EFFECTS OF POTASSIUM ON DIGITALIS-INDUCED INOTROPIC RESPONSE AND DIGITALIS-Na^+,K^+-ATPase INTERACTION

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ABSTRACT

EFFECTS OF POTASSIUM ON DIGITALIS-INDUCED INOTROPIC RESPONSE AND DIGITALIS-Na^+,K^+-ATPase INTERACTION

By

Sally Ann Wiest

There is considerable evidence which indicates that the positive inotropic action of digitalis is related to the interaction of drug with cardiac Na^+,K^+-ATPase. Theories currently proposed to explain the mechanism of digitalis action suggest that drug first binds to Na^+,K^+-ATPase which concomitantly causes enzyme inhibition. It is not known whether the binding of digitalis to enzyme is required to transport drug to an intracellular receptor site or whether digitalis interaction with Na^+,K^+-ATPase is responsible for initiation of the pharmacologic response. On the other hand, several investigators report that the inotropic responses of cardiac glycosides do not follow the time course of the inhibition of Na^+,K^+-ATPase during drug washout and, therefore, that Na^+,K^+-ATPase is not causally related to the pharmacologic response of digitalis.

The present studies were conducted to test the hypothesis that cardiac Na^+,K^+-ATPase is intimately involved in the inotropic response of digitalis. Two groups of experiments were used to investigate the effects of potassium on

the inotropic action of a cardiac glycoside and aglycone and to compare these with the effects of potassium on the interaction of glycosides and aglycones with Na^+,K^+-ATPase

It is suggested that potassium reduces binding and dissociation of cardiac glycosides with Na^+,K^+-ATPase by two separate mechanisms. The decreased rate of association of glycoside to enzyme is due to a decreased binding form of the enzyme, whereas the decreased dissociation of drug from enzyme results from a potassium-induced lipid barrier to the bound drug. If this postulate is correct, then potassium should affect the interaction of cardiac glycosides and aglycones with Na^+,K^+-ATPase in a different manner since lipid soluble aglycones would be highly permeable to lipid barriers. The first group of experiments was performed to determine the effect of potassium on the steady state level of digitoxin and digitoxigenin binding to purified Na^+,K^+-ATPase. Results indicated that potassium decreases the steady state level of the aglycone interaction with Na^+,K^+-ATPase but has only a slight effect on the equilibrium level of the cardiac glycoside bound to enzyme. Therefore, it appears that potassium reduces the forward and reverse reaction of cardiac glycoside binding to Na^+,K^+-ATPase to a similar extent but that potassium decreases the association rate to a greater extent than the dissociation rate for the aglycone-enzyme interaction.

If Na^+,K^+-ATPase is intimately related to the positive inotropic action of digitalis, then it is expected that potassium would also affect the glycoside- and

aglycone-induced inotropic response differentially. In the second group of experiments the effects of potassium on the onset, steady state and washout of the digoxin- and digoxigenin-induced inotropic response were studied using isolated heart preparations. Results from these experiments indicated that potassium delays the rate of onset and offset but has little effect on the steady state level of the cardiac glycoside-induced inotropic response. Also, potassium has little effect on the rapid onset and offset of the aglycone-induced inotropic response but markedly reduces the steady state level of the aglycone-induced inotropic response. These data demonstrate that the effects of potassium on the digitalis-induced inotropic response are closely related to the effects of potassium on the digitalis-Na^+,K^+-ATPase interaction.

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I would like to dedicate this thesis to my parents
in appreciation of their unfailing
confidence and encouragement.

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I would like to thank Dr. Akera for his guidance throughout this project and for his encouragement towards becoming a more independent scientist. Also, I would like to thank Drs. Brody, Hook and Suelter for their time and interest.

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INTRODUCTION

A. General Background

The digitalis compounds encompass the large group of cardiac glycosides and aglycones whose primary effect is to increase contractile force in both normal and diseased hearts. These drugs were first introduced in 1785 by William Withering as therapeutic agents in the treatment of certain forms of dropsy. It was not until after 1938, when Cattell and Gold demonstrated that digitalis increases contractile force in the isolated cat papillary muscle, that these compounds were considered primarily as cardiotonic drugs. At present, digitalis is unrivaled as the drug of choice in the treatment of congestive heart failure. The drug preparations commonly employed are obtained from digitalis leaf and strophanthus.

Although an important and widely used class of drugs, digitalis has an extremely narrow margin of safety. Thus, it is important to know the mechanisms which are responsible for therapeutic and toxic actions of these compounds. The sequence of events leading to myocardial contraction is generally referred to as the excitation-contraction coupled mechanism. Digitalis appears to increase cardiac contractile force by modifying this mechanism. Membrane excitation, which is characterized by increased membrane

permeability to Na^+ and Ca^{2+} followed by an increased permeability to K^+, results in an increased intracellular calcium concentration. When intracellular free calcium ion concentration increases to approximately 5 μM, a significant amount of Ca^{2+} binds to troponin and releases inhibition of the myofibril proteins which subsequently interact and cause muscle contraction. The exact manner by which intracellular Ca^{2+} concentrations are increased after membrane excitation is not known. It is recognized that extracellular calcium is involved, but in addition, excitation may labilize a larger internal store of calcium. After exposure to a cardiac glycoside, these levels of exchangeable calcium are increased even further (Langer and Serena, 1970; Lee and Klaus, 1971). Since digitalis fails to affect any of the known steps linking increases in intracellular calcium concentration with the contractile event, digitalis appears to affect the steps which link membrane excitation to the increased intracellular Ca^{2+} concentration.

Na^+,K^+-ATPase, the enzyme system which is responsible for the active reestablishment of the sodium and potassium concentration gradients disrupted during membrane excitation, is the only biochemical process in the contractile event shown to be specifically altered by therapeutic doses of digitalis (Lee and Klaus, 1971). When a cardiac glycoside is bound to Na^+,K^+-ATPase, the enzyme loses its ability to bind ATP resulting in loss of enzyme activity (Post et al., 1969; Hansen et al., 1971). Release of the

cardiac glycoside from the enzyme is accompanied by reactivation of enzyme activity (Akera and Brody, 1971). It follows, then, that delineation of the relationship between enzyme inhibition and inotropic response is considered a key factor in determining the mechanism of digitalis action as well as the missing links in the excitation-contraction coupling mechanism.

B. Relationship between Na^+,K^+-ATPase Interaction and Positive Inotropic Response of Cardiac Glycosides

There is considerable evidence which suggests that the interaction of digitalis with Na^+,K^+-ATPase (which results in enzyme inhibition) is intimately related to the inotropic response. The causality of these two events, however, has not been unequivocally established.

Both *in vivo* and isolated heart studies have been used to demonstrate that Na^+,K^+-ATPase is inhibited at the time of the inotropic response to cardiac glycosides. Inhibition of the cardiac enzyme after ouabain administration in the intact animal was first reported by Akera et al. (1969, 1970). Enzyme isolated from dog heart exposed to pharmacologic doses of the cardiac glycoside showed a mild to moderate (20 to 40%) decrease in Na^+,K^+-ATPase activity. Similar experiments by Goldman et al. (1975) demonstrated about a 40% inhibition of Na^+,K^+-ATPase activity in animals given digoxin at a dose which increased left ventricular contractile force by 50%. Besch et al. (1970) also reported a 59% decrease in Na^+,K^+-ATPase

activity in enzyme purified from ouabain-perfused isolated dog hearts exhibiting a 45% increase in contractile force. These studies indicate that the enzyme was inhibited at the time of the inotropic response.

Investigation into the basis for the difference in sensitivity to digitalis exhibited by various animal species resulted in additional evidence supporting an intimate relationship between digitalis binding to Na^+,K^+-ATPase and inotropic response. It was found that Na^+,K^+-ATPase obtained from digitalis-insensitive species such as the rat had a low affinity for digitalis, whereas that obtained from digitalis-sensitive species such as man, dog or cat had a high affinity for digitalis (Repke, 1965; Akera et al., 1969; Allen and Schwartz, 1969; Ku et al., 1976). The difference in the affinity has been shown to result from a difference in the dissociation rate constants of the digitalis-enzyme complexes (Tobin and Brody, 1972; Akera et al., 1973). The rate of release of the glycoside from enzyme prepared from hearts of a moderately digitalis-sensitive species, such as the guinea pig or rabbit, is much faster than that observed with enzyme prepared from a highly glycoside-sensitive species such as the cat or dog. In a similar fashion, the washout of the positive inotropic action of ouabain in isolated perfused heart preparations is faster in hearts obtained from guinea pigs and rabbits than in those from dogs or cats.

In subsequent studies, isolated perfused heart preparations were employed to determine if there was a temporal

relationship between enzyme inhibition and the inotropic response to digitalis. In studies using isolated guinea pig hearts, Ku et al. (1974) demonstrated that there was a reduction of enzyme activity during the inotropic response and a recovery of that enzyme activity following washout. In these experiments, inhibition of Na^+,K^+-ATPase by digitoxin was measured immediately after homogenization by estimating the initial velocity of specific (^3H)-ouabain binding. This technique, because it can be accomplished rapidly, minimizes the dissociation of drug from the enzyme and thus estimates, reasonably well, the number of sites not occupied by digitoxin. These studies also indicated that the onset and offset of the inotropic action of the glycoside was accompanied not only by inhibition and recovery, respectively, of cardiac Na^+,K^+-ATPase activity but also by inhibition and recovery of Na-pump activity (estimated from ^{86}Rb-uptake into ventricular slices). It was suggested that inhibition of sodium pump activity during the inotropic response to cardiac glycosides resulted in an alteration in transmembrane sodium movement leading to a greater calcium influx during the early phase of each cycle of myocardial function and thus causing a greater contraction.

Earlier experiments which attempted to establish a temporal relationship between enzyme inhibition and inotropic response found that enzyme inhibition and inotropic action of digitalis were unrelated (Okita et al., 1973; Ten Eick et al., 1973; Peters et al., 1974; Murthy et al.,

1974; Rhee et al., 1976). Results from Okita's studies indicated either that the enzyme was inhibited after wash-out of the inotropic response or that the enzyme was not inhibited at the time of the inotropic response. Rhee et al. (1976) reported that, in anesthetized dogs, ouabain infused over a 6-hour period caused a significant inhibition of myocardial Na^+,K^+-ATPase after the infusion of toxic doses but not following the infusion of non-toxic doses of the glycoside. They concluded that Na^+,K^+-ATPase inhibition could not be the cause of the inotropic action of digitalis since the lower dose exhibiting increased contractile force did not cause enzyme inhibition. However, it should be noted that with non-toxic doses of ouabain, there was a reduction in the cardiac Na^+,K^+-ATPase even though this reduction was not significantly different from control. Since the Na^+,K^+-ATPase activity was reduced only 29% in animals receiving the toxic doses of ouabain, it is reasonable to expect a smaller magnitude of drug effect when a lower concentration of drug is used. A demonstration of statistical significance at the low drug concentration depends on the variability of the data and the sample size. Thus, the failure to demonstrate a significant change when comparing variable data from groups containing a small number of animals may not necessarily justify the conclusion that there is no difference in the data.

It is obvious that additional research will aid in establishing the exact role of Na^+,K^+-ATPase in the genesis

of the digitalis-induced inotropic response. In several cases, factors affecting drug-enzyme interaction have also been recognized to affect digitalis action. The correlation of these effects is a useful model to determine the relationship between the two events. Therefore, the next sections will serve to introduce the characteristics of Na^+,K^+-ATPase and some of the factors which affect the digitalis-enzyme interaction.

C. Characteristics of the Na^+,K^+-ATPase System

The enzyme system, Na^+,K^+-ATPase, which was discovered by Skou in 1957, is responsible for the active transport of Na^+ and K^+ across the cell membrane. This system is found in all cells which exhibit a significant gradient between intracellular and extracellular concentrations of sodium and potassium ions (Bonting et al., 1961). Its ability to hydrolyze adenosinetriphosphate (ATP) to adenosinediphosphate (ADP) and orthophosphate (Pi) differs from other ATP-hydrolyzing enzymes by requiring both sodium and potassium in addition to magnesium for maximum activation (Skou, 1957). If magnesium is present in the absence of sodium and potassium, enzyme activity is low. On the basis of studies of the effects of magnesium ions and several inhibitors on Na^+,K^+-ATPase, a mechanism for enzyme activity has been incorporated into a model for coupled active Na^+ and K^+ transport (Albers et al., 1968). In this model, a phosphorylated enzyme mediates the vectorial work of transport through allosteric transitions.

The Na^+,K^+-ATPase system (MW 280,000) occupies the space of an 80 A unit-diameter sphere (if it is a globular protein). Since the thickness of the cell membrane is only 70 A units, the enzyme is probably facing both sides of the cell membrane simultaneously. Early kinetic analysis of the effect of sodium and potassium on enzyme activity suggested that the enzyme system had two sites for cation binding (one site with high affinity for sodium and a second site with high affinity for potassium). Subsequent experiments using intact cells showed that potassium was required on the outside and sodium on the inside of the membrane for enzyme activity (Baker, 1963; Glynn, 1962). A current hypothesis for sodium and potassium transport fitting these observations suggests that intracellular sodium binds to the enzyme (E_1 conformation) in the presence of magnesium and ATP (forming a sodium-bound phosphorylated enzyme, E_1P) and is transported to the outside of the cell (possibly via a magnesium-induced conformational change to the E_2P form). Extracellular potassium binds to the phosphorylated enzyme (causing a conformational change from E_2P to an E_xP form) resulting in hydrolytic dephosphorylation of the enzyme and subsequent release of potassium on the inside of the cell membrane. After this cycle of ion transport, the enzyme (E_x conformation) spontaneously relaxes to the original form (E_1 conformation). It is not known whether sodium and potassium are transported in a sequential manner or whether they are transported in a simultaneous manner. However, it seems clear from

extensive investigation that this enzymatic system is an allosteric type. Ligands, such as Mg^{2+}, ATP, Na^+, K^+, Ca^{2+} and digitalis, bind to various sites on the Na^+,K^+-ATPase producing specific conformational changes which lead to changes in activity of the pump. The locations of these sites are not entirely clear. However, it seems reasonable to postulate that potassium sites are located on the outside portion of the membrane and that the sodium, magnesium and ATP sites are located on the inside.

D. Factors Affecting the Digitalis-Na^+,K^+-ATPase Interaction

Early studies indicated that cardiac glycosides apparently inhibited the sodium and potassium transport in red blood cells only when the drug was applied externally but not when it was in contact only with the intracellular surface of the sarcolemma (Caldwell and Keynes, 1959). From this it was postulated that the receptor for digitalis was on the external surface of the membrane (Hoffman, 1966). It seems clear now that digitalis inhibits the Na^+,K^+-ATPase by first binding to a specific site (or region) which produces a conformational change that leads to an inhibition of enzyme activity (Schwartz et al., 1975).

Matsui and Schwartz (1968) quantitatively measured the interaction of cardiac glycosides with Na^+,K^+-ATPase of fragmented membrane preparations isolated from calf heart. They found that the optimal conditions for the binding of glycosides to Na^+,K^+-ATPase were the simultaneous presence of ATP, Na^+ and Mg^{2+} or the presence of inorganic phosphate

(P_i) and Mg^{2+}. In addition, only the cardioactive glycosides such as digoxin and ouabain would bind to the enzyme and these could not be displaced by non-active steroids.

Several factors influence the cardiac glycoside-Na^+,K^+-ATPase interaction. As mentioned before, the stability of a digitalis-enzyme complex is affected by the source of the enzyme. The marked species differences to digitalis can be explained by the differences in the dissociation rates of the drug from the enzyme. In addition, the stability of the cardiac glycoside-Na^+,K^+-ATPase interaction is dependent on the particular glycoside (Yoda, 1973; Akera et al., 1974b). It has been shown that when compared to the complex formed with ouabain, those formed with digoxin and digitoxin are significantly more stable. Aglycones, which lack the sugar moieties of cardiac glycosides, also bind to Na^+,K^+-ATPase in the presence of Mg^{2+} and P_i or Na^+, Mg^{2+} and ATP to form highly unstable complexes (Yoda, 1976). Both the association and dissociation rates of the aglycones are more rapid than those for cardiac glycosides binding to Na^+,K^+-ATPase (Yoda and Yoda, 1977).

The glycoside-Na^+,K^+-ATPase complexes formed *in vitro* seem to have different properties dependent upon the ligand conditions which prevail during the binding reaction (Akera and Brody, 1971). These differences were detected by monitoring the dissociation reaction of the drug-enzyme complex in a mixture of low ionic strength. It appears that there are at least three different forms of the ouabain-enzyme complex prepared *in vitro* with rat

brain enzyme (Akera et al., 1974a). Complexes formed in the presence of Mg^{2+} and P_i or Mg^{2+} and ATP are relatively stable and unaffected by the addition of potassium to the dissociation mixture, whereas the ouabain-enzyme complex formed in the presence of Na^+, Mg^{2+} and ATP has a fast dissociation which is slowed by potassium. When the ouabain-enzyme complex was formed in the beating heart (Langendorff preparation) and the dissociation was monitored *in vitro*, it also exhibited a fast dissociation rate which could be stabilized by potassium. Therefore, it was suggested that the binding of cardiac glycosides to enzyme in the presence of Na^+, Mg^+ and ATP is the best model for conditions of drug binding Na^+,K^+-ATPase in the beating heart (Akera et al., 1976b).

Since Na^+,K^+-ATPase has been shown to be an allosteric enzyme whose conformation is determined by monovalent cations and phosphate ligands, it is reasonable to assume that these ligands affect the cardiac glycoside-enzyme interaction. This was first demonstrated in experiments using (^3H)-digoxin and partially purified calf heart enzyme. It was shown that digitalis preferentially binds to a phosphorylated form of the Na^+,K^+-ATPase and that ATP, Mg^{2+} and Na^+ increase this form, thereby enhancing cardiac glycoside binding, whereas potassium decreases the phosphoenzyme, thereby inhibiting cardiac glycoside binding to the Na^+,K^+-ATPase. Evidence for the facilitation of digitalis inhibition of Na^+,K^+-ATPase by sodium led to experiments investigating the influence of sodium

on the digitalis-induced inotropic response in the beating heart. A sodium-dependence of the rate of development of the positive inotropic action of cardiac glycosides was demonstrated. Drugs which increased transmembrane Na^+ influx enhanced the onset of the inotropic response, whereas conditions which decreased transmembrane Na^+ influx delayed the development of the positive inotropic action of digitalis (Caprio and Farah, 1967; Wasserman and Holland, 1969; Akera et al., 1976a).

Likewise, the effects of potassium on the interaction of cardiac glycosides with Na^+,K^+-ATPase can be used as a basis to correlate effects of potassium on the inotropic action of digitalis. In the above discussion it has been indicated that potassium plays two distinct roles in the interaction of cardiac glycosides with Na^+,K^+-ATPase. The effect of potassium to reduce the rate of the drug binding reaction has been well documented (Matsui and Schwartz, 1968; Allen and Schwartz, 1970; Akera and Brody, 1971). This antagonism of the cardiac glycoside inhibition of enzyme by potassium does not fit kinetics of simple competitive inhibition but, instead, reflects a decrease in the binding (phosphorylated) form of the enzyme (Schwartz et al., 1968). Potassium binds to the E_2P form of the enzyme causing a conformational transition to an E_xP form which can be easily attacked by H_2O to release P_i. The conformational change results in an enzyme which is unable to effectively bind the glycoside. The effect of potassium to reduce the rate of glycoside release from Na^+,K^+-ATPase

has been postulated (Akera et al., 1976b) to result from a potassium-induced lipid barrier limiting access to and from the glycoside binding sites. If this hypothesis is true, then potassium should not decrease the rate of release of highly lipid soluble compounds, such as aglycones, to an extent comparable with the reduction in cardiac glycoside dissociation. However, the effect of potassium on the aglycone binding to Na^+,K^+-ATPase should be reduced to a similar degree. Since the equilibrium level of drug binding is determined by the ratio of the association and dissociation rates of glycoside with enzyme, it is possible to determine the relative effects of potassium on the two rates by measuring steady state levels of drug binding. Reports in the literature indicate both a decrease and no change in the steady state levels of ouabain binding to Na^+,K^+-ATPase in the presence of potassium. Reports on aglycone binding are unavailable. It would be of interest to determine if, indeed, potassium does decrease the equilibrium level of aglycone binding to Na^+,K^+-ATPase to a greater extent than the steady state level of cardiac glycoside binding to enzyme. These results could then be compared to the effects of potassium on the maximal level of the glycoside- and aglycone-induced inotropic response. Studies by Prindle et al. (1971) indicate that potassium delays the rate of onset of the digoxin-induced inotropic response in cat papillary muscle but has little effect on the maximal level of that response. Similar experiments with aglycones have not been reported.

E. Specific Objectives

The purpose of this study was to investigate the effects of potassium on the inotropic action of a cardiac glycoside and aglycone and to compare these with the effects of potassium on the interaction of glycosides and aglycones with Na^+,K^+-ATPase. Changing the ionic environment surrounding the drug-receptor and drug-enzyme interaction is a useful means to indirectly assess the causality of the two events. Similar actions of potassium on the digitalis-Na^+,K^+-ATPase interaction and on the digitalis-inotropic receptor interaction would strengthen the hypothesis that the enzyme is the inotropic receptor for cardiac glycosides while dissimilar actions of potassium on these two events would weaken the hypothesis.

The first group of experiments included determinations of the effects of potassium on the binding of digoxin, digitoxin and digitoxigenin to Na^+,K^+-ATPase. The second group of experiments was designed to investigate the influence of potassium on the digoxin- and digoxigenin-induced inotropic response in isolated tissue preparations. From these two studies it was anticipated that a correlation, or lack of correlation, between the actions of potassium on the drug-receptor interaction and the drug-enzyme interaction could be established.

MATERIALS AND METHODS

A. Materials

Ouabain (Strophanthin-G) (^{3}H-labelled with a specific radioactivity of 19 Ci/mmole) was purchased from Amersham/Searle, Arlington Heights, IL. Digoxin (^{3}H-labelled with a specific radioactivity of 4.85 Ci/mmole) and digitoxigenin (^{3}H-labelled with a specific radioactivity of 12 Ci/mmole) were obtained from New England Nuclear, Boston, MA. Ouabain octahydrate (Strophanthin-G), Tris ATP [Tris (hydroxymethyl)-aminomethane-ATP], digoxin, digitoxin and digitoxigenin were purchased from Sigma Chemical Company, St. Louis, MO. Digoxigenin was obtained from Aldrich Chemical Company, Milwaukee, WI. Other chemicals were of analytical reagent grade.

B. Na^+,K^+-ATPase Purification

Cardiac and brain Na^+,K^+-ATPase preparations were partially purified as previously described by Akera et al. (1969). Ventricular muscle of guinea pig heart (10 g) or rat brain (10 g) was homogenized with a Dounce ball-type homogenizer, in 4 volumes of a solution containing 0.25 M sucrose, 5 mM histidine, 5 mM disodium ethylene diamine tetraacetate (EDTA), 0.15% sodium deoxycholate and 0.01 mM dithiothreitol (pH adjusted to 6.8 with Tris base). The

homogenate was centrifuged at 10,000 x g for 30 minutes. The resulting supernatant was centrifuged for 60 minutes at 100,000 x g. The residue was suspended and recentrifuged at the same speed for 30 minutes and the final pellet suspended in 10 ml of a solution containing 0.25 M sucrose, 5 mM histidine and 1 mM Tris-EDTA. This mixture was then added to an equal volume of 2.0 M NaI solution, stirred for 1 hour and diluted with 2.5 volumes of 1 mM Na_2EDTA. The resulting solution was centrifuged twice for 30 minutes at 100,000 x g, resuspending each time with sucrose-histidine-EDTA suspending solution. All procedures were carried out at 2°C. The final resuspension was frozen until use.

The ATPase activity of the purified enzyme preparations was assayed by measuring the inorganic phosphate liberated from ATP (Akera et al., 1969). The incubation mixture contained enzyme (0.1 mg of protein) in a 1.0 ml solution of 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$ and 5 mM Tris-ATP in the presence and absence of NaCl and KCl. In addition, incubation mixtures without enzyme were used to determine background inorganic phosphate concentrations. Mg-ATPase activity assayed in the absence of NaCl and KCl was subtracted from total ATPase activity assayed in the presence of NaCl and KCl to calculate Na^+,K^+-ATPase activity. After a 5-minute incubation period, the ATPase reaction was started by the addition of ATP and terminated 10 minutes later by the addition of 1.0 ml of ice-cold 0.8 N perchloric acid. After

centrifugation at 800 x g for 15 minutes, an aliquot of the protein free supernatant was added to color reagent (1 g ammonium molybdate, 3.3 ml conc H_2SO_4, 7.3 g $FeSO_4 \cdot 7H_2O$ in a total volume of 100 ml water). The color change was quantified spectrophotometrically at a wavelength of 700 mμ. Activity was expressed as μmoles inorganic phosphate/mg protein/hour.

C. (^{3}H)-Digitalis Binding Studies

The effect of potassium on the binding of digoxin to purified guinea pig heart Na^+,K^+-ATPase was studied using ^{3}H-labelled drug. (^{3}H)-Digoxin (0.1 μM) was incubated with enzyme at 37°C in the presence of 50 mM Tris-HCl buffer, 30 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-ATP and either 0, 1, 3.5, 5.8 or 9.5 mM KCl. Aliquots of the binding mixture were taken at 1, 3, 5, 10 and 15 minutes. The binding reaction was started by adding enzyme (0.08 mg/ml) to the prewarmed incubation mixture and was terminated by adding a 0.4 ml aliquot to 4 ml of ice-cold "stopping solution" containing 15 mM KCl and 0.1 mM unlabelled ouabain. The mixture was immediately passed through a Millipore filter to separate bound and unbound (^{3}H)-digoxin. The filters were washed twice with 4 ml each of ice-cold stopping solution. Filters were then dissolved in ethylene glycol monomethyl ether and radioactivity assayed using liquid scintillation counting. The counting cocktail contained 16 g 2,5-diphenyloxazole (PPO), 668 mg 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene (POPOP),

1000 ml ethylene glycol monomethyl ether ("Piersolve", Pierce) and 3000 ml toluene. Counting efficiency, approximately 35%, was monitored by the external standard channel ratio method. It has been shown that in the presence of sodium and in a medium of high ionic strength, the binding of digitalis to steroid binding sites on Na^+,K^+-ATPase requires Mg^{2+} and ATP (Allen et al., 1971). Therefore, non-specific binding of (^{3}H)-digoxin was estimated in the absence of ATP. In addition, binding mixtures used for the assay of non-specific digoxin binding contained a large excess of non-radiolabelled ouabain. This value was subtracted from total binding values to calculate the specific digoxin binding.

In another series of experiments, the effects of potassium on the affinity of the *in vitro* interaction of a glycoside and aglycone with Na^+,K^+-ATPase were studied. Various concentrations of digitoxin and digitoxigenin, dissolved in 30% ethanol, were incubated with rat brain Na^+,K^+-ATPase (0.01 mg/ml) at 37°C in the presence of 50 mM Tris-HCl buffer, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-ATP and either 0, 0.1, 1.0 or 10 mM KCl. At each potassium concentration 3 tubes contained 3% ethanol (final concentration) in place of digitalis and were used as controls. Fractional occupancy of the cardiotonic steroid binding sites on the Na^+,K^+-ATPase by the test drugs was estimated after a 20-minute incubation period by measuring initial velocity of (^{3}H)-ouabain binding. (^{3}H)-Ouabain (0.1 µM) was added to the incubation mixture and after 1 1/2 minutes

the binding reaction was stopped by adding 5 ml of ice-cold stopping solution containing 15 mM KCl and 1 mM non-radiolabelled ouabain. The mixture was immediately passed through a Millipore filter to separate bound and unbound (^{3}H)-ouabain. The filters were washed twice with an additional 15 ml of ice-cold stopping solution. Radioactivity was assayed using liquid scintillation counting. Values of bound (^{3}H)-ouabain after a 1 1/2-minute incubation are representative of the initial velocity for (^{3}H)-ouabain binding since the ATP-dependent (^{3}H)-ouabain binding is essentially linear with time over the first 3-minute period. By use of a linear regression analysis of the plot of percent inhibition of (^{3}H)-ouabain binding versus log drug concentration, an accurate estimate of the drug concentration needed to occupy 50% of the binding sites on the enzyme (I50) was obtained at each potassium concentration. Initial (^{3}H)-ouabain binding velocity is proportional to the product of the (^{3}H)-ouabain concentration and the concentration of free steroid binding sites on the Na^+,K^+-ATPase. With (^{3}H)-ouabain concentration held constant, the initial binding velocity is proportional to free binding site concentration. Since ouabain and digitoxin bind to the same site on the Na^+,K^+-ATPase (Matsui and Schwartz, 1968), initial (^{3}H)-ouabain binding velocity is a good estimate of the fractional occupancy of the steroid binding sites on the enzyme.

Affinity can be expressed as the reciprocal of the dissociation constant for the drug-enzyme interaction;

i.e., affinity = 1/K(diss) = [DR]/[D][R] where [D] is the free drug concentration, [R] is the concentration of free binding sites and [DR] is the concentration of the bound drug or occupied enzyme. When 50% of the binding sites are occupied (i.e., [R] = [DR]), affinity is equal to the reciprocal of the drug concentration (I50). Therefore, determination of the I50 value for the digitoxin or digitoxigenin binding to Na^+,K^+-ATPase at each potassium concentration was used as a direct estimate of the affinity of the enzyme for the digitalis compound.

Similar binding studies have been attempted using digoxin and digoxigenin and guinea pig heart enzyme. In these experiments, various concentrations of the glycoside or aglycone dissolved in 5% ethanol (final concentration, 0.5%) were incubated with guinea pig heart homogenate (0.2 mg/ml) or purified cardiac enzyme (0.075 mg/ml) at 37°C in the presence of 50 mM Tris-HCl buffer, 30 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-ATP and either 0, 3.5, 5.8, or 9.5 mM KCl. Control tubes were added with 0.5% ethanol (final concentration) in place of digitalis. After a 15- or 10-minute incubation period for digoxin and digoxigenin, respectively, (^3H)-ouabain (1 μM) was added and allowed to bind for 1 1/2 minutes. The Millipore filter system was used to separate bound from unbound drug and radioactivity was assayed by liquid scintillation counting. Data were analyzed in the same manner as those obtained in the digitoxin and digitoxigenin binding studies.

D. Isolated Heart Preparations

Electrically stimulated isolated perfused heart (Langendorff) preparations and electrically driven left atrial preparations were used to study the effects of potassium on the digoxin- and digoxigenin-induced inotropic response. The guinea pig was chosen as the experimental animal because of its intermediate sensitivity to digitalis and its suitable time course for development and dissipation of the glycoside and aglycone-induced inotropic response. In addition, the guinea pig is practical in terms of cost, size and availability.

For the studies on the effects of potassium on the onset and maximal level of the digoxin- and digoxigenin-induced inotropic response, electrically stimulated left atrial preparations were used. Guinea pigs of either sex weighing between 300 and 500 g were killed by a blow to the head. Hearts were immediately excised and placed in a Krebs-Henseleit solution of 118 mM NaCl, 27.2 mM $NaHCO_3$, 1.0 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11.1 mM glucose and either 2.5, 4.8 or 8.5 mM KCl. Left atria were dissected free from the heart, mounted on a fixed electrode-clamp apparatus and placed in a 30°C bath of modified Krebs-Henseleit solution containing either 3.5, 5.8 or 9.5 mM potassium. The bicarbonate-buffered solution was aerated with a 95% O_2-5% CO_2 gas mixture before addition of atria and throughout the experiment to maintain pH at 7.4. The atria were electrically stimulated with 2 platinum field electrodes at 1 Hz with square-wave pulses

of 3 msec duration and voltage not exceeding 15% above threshold. Isometric contractile force was recorded with an FT-03C force-displacement transducer and Grass polygraph. Resting tension was adjusted to 1 gram. Either digoxin or digoxigenin was added after a 60-minute equilibration period in a volume of less than 3% of the total bath. The change in isometric contractile force monitored for 45 minutes after addition of drug was expressed as the percent change in contractile force setting pre-drug levels as zero percent. Drug concentrations (0.4 μM digoxin and 5.0 μM digoxigenin) were selected so that both drugs produced approximately the same maximal inotropic response (70% above control values) in atrial preparations equilibrated in baths containing 5.8 mM potassium.

The effects of potassium on the half-time of the washout of the digoxin- and digoxigenin-induced inotropic response were studied using electrically driven Langendorff preparations. Use of a constant flow Langendorff preparation permitted precise control of extracellular cations in contact with myocardial tissue, immediate monitoring of loss of contractile force after termination of drug perfusion and efficient removal of dissociated drug minimizing rebinding to myocardial tissue. Hearts excised from guinea pigs after a blow to the head were cannulated via the aortic root on a modified Langendorff apparatus (Akera et al., 1973) and perfused with aerated Krebs-Henseleit solution containing either 3.5, 5.8 or 9.5 mM potassium. Temperature was maintained at 30°C. When

hearts had a regular rhythm, both atria were removed and the ventricles were electrically driven by an electrode placed at or close to the A-V node with 4 msec pulses from a Grass S44 stimulator at 1.5 Hz and a voltage of 10-15% above threshold. Isometric contractile force was continuously monitored using a force-displacement transducer (Grass FT-03C) attached via a silk thread to the apex of the heart with a resting tension of 2 grams. After a 45-minute equilibration period, digoxin (0.6 μM) or digoxigenin (3 μM) was perfused until steady state inotropic response was obtained. The perfusion time was approximately 20 and 10 minutes for digoxin and digoxigenin, respectively. After termination of drug perfusion, drug-free solution was continued. Contractile force was monitored until at least 50% loss of the drug-induced inotropic response was observed. Drug response during washout was expressed as percent of maximal inotropic response. Since washout of drug response followed first order reaction kinetics, a plot of log percent contractile force versus time was used to compare data.

RESULTS

A. The Effects of Potassium on the *in vitro* Binding of Cardiac Glycosides and Aglycones to Na^+,K^+-ATPase

It is known that potassium reduces the binding of cardiac glycosides to Na^+,K^+-ATPase (Schwartz et al., 1968; Allen et al., 1970; Akera and Brody, 1971) and also the dissociation of bound glycoside from the enzyme (Akera and Brody, 1971; Schwartz et al., 1974; Allen et al., 1971). The relative association and dissociation rate constants of the glycosides in the presence of potassium determine the steady state level of the drug-enzyme interaction *in vitro*. The effects of potassium on the association and dissociation rates of an aglycone-enzyme interaction are less well known. Aglycones form highly unstable complexes with Na^+,K^+-ATPase whose formation and dissociation are difficult to assess (Yoda, 1977).

Digoxin and digoxigenin were the glycoside/aglycone pair used to study the effects of potassium on the inotropic response in isolated guinea pig heart preparations. Because of high non-specific binding (Schwartz et al., 1968), *in vitro* (^3H)-labelled drug binding studies with these two drugs required relatively purified Na^+,K^+-ATPase preparations.

1. (^{3}H)-Digitalis Binding. Studies on the effects of potassium on the (^{3}H)-digoxin binding reaction were performed using purified guinea pig heart Na^+,K^+-ATPase. The results of such studies are shown in Figure 1. Values presented are expressed as the percentage of specific (^{3}H)-digoxin binding observed after 15 minutes of incubation in the absence of potassium (maximal binding). Total binding was assayed in the presence of 50 mM Tris-HCl buffer, 30 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-ATP and either 0, 1, 3.5, 5.8 or 9.5 mM KCl. Non-specific binding assayed without ATP in the presence of a high concentration of non-radiolabelled ouabain was subtracted from total binding to calculate specific (^{3}H)-digoxin binding to Na^+,K^+-ATPase. The presence of potassium in the binding mixture markedly reduced (^{3}H)-digoxin binding to cardiac Na^+,K^+-ATPase. With the experimental time period used, it could not be determined if binding in the presence of potassium had reached its maximum steady state level. The binding of (^{3}H)-digoxin to Na^+,K^+-ATPase in the presence of 3.5, 5.8 and 9.5 mM potassium reached approximately the same level (20-30% maximal binding) after a 10-minute incubation period. However, the rate of binding in solutions containing 5.8 and 9.5 mM potassium was significantly delayed at 1, 3 and 5 minutes compared to values obtained from solutions containing 3.5 mM potassium.

Further attempts were made to determine the effects of potassium on the dissociation of (^{3}H)-digoxin and the binding and dissociation of (^{3}H)-digitoxigenin. Such

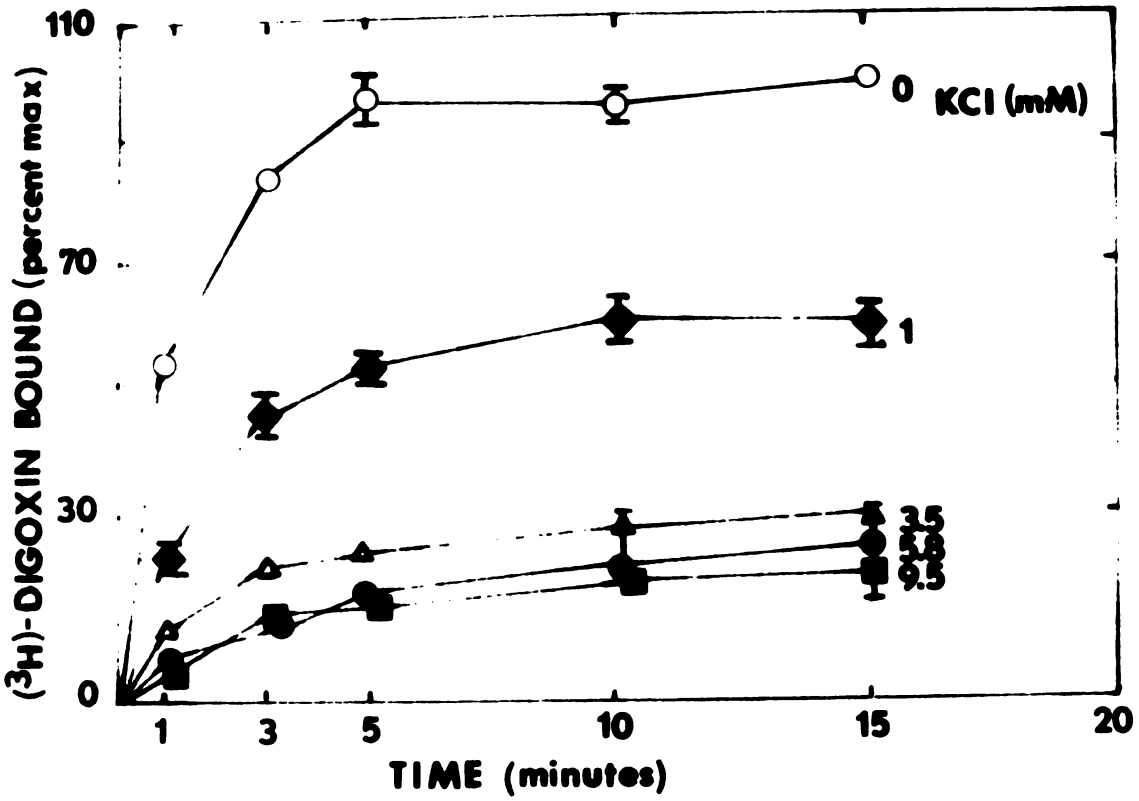

Figure 1. ATP-dependent (^{3}H)-digoxin binding to partially purified guinea pig heart Na^+,K^+-ATPase. Enzyme (0.08 mg/ml) was incubated with (^{3}H)-digoxin (0.1 μM) in the presence of Na^+, Mg^{2+}, ATP and various concentrations of K^+ to determine total binding. Non-specific binding without ATP in the presence of a high concentration of non-radiolabelled ouabain was subtracted from total binding to calculate specific (^{3}H)-digoxin binding. Data are expressed as the percent of maximal binding (pmol bound/mg protein after 15 minutes in mixtures containing no potassium). Values are means of 5 experiments with different enzyme preparations. Vertical lines represent standard error.

studies, however, were not completed due to experimental difficulties. There was great variability in both (^{3}H)-digoxin and (^{3}H)-ouabain binding to purified cardiac Na^+,K^+-ATPase from one preparation to another. This variability was not observed using purified rat brain Na^+,K^+-ATPase and therefore might be attributed to the cardiac Na^+,K^+ ATPase preparations. Enzyme activity (approximately 9.0 and 4.2 μmol P_i/mg protein/hr for Na^+,K^+-ATPase and Mg^{2+}-ATPase activity, respectively) was consistent in all cardiac preparations. Differences in digitalis binding with enzyme preparations possessing similar specific enzyme activities would occur if the lipid moieties associated with the enzyme were disrupted to various extents. Overtreatment with deoxycholate or dithiothreitol when isolating the enzyme from the cell membrane would result in such a destruction of the lipid moieties. Since it is postulated that potassium decreases the dissociation of cardiac glycosides from Na^+,K^+-ATPase by inducing a lipid barrier to the bound drug, these purified cardiac enzyme preparations with altered lipid moieties could not be used.

2. Equilibrium of Digitalis-Enzyme Interaction. Experiments to determine the effects of potassium on the steady state level of digoxin and digoxigenin binding to Na^+,K^+-ATPase were initially attempted using purified guinea pig heart enzyme. Various concentrations of digoxin were incubated with enzyme (0.075 mg/ml) in

solutions containing 50 mM Tris-HCl buffer, 30 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-ATP and either 0, 3.5, 5.8 or 9.5 mM KCl. Fractional occupancy of the cardiotonic steroid binding site on Na^+,K^+-ATPase by digoxin was estimated after a 15-minute incubation period by measuring initial velocity of (^3H)-ouabain binding. After the 15-minute incubation period, (^3H)-ouabain (final concentration, 0.07 μM) was added to the mixture and allowed to bind for 1 1/2 minutes. Specific (^3H)-ouabain binding was calculated by subtracting non-specific binding (assayed without ATP in the presence of excess non-radiolabelled ouabain) from total binding. A plot of the percent inhibition of (^3H)-ouabain binding to enzyme versus log of digoxin concentration at various potassium concentrations is shown in Figure 2. The drug concentration needed to occupy 50% of the binding sites on the enzyme (I50) was increased as the potassium concentration increased. The slopes of the regression lines at 0, 3.5, 5.8 and 9.5 mM potassium are -47, -41, -62 and -40, respectively. Any slope between -28 and -115 is compatible with mass law theory which assumes that the response is proportional to receptor occupancy, that the drug/receptor combining ratio may be 1/2, 1/1 or 2/1 and that a negligible fraction of total drug is combined with the binding sites (Goldstein, 1974). Therefore, the values of these slopes indicate that the decreased (^3H)-ouabain binding is due to the occupancy of enzyme binding sites by non-labelled drug. Comparable studies using the aglycone, digoxigenin, have not been

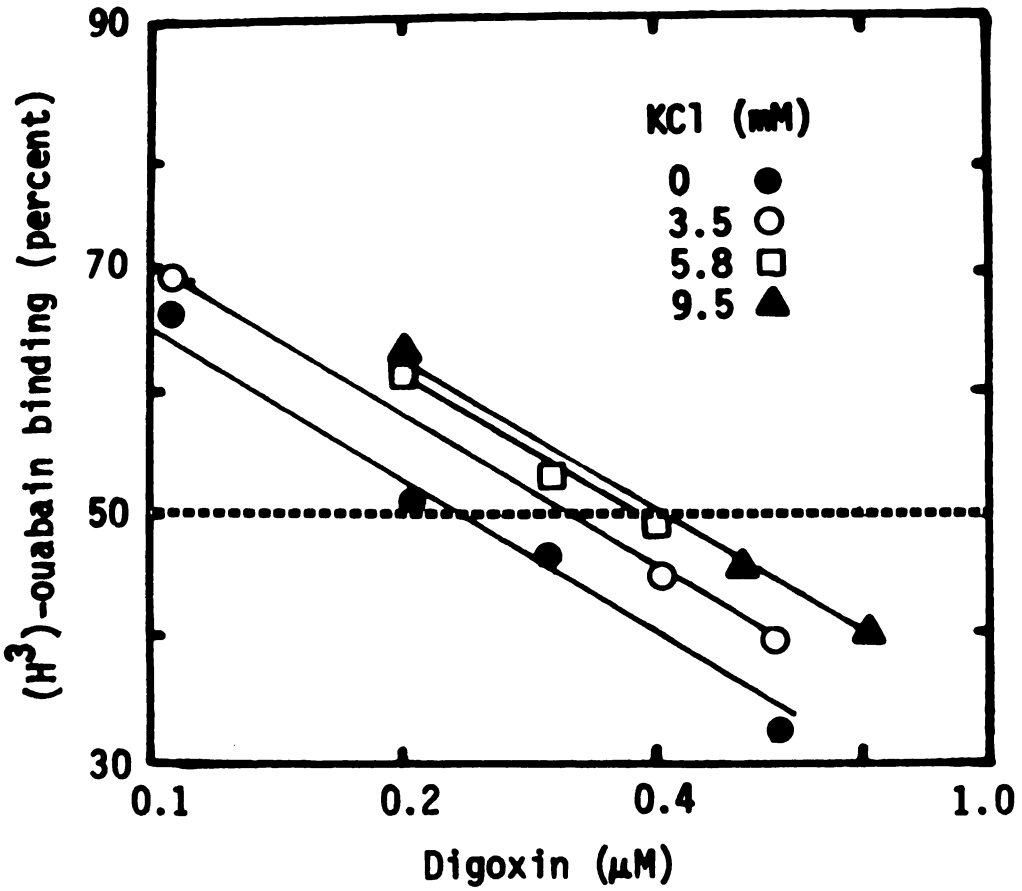

Figure 2. Specific (^{3}H)-ouabain binding to purified guinea pig heart Na^+,K^+-ATPase after preincubation with digoxin. Enzyme (0.075 mg/ml) was incubated with digoxin (concentrations indicated on abscissa) in the presence of Na^+, Mg^{2+}, ATP and K^+ (0, 3.5, 5.8, 9.5 mM). After addition of (^{3}H)-ouabain for 1 1/2 minutes, the binding reaction was stopped by addition of excess non-radiolabelled ouabain and bound drug separated from unbound using the Millipore filter system. Non-specific (^{3}H)-ouabain binding measured without ATP in the presence of a high concentration of non-radiolabelled ouabain was subtracted from total binding to calculate specific (^{3}H)-ouabain binding. Fractional occupancy of the Na^+,K^+-ATPase digitalis binding sites by digoxin, measured by (^{3}H)-ouabain binding, is expressed as percent (^{3}H)-ouabain bound. Values are the means of 4 experiments with different enzyme preparations. Regression lines connect data points at each potassium concentration. Concentration of digoxin needed to reduce (^{3}H)-ouabain binding to Na^+,K^+-ATPase by 50% (I50) can be read on the abscissa.

completed due to problems with the enzyme preparation. Therefore, experiments to determine the relative effects of potassium on the steady state level of digoxin and digoxigenin binding to Na^+,K^+-ATPase were initiated using guinea pig heart homogenates instead of partially purified cardiac enzyme. The specific fraction of (^{3}H)-ouabain binding to homogenate preparations in incubation mixtures containing no potassium could be studied. However, in the presence of potassium, the specific binding of (^{3}H)-ouabain was too low to determine with reasonable accuracy.

Since experiments could not be performed with cardiac Na^+,K^+- ATPase, the effects of potassium on the equilibrium level of Na^+,K^+-ATPase interaction with the glycoside, digitoxin, and the aglycone, digitoxigenin, were studied using purified rat brain enzyme preparations. Experimental protocol was similar to that described previously with digoxin. Rat brain enzyme (0.01 mg/ml) was incubated with digitoxin or digitoxigenin in the presence of 50 mM Tris-HCl buffer, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-ATP and either 0, 0.3, 1 or 10 mM KCl. After a 20-minute incubation period, (^{3}H)-ouabain (final concentration, 0.1 μM) was added to the mixture and allowed to bind for 1 1/2 minutes. Figures 3 and 4 show the percent inhibition of (^{3}H)-ouabain binding to rat brain enzyme after preincubation with digitoxin and digitoxigenin, respectively. Slopes of the regression lines in Figure 3 for 0, 0.3, 1.0 and 10 mM potassium are -41, -47, -46 and -54,

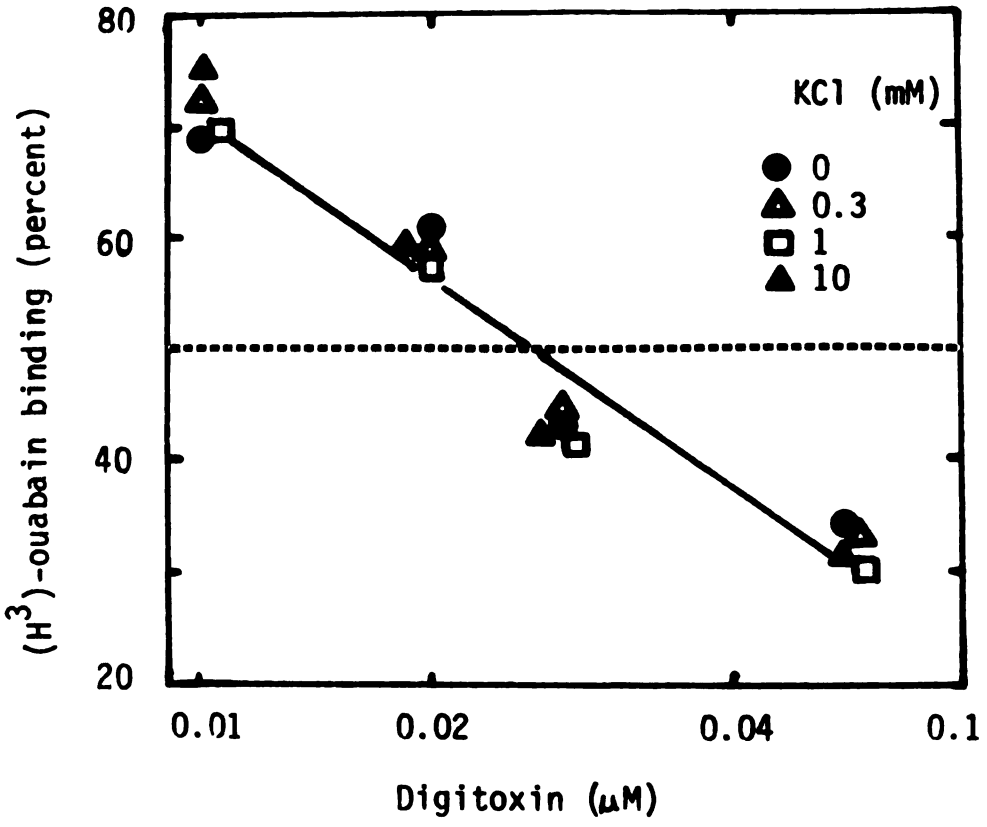

Figure 3. Specific (^{3}H)-ouabain binding to purified rat brain Na^+,K^+-ATPase after preincubation with digitoxin. Enzyme (0.01 mg/ml) was incubated with digitoxin (concentrations indicated on abscissa) in the presence of Na^+, Mg^{2+}, ATP and K^+ (0, 0.1, 1.0, 10.0 mM). After a 20-minute incubation period, (^{3}H)-ouabain (0.05 μM, final concentration) was added to the mixture and allowed to bind for 1 1/2 minutes. See legend for Figure 2. Values are the means of 4 experiments with different enzyme preparations.

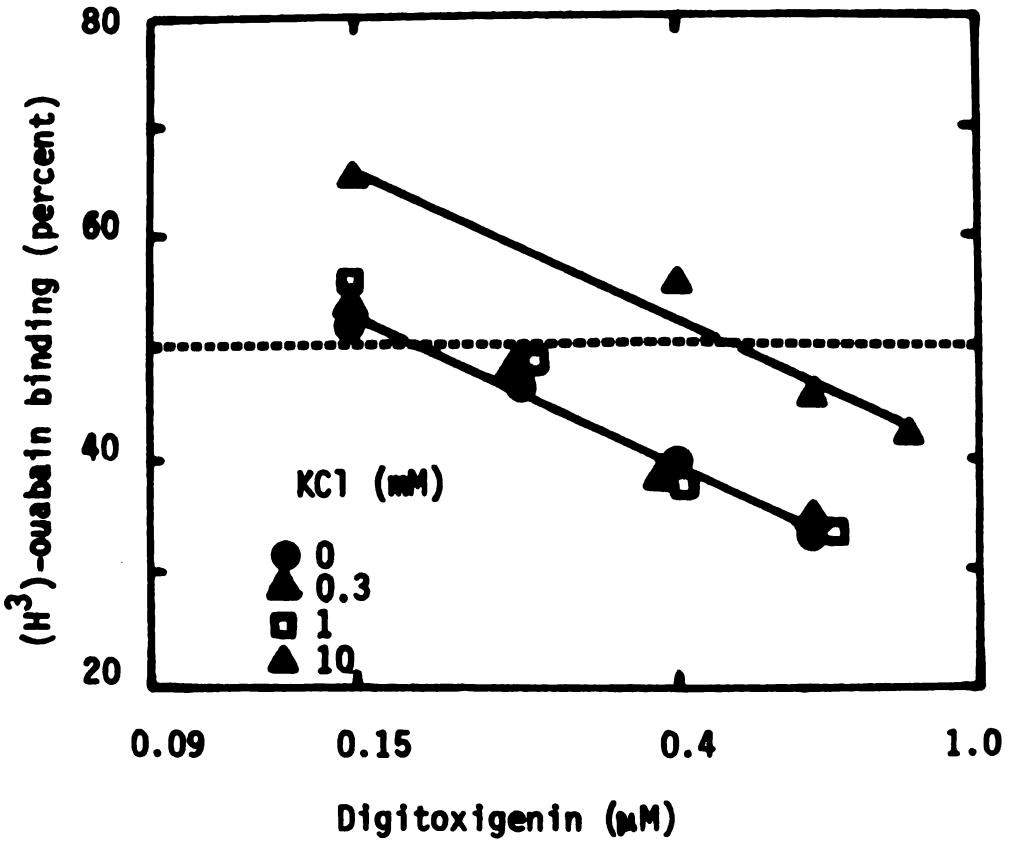

Figure 4. Specific (^{3}H)-ouabain binding to purified rat brain Na^+,K^+-ATPase after preincubation with digitoxigenin. See legend for Figure 3. Values are the means of 4 experiments with different enzyme preparations.

respectively. In Figure 4, slopes of the regression lines are -34, -38, -39 and -48 for 0, 0.3, 1.0 and 10 mM potassium, respectively. Potassium had little effect on the I50 for (^{3}H)-ouabain binding after incubation with the glycoside (digitoxin). On the other hand, 10 mM potassium markedly increased the amount of aglycone (digitoxigenin) needed to inhibit 50% of (^{3}H)-ouabain binding to rat brain enzyme. These results were replotted in Figure 5. In this figure, the I50 values at increasing potassium concentrations are expressed relative to the I50 in solutions containing no potassium. Analysis of variance-completely random design with p 0.05 showed that potassium had no significant effect on the I50 values for the digitoxin-pretreated enzyme. However, the I50 values for the digitoxigenin-pretreated enzyme were significantly affected by potassium. Since the concentration of drug needed to occupy 50% of the enzyme binding sites is related to the ratio of the association and dissociation rate constants for drug binding to enzyme, these data indicate that potassium had a lesser effect on the equilibrium level of the Na^+,K^+-ATPase interaction with glycoside than with aglycone.

B. The Effects of Potassium on the Inotropic Response of Digoxin and Digoxigenin

1. Onset and Equilibrium Level of Inotropic Response. Guinea pig left atrial preparations were used to study the effect of potassium on the onset and the equilibrium

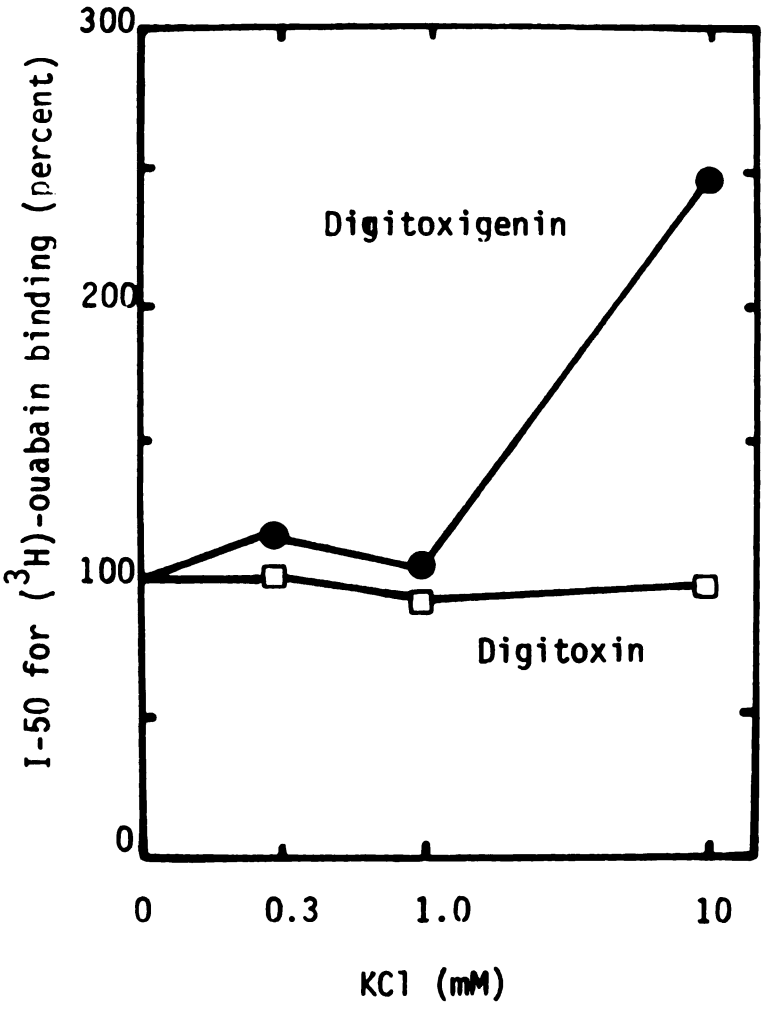

Figure 5. Effect of potassium on the I50 concentration for (^{3}H)-ouabain binding to purified rat brain Na^+,K^+-ATPase after preincubation with digitoxin or digitoxigenin. Data were obtained from experiments shown in Figures 3 and 4. Concentration of digitoxin or digitoxigenin needed to cause a 50% reduction of (^{3}H)-ouabain binding to Na^+,K^+-ATPase (I50) is expressed relative to I50 values in solutions containing no potassium.

level of inotropic response to digoxin or digoxigenin. Atria were incubated in a 30°C bath of Krebs-Henseleit solution containing 3.5, 5.8 or 9.5 mM potassium and electrically stimulated at 1 Hz. Figure 6 is a plot of the percent increase in contractile force above control value versus time in minutes. The control value is the contractile force observed after a 60-minute equilibration period and immediately before the addition of digoxin (0.4 μM). The onset of the inotropic response followed a slow time course. In 3 potassium concentrations, the rates of development of the inotropic response were compared by using the time required for development of half maximal inotropic effect. This value, defined as T50, is smaller if the rates of onset of inotropic response are rapid. The T50 values for the digoxin-induced inotropic response in solutions containing 3.5, 5.8 and 9.5 mM potassium are 15, 17 and 25 minutes, respectively. Thus, the presence of a higher potassium concentration in the incubation medium delayed the development of the inotropic response. On the other hand, the maximal contractile force (approximately 70% above control) was relatively unaffected by the potassium concentration in the tissue bath. At 50 minutes, there was no significant effect of potassium on the digoxin-induced inotropic response. Statistical significance was tested using an analysis of variance-completely random design at $p<0.05$. These results are in agreement with Prindle et al. (1971), who reported that the presence of higher potassium concentration in the

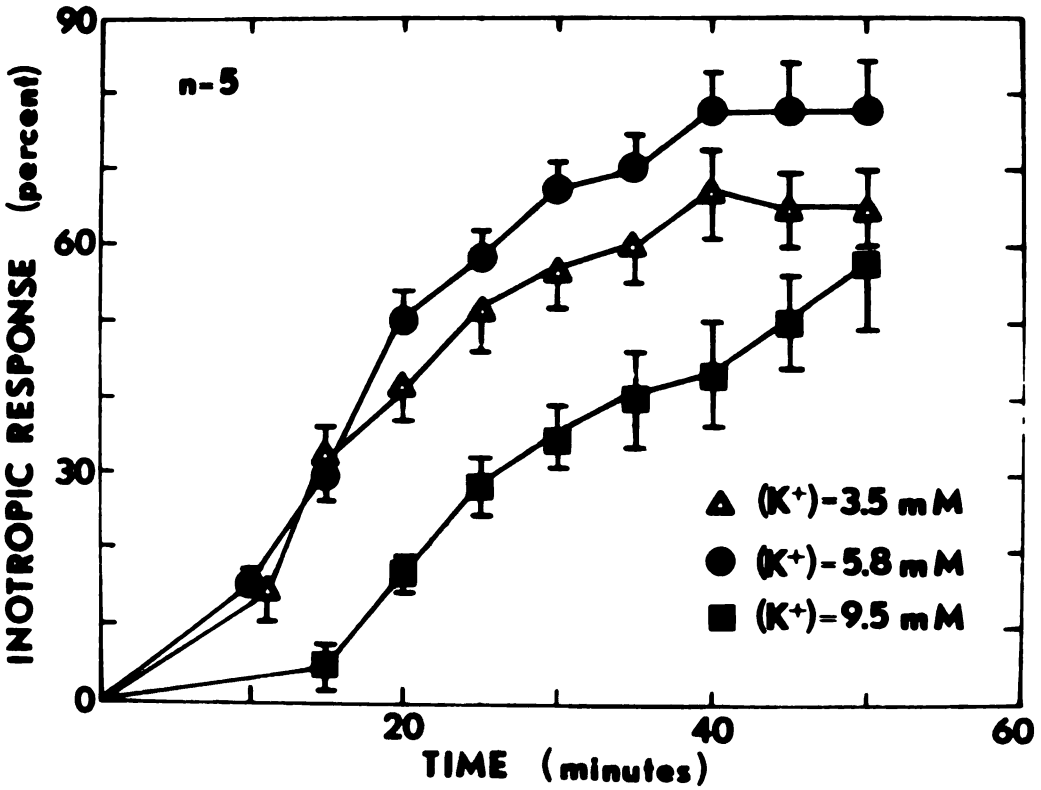

Figure 6. Positive inotropic effect of digoxin in guinea pig left atrial preparations. Atrial preparations were electrically driven at 1 Hz. Following a 60-minute equilibration period at 30°C in a Krebs-Henseleit solution containing 3.5, 5.8 or 9.5 mM potassium, 0.4 μM digoxin was added to the bath. Inotropic response is expressed as the percent increase in contractile force compared to control levels before the addition of drug. Each point represents the mean of 5 experiments. Vertical lines indicate the standard error.

incubation medium delays the development of the positive inotropic response to digoxin but does not affect the ultimate magnitude of that response.

Results of similar experiments with left atrial preparations using the aglycone, digoxigenin (5 μM) are shown in Figure 7. The rate of onset of digoxigenin-induced inotropic response was very fast and relatively unaffected by potassium. The T50 values in solutions containing 3.5, 5.8 and 9.5 mM potassium are 6, 7 and 6 minutes, respectively. However, the maximal level of the aglycone-induced inotropic effect was significantly ($p<0.05$) reduced at higher potassium concentrations. At steady state, the inotropic response in Krebs-Henseleit solutions containing 3.5, 5.8 and 9.5 mM potassium was 120, 80 and 50% above control values. Therefore, in contrast to the glycoside-induced inotropic response, the aglycone-induced inotropic response at steady state was markedly influenced by potassium concentration.

2. Time Course of the Loss of Inotropic Effects. Potassium reduces the rate of digitalis binding to Na^+,K^+-ATPase *in vitro*. In addition, potassium reduces the rate of release of cardiac glycosides from Na^+,K^+-ATPase. If the digitalis-glycoside interaction is intimately involved in the drug-induced inotropic response, then washout of the cardiac glycoside-induced inotropic effect should also be delayed by potassium.

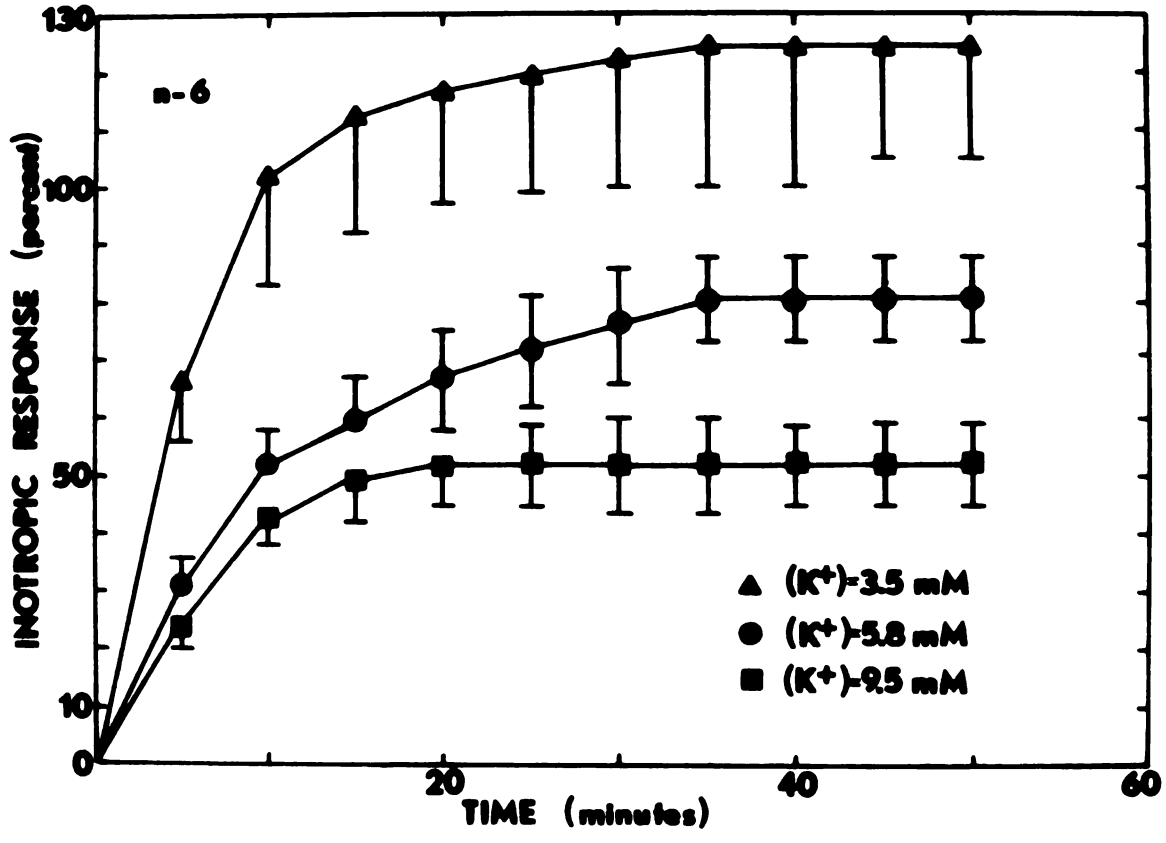

Figure 7. Positive inotropic effect of digoxigenin in guinea pig left atrial preparations. See legend for Figure 5. Digoxigenin concentration was 5 μM. Each point represents the mean of 6 experiments.

Isolated guinea pig hearts were perfused with Krebs-Henseleit solution containing either 3.5, 5.8 or 9.5 mM potassium. After a 45-minute equilibration period, hearts were perfused with digoxin (0.6 μM) which produced a marked positive inotropic effect (approximately 45% above control values in all 3 potassium concentrations). After a 20-minute drug perfusion, hearts were perfused with drug-free solution and the loss of the inotropic response was monitored. Figure 8 shows a graph of the dissipation of the digoxin-induced inotropic effect expressed as percent maximal response versus time on a semi-logarithmic plot. Washout of the digoxin-induced response followed a slow time course. After a 10-minute wash, only 25% of the maximal response could be reversed using solutions containing 5.8 mM potassium. Higher potassium concentration in the perfusate further delayed washout of drug response while lower potassium concentration increased dissipation of the inotropic response.

Similar studies were conducted using a second cardiac glycoside, ouabain (Figure 9). Again, the washout of the inotropic response was slow with approximately 35% dissipation after 10 minutes of perfusion with drug-free solution containing 5.8 mM potassium. Lower potassium concentration resulted in a faster reversal and a higher potassium concentration delayed the loss of the ouabain-induced inotropic response.

It is postulated that potassium causes a conformational change in the drug-bound enzyme imposing a lipid

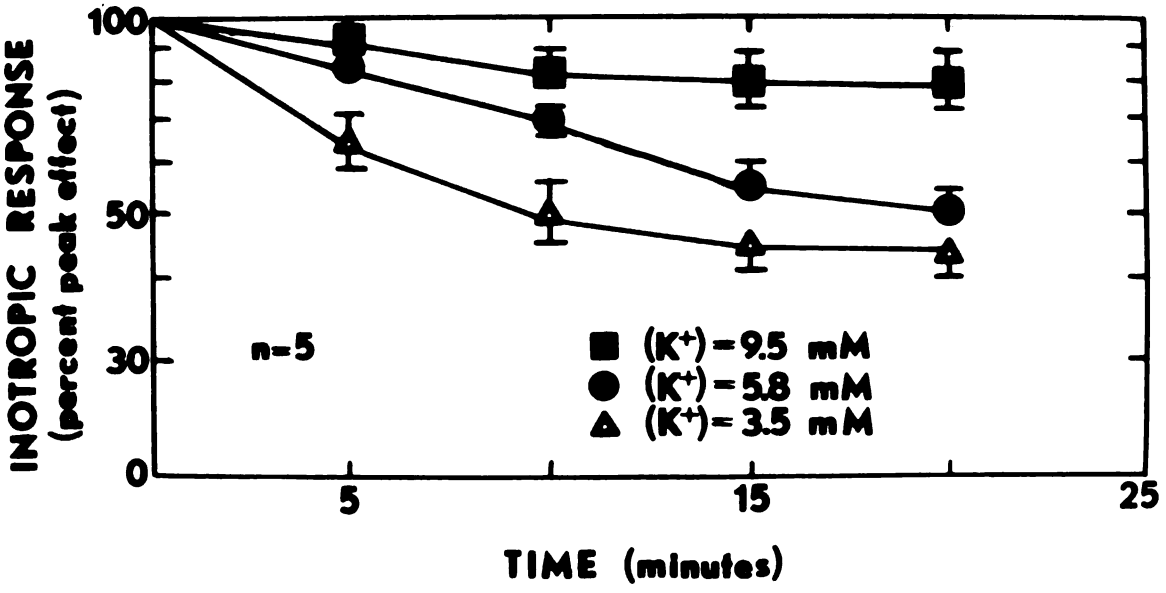

Figure 8. Washout of digoxin-induced inotropic response in isolated perfused heart preparations. Hearts were paced at 1.5 Hz and perfused at 4 ml/min with Krebs-Henseleit solution containing 3.5, 5.8 or 9.5 mM potassium at 30°C. After a 45-minute equilibration period, 0.6 μM digoxin was perfused for 20 minutes followed by perfusion with drug-free solution. Washout of inotropic response is plotted as the percent maximal response. Each point represents the mean of 5 experiments. Vertical lines indicate the standard error.

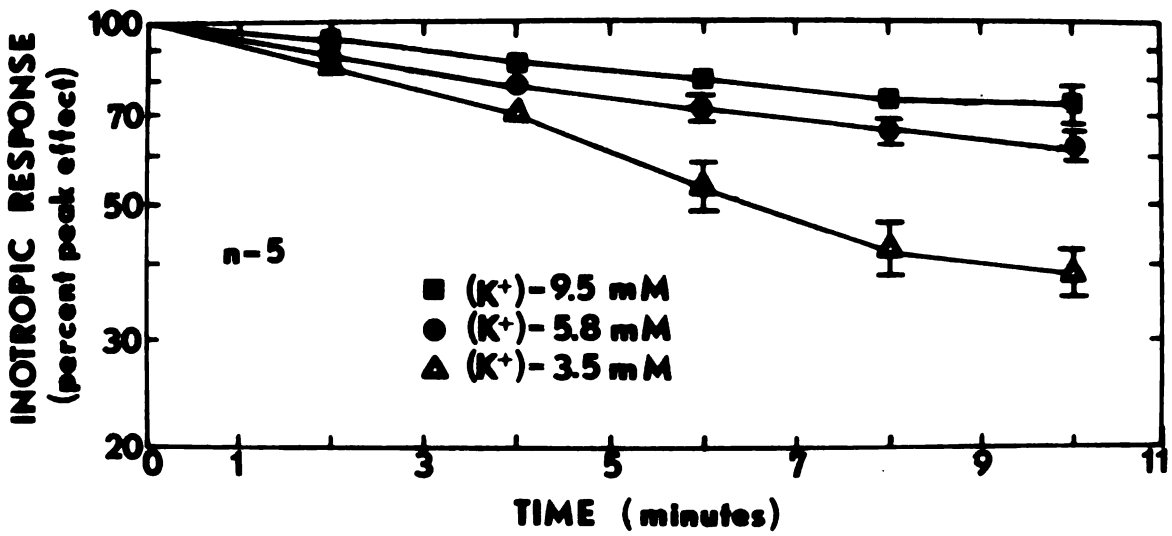

Figure 9. Washout of ouabain-induced inotropic response in isolated perfused heart preparations. See legend for Figure 7. Ouabain (0.4 μM) was perfused for 20 minutes before washout with drug-free solution. Each point represents the mean of 5 experiments.

to the dissociation of drug from binding sites on the enzyme. Aglycones which are the highly lipid-soluble, steroid nucleus of cardiac glycosides should be relatively permeable to a potassium-induced lipid barrier.

In order to study the effect of potassium on the dissipation of the inotropic action of an aglycone, isolated guinea pig hearts were perfused for 10 minutes with digoxigenin (3 μM). Digoxigenin caused a marked increase in inotropic response, 40, 27, and 22% above control values in solutions containing 3.5, 5.8 and 9.5 mM potassium, respectively. After the 10-minute perfusion of the aglycone, Langendorff preparations were perfused with a drug-free solution and the dissipation of the inotropic effect was monitored. In contrast to the results observed with glycosides, loss of the aglycone-induced inotropic response was rapid (Figure 10). Fifty percent of the response was lost after 1 minute of perfusion with drug-free solution. In addition, the effect of potassium on the rate of dissipation of inotropy was markedly smaller than that of potassium on the rate of reversal of the glycoside-induced inotropic response.

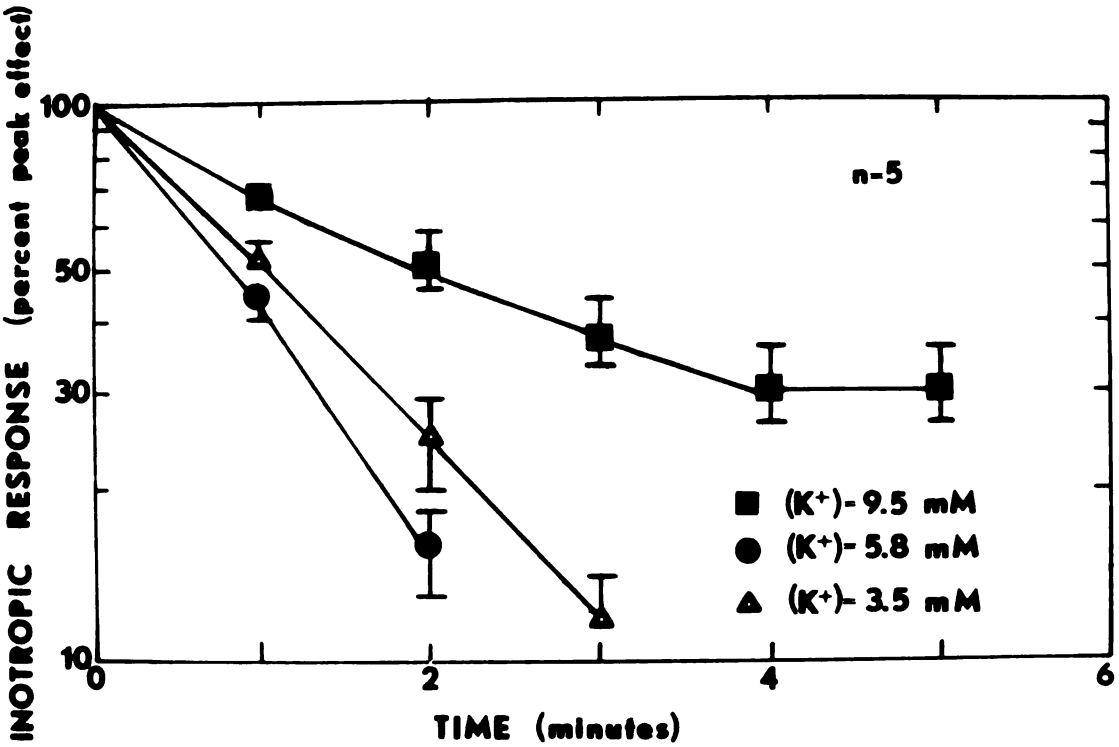

Figure 10. Washout of digoxigenin-induced inotropic response in isolated perfused heart preparations. See legend for Figure 7. Digoxigenin was perfused for 10 minutes followed by the perfusion of a drug-free solution. Each point represents the mean of 5 experiments.

DISCUSSION

One of the major views regarding the mechanism of digitalis action is based on the premise that digitalis acts to inhibit membrane-bound Na^+,K^+-ATPase (Repke, 1965). This idea is strengthened by findings which show that Na^+,K^+-ATPase is inhibited when isolated from hearts exhibiting a glycoside-induced positive inotropic response (Akera et al., 1970; Besch et al., 1970). In addition, species differences in the sensitivity to the inotropic effects of cardiac glycosides can be correlated with the affinity of cardiac glycosides to their receptors and with the glycoside sensitivity of the Na^+,K^+-ATPase preparations (Repke, 1965; Tobin and Brody, 1972). On the other hand, it has been reported that Na^+,K^+-ATPase may not be inhibited when the inotropic response is produced (Rhee et al., 1976) or that the positive inotropic effect may be dissipated without recovery of the Na^+,K^+-ATPase activity (Okita et al., 1973). Factors affecting the digitalis-Na^+,K^+-ATPase interaction have been extensively investigated. Of particular interest here is the role of potassium in the drug-enzyme interaction. *In vitro* binding studies indicate that potassium reduces both the rate of association (Schwartz et al., 1968) and the rate of release of cardiac glycosides from Na^+,K^+-ATPase (Akera

and Brody, 1971). If potassium alters the drug-receptor interaction (inotropic response) in a manner consistent with its effect on the drug-enzyme interaction, this would support the theory that Na^+,K^+-ATPase is the receptor for the inotropic action of digitalis.

A. The Effects of Potassium on the *in vitro* Binding of Cardiac Glycosides and Aglycones to Na^+,K^+-ATPase

Effects of potassium on the interaction of glycosides and aglycones with Na^+,K^+-ATPase were examined first. The ability of potassium to reduce the binding of cardiac glycosides to Na^+,K^+-ATPase is a consistently observed phenomenon. Potassium reduces (^3H)-ouabain binding to rat brain and guinea pig heart enzyme (Akera and Brody, 1971; Choi and Akera, 1977) and (^3H)-digoxin binding to calf heart Na^+,K^+-ATPase (Schwartz et al., 1968). These observations were confirmed in the present study using (^3H)-digoxin and enzyme isolated from guinea pig heart (see Figure 1). In addition, potassium reduces the rate of release of drug from the glycoside-Na^+,K^+-ATPase complex formed under certain ligand conditions. Ouabain binds to different forms of the phosphoenzyme under the following conditions: 1) Na^+, Mg^{2+} and ATP, 2) Mg^{2+} and ATP, and 3) Mg^{2+} and P_i (Akera et al., 1974b, 1976b). Addition of potassium to the dissociation mixture slows the rate of release of ouabain from the drug-enzyme complex formed in the presence of Na^+, Mg^{2+} and ATP but has no effect on the release of drug from the complex formed in the presence of

Mg^{2+} and P_i or Mg^{2+} and ATP. The *in vitro* dissociation of (^{3}H)-ouabain bound to ventricular tissue during a Langendorff perfusion of isolated puppy heart was also stabilized by the addition of potassium. Thus, the drug-enzyme complex formed in the presence of Na^+, Mg^{2+} and ATP is the best model for the drug-enzyme interaction which occurs in the beating heart. The reduction in both the association and dissociation rates of cardiac glycoside binding to Na^+,K^+-ATPase results, presumably, from the involvement of potassium at two distinct steps in the drug-enzyme interaction. It is postulated that the reduced rate of binding is due to a potassium-induced decrease in the binding form of the enzyme (Schwartz et al., 1968), whereas the decreased rate of release is due to a potassium-induced lipid barrier surrounding drug binding sites (Akera and Brody, 1971).

The effect of potassium on the steady state level of cardiac glycoside binding to Na^+,K^+-ATPase, however, is still unclear. Allen and Schwartz (1970) reported that potassium reduced the rate of binding of (^{3}H)-ouabain to isolated enzyme preparations but failed to influence the ultimate level of that binding. Similar findings have been reported by other investigators (Barnett, 1970; Lindenmayer and Schwartz, 1973). On the other hand, based on a kinetic analysis of (^{3}H)-ouabain binding to purified rat brain enzyme, Choi and Akera (1977) found that potassium reduced the apparent association rate constant to a greater extent than the dissociation rate

constant for the glycoside-enzyme interaction. Since the equilibrium level of digitalis is determined by the ratio of these two rate constants (at equilibrium the rate of binding equals the rate of release), these data suggest that increasing potassium concentration from 0 to 5 mM decreases the steady state concentration of bound drug. The cause of this apparent difference with respect to earlier studies is not known. However, aside from the possibility that there might be differences in experimental design or in the source of enzyme preparation, other factors, such as the concentration of drug, may contribute to the variability in results. When the drug concentration is high, ouabain binding may be close to saturating all the enzyme binding sites. Under such conditions, the concentration of bound drug is primarily determined by the concentration of the enzyme and is relatively insensitive to changes in association and dissociation rate constants.

The relative effect of potassium on the association and dissociation rates of the digitalis-enzyme interaction can be studied by investigating the effect of potassium on the affinity of the enzyme for the drug. Affinity can be expressed as the ratio of the two rate constants (k_a/k_d), or as the inverse of the dissociation constant ($1/K_D$). In the latter case, affinity is equal to the inverse of the drug concentration needed to bind 50% of the cardiotonic steroid binding sites on the enzyme (I50). An increase in the ratio of the association and dissociation

rate constants would decrease the I50 concentration and, conversely, a decrease in this ratio would increase the I50 concentration. In experiments shown in Figures 3 and 4, several concentrations of digitoxin and digitoxigenin were allowed to bind to rat brain Na^+,K^+-ATPase for 20 minutes and then challenged with (^3H)-ouabain for 1 1/2 minutes. This method, which measures initial (^3H)-ouabain binding velocity, is used to determine free binding site concentration. Initial velocity is proportional to the concentration of unoccupied binding sites when (^3H)-ouabain concentration is constant. From plots of the percent inhibition of (^3H)-ouabain binding versus the logarithmic drug concentration, the I50 for digitoxin and digitoxigenin was obtained at the various potassium concentrations. Potassium (0-10 mM) had little effect on the concentration of digitoxin needed to occupy 50% of the enzyme binding sites (approximately 0.03 μM). However, it took 0.51 μM digitoxigenin to inhibit (^3H)-ouabain binding by 50% with 10 mM potassium as compared to 0.2 μM with no added potassium. This indicates that potassium decreases the steady state level of the aglycone-Na^+,K^+-ATPase interaction but has little effect on the cardiac glycoside-enzyme interaction. These results suggest that potassium causes a differential effect on the relative reduction of the association and dissociation rates for a glycoside- and an aglycone-enzyme interaction. Such findings strongly support the contention that potassium affects the rate of digitalis binding and digitalis

dissociation by two distinct mechanisms. Based on these observations, it would be expected that potassium would also affect the inotropic response of glycosides and aglycones in a different manner if, indeed, the binding of drug to Na^+,K^+-ATPase is intimately related to the inotropic response.

B. The Effects of Potassium on the Inotropic Response of a Glycoside and an Aglycone

Several reports in the literature describe the effects of potassium on the onset and maximal level of the inotropic response to cardiac glycosides. However, the results from these studies are conflicting. Garb and Venturi (1954) reported that potassium ranging in concentration between 3.5 and 8.5 mM did not alter the inotropic effects of ouabain in the failing cat papillary muscle. Similarly, Leonard and Hadju (1959) found no change in the inotropic effects of ouabain when potassium concentration was varied between 2.5 and 5.0 mM in the frog heart or between 4.7 and 7.5 mM in the guinea pig or rabbit heart. On the other hand, Lee et al. (1961) showed that in cat papillary muscle, 24 mM potassium delayed the onset of the inotropic effects of ouabain and diminished the peak inotropic response. Similarly, Cohn et al. (1967) found that 10 mM potassium delayed the onset and peak of the ouabain-induced inotropic response in right ventricular guinea pig heart strips. More recently, Prindle et al. (1971) reported the results of experiments monitoring the effects

of potassium (1.5, 4.5 and 7.5 mM) on the inotropic response to digoxin in the isolated cat papillary muscle. In these studies it was found that potassium delayed the onset of inotropic response but had little influence on the maximal level of that response.

From the above studies it appears that potassium does modulate the inotropic response to cardiac glycosides in isolated tissue preparations. However, it is unclear as to the extent of this effect on the onset and maximal levels of the digitalis response. The present experiments were conducted to explore the effect of potassium on the digitalis-induced inotropic effect in detail. It was found that 9.5 mM potassium delayed the onset of the digoxin-induced inotropic response but had relatively little effect on the ultimate drug response (see Figure 6). These results are similar to those reported by Prindle et al. (1971). In addition, such findings correlate well with the expected results predicted by the effects of potassium on cardiac glycoside binding to Na^+,K^+-ATPase. In the digitoxin-enzyme interaction studies it was found that the amount of cardiac glycoside bound to Na^+,K^+-ATPase at steady state (which can be expressed as the ratio of the association and dissociation constants) was unaffected by potassium concentration in the binding mixture (see Figure 3). Since potassium reduces both association and dissociation rate constants of glycoside binding to enzyme, these results indicate a similar degree of reduction in the two rate constants. In the inotropic

studies with digoxin the steady state level of cardiac glycoside-induced inotropic response was not influenced by potassium (see Figure 6), whereas both the onset (Figure 6) and offset (Figure 8) were markedly reduced. It appears, then, that the rates of onset and offset of the drug-receptor interaction also are similarly delayed by potassium. These findings can be explained if the inotropic response does, indeed, result from binding of cardiac glycosides to Na^+,K^+-ATPase.

As described before, the binding of aglycones to Na^+,K^+-ATPase is also influenced by potassium. In contrast to studies on the glycoside-enzyme interaction, *in vitro* binding studies with digitoxigenin indicated that potassium markedly reduced the steady state level of aglycone binding to Na^+,K^+-ATPase (see Figure 4). These results were explained as a greater reduction by potassium of the association rate constant than the dissociation rate constant for the aglycone-enzyme interaction. When the effect of potassium on the digoxigenin-induced inotropic response was investigated using guinea pig left atrial preparations, it was found that maximal inotropic response was reduced at higher potassium concentrations (see Figure 7). Further studies to monitor the effect of potassium on the washout of this response in isolated perfused heart preparations showed that potassium had only a slight stabilizing influence on the fast dissipation of the inotropic response (see Figure 10). These data indicate that potassium reduces the association of aglycone with inotropic

receptor to a greater extent than the dissociation of drug from receptor. Again, such findings support the theory that binding of digitalis to Na^+,K^+-ATPase is intimately involved in the inotropic response.

C. Drug-Enzyme Interaction *in vitro* and Drug-Receptor Interaction in Beating Hearts

In the preceding discussion the effects of potassium on the steady state levels of drug-enzyme and drug-receptor interactions were compared. Since the steady state levels of a drug-enzyme and a drug-receptor interaction are determined by two independent variables (rate of association and rate of release), attempts were made to study these two parameters separately. Comparison of the time course for the binding of digitalis to Na^+,K^+-ATPase and for the onset of the digitalis-induced inotropic response, however, can be limited by several factors. A direct correlation of these two events is based not only on the assumption that drug interacts with Na^+,K^+-ATPase to produce the positive inotropic effect, but it is also based on the assumption that this interaction is the rate limiting step in the genesis of the response.

If we assume that Na^+,K^+-ATPase is the inotropic receptor, then binding of drug to enzyme would initiate a sequence of events leading to the pharmacologic response. Factors which influence the binding of digitalis to Na^+,K^+-ATPase would be expected to similarly affect the onset of the inotropic response. However, the absence of

such confirmatory data would still be subject to evaluation. If a subsequent step in the sequence of events leading to the pharmacologic response is slower than the initial drug-receptor interaction, then factors affecting the rate of receptor binding may not be reflected in the inotropic response. On the other hand, if binding to the receptor is rate limiting, then factors affecting this interaction would influence the onset of the inotropic response. In simple enzyme kinetics, the binding of ligand to enzyme is determined by the concentration of drug and the concentration of available binding sites. In *in vitro* binding studies these concentrations can be accurately controlled. The effect of potassium on the "apparent" association rate constant for the binding of a cardiac glycoside, like ouabain, to Na^+,K^+-ATPase can be estimated from the initial velocity of drug binding to enzyme in the presence and absence of potassium (Choi and Akera, 1977). The slope of the regression line for the plot of initial binding velocity versus drug concentration corresponds to the "apparent" association rate constant or the $k_a \cdot (E)$ value. Therefore, a potassium-induced change in the $k_a \cdot (E)$ value does not necessarily indicate a change in the true association rate constant. Alternatively, it might indicate a change in the concentration of the binding form of the enzyme. The true association rate constant should be expressed in $(M \cdot min)^{-1}$ when the concentration of the binding form of the enzyme is expressed in molar concentrations. However, an apparent

association rate constant may be expressed in the same unit, $(M \cdot min)^{-1}$, when the concentration of binding sites on the enzyme is expressed in molar concentrations. By keeping the drug concentration constant, the effect of potassium on the concentration of available binding sites and hence on the "apparent" association rate constant can be adequately determined. In the case of the drug-receptor interaction in the beating heart, however, conditions cannot be as precisely controlled. It is possible that besides decreasing the binding form of the receptor, potassium might also influence the concentration of free drug at the receptor sites. In the latter case, it would be difficult to correlate the initial velocity or time course of onset of the inotropic response to digitalis with those of the *in vitro* binding of drug to enzyme. However, since the membrane-bound Na^+,K^+-ATPase is in constant contact with the extracellular fluid, it is likely that results indicating a similar effect of potassium on the drug-enzyme and the drug-receptor interaction actually do reflect a change in concentration of the binding form of the receptor rather than a change in the concentration of available drug.

On the other hand, we can also postulate that the binding of digitalis to Na^+,K^+-ATPase is not the drug-receptor interaction but may be an interaction prior to receptor binding. Such an interaction, for example, might serve to transport cardiac glycosides to an inotropic receptor inside the cell. In this case, then, factors

which would influence the amount of drug bound to Na^+,K^+-ATPase or the activity of the sodium-potassium pump would likewise influence the delivery of drug to intracellular receptor and consequently the onset of the inotropic response. This concept has been sustained by studies of the effects of temperature and beat interval on the onset of the glycoside- and aglycone-induced pharmacologic response (Parks and Vincenzi, 1975). Both temperature and stimulus rate influence the rate of onset of the glycoside-induced inotropic response but neither factor influenced the onset of the aglycone-induced inotropic response in isolated rabbit atrial preparations. The authors suggested that these data might indicate that cardiac glycosides are transported to the digitalis inotropic receptor by the active sodium-potassium pump (Na^+,K^+-ATPase) but that the aglycones gain access to the digitalis receptor via passive diffusion. Studies such as these, however, do not allow one to decide whether the binding of the glycoside to Na^+,K^+-ATPase is merely to transport these agents to an ultimate site of action or if the binding to the enzyme is the first step in the sequence of events which lead to the inotropic response.

Correlation of the effect of potassium on the dissipation of the inotropic response by washout and release of drug from Na^+,K^+-ATPase may be more easily interpreted. If Na^+,K^+-ATPase is the inotropic receptor, then release of drug from the enzyme would be the rate-limiting step for the loss of the drug response. If the

drug-Na^+,K^+-ATPase interaction is a step prior to receptor binding and the receptor for inotropic action of digitalis is an entity unrelated to Na^+,K^+-ATPase, then factors modulating the release of the drug from the enzyme might influence the onset of the inotropic response but would not affect the loss of this response. Therefore, a similar effect of potassium on these two events would indicate that Na^+,K^+-ATPase plays an intimate role in the inotropic response.

D. Present Findings and the Theories on the Mechanism of the Inotropic Action of Digitalis

The present findings support the concept that Na^+,K^+-ATPase is the inotropic receptor. Inotropic studies on the onset and equilibrium response to digoxin and digoxigenin show that potassium affects the drug-receptor and the drug-enzyme interaction in a similar manner, indicating that binding of digitalis is related to the drug response. However, these data could be used to support any of the three theories on the mechanism of digitalis action which involve binding of drug to Na^+,K^+-ATPase. One theory postulates that digitalis binds to Na^+,K^+-ATPase and is transported to an intracellular receptor site (Dutta et al., 1968). This theory, implying that enzyme inhibition, alone, is not sufficient for increased contractile force, supports a non-causal relationship between the two events. At present, evidence for a digitalis receptor localized inside the cell membrane

is lacking. A second hypothesis proposes that binding of digitalis to Na^+,K^+-ATPase causes the enzyme to assume a specific conformation which promotes a decreased affinity for calcium of enzyme-associated lipids and an increased calcium influx (Schwartz, 1976; Gervais et al., 1977). In this case, the interaction of the glycoside with enzyme would be necessary. However, inhibition of the sodium pump would not be required. The third theory requires binding of drug to Na^+,K^+-ATPase and inhibition of the active exchange of sodium and potassium (Akera et al., 1976c). Inhibition of the sodium pump by digitalis would thus enhance the sodium transient resulting in a greater calcium influx during the early phase of each cycle of the myocardial contractile event and causing a greater contraction. Lack of confirmation of an increased myocardial sodium concentration after Na^+,K^+-ATPase inhibition has been a major block in the acceptance of this theory. The inotropic studies on the onset and equilibrium response to digoxin and digoxigenin cannot distinguish whether Na^+,K^+-ATPase-drug interaction is required for the transport of digitalis to its receptor or for the initiation of the inotropic response. The time course of inotropic response in both cases would be limited by the Na^+,K^+-ATPase interaction and, as such, influenced by factors affecting this interaction.

The study on the dissipation of the inotropic response, however, tends to rule out the possibility that Na^+,K^+-ATPase is a carrier to move digitalis to an intracellular site of

action. Similar influence of potassium on the washout of the inotropic response and release of drug from Na^+,K^+-ATPase indicates that release of drug from enzyme is the rate limiting step in the dissipation of the drug effect.

The present results cannot be used to further differentiate whether Na^+,K^+-ATPase inhibition is important or whether conformational change in the enzyme leading to altered calcium affinity is important in the genesis of the inotropic response. In either case, however, Na^+,K^+-ATPase may be considered as the receptor for the inotropic action of digitalis since the binding of these agents to this enzyme system ultimately leads to the drug response.

SUMMARY AND CONCLUSION

Data from this study indicate that potassium has only a slight effect on the steady state level of the interaction of digitoxin with Na^+,K^+-ATPase, whereas potassium reduces the steady state level of digitoxigenin bound to enzyme. Since it is known that potassium reduces both the association rate constant and the dissociation rate constant of a cardiac glycoside-enzyme interaction, these results suggest that potassium has a similar effect on the forward and reverse reaction velocities of a cardiac glycoside binding to Na^+,K^+-ATPase but potassium has a differential effect on the two rate constants for an aglycone binding to enzyme. This can be explained by the postulate that potassium affects two distinct steps in the drug binding reaction.

Further results of the effects of potassium on the inotropic response to the glycoside, digoxin, and the aglycone, digoxigenin, showed that potassium reduces both the rate of onset and the rate of offset of the glycoside-induced inotropic response but has little influence on the steady state level of that response. On the other hand, potassium markedly reduces the maximal level of the aglycone-induced inotropic response but has only a slight

effect on the rapid onset and dissipation of the aglycone-induced inotropic response.

These data indicate that the effects of potassium on the interaction of cardiac glycosides and aglycones with Na^+,K^+-ATPase are closely related to the effects of potassium on the glycoside- and aglycone-induced inotropic response. Therefore, this study supports the hypothesis that Na^+,K^+-ATPase is intimately involved in the therapeutic response to the digitalis compounds.

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