitalis

Ouabain Action on Calcium Binding to Sites Associated with a Purified Sodium-Potassium-Activated Adenosine Triphosphatase from Kidney

ANDRE GERVAIS, LOIS K. LANE, BEATRICE M. ANNER, GEORGE E. LINDENMAYER, AND ARNOLD SCHWARTZ

SUMMARY Calcium binding at 0°C to a purified sheep kidney Na⁺,K⁺-ATPase was described by linear Scatchard plots. Binding at saturating free calcium was 65–80 nmol/mg of protein, or 30–40 mol of calcium/mol of enzyme. Aqueous emulsions of lipids extracted from Na⁺,K⁺-ATPase yielded dissociation constants and maximum calcium-binding values that were similar to those for native Na⁺,K⁺-ATPase. Phospholipase A treatment markedly reduced calcium binding. Pretreatment of native Na⁺,K⁺-ATPase with ouabain increased the dissociation constant for calcium binding from 131 ± 7 to 192 ± 7 μM without altering maximum calcium binding. Ouabain pretreatment did not affect calcium binding to extracted phospholipids, ouabain-insensitive ATPases, or heat-denatured Na⁺,K⁺-ATPase. Na⁺ and K⁺ (5–20 mM) increased the dissociation constants for calcium, which suggests competition between the monovalent cations and calcium for the binding sites. At higher concentrations of monovalent cations, ouabain increased the apparent affinity of binding sites for calcium. Extrapolation to physiological cation concentrations revealed that the ouabain-induced increase in apparent affinity for calcium may be as much as 2- to 3-fold. These results suggest: (1) calcium binds to phospholipids associated with Na⁺,K⁺-ATPase; (2) ouabain interaction with Na⁺,K⁺-ATPase induces a perturbation that is transmitted to adjacent phospholipids, altering their affinity for calcium; and (3) at physiological concentrations of Na⁺ or K⁺, ouabain interaction with Na⁺,K⁺-ATPase may lead to an increased pool of membrane-bound calcium.

THE SODIUM-POTASSIUM pump and its in vitro manifestation, sodium-potassium-activated adenosine triphosphatase (Na⁺,K⁺-ATPase), react with and are inhibited by cardiac glycosides. It seems reasonable that such interaction is responsible for the positive inotropic effect of glycosides on the heart. Several hypotheses have been proposed to explain how binding of cardiac glycosides to the Na⁺,K⁺-ATPase causes intracellular responses. All are based on the assumption that cardiac glycosides induce a positive inotropic effect by raising intracellular calcium concentration secondary to an interaction at the cell membrane. One popular hypothesis suggests that a membrane interaction of the pump that favors the exchange of internal potassium for external calcium through the glycoside-pump complex. A third hypothesis is that glycosides affect a potassium-calcium exchange occurring during the cardiac action potential. The latter two hypotheses have been challenged by a recent report showing a dissociation of the positive inotropic response to cardiac glycosides from net potassium loss.

Calcium has direct effects on Na⁺,K⁺-ATPase. It inhibits activity through (1) formation of a complex with ATP, (2) reduction in the apparent affinity of a site on the enzyme for magnesium, and (3) reduction in the apparent affinity of sodium with respect to sodium activation. Calcium may reduce the amount of potassium required for half-maximal activation of Na⁺,K⁺-ATPase and, at very low concentrations, may also "substitute" for sodium in stimulating the binding of ouabain to Na⁺,K⁺-ATPase.
ase. These effects, while complex, do suggest that calcium can react with sites on the enzyme per se or with sites closely associated with Na⁺,K⁺-ATPase in its native lipoprotein (i.e., protein-membrane) configuration. On the basis of such considerations, Besch and Schwartz suggested that the inotropic action of digitalis glycosides may be due to a conformational change in Na⁺,K⁺-ATPase such that sites associated with the system would have increased affinity for calcium. In effect it was suggested that glycoside interaction with Na⁺,K⁺-ATPase induces an increase in a pool of calcium bound to the cell membrane. Also, inherent in their proposal was the concept that the number of calcium-binding sites could be greater than, for example, the number of sodium-activation sites (i.e., thought to be three per molecule of enzyme). This hypothesis could not be tested at the time of its proposal, because of the impurity of Na⁺,K⁺-ATPase preparations. With the availability of purified Na⁺,K⁺-ATPases, the concept can be examined, and the results presented herein confirm the suggestion that cardiac glycoside interaction with Na⁺,K⁺-ATPase modulates the affinity for calcium of sites on the purified enzyme preparation.

Methods

ENZYME ISOLATION

Highly active Na⁺,K⁺-ATPase preparations were isolated from the outer medulla of frozen lamb kidneys by the method of Lane et al. Na⁺,K⁺-ATPase activities were measured at 37°C by a spectrophotometric, coupled-enzyme assay in a medium containing histidine, 25 mM, pH 7.2; Na₂ATP, 2.5 mM; MgCl₂, 5 mM; NaCl, 100 mM; KCl, 10 mM; tris(hydroxymethyl)aminomethane-ethylene-diaminetetraacetic acid (Tris-EDTA), 1 mM; NADH, 0.40 mM; phosphoenolpyruvate, 2 mM; and pyruvate kinase/lactic acid dehydrogenase (Sigma). Protein concentrations of these preparations were determined by the method of Lowry et al. Using bovine serum albumin as the standard, specific activities ranged from 900 to 1,200 μmol of ATP hydrolyzed per mg of protein per hour. Maximal ouabain binding, using [3H]ouabain, was carried out in the presence of ATP, magnesium, sodium, or magnesium and inorganic phosphate, as previously described.

Ca²⁺ BINDING TO Na⁺,K⁺-ATPase PREPARATION

Assays were carried out at 0°C in the presence of imidazole, 25 mM, pH 7.3; CaCl₂, 10–1,000 μM, containing [⁴⁵Ca], 0.4 mCi/mmol was added, in a final volume of 2 ml. The radioactivity in each tube varied proportionally to the concentration of CaCl₂. The reaction was initiated by the addition of 200 μg (protein) of Na⁺,K⁺-ATPase, and the tubes were immediately centrifuged at 105,000 g for 15 minutes. The supernatant fraction was removed, the pellet was solubilized with NaOH, added to a scintillation medium, and assayed for radioactivity. This yielded total radioactivity in the pellet. Radioactivity in the water space of the pellets was determined by separate assays which included the amount of [⁴⁵Ca] normally present in assays with 10 μM CaCl₂ for total binding plus additional unlabeled CaCl₂ at 1 or 10 mM. The counts per minute (cpm) found in this pellet were equivalent to radioactivity not bound to the specific binding sites but trapped by the pellet or associated with the water space. The radioactivity contained in the water space for each concentration of CaCl₂ was calculated, assuming that the amount of radioactivity in the water space was proportional to the cpm added. The cpm calculated for each concentration was subtracted from the total amount to obtain the net radioactivity, which was taken to represent calcium bound to the membrane preparation. Equivalent values for the isotope content in the water space were obtained with 1 and 10 mM unlabeled calcium, and these values were similar to those obtained in independent experiments by the use of [⁴⁵Na]. We assumed that the amount of isotope contained in any fraction of solvent is independent of calcium concentration, whereas the amount of isotope bound to the enzyme is a function of the specific activity. Endogenous calcium in the enzyme preparation was determined by atomic absorption spectroscopy of HCI-LaCl₃ extracts of the enzyme preparation and buffer solutions. The amount was usually 1–2 μM per assay. The values for total and water space were unaffected by longer incubation or centrifugation times.

PREPARATION OF A OUABAIN-Na⁺,K⁺-ATPase COMPLEX

The Na⁺,K⁺-ATPase preparation (about 3 mg) was incubated with 1.8 mM ouabain in the presence of imidazole, 25 mM, pH 7.4, and MgCl₂, 1.1 mM, for 30 minutes at room temperature. This condition yields maximum ouabain binding to the Na⁺,K⁺-ATPase. Binding was promoted by magnesium alone in these experiments, because other binding conditions contained ligands that complex with calcium, thereby reducing free calcium concentrations in the assays for calcium binding. Controls were treated in an identical way except that ouabain was absent. Subsequently, samples were taken to measure calcium binding as described above. The final magnesium concentration in the calcium binding assays was 57 μM.

EXTRACTION OF LIPIDS FROM Na⁺,K⁺-ATPase PREPARATIONS

Lipids were extracted with chloroform-methanol (2:1). After removal of the solvent by rotary evaporation, the lipids were redispersed in Tris-EDTA, 1 mM, by homogenization, and total lipid phosphorus was determined by the method of Bartlett. Samples were taken to measure calcium binding as described above. PHOSPHOLIPASE A TREATMENT OF THE Na⁺,K⁺-ATPase

Phospholipase A was partially purified from Crotalus adamanteus venom and reacted with the Na⁺,K⁺-ATPase preparations. The reaction consisted of a 10-minute incubation at 37°C in the presence of Na⁺,K⁺-ATPase (25 mg protein), phospholipase A (5 mg), CaCl₂ (5 mM), imidazole (0.25 mM, pH 7.2), and 1% fatty acid-poor bovine serum albumin. The reaction was terminated by dilution and centrifugation followed by multiple washes. Controls were treated in a similar manner except for the absence of the phospholipase A.
FIGURE 1  Calcium binding to a purified Na⁺,K⁺-ATPase preparation from the outer medulla of sheep kidney. The assays were carried out at 0°C in the presence of imidazole, 25 mM, pH 7.3, CaCl₂, and [¹⁴C]Ca as shown. The reactions were started by addition of 200 μg (protein) of the membrane preparation (total volume = 2 ml). Subsequently, the membranes were collected in pellets and assayed for [¹⁴C]Ca content. “Water Space” and “Net” cpm were calculated as described under Methods. Results depicted in Figures 1-7 are individual experiments which are representative of four to eight experiments.

HEAT-DENATURED Na⁺,K⁺-ATPase PREPARATIONS AND ISOLATION OF GLYCOSIDE-INSENSITIVE ATPase PREPARATIONS

The Na⁺,K⁺-ATPase was denatured by placing it in a boiling water bath for 5 minutes; this resulted in a loss of catalytic activity without coagulation of the preparation. Subsequently, the preparation was resuspended by hand with a glass homogenizer fitted with a Teflon pestle. Glyc oside-insensitive ATPases employed were those present in isolated cardiac sarcoplasmic reticulum and in mitochondria.

Results

CALCIUM BINDING TO SHEEP KIDNEY Na⁺,K⁺-ATPase PREPARATIONS

Calcium, in a concentration range of 10-1,000 μM, adsorbed to the sheep kidney preparations in a manner suggesting saturation of the adsorption sites at higher concentrations of free calcium (Fig. 1). A Scatchard plot of the data revealed that adsorption occurs, at least predominantly, to a single species of noninteracting sites on the membrane preparation (Fig. 2), with a dissociation constant (Kd) for the adsorption process of approximately 100 μM (i.e., in the absence of magnesium; see below). Extrapolation to the abscissa suggested that the number of calcium-binding sites in the preparations was 50-70 nmol/mg of protein. In other experiments (not shown) which employed free calcium concentrations of less than 10 μM or greater than 1,000 μM, there was evidence of additional calcium-binding species in the preparation. The reliability of the binding technique employed in the present study, however, was significantly reduced at these concentration extremes, since in the lower range contaminating (endogenous) calcium became significant, while in the upper range, [¹⁴C]Ca in the water space comprised most of the [¹⁴C]Ca in the pellet (Fig. 1). The amount of binding was unaffected by longer incubation times or longer centrifugation times.

THE EFFECT OF OUABAIN ON CALCIUM BINDING TO THE ATPase PREPARATION

We took advantage of the fact that ouabain interacts with a site on the Na⁺,K⁺-ATPase in a pseudoirreversible manner to prepare a ouabain-membrane complex that subsequently could be employed in calcium-binding assays. Magnesium was used to stimulate ouabain binding in these experiments, and a high ouabain concentration (1.8 mM) was employed to ensure that the receptor sites on the Na⁺,K⁺-ATPase were saturated with the drug. The magnesium concentration in the assays for calcium binding was 57 μM, which increased the dissociation constant for calcium binding to the control preparation (i.e., not exposed to ouabain) by 1.3-fold. Ouabain pretreatment increased the dissociation constant for calcium binding from 131 μM to 192 μM without changing the number of calcium-binding sites (i.e., 68 and 71 nmol/mg of protein for control and drug-treated preparations, respectively) (Fig. 2 and Table 1 A). Experiments carried out at 22°C with sheep or dog kidney as sources for the ATPase yielded similar results (Tables 2 and 3). Prednisolone was used to test the possibility that the ouabain effect was due to the steroid nature of the compound rather than to a reaction with a specific cardiac glycoside receptor site on or in the

FIGURE 2  Scatchard plots of calcium binding to control (O), ouabain-pretreated (●) and prednisolone-pretreated (▲) Na⁺,K⁺-ATPase preparations. Pretreatment of approximately 3 mg protein of the Na⁺,K⁺-ATPase preparation was carried out at room temperature for 30 minutes in the presence of imidazole, 25 mM, pH 7.4, MgCl₂, 1.1 mM, and, where indicated, ouabain, 1.8 mM, or prednisolone sodium succinate, 1.8 mM. Subsequently, the control and drug-treated preparations were used for calcium-binding experiments as described in Figure 1 and Methods. Final magnesium and drug (where present) concentrations in the assays for calcium binding were 57 and 93 μM, respectively. Least squares analysis was used to determine the lines presented.
Effects of Ouabain Pretreatment on Calcium Binding to Purified Sheep Kidney Na⁺,K⁺-ATPase Preparations at 0°C

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>K_D (µM)</th>
<th>B_max (nmol/mg)</th>
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<tbody>
<tr>
<td>Control</td>
<td>Ouabain</td>
<td>Control</td>
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<tr>
<td>1</td>
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<td>192</td>
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Results from eight experiments carried out as in Figure 2. Least squares analysis of each data set was used to calculate K_D and B_max.

Effects of Ouabain Pretreatment on Calcium Binding to Purified Sheep or Dog Kidney Na⁺,K⁺-ATPase Preparations in the Absence of Sodium at 22°C

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>K_D (µM)</th>
<th>B_max (nmol/mg)</th>
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</thead>
<tbody>
<tr>
<td>Sheep Kidney</td>
<td>Na⁺,K⁺-ATPase</td>
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<td>Ouabain</td>
<td>Control</td>
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<td>137</td>
</tr>
<tr>
<td>SEM</td>
<td>7</td>
<td>19</td>
</tr>
</tbody>
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| Dog Kidney    | Na⁺,K⁺-ATPase |
| Control       | Ouabain  | Control         | Ouabain |
| 1             | 96       | 111             | 91      | 67    |
| 2             | 83       | 128             | 84      | 87    |
| 3             | 70       | 72              | 73      | 61    |
| Mean          | 83       | 104             | 83      | 72    |
| SEM           | 8        | 17              | 5       | 8     |

The enzyme was pretreated with 10⁻⁴ M ouabain and 1.5 mM Mg in 25 mM imidazole, centrifuged for 20 minutes at 100,000 g and, after removal of the supernatant fraction, the pellets were resuspended in 25 mM imidazole for calcium binding. The temperature was maintained at 22°C during the entire experiment.

The effect of monovalent, divalent, and trivalent cations on calcium binding to the Na⁺,K⁺-ATPase

Sodium (Fig. 5 and Table 3) and potassium (similar to sodium, data not shown) appeared to compete with calcium for the binding sites, at least in a concentration range of 5–20 mM. Pretreatment with ouabain markedly affected the ability of sodium to antagonize calcium binding. Ouabain increased the "inhibitory constant" for sodium from 9 to 41 mM and had a similar effect on potassium as an inhibitor of calcium binding. Thus, ouabain reduced the affinity for sodium by 78% (Fig. 5), whereas it reduced the affinity for calcium in the absence of sodium by only 32%. This difference led to the conclusion that ouabain increased the affinity of the sites for calcium when high sodium (or potassium) was present. At 20 mM sodium, a 1.45-fold increase in affinity for calcium was observed (Fig. 5). Extrapolation to a physiological concentration of sodium (e.g., 140 mM) suggested that ouabain may induce as much as a 2- to 3-fold increase in the affinity for calcium. Sodium and potassium also appeared to decrease, to some extent, the number of calcium-binding sites (inset of Fig. 5).

In other studies, 0.1–10 mM magnesium and 10 µM lanthanum were found to have a mixed effect on calcium binding by increasing the K_D and decreasing the number of binding sites; but 10 µM verapamil, a potent calcium antagonist with respect to myocardial contractility, had no effect (Fig. 6).
NATURE OF THE ADSORPTION SITES FOR CALCIUM

The number of calcium binding sites is 25-40 times greater than the number of Na⁺,K⁺-ATPase molecules in the preparation, as measured by ouabain-binding experiments. There are at least three possibilities as to the chemical nature of these sites. The sites could be (1) the negatively charged amino acid residues on the external surfaces of the polypeptides, (2) the glycoprotein which contains negatively charged sialic acid residues, or (3) membrane lipids. Recent experiments on the same Na⁺,K⁺-ATPase preparations that were employed for these studies showed that the preparations contained 133-266 mol of lipid phosphate per mol of ouabain-binding sites. Other studies revealed the presence of a significant amount of phosphatidylyserine, which carries a net negative charge and, as a consequence, could serve as a binding site for calcium. In an attempt to identify the adsorption site for calcium, interaction of calcium with extracted phospholipids was measured. Binding to these lipids was described by adsorption to a single species of binding sites characterized by a dissociation constant of 147 μM and maximal binding of 86 nmol (Fig. 4). This compares favor-
ably with the values obtained for the native preparation (Table 1). (Equivalent amounts of phospholipids were present in assays of the two preparations.) These similarities strongly suggested that calcium was binding to the membrane lipids in the Na+,K+-ATPase preparation.

The result of a second experiment also was consistent with the conclusion that calcium was adsorbing to the membrane phospholipids. Phospholipase A treatment of the Na+,K+-ATPase preparation was found to reduce the phospholipid content and Na+,K+-ATPase activity to 40% and 28%, respectively, of control values. The treated preparation, however, retained the ability to bind ouabain (maximal binding was 99% of control) and to be phosphorylated by ATP in the presence of magnesium and sodium (71% of control). The latter data are compatible with the suggestion that significant native configurations of the enzyme are retained after phospholipase A treatment. Calcium binding to the treated preparations, however, was markedly reduced compared to the native preparation (Fig. 7).

Discussion

It generally has been recognized that in order to catalyze the hydrolysis of ATP, the Na+,K+-ATPase must be associated with lipids or at least a lipid-like environment (e.g., some detergents). Arrhenius plots of Na+,K+-ATPase activity are characterized by an inflection point between 15°C and 20°C; the plots become linear on partial removal of the lipids and are reconverted to a nonlinear form when phospholipids are added back to the preparations. The discontinuity in the Arrhenius plot was found by calorimetric and electron spin resonance studies to be related to temperature-induced transitions in the physical state of the lipids. In molecular terms this means that the macromolecular structure of the Na+,K+-ATPase can be modified by the composition and physical state of the surrounding lipids.

The transfer of structural information from membrane lipids to the Na+,K+-ATPase may also occur in the opposite direction (i.e., from enzyme to lipids). Grisham and Barnett found that in a partially purified preparation the phospholipid bilayer adjacent to the enzyme was more ordered than that of the overall membrane but the interior of the bilayer next to the Na+,K+-ATPase was more fluid. Therefore, conformation changes in the Na+,K+-ATPase could perturb the structure of its adjacent lipids. The studies reported herein support these suggestions. Ouabain reacts with a specific site on the Na+,K+-ATPase, and Ruoho and Kyte have tentatively localized the receptor region on the higher molecular weight polypeptide in the purified Na+,K+-ATPase preparations. In the present study, the ouabain effect on calcium binding was shown to require the native integrity of the preparation. Ouabain did not affect calcium binding to extracted phospholipids, and the effect on calcium binding and inhibition of catalytic activity persisted after removal of unbound drug. These characteristics are consistent with the conclusion that ouabain affects calcium binding by reacting with a site on a protein moiety of the Na+,K+-ATPase. Furthermore, it had previously been concluded that glycoside interaction with this receptor stabilized the Na+,K+-ATPase in a particular conformation. It is reasonable, therefore, to suppose that such an action would result in an altered reactivity of sites on or associated with the Na+,K+-ATPase.

The conclusion that the ouabain effect is transmitted from protein to lipids, the latter serving as sites for calcium binding, is less secure. The possibility that calcium binds to negatively charged residues on certain surfaces (i.e., those exposed to the aqueous environment) of the protein is not eliminated by these studies. It is important to note that any procedure required to remove all of the lipids in Na+,K+-ATPase denatures the preparation.

Based on Kyte's analysis, it appears that the number of sialic acid residues on the smaller polypeptide in the preparation is too small to account for the number of calcium-binding sites. On the other hand, several experiments strongly suggest that calcium binding occurred to phospholipids in the preparation. First, there were more than enough phospholipids in the preparation to account for calcium binding and, at least in semiquantitative terms, about 17% of the phospholipid was found to consist of phosphatidyleserine. This species has net negative charges at pH 7.4 and, therefore, could serve as a site for calcium binding. Second, lipid extracts bound calcium in a manner similar to that of the native preparation. Third, phospholipase A treatment reduced the phospholipid content and calcium binding while leaving the Na+,K+-ATPase with considerable ability to react with ATP and ouabain.

Sodium and potassium inhibited calcium binding in a similar manner, with "inhibitory" dissociation constants of approximately 9 mM. Pretreatment with ouabain increased the constants to 41 mM for sodium and 27 mM for potassium. These constants were much greater than those of the sodium and potassium activation sites for sodium and potassium, respectively, but were in the range of those which characterize sodium and potassium interaction with phospholipids. Thus, the basis of the antago-
nism between monovalent cations and calcium may be a competition for negatively charged headpieces of phospholipids in the bilayer around the Na+ K+-ATPase. An alternate possibility is that the monovalent cations are modifying the surface charge of the bilayer without actually binding to the negative charges in the sense visualized for calcium adsorption.

Regardless of the nature of the calcium-binding site, the present studies may pertain to the question of how the digitalis glycosides produce an inotropic effect in the heart. Glycoside interaction with the sodium-potassium pump potentially could result in an increase in a membrane-bound pool of calcium. Attempts to extrapolate these considerations to the action of cardiac glycosides on the heart must be tempered by the fact that the studies employed a highly purified Na+, K+-ATPase preparation from kidney. Furthermore, if the results do apply to heart, some as yet unknown mechanism is still required to move the calcium from the membrane into the cell in a nonelectrogenic manner, since voltage clamp experiments have yielded data confirming the lack of direct effect of digitalis on a calcium current.

It is therefore possible that an electroneutral exchange diffusion system is operative in moving calcium into the cell.

Acknowledgments

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