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^{3}H-Ouabain Binding and Sodium-Pump Activity Measured in Myocytes Isolated from Guinea-pig Heart

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^{3}H-OUABAIN BINDING AND SODIUM-PUMP ACTIVITY MEASURED IN MYOCYTES ISOLATED FROM GUINEA-PIG HEART

By

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ABSTRACT

^{3}H-Ouabain Binding and Sodium-Pump Activity Measured in Myocytes Isolated from Guinea-Pig Heart

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Myocytes, quiescent in millimolar concentrations of Ca^{2+}, were isolated from guinea-pig heart by treatment with collagenase and hyaluronidase; greater than 80% of cells were rod-shaped. One micromolar ouabain protected isolated myocyte preparations from loss of viable cells during a 60-min incubation. One millimolar ouabain was toxic to cells within 60 min as determined by loss of the rod shape. Because of toxicity of millimolar ouabain, non-specific ^{3}H-ouabain binding was assessed by monitoring dissociation of bound drug. Analysis of specific ^{3}H-ouabain binding to myocytes yielded non-linear Scatchard plots. Non-linearity appears to result from reduced ^{3}H-ouabain binding due to low intracellular Na^{+} concentration. Addition of 2 μM monensin, a Na^{+} ionophore, significantly increased ^{3}H-ouabain binding. Incubation in Ca^{2+}-free solution (0.25 mM EGTA) stimulated ^{3}H-ouabain binding to a greater degree than monensin and caused Scatchard plots to have two distinct linear components. Monensin had no significant effects when ^{3}H-ouabain binding occurred in Ca^{2+}-free solution. Effects of Ca^{2+}-free incubation to increase ^{3}H-ouabain binding suggest that Ca^{2+} has a direct effect on ^{3}H-ouabain binding. Alternatively, Ca^{2+}-free incubation may increase Na^{+} permeability of the sarcolemma. Isoproterenol, phenylephrine,

TPA (phorbol 12-myristate 13-acetate), La^{3+}, and the Ca^{2+}-ionophore A23187 failed to cause significant changes in ^{3}H-ouabain binding when myocytes were incubated in a solution containing 0.5 or 2.5 µM ^{3}H-ouabain, 0.1 mM Ca^{2+} and 1 mM K^{+}. Caffeine decreased binding of ^{3}H-ouabain and the percentage of rod-shaped cells. Activity of the sodium-pump was estimated in myocytes using ouabain-sensitive $^{86}Rb^{+}$ uptake. Monensin or Na^{+}-loading in a K^{+}-free, Rb^{+}-free solution caused up to a four-fold increase in $^{86}Rb^{+}$ uptake by myocytes, indicating enhanced Na^{+}-pump activity. Stimulation of pump activity by monensin peaked at 30 µM. Stimulation by Na^{+}-loading plateaued after a 45-min incubation for Na^{+}-loading at 37°C. Under the above conditions, intracellular Na^{+} is apparently no longer rate-limiting for turnover of the sodium-pump. In addition, these interventions caused concentration-response curves for ouabain-induced Na^{+}-pump inhibition to be shifted to the left. The presence of Ca^{2+} during $^{86}Rb^{+}$ uptake reduced this measure of sodium-pump activity in non-treated and stimulated myocytes indicating that extracellular Ca^{2+} reduces the Na^{+} load to the sodium-pump or acts directly to inhibit pump activity.

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INTRODUCTION

A. General Background

It is generally accepted that Na,K-ATPase is the pharmacological receptor for the therapeutic and toxic effects of cardiac glycosides (Lee and Klaus, 1971; Akera and Brody, 1978, 1982). The positive inotropic effect of these agents is believed to result from inhibition of the Na,K-ATPase. Inhibition of the Na,K-ATPase causes Na^+ transients associated with membrane depolarization to be larger in magnitude and of longer duration (Bentfeld et al., 1977; Akera and Brody, 1978). Also associated with Na,K-ATPase inhibition is an increase in resting intracellular Na^+ concentration (Lee et al., 1980; Lee and Dagostino, 1982). Changes in the sodium transient and resting intracellular Na^+ concentration affect force of contraction by altering the dynamic and equilibrium activity of the Na/Ca exchange mechanism which in turn affects Ca^{2+} transients and the magnitude of intracellular Ca^{2+} stores (Akera and Brody, 1985; Lee et al., 1985). Minor changes in intracellular Na^+ concentration are able to cause significant changes in the transport of Ca^{2+} via the Na/Ca exchange mechanism because this system is extremely sensitive to changes in the Na^+ gradient caused by changes in intracellular Na^+ concentration (Mullins, 1979; Langer, 1982). Larger changes in the Na^+ transient and intracellular Na^+ concentration which are associated with 60 to 80% inhibition of the Na,K-ATPase (Akera and Brody, 1978) result in "Ca^{2+} overload" which causes toxic effects of the cardiac glycosides.

Although an overwhelming amount of evidence supports the theory that all direct effects of cardiac glycosides on the heart are the result of Na,K-ATPase

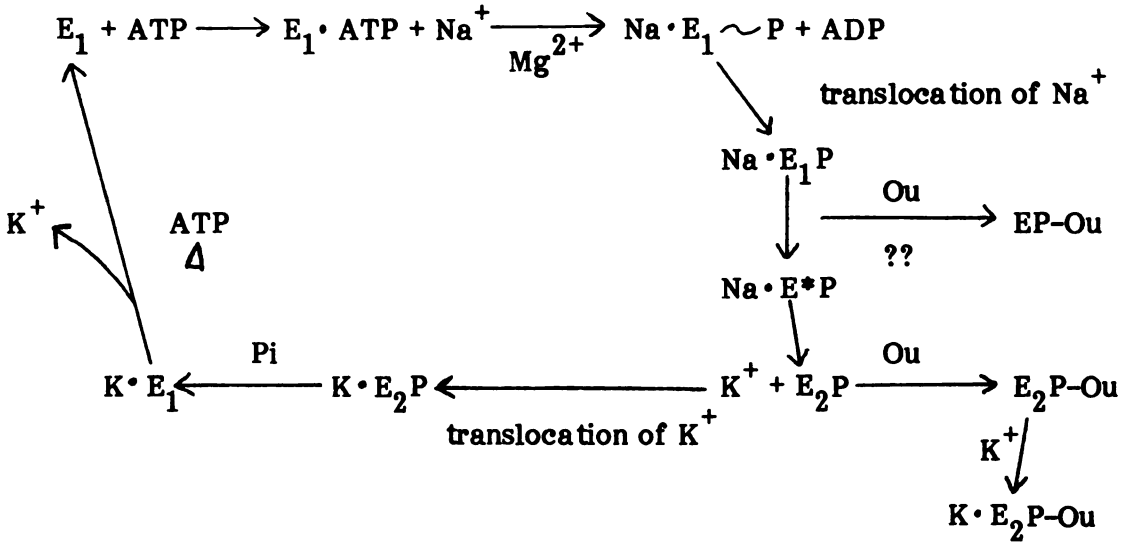
inhibition, other theories have been proposed to explain the inotropic effects of low concentrations of cardiac glycosides. These are that glycoside binding to the Na,K-ATPase affects Ca^{2+} binding to the intracellular side of the sarcolemmal membrane (Schwartz, 1976; Gervais et al., 1977; Lullmann and Peters, 1979; Preuner, 1979; Schwartz and Adams, 1980) or that binding to a different receptor or different site on the Na,K-ATPase than the one which causes enzyme inhibition results in a positive inotropic effect which is associated with a stimulation of the Na,K-ATPase (Ghysel-Burton and Godfraind, 1979; Godfraind and Ghysel-Burton, 1980; Noble, 1980; Godfraind et al., 1982). Decreases in tissue Na^+ content and increases in tissue K^+ content of cardiac muscle exposed to low concentrations of cardiac glycosides have been offered as evidence for stimulation of the Na,K-ATPase (Godfraind and Ghysel-Burton, 1977; Ghysel-Burton and Godfraind, 1979); however, a mechanism by which stimulation of the Na,K-ATPase could produce or be caused by an increase in the intracellular Ca^{2+} concentration is unknown. Therefore, the theory is not generally accepted. High affinity binding sites other than the Na,K-ATPase for the cardiac glycosides have not been found in homogenates of cardiac muscle. This, however, could be due to some factor in intact tissue which is essential for binding of these agents to a site other than the Na,K-ATPase being lost during tissue homogenization.

In order to unequivocally confirm that all direct actions of cardiac glycosides are due to the binding to and inhibition of Na,K-ATPase, glycoside binding must be studied in intact tissue or intact cardiac muscle cells. Binding must then be shown to correlate with inhibition of the sodium-pump. The sodium-pump is the physiological representation of the Na,K-ATPase. The goal of this project was to characterize binding of one cardiac glycoside, ^{3}H-ouabain, to Ca^{2+} tolerant myocytes from guinea-pig heart and then to further determine if intracellular Ca^{2+} concentration or phosphorylation of membrane constituents can

affect either glycoside binding or sodium-pump function in a manner which could affect the sensitivity of the heart to therapeutic or toxic effects of the cardiac glycosides.

B. Factors Affecting Glycoside Binding to Na,K-ATPase

Binding of cardiac glycosides is believed to be a second order reaction which can be considered to be a pseudo first-order reaction, described kinetically as a first-order reaction, when an excess of glycoside is present in the reaction mixture (Gelbart and Goldman, 1977). Binding of the glycosides is dependent on the conformation of the Na,K-ATPase (Akera et al., 1976). Enzyme conformation changes as the Na,K-ATPase binds different ligands and promotes the transport of ions. The reaction cycle of Na,K-ATPase is considered to be represented by the following scheme (Akera and Brody, 1978; Yoda and Yoda, 1986).



Ouabain binds to phosphorylated intermediates of the enzyme particularly the E_2P intermediate (Matsui and Schwartz, 1968). It has not been shown that ^{3}H-ouabain binds to intermediates other than the E_2P form of the Na,K-ATPase, however, curved Scatchard plots describing ^{3}H-ouabain binding to isolated Na,K-ATPase (Hansen, 1976; Wellsmith and Lindenmayer, 1980; Godfraind et al., 1980) indicate

that this ligand is binding to more than a single class of binding site. Dependent on the presence or absence of K^+ during binding, the Scatchard plots describing the binding become linear (Hansen, 1976; Godfraind et al., 1980). Hansen (1976) showed that addition of 2 mM K^+ caused Scatchard plots to become linear when the ^{3}H-ouabain binding to Na,K-ATPase occurred in a Mg^{2+} plus phosphate system. Godfraind and coworkers (1980) showed that in an incubation mixture containing Na^+, Mg^{2+}, ATP, phosphate and vanadate, linear Scatchard plots were obtained when ^{3}H-ouabain binding occurred in the absence but not in the presence of K^+. The ability of K^+ to affect ^{3}H-ouabain binding under each incubation condition suggests that these sites are interconvertible and represent different conformations of the Na,K-ATPase. Wellsmith and Lindenmayer (1980) found the ^{3}H-ouabain binding sites in their preparation were not interconvertible, but that binding to each site was affected by the presence of K^+. Therefore, these investigators propose that one of the binding sites is an inactive form of Na,K-ATPase. Other evidence indicating ^{3}H-ouabain can be bound to different conformations of Na,K-ATPase is that dissociation of ^{3}H-ouabain from isolated enzyme is described by two exponential equations. These equations are believed to describe dissociation of ^{3}H-ouabain from different forms of the Na,K-ATPase. The relative abundance of each conformation of the enzyme is determined by the presence of K^+ (Choi and Akera, 1977; Godfraind et al., 1980).

Ligand environment is the ultimate determinant of the kinetics of ^{3}H-ouabain binding. Glycoside binding is supported by ligands which promote conformation changes of the enzyme to a binding conformation (Matsui and Schwartz, 1968; Akera et al., 1974). These ligands are Na^+, Mg^{2+} and ATP together or Mg^{2+} and inorganic phosphate together. Other combinations of ligands, such as Mg^{2+} and ATP, can support glycoside binding to a limited extent by affecting enzyme conformation. Alternatively, glycoside binding is inhibited

by ligands which promote changes which take the enzyme out of a binding conformation (Akera and Brody, 1970; Tobin et al., 1974; Inagaki et al., 1974). The species which do this are K^+ itself, or ions such as Rb^+ or Li^+ which substitute for K^+ and promote dephosphorylation of the E_2P intermediate. Inhibition of Na,K-ATPase activity by Ca^{2+} has been shown to occur and be caused by competitive inhibition of Na^+ binding (Tobin et al., 1973). This inhibition requires high concentrations of Ca^{2+} (IC_{50} = 0.5 mM) relative to what is known to be present in cardiac muscle where maximum interaction of contractile filaments occurs when Ca^{2+} reaches a concentration of 10 μM (Fabiato and Fabiato, 1970; Solaro et al., 1974). Therefore, Ca^{2+} itself is not considered to have direct effects on Na,K-ATPase.

In intact cells, affinity of Na,K-ATPase for cardiac glycosides is also affected by the ligand environment. The same principles which apply to the isolated enzyme apply to the Na,K-ATPase in intact cells. Those are, ligands which cause changes to a binding conformation (e.g., intracellular Na^+) stimulate glycoside binding and those which promote changes out of a binding conformation (e.g., extracellular K^+) inhibit glycoside binding (Akera and Brody, 1971; Yamamoto et al., 1980; Temma and Akera, 1982; Kennedy et al., 1983). It is possible however, that in intact cells other mechanisms exist which can affect the affinity of the enzyme for cardiac glycosides or the activity of the sodium-pump at given Na^+ and K^+ concentrations. If such mechanisms exist, then their actions could have significant effects on the therapeutic and/or toxic actions of the cardiac glycosides.

One factor, which is intrinsically tied to the positive inotropic effect of cardiac glycosides and has been reported to affect Na,K-ATPase activity and ^3H-ouabain binding under very specific conditions, is Ca^{2+}. Reports implicating Ca^{2+} as a factor involved in modulation of the sodium-pump or cardiac glycoside

binding are not supported by experiments examining the effect of Ca^{2+} on isolated Na,K-ATPase. Therefore, another factor, such as a Ca^{2+}-binding protein, is likely to be involved in any effect of Ca^{2+} on the Na,K-ATPase which occurs at physiological Ca^{2+} concentrations. The potential interaction between Ca^{2+} and Na,K-ATPase activity or Ca^{2+} and cardiac glycoside binding is very intriguing. If intracellular Ca^{2+} stimulates Na,K-ATPase activity, either by itself or via a Ca^{2+}-binding protein, it would provide a feedback mechanism to control the effects of sodium-pump inhibition on Ca^{2+} transients which affect force of contraction. Alternatively, if intracellular Ca^{2+} inhibits glycoside binding, it may provide a feedback mechanism to control the effects of any endogenous digitalis-like substances.

Removal of Ca^{2+} has been reported to increase the affinity of the Na,K-ATPase from murine plasmocytoma cell membranes for ouabain as determined by a shift in the concentration response curve for ouabain inhibition of Na,K-ATPase activity (Zachowski et al., 1977; Lelievre et al., 1979). Also, short exposure (6 min) of rat hearts to Ca^{2+}-free medium has been reported to increase the affinity of Na,K-ATPase purified from those hearts for ouabain as determined by the same assay (Mansier and Lelievre, 1982; Mansier et al., 1983). The increase in affinity, caused by removal of Ca^{2+} from murine plasmocytoma cell membranes, may be due to removal of some Ca^{2+}-binding protein from the membrane. Evidence for this is that the normal affinity of the Na,K-ATPase for ouabain in these membranes depleted of Ca^{2+} with EDTA is restored by addition of Ca^{2+} and Ca^{2+}-binding proteins (Charlemagne et al., 1980; Geny et al., 1982) in a calmodulin-dependent manner (Lelievre et al., 1985). The Ca^{2+}-binding proteins which decreased the affinity of this Na,K-ATPase for ouabain in a Ca^{2+} and calmodulin-dependent manner were tropomyosin (Charlemagne et al., 1980; Lelievre et al., 1985) and a β-actinin-like protein purified from the cells (Geny et al.,

1982). The effective concentration of Ca^{2+} was less than 1 μM (Lelievre et al., 1985). These experiments demonstrate that, in at least one cell type, it is possible to modulate affinity of the Na,K-ATPase for ouabain in a manner dependent on Ca^{2+} at concentrations which occur within heart muscle and which do not affect activity of the enzyme.

Isolated Na,K-ATPase from rat hearts exposed to Ca^{2+}-free medium also has an increased affinity for ouabain (Mansier and Lelievre, 1982; Mansier et al., 1983). The significance of this is more difficult to evaluate because rat heart is known to contain two forms of Na,K-ATPase with different affinities for the cardiac glycosides (Erdmann et al., 1980; Noel and Godfraind, 1984). Therefore, it is possible that removing Ca^{2+} somehow caused the high affinity form of the enzyme to be selectively retained during isolation. This appears to be extremely unlikely, however, and is seemingly refuted by the observation that preincubation of the ouabain-sensitive enzyme with 10 mM K^+ totally reverses the high affinity inhibition of Na,K-ATPase activity by ouabain of a small percentage of the enzyme (Mansier et al., 1983). Efforts to reverse the effect of Ca^{2+}-free perfusion to increase affinity of Na,K-ATPase, by incubation with Ca^{2+} or Ca^{2+}-binding proteins after the enzyme isolation, were not reported. These studies suggest a link between Ca^{2+} and affinity of Na,K-ATPase from cardiac muscle for cardiac glycosides.

C. Factors Affecting Sodium-Pump Activity

Activity of the Na,K-ATPase can also be studied with isolated enzyme preparations or in intact tissue. From experiments done with isolated enzyme it is known that Na^+ and K^+ each stimulate sodium-pump activity in a concentration-dependent manner. The maximum effect of Na^+ is not achieved unless the Na^+ concentration is greater than 100 mM (Akera et al., 1985) whereas the K_m

for K^+ activation has been estimated to be between 0.9 and 6.3 mM (Cohen et al., 1984).

Experiments examining effects of Ca^{2+} on Na,K-ATPase activity have shown that Ca^{2+} inhibits enzyme activity (Tobin et al., 1973; Godfraind et al., 1977; Beauge and Campos, 1983). A single report that Ca^{2+} at micromolar concentrations stimulates activity of Na,K-ATPase in brain homogenates has been presented (Powis et al., 1983). The latter investigators found that if Ca^{2+} was present at a concentration of 0.1 to 1.0 μM in the absence of any chelating agents, the Na,K-ATPase activity of rat brain homogenates was increased approximately 25%. The presence of calmodulin with 0.1 to 1.0 μM concentrations of Ca^{2+} caused enzyme activity to increase approximately 55%. In the presence of EDTA, basal Na,K-ATPase activity was increased 120% and the effect of Ca^{2+} to stimulate the enzyme was eliminated. Higher concentrations of Ca^{2+} inhibited enzyme activity with an IC_{50} of approximately 200 μM, irrespective of the presence of EDTA. This inhibitory effect of Ca^{2+} on Na,K-ATPase activity is consistent with the work of other investigators who find that the IC_{50} for inhibition of enzyme activity by Ca^{2+} is approximtely 0.5 mM (Tobin et al., 1973; Godfraind et al., 1977; Beauge and Campos, 1983). The report that low concentrations of Ca^{2+} stimulate Na,K-ATPase activity (Powis et al., 1983) is unique but suggests there may be a feedback mechanism other than the Na^+ concentration to regulate the Na,K-ATPase. The ability of calmodulin to amplify the stimulatory effect of Ca^{2+} suggests that it is mediated by a Ca^{2+}-binding protein.

An opposite effect of a factor from human red blood cells has been reported. This factor decreases the IC_{50} for Ca^{2+} inhibition of Na,K-ATPase activity to 1 μM (Yingst and Marcovitz, 1983; Yingst, 1983; Yingst and Polasek, 1985). The nature of the inhibitory factor and the mechanism by which it

interacts with Ca^{2+} to inhibit the Na,K-ATPase are unknown. The presence of factors in cardiac muscle which can interact with Ca^{2+} to stimulate or inhibit Na,K-ATPase in that tissue has not been demonstrated. However, their reported presence in other tissues suggests that similar mechanisms for control of the sodium-pump may exist in the heart.

Sodium-pump activity is the physiological representation of the Na,K-ATPase. Measurement of sodium-pump activity can be accomplished in sodium-loaded cardiac muscle by monitoring a transient outward current upon exposure of those tissues to K^+, Rb^+ or Cs^+ (Gadsby and Cranefield, 1979; Eisner and Lederer, 1980; Daut and Rudel, 1982). This current is inhibited by cardiac glycosides and, therefore, is attributed to activity of the Na,K-ATPase.

The more commonly used method to monitor Na,K-ATPase activity in intact cardiac muscle is by counter-ion transport. This method requires measurement of the uptake of $^{86}Rb^+$ or $^{42}K^+$ with time in the presence and absence of ouabain. The ouabain-sensitive $^{86}Rb^+$ or $^{42}K^+$ uptake is believed to represent ion uptake which is dependent on Na,K-ATPase activity.

In intact cardiac muscle, the intracellular Na^+ activity is less than 10 mM. Activation of Na,K-ATPase, however, is not maximal at less than 100 mM Na^+. Therefore, as the Na^+ influx rate increases, sodium-pump activity will also increase so that only modest increases in intracellular Na^+ concentration occur (Akera and Brody, 1985). This feature of the sodium-pump is responsible for the observed reserve capacity of this enzyme system in cardiac muscle. The fact that the sodium-pump has a reserve capacity means that the measurement of Na,K-ATPase activity in intact tissue is really a measure of Na^+ influx rate unless Na^+ is accumulating in the tissue or has accumulated to the point that the Na^+ concentration is not rate-limiting for Na,K-ATPase activation (Yamamoto et al., 1979; Akera et al., 1981; Akera and Brody, 1985).

In order to determine if an intervention has a direct effect on the Na,K-ATPase an increase or decrease in reserve capacity of the sodium-pump must be documented. Direct modulation of the Na,K-ATPase, other than by specific inhibitors, has not been demonstrated in intact tissue although a decrease in reserve capacity has been shown to occur when Na^+ influx is increased by electrical stimulation (Yamamoto et al., 1980). The decrease in reserve capacity is seen as a shift to the left of the digoxigenin concentration response curve for inhibition of ouabain-sensitive $^{86}Rb^+$ uptake. Stimulation of sodium-pump activity in isolated cardiac myocytes, by interventions causing an increase in Na^+ influx or by sodium-loading the cells, has not been demonstrated. Measurement of sodium-pump activity in Ca^{2+}-tolerant myocytes rather than in intact tissue offers the advantage of only one cell type being present. Additionally, removal of all barriers for ion diffusion may allow for a more accurate determination of sodium-pump activity.

D. Isolated Myocytes as a Preparation for ^{3}H-Ouabain Binding and Ion Flux Studies

Quantitating cardiac glycoside binding to intact cardiac muscle has been attempted with only limited success (Busse et al., 1979; Kjeldsen et al., 1985; Herzig et al., 1985b). Problems associated with this are a high degree of non-specific binding (50% of total binding) and that binding occurs to receptors on many different cell types.

Analysis of kinetic parameters for ^{3}H-ouabain binding has been successfully accomplished using data describing binding to myocytes dissociated from hearts of adult animals (Onji and Liu, 1981; Adams et al., 1982) or cultured cells derived from hearts in the embryonic stage of development (Friedman et al., 1980; Kazazoglou et al., 1983; Kinor et al., 1984; Werden et al., 1984). Each of these preparations appear to be better suited than intact muscle for experiments where

determination of kinetic parameters for drug binding is desired. Each of these preparations also has a drawback, however. Myocytes from adult animals always exist as a mixed population of living, dead and dying cells. Therefore, it is necessary to develop a selection procedure to isolate viable cells with good stability in the presence of millimolar concentrations of Ca^2 if effects of Ca^2 on ^{3}H-ouabain binding are to be examined. Cultured cells have the disadvantage of originating from embryonic tissue which may have very different properties from adult tissue. Additionally, cell cultures usually exist as a mixed population of muscle and non-muscle cell types and while in culture the properties of the cells, including the number of receptors for drugs, may change.

In order to examine effects of Ca^{2+} on ^{3}H-ouabain binding and sodium-pump activity, improved techniques for isolation and selection of Ca^{2+}-tolerant myocytes have been developed. The preparations we use contain greater than 80% rod-shaped cells. The remainder of the cells are dead or dying as determined by a rounded appearance. Although these preparations are not homogeneous, they are currently the best available. Direct effects of interventions on cardiac glycoside binding and sodium-pump activity should be discernable using this myocyte preparation.

E. Objectives

The objective of this study was to develop a preparation with which direct effects of agents or interventions on cardiac glycoside binding to Na,K-ATPase and on sodium-pump activity could be examined in intact cardiac muscle cells. This preparation was then to be used to examine effects of intracellular Ca^{2+} and sarcolemmal phosphorylation on ^{3}H-ouabain binding and sodium-pump activity.

MATERIALS AND METHODS

A. Myocyte Isolation

Isolation of viable myocytes from mammalian heart in high yield is a difficult task which on several occasions during the course of these experiments seemed to be impossible. Reasons for variable degrees of success could often be attributed to the collagenase used for the digestion. The protocol for isolation as written here has evolved over two years of experiments, during which time numerous variations were attempted to increase yield and viability of myocytes. The basic procedure, however, has remained intact over the entire time. This procedure for myocyte isolation has been used successfully to isolate viable myocytes in high yield from hearts of guinea-pig, rat, ferret and rabbit.

Myocytes were isolated from guinea-pig hearts by digestion with collagenase plus hyaluronidase. Male animals weighing 450 to 600 g were injected with heparin (700 units, i.p.) and sacrificed 40 min later by a sharp blow to the neck. Heart and lungs were quickly removed and immediately perfused with a modified Krebs-Henseleit bicarbonate (KHB) buffer solution at 37°C with a constant flow rate of 6.5 ml/min. The composition of KHB solution was 118 mM NaCl, 27.1 mM $NaHCO_3$, 2.8 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 2.5 mM Na-pyruvate and 10.0 mM dextrose. The solution was saturated with a 95% O_2, 5% CO_2 gas mixture yielding a final pH value of 7.4. We found it important to prepare the heart so that circulation would be maintained during perfusion with solutions containing low concentrations of Ca^{2+}. This was achieved by leaving as much of the free aorta as possible below the inlet cannula, cutting the pulmonary

artery close to its origin and occluding the pulmonary veins with a silk suture tied between the heart and lungs before removal of the lungs. After a 15 min perfusion with KHB buffer, perfusion solution was changed to one containing 105.1 mM NaCl, 20.0 mM $NaHCO_3$, 2.8 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.01 mM $CaCl_2$, 5.0 mM mannitol, 10.0 mM taurine (2-aminoethanesulfonic acid), 10.0 mM dextrose, 5.0 mM Na-pyruvate and saturated with a 95% O_2, 5% CO_2 gas mixture. During the perfusion with this low Ca^{2+} solution, contractions ceased and left atria became swollen. Time of perfusion with low Ca^{2+} solution before addition of enzymes was usually 8 min but was occasionally decreased to as little as 5 min to enhance subsequent digestion.

Following the initial perfusion with a low Ca^{2+} solution, perfusion with low Ca^{2+} solution containing collagenase (0.52 mg/ml) and hyaluronidase (0.2 mg/ml) was started. Effluent from the hearts was discarded during the first 3 min of perfusion with solution containing enzymes. After the first 3 min, the effluent was collected and recirculated. During this perfusion, hearts were immersed in the recirculating medium and flow-rate was reduced to 5 ml/min for each heart. Perfusing solution was continuously bubbled with a 95% O_2, 5% CO_2 gas mixture during the 52 min digestion period.

Subsequent to digestion, ventricular muscle was excised from each heart and minced into 12 ml of a solution composed of 3 parts KHB solution and 1 part fresh low Ca^{2+} solution containing collagenase and hyaluronidase and maintained at 37°C. The minced muscle (small chunks of 1 to 2 mm diameter and a fluffy layer of single cells and cell aggregates) was permitted to sediment and most of the supernatant solution was aspirated away. Additional KHB solution (4 ml, 37°C) was added to the sediment of minced muscle. Fresh KHB solution (4 ml, 37°C) was added twice more without removal of supernatant. Between additions of KHB

solution, sedimented materials were drawn in and out of a wide mouthed pipet to disaggregate the myocytes.

Following disaggregation, cell suspensions from individual hearts were filtered through a stainless steel mesh (pore size, 400 μm) and resulting suspensions were diluted to a final volume of approximately 30 ml with KHB solution containing 1.8 mM $CaCl_2$. The suspension was centrifuged at 60 x g for 15 sec. Supernatant solution was discarded and rod-shaped myocytes were selected for by gravity sedimentation through KHB solution. Dispersed cells were separated from cell aggregates and tissue debris by filtration through a nylon mesh (pore size, 200 μm). Myocytes passing thrugh the nylon mesh were collected by gravity sedimentation and loaded into a glass column (29 cm long; 0.8 cm internal diameter). The column was adjusted to an angle of 2 to 5 degrees from vertical. Flow of KHB solution at a rate of 3.0 ml/min from the bottom to the top of the column maintained viable cells within the column. Viable cells preferentially aggregated into loose clusters which filled 40 to 60% of the column.

Cells were maintained in the column for 60 min, during which time the flow rate was periodically increased to 10 ml/min for short periods to prevent the cells from forming a packed pellet at the bottom of the column. Myocytes were removed from the column 10 min before the start of an experiment, and rod-shaped cells were selected for by 2 to 3 gravity sedimentations in KHB solution.

The single most important part of myocyte isolation is to ensure that tissue digestion is sufficient so that minimum mechanical disaggregation is necessary. Best results were obtained when hearts were digested to such an extent that some hearts would fall from the cannula when the support of the enzyme solution they were immersed in was removed.

Selection of collagenase was by trial and error. Samples from all available sources were tested using a single protocol. The collagenase producing the best

results was purchased and the digestion procedure "fine tuned" for optimal results with that batch of enzyme. The following points were modified to enhance digestion: time of perfusion with low Ca^{2+} solution prior to addition of enzymes; time of recirculating perfusion with enzymes; and volume of recirculating perfusate with enzymes. Increasing the time of recirculating perfusion with enzymes always increased digestion of the heart, but this was avoided because extended time in low Ca^{2+} solution appeared to decrease cell viability. Time of perfusion with low Ca^{2+} solution prior to addition of enzymes was decreased from the standard 8 min to as little as 5 min to enhance subsequent digestion. The mechanism responsible for enhancement of digestion is unknown but may be a decrease in time for efflux of some factor from the heart before the effluent is collected for recirculation. This same mechanism may also be involved in enhancement of digestion when volume of recirculating perfusate is reduced.

B. ^{3}H-Ouabain Equilibrium Binding Studies

Specific binding of ^{3}H-ouabain to Na,K-ATPase from cardiac muscle has been shown to correlate well with inhibition of the enzyme (Erdmann et al., 1976). Specific binding of ^{3}H-ouabain causes inhibition of the sodium-pump in intact tissue and isolated myocyte preparations (Werden et al., 1983). Inhibition of the sodium-pump by cardiac glycoside binding to the Na,K-ATPase is believed to be responsible for the positive inotropic effect of these agents (Akera et al., 1974). Binding of ^{3}H-ouabain to cardiac myocytes was examined to characterize the binding of this glycoside to the Na,K-ATPase in intact cardiac muscle cells and to determine if affinity of the Na,K-ATPase or number of ^{3}H-ouabain binding sites in intact cells could be altered by acute or chronic interventions.

Affinity of binding sites for ^{3}H-ouabain and the number of binding sites were estimated using the method of Akera and Cheng (1977) or by analysis of

Scatchard plots. For analysis by the method of Akera and Cheng, myocytes (0.2-0.6 mg protein) were incubated in 1.2 to 2.0 ml of KHB solution or homogenates (0.3-0.4 mg protein), were incubated in 1.5 ml of a solution containing 1.0 mM $MgCl_2$, 1.0 mM Tris-phosphate, and 10.0 mM Tris-HCl (pH 7.5 with Tris-base). Homogenates were prepared from myocytes which had been frozen. Myocytes were homogenized by several strokes with a Dounce ball-type homogenizer.

Incubation tubes were prewarmed to 37°C and maintained at that temperature for the duration of the incubation (35-90 min). Solutions for cell incubation were saturated with 95% O_2, 5% CO_2 before addition to incubation tubes. Tubes containing cells were filled with the same gas mixture and sealed with marbles. Solutions for homogenate incubation were not exposed to any gas except room air.

Each incubation tube contained ^{3}H-ouabain (50 or 100 nM, final concentration), and various concentrations of non-labeled ouabain (0, 0.05, 0.1, 0.3, 1.0, 3.0 or 300 μM) were present in equal numbers of tubes. After incubation, the binding reaction was stopped and cells were collected on GF/C or homogenates were collected on GF/B fiberglass filters (Whatman). Specific ^{3}H-ouabain binding was calculated by subtracting the binding observed in the presence of 300 μM non-labeled ouabain. Affinity (K_D) and ^{3}H-ouabain binding site number (B_{max}) were then estimated from linear plots on logarithmic probability paper describing the inhibition of specific ^{3}H-ouabain binding by the non-labeled ouabain.

For determination of kinetic parameters of ^{3}H-ouabain binding by Scatchard analysis, myocytes (0.2-0.4 mg protein) were incubated in 1.2 ml of KHB solution containing various concentrations (0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, 5.0, 10.0 or 20.0 μM) of ^{3}H-ouabain. Cells were incubated in the presence of ^{3}H-ouabain for 60 min then either collected by a centrifugation method, which is described in detail later, or resuspended in a 100-fold volume (30 ml) of KHB solution containing a concentration of non-labeled ouabain equal to the concentration of

^{3}H-ouabain the cells had been incubated with and 13.8 mM KCl. Dissociation of ^{3}H-ouabain was allowed to proceed for 60 min then cells were collected by centrifugation. Samples of cells collected immediately following the 60 min incubation with ^{3}H-ouabain or after that incubation followed by a 60 min incubation for dissociation of bound drug were analyzed for determination of total binding and non-specific binding, respectively. Specific binding to the Na,K-ATPase was calculated as the difference in these values.

C. Dissociation of ^{3}H-Ouabain from Myocytes

Differential rates for dissociation of bound ^{3}H-ouabain from specific and non-specific binding sites allow for estimates of the relative incorporation of ^{3}H-ouabain into each of these sites in whole tissue by monitoring dissociation after binding (Kjeldsen et al., 1985). Relative amounts of ^{3}H-ouabain bound to different conformations of Na,K-ATPase can also be determined by monitoring dissociation of drug bound to the isolated enzyme (Choi and Akera, 1976). Dissociation of ^{3}H-ouabain from myocytes was examined to determine the relative amount of non-specific binding and to substantiate that ^{3}H-ouabain can bind to more than one conformation of the Na,K-ATPase in intact myocytes.

Time courses for dissociation of ^{3}H-ouabain from myocytes were monitored following incubation of cells in 0.2, 0.5 or 2.5 μM ^{3}H-ouabain for 40 or 60 min. Cell pellets were collected by gravity sedimentation after the binding reaction and sampled by centrifugation at 1500 x g for 2 min to determine total ^{3}H-ouabain bound before the initiation of dissociation. The remainder of the cell pellet was dispersed in a 100- or 200-fold volume of KHB solution containing a concentration of non-labeled ouabain equal to the concentration of ^{3}H-ouabain the cells had been incubated with. Concentration of K^+ in the binding and dissociation media were varied as described for individual experiments. After

resuspension of myocytes to initiate dissociation 6.0 ml of well-mixed cell suspension was removed at regular time intervals for up to 3 hours. Aliquots of cell suspension were taken with an adjustable volume pipet and cells collected by filtering the suspension through silanized GF/C fiberglass filters (Boehringer Mannheim). Supernatants from the KHB solution in which cells had been incubated for binding of ^{3}H-ouabain were centrifuged for 2 min at 1500 x g to remove cells. These supernatants were then added to an aliquot of solution identical to the dissociation medium in proportion to the volume of cells which had been added. This solution was then sampled four times in a manner identical to that used for sampling cell suspensions. The mean amount of radioactive material retained on the filters was used as an estimate of the background ^{3}H-ouabain binding to filters. Dissociation rates from different sites were determined to be linear exponential processes by use of a curve-peeling procedure or by subtracting the ^{3}H-ouabain remaining bound after 60 min of dissociation from the amount remaining bound at all earlier time points. Dissociation rate constants (k^{-1}) were estimated from the slopes of the linear exponential processes.

D. $^{86}Rb^{+}$ Uptake Studies

Sodium-pump activity was estimated in myocytes as the difference in $^{86}Rb^{+}$ uptake observed in the absence and presence of 1 mM ouabain. Myocytes used in $^{86}Rb^{+}$ uptake studies were prepared in the normal manner except that a modified KHB solution containing 5.0 mM RbCl and 1.0 mM NaH_2PO_4 was used instead of one containing 2.8 mM KCl and 1.0 mM KH_2PO_4 from the time cells were loaded on columns for the 60 min elutriation. Uptake of $^{86}Rb^{+}$ was initiated by adding cells to prewarmed (37°C) incubation tubes containing 2.0 ml of the modified KHB solution with 5.0 mM RbCl and tracer amounts of $^{86}Rb^{+}$. Non-specific $^{86}Rb^{+}$ uptake was estimated as the uptake of $^{86}Rb^{+}$ not inhibited by the presence of 1

mM ouabain. Exposure of myocytes to ouabain and $^{86}Rb^+$ were simultaneous when estimates of non-specific uptake were made. After 6 min of exposure to $^{86}Rb^+$, myocytes were collected by centrifugation and then sampled for determination of $^{86}Rb^+$ and protein content. Specific activity of $^{86}Rb^+$ was measured directly by sampling the incubation media after removing cells.

Sodium-pump capacity can only be measured when the intracellular Na^+ concentration is not the factor limiting sodium-pump activity (Akera et al., 1981). Capacity of the sodium-pump was examined using cells which had been preincubated for 5 min with maximally effective concentrations of the Na^+-ionophore monensin, or using cells which had been sodium-loaded by incubation in medium devoid of K^+ and Rb^+. These interventions each increase sodium-pump activity as estimated by ouabain sensitive $^{86}Rb^+$ uptake.

Ouabain sensitive $^{86}Rb^+$ uptake was estimated using KHB solutions containing 2.0 mM RbCl when determining sodium-pump capacity. For these experiments the cells were preincubated with monensin. Myocytes were added to prewarmed (37°C) incubation tubes containing monensin. After a 5-min incubation in the presence of monensin, tracer amounts of $^{86}Rb^+$ were added to the incubation tubes. Cells were collected by centrifugation after a 3-min exposure to $^{86}Rb^+$ then sampled for determination of $^{86}Rb^+$ and protein content. Non-specific $^{86}Rb^+$ uptake was estimated by adding 1 mM ouabain (final concentration) in solution with the $^{86}Rb^+$.

Myocytes were sodium-loaded by elutriation in a glass column with KHB solution devoid of K^+ and Rb^+ and containing only 10 μM Ca^{2+}. Cells were maintained in the column at 37°C with a continuous flow (3 ml/min) of fresh solution to remove any K^+ or Rb^+ released by the cells. Under these conditions, Na^+ accumulates in the cells because a counter-ion to support efflux of Na^+ from

myocytes by the Na,K-ATPase is not available. Effectiveness of the sodium-loading procedure was determined by removing cells from the column at 15 min intervals and measuring ouabain-sensitive $^{86}Rb^+$ uptake as described earlier, with incubations taking place in 5.0 mM Rb^+ KHB solution containing tracer amounts of $^{86}Rb^+$. Incubations were 2 min in duration for $^{86}Rb^+$ uptake into Na^+-loaded cells.

Reserve capacity of the sodium-pump is decreased by interventions which increase the Na^+ concentration inside cells. Concentration dependence for ouabain inhibition of sodium-pump activity in non-stimulated myocytes or myocytes exposed to 50 μM monensin was determined to demonstrate that increased Na^+ influx reduces sodium-pump reserve capacity. Myocytes were exposed to 0.1, 0.3, 1.0 or 3.0 μM ouabain for 60 min before initiation of $^{86}Rb^+$ uptake in solutions containing the same concentration of ouabain. Ouabain-sensitive $^{86}Rb^+$ uptake was measured as described earlier with incubations taking place in 5.0 mM Rb^+ KHB solution with tracer amounts of $^{86}Rb^+$ for 6 min.

E. Separation of Myocytes and Bound Ligands from Non-bound Ligands

Rapid filtration and centrifugation methods were utilized to collect myocytes and separate bound from non-bound ligands. Collecting samples by rapid filtration is the standard method used in studies examining ^3H-ouabain binding to isolated enzyme or tissue homogenate preparations. This method also worked well for collection of isolated myocytes after ^3H-ouabain binding. Myocytes incubated with ^3H-ouabain were poured onto fiberglass filters after stopping the binding reaction by the addition of 5 ml of an ice-cold solution, comprised of 0.1 mM non-labeled ouabain, 15 mM KCl and 50 mM Tris-HCl buffer (pH 7.5). Filters and myocytes were washed twice with 5 ml volumes of the same solution used to stop the reaction. Cells were always collected on fiberglass filters. Effective pore

size and preconditioning of filters with dimethyldichlorosilane varied according to the experiment. Myocytes trapped on filters were digested with 0.6 ml of tissue solubilizer (Protosol, NEN). Scintillation fluid (9 ml) using PPO (2.5 diphenyloxazole) and POPOP (1,4-bis [2-(4-methyl-5-phenyloxazolyl)]-benzene) as scintillants was added to each vial and radioactivity was assayed using a liquid scintillation spectrophotometer. Counting efficiency (approximately 30%) was monitored by the external standard channel ratio.

Collection of myocytes by centrifugation was a necessity for experiments examining $^{86}Rb^{+}$ uptake because background radioactive material trapped by fiberglass filters in these experiments often exceeded the total $^{86}Rb^{+}$ in samples of myocytes. The centrifugation method was also advantageous because both label and protein were determined after the binding or uptake reaction was complete. Therefore, protein concentration in incubation tubes could vary somewhat. Protein concentration in incubation tubes was difficult to control because myocytes settle quickly in KHB solution and repeated agitation in a manner vigorous enough to uniformly disperse cells resulted in damage to myocytes.

Centrifuge tubes for sampling were prepared by pipeting 4.0 ml of a sucrose solution containing 280 mM sucrose, 10.0 mM procaine HCl and 0.9 mM $CaCl_2$ into 15 ml polypropylene tubes (Becton-Dickinson), then slowly adding 9.0 ml of a less dense solution containing 99.0 mM NaCl, 30.0 mM RbCl, 20.0 mM procaine HCl, 10.0 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 2.0 mM $BaCl_2$, 0.9 mM $CaCl_2$, 0.1 mM ouabain and a trace of Patent Blue Violet dye (pH 7.1 with 2 M NaOH). The less dense solution was added slowly so at least 3 ml of the sucrose layer remained unmixed with the less dense layer. These "sampling tubes" were placed in a NaCl-ice bath with the temperature adjusted to $-2^{o}C$ and were allowed to stand at least 1 hour before use.

Prior to sampling, myocytes incubated with ^{3}H-ouabain or $^{86}Rb^{+}$ were allowed to settle in incubation tubes to as great an extent as possible within the confines of individual experiments. At the time ^{3}H-ouabain binding or $^{86}Rb^{+}$ uptake reactions were to be stopped, 0.5 ml of solution containing cells was taken from the bottom of incubation tubes and added to a centrifuge tube prepared as described earlier. Homogeneous dispersion of the sample is calculated to increase the temperature of the less dense solution to 0°C. Therefore, all reactions involving Na,K-ATPase would be virtually stopped. Preliminary experiments showed ^{3}H-ouabain or $^{86}Rb^{+}$ content of myocytes did not change significantly when centrifugation was delayed for up to 15 min after samples were added to centrifuge tubes.

Cells were collected by centrifuging the samples for 2 min at 1500 x g in a refrigerated centrifuge maintained at 2°C. The less dense solution was identified by its blue color. The blue solution containing non-bound ^{3}H-ouabain or $^{86}Rb^{+}$ was aspirated from the centrifuge tube leaving approximately 3 ml of sucrose solution covering the cell pellet. Tubes with cell pellets were frozen rapidly by immersing them in methanol at -30°C. Cell pellets were collected by cutting the bottom from the frozen tubes and then analyzed to determine content of radioactive material and protein.

Samples for determination of $^{86}Rb^{+}$ content were analyzed without further treatment using a gamma counter. Samples for determination of ^{3}H-ouabain content were dried overnight at room temperature then wetted with 75 µl of distilled water before digesting and counting as described for filtered samples. Wetting of samples was necessary to maintain the sucrose in solution after addition of scintillation fluid.

F. Silane Treatment for Fiberglass Filters

Estimates of non-specific binding of ^{3}H-ouabain to myocytes and fiberglass filters were large and varied to an extent that it was difficult to have confidence in these estimates. Accurate estimation of non-specific ^{3}H-ouabain binding to filters was particularly important when determining non-specific ^{3}H-ouabain binding to cells. Silane treatment of filters reduced average non-specific binding to GF/C filters from Boehringer Mannheim only slightly but significantly reduced variation in ^{3}H-ouabain binding to the filters.

Silanization was accomplished in the following manner. Approximately 100 filters were stacked on a stainless-steel mesh inside a 50-ml plastic syringe fitted with a stopcock. The syringe was suspended vertically and a second mesh placed on top of the filters. Dimethyldichlorosilane (1% in hexane) was poured over the filters until filters and mesh overlay were immersed. The silane solution was pressed out of filters 30 min after its addition and the residual silane was removed by two washes with hexane. Following each hexane wash, the hexane was also pressed out of the filters. Methanol was poured over the filters after the second rinse with hexane. Filters remained immersed in methanol for 30 min then methanol was pressed out of the filters and a final hexane rinse was done in a manner identical to the initial hexane rinse. Filters were dried under vacuum and those in contact with the stainless steel mesh during the silanization procedure were discarded.

This procedure could not be used for Whatman fiberglass filters.

G. Experiments with K^{+}-Deficient Animals

Changes in the density of active sodium-pump sites or activity of the Na,K-ATPase may occur in animals with reduced serum K^{+} concentration (Ward and Cameron, 1984). To substantiate that the sodium-pump was operating at capacity

in myocytes exposed to high concentrations of monensin or following loading with Na^+ in solutions devoid of K^+ and Rb^+, attempts were made to increase sodium-pump capacity by increasing the density of Na,K-ATPase by depleting animals of K^+.

Guinea pigs were depleted of K^+ by maintaining the animals on a K^+-deficient diet. Animals were housed in individual cages and were fed a diet low in K^+. Control animals were fed a diet of the same composition supplemented with K^+. Animals were maintained on either of these diets for 2 to 3 weeks before hearts were removed for cell preparation.

Depletion of K^+ was assessed by measuring activities of Na^+ and K^+ in serum using a Na^+ and K^+ activity analyzer (Orion). Serum was prepared from blood drawn from ketamine (40 mg/kg, i.m.) anesthetized animals.

Kinetic parameters for ^3H-ouabain binding to myocytes were determined by the method of Akera and Cheng (1977) as previously described. Sodium-pump activity in the absence and presence of monensin was measured as previously described.

H. Miscellaneous Methods

Protein determinations were by the method of Bradford (1975) using a dye concentrate purchased from Bio-Rad. This method was necessary due to the variable sucrose content in samples obtained by the centrifugation method. Sucrose has been shown to interfere with other procedures for the determination of protein such as the Lowry and the Biuret procedures (Bradford, 1975).

Statistical analysis was by grouped t-test, paired t-test or analysis of variance. Significance was established by a P value of < 0.05.

I. Materials

Tritium-labeled ouabain (generally labeled, specific radioactivity 20.0 Ci/mmol) and ^{86}RbCl (concentration and specific radioactivity variable) were purchased from New England Nuclear, Boston, MA.

Collagenase was purchased from Cooper Biomedical, Malvern, PA, Boehringer Mannheim, Indianapolis, IN, and Sigma Chemical Company, St. Louis, MO. Hyaluronidase, dimethyldichlorosilane and Patent Blue Violet dye were all purchased from Sigma Chemical Company.

Filters were purchased from Boehringer Mannheim (fiberglass filters, 24 mm), or Fisher Scientific Company (Whatman GF/C and GF/B fiberglass filters), Pittsburgh, PA.

Tissue solubilizer was purchased from New England Nuclear. Dye reagent concentrate for protein analysis was purchased from Bio-Rad Laboratories, Richmond, CA. Polypropylene centrifuge tubes (Becton-Dickinson) were purchased from Sargent-Welch, Livonia, MI. Heparin sodium (from beef lung) was purchased from the Upjohn Company, Kalamazoo, MI. The K^+-deficient diet was purchased from BioServ Inc., French Town, NJ.

RESULTS

A. Isolation of Myocytes from Guinea-pig Heart

Isolation of Ca^{2+} tolerant myocytes from mammalian heart has been described by many investigators (eg: Farmer et al., 1977; Isenberg and Klockner, 1982). The procedure described here which was used to isolate myocytes from guinea-pig heart is not substantially different from others which have been published. Populations of myocytes used for experiments contained greater than 80% of cells which were rod-shaped when incubated in solutions containing millimolar concentrations of Ca^{2+}. Rounded cells in the preparations accounted for less than 20% of all cells and were apparently incapable of maintaining a low intracellular Ca^{2+} concentration, as shown by their hypercontracted state. Approximately 50% of rounded cells excluded the dye Trypan Blue; therefore, it is possible that some rounded cells had not completely lost membrane integrity. Photographs of typical myocytes are shown in Figure 1. Rod-shaped cells have clear striations exclude trypan blue, have normal resting membrane potential[a] and respond to electrical stimulation with discrete contractions. These features indicate that the cells have intact membranes, are alive and have normal responses to stimuli. Details on the surface of these myocyes were examined by transmission electron microscopy.[b] An electron photomicrograph of one cell is shown in Figure 2.

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[a]Membrane potentials of myocytes were measured using microelectrodes by Robert W. Hadley working in the laboratory of Dr. Joseph R. Hume at Michigan State University.

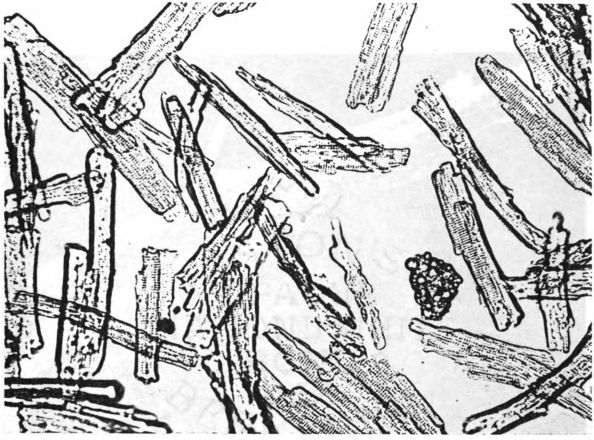

Figure 1. Photograph of ventricular myocytes isolated from guinea-pig heart by treatment with collagenase and hyaluronidase. Typical preparations had greater than 80% of cells rod-shaped and quiescent in solution containing 1.8 mM Ca^{2+}.

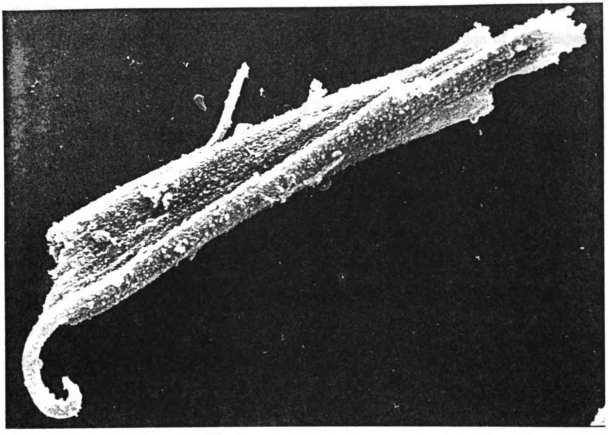

Figure 2. Electron micrograph of a single cardiac myocyte. Myocytes were fixed in 10% glutaraldehyde solution then prepared for examination by scanning electron microscopy using standard techniques.

Myocytes were isolated and rod-shaped cells selected as described in Methods. Purification of myocytes was repeated until microscopic examination indicated greater than 80% of the cells in the preparation were quiescent and maintained a rod shape. This usually required two or three sedimentations of myocytes through KHB solution at unit gravity. Further selection of rod-shaped cells by gravity sedimentation is possible but was usually not attempted due to excessive loss of tissue. Isolated myocytes were very sensitive to mechanical stimuli. The fragile nature of these cells precludes more vigorous methods for selection of rod-shaped cells. The relative percentage of rod-shaped cells in preparations was very consistent due to it being controlled. Also, each population of myocytes acted as its own control as experiments were designed so each intervention or concentration of drug was tested in every population of myocytes used for a particular experiment. Therefore, no attempt was made to quantitate the percentage of rod-shaped cells in individual preparations unless the viability of cells was being examined.

1. Viability of Guinea-Pig Myocytes Incubated in the Presence or Absence of Ouabain

Equilibrium of ouabain binding to Na,K-ATPase from guinea-pig heart requires greater than a 30-min incubation of the glycoside with the enzyme as judged by the inotropic response of guinea-pig atrial muscle to ouabain (Ku et al., 1976). It is well documented however, that high concentrations of cardiac glycosides are toxic to cardiac muscle preparations. To determine if myocytes isolated from guinea-pig heart were stable in solutions containing ouabain, cells were incubated for 60 min in KHB solution containing 1.0 μM or 1.0 mM ouabain.

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[b]Electron microscopy was performed by Vivion E. Shull and Dr. Karen Baker at the Electron Optics Center at MSU.

Myocytes from the same preparation were incubated for 60 min in KHB solution without ouabain or sampled immediately after addition to an incubation solution. Myocytes were sampled by dispersing 0.3 ml of cell suspension into 2.0 ml of 10% formalin. Dispersing of myocytes in formalin caused cells to be fixed in either a rounded or a rod shape. Fixed cells were counted using a hemocytometer. Relative amounts of rod-shaped and rounded cells were determined after counting at least 100 cells.

Incubation of guinea-pig myocytes in KHB solution at 37°C for 60 min caused a significant decrease in the percentage of cells retaining a rod shape (Figure 3). Myocytes incubated with 1.0 μM ouabain, however, showed no loss of viable cells as determined by maintenance of the rod shape. Since a concentration of 1.0 μM ouabain can be toxic to preparations of contracting cardiac muscle from guinea pig, the protective effect on myocytes is surprising. It is conceivable that this reflects a decreased energy demand on the cells due to inhibition of some pump units, or a decrease in Na^+ influx because the cells are quiescent. Complete inhibition of the sodium-pump by 1 mM ouabain caused almost complete loss of viability after a 60 min exposure (Figure 3). Microscopic examination of these cells prior to fixing in formalin showed that cells still retaining a rod shape were contracting spontaneously. The contractions were not discrete but more closely resembled a writhing motion indicating that the cells were dying.

B. Binding of ^{3}H-Ouabain to Myocytes Isolated from Guinea-pig Heart

Inhibition of Na,K-ATPase is believed to be responsible for the positive inotropic effect of cardiac glycosides on cardiac muscle (Akera et al., 1970; Ku et al., 1976). Binding of cardiac glycosides to the Na,K-ATPase results in inhibition of this enzyme (Matsui and Schwartz, 1968; Gelbart and Goldman, 1977). Kinetic

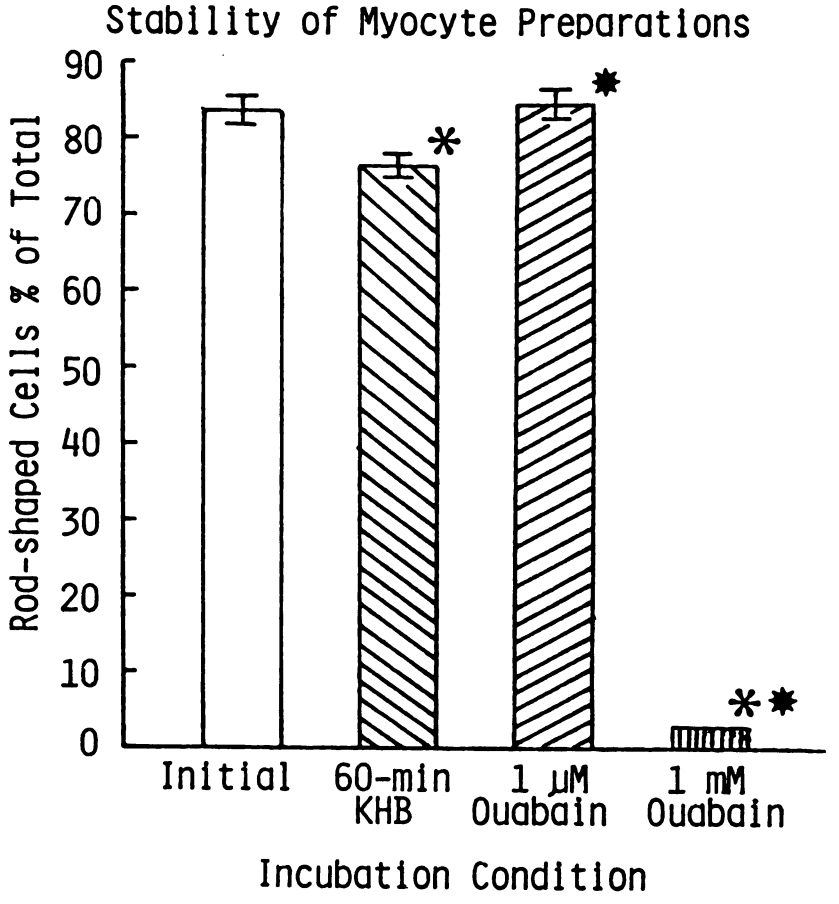

Figure 3. Viability of myocytes from guinea-pig heart in the presence of ouabain. Myocytes were isolated and rod-shaped cells were selected as described in the text. Bars represent the percentage of myocytes in the entire population which were rod-shaped at initiation of incubation ☐ , following a 60-min incubation ▨ , following a 60-min incubation with 1.0 µm ouabain ▨ , following a 60-min incubation with 1.0 mM ouabain ▥ . All incubations were in KHB solution containing 1.8 mM Ca^{2+}. Percentages were calculated after counting at least 100 cells which had been fixed by being sampled into 10% formalin at the indicated time (n=5). * significantly different from the percentage rod-shaped at the initiation of incubation, ✸ significantly different from the percentage rod-shaped after a 60-min incubation in KHB solution. Significance ($p < .05$) determined by analysis of variance with individual comparisons by method of least significant differences.

parameters of binding (association rate constant, k^1; and dissociation rate constant, k^{-1}) determine the extent of enzyme inhibition at any given concentration of a cardiac glycoside and may be subject to modification by many factors. These factors would, therefore, contribute to the regulation of the pharmacological effects of the glycoside.

Many factors which affect glycoside binding to Na,K-ATPase can be studied using preparations of isolated enzyme. Other factors may be lost or inactivated during preparatory procedures or tissue homogenization and therefore, must be examined in intact cells. Possible binding sites for glycosides other than the Na,K-ATPase may also be lost or exposed during tissue disruption and the question of their existence must be addressed using intact cells. Another advantage of intact cells over tissue fractions for examining glycoside binding to Na,K-ATPase is the maintenance of ionic gradients across the sarcolemmal membrane. This is advantageous because ionic environments on the internal and the external sides of the sarcolemma can be changed individually. Also, living myocytes contain an ATP regenerating system which ensures an adequate supply of substrate for the ATPase. The presence of ionic gradients in intact cells is an aspect of this preparation which must be considered during the design of experiments. In comparison to isolated enzyme preparations, the presence of ionic gradients and possible changes in ionic gradients subsequent to sodium-pump inhibition in intact cells, can confound interpretation of binding data.

Examining glycoside binding in isolated myocytes is a vastly superior technique to examining the binding in intact cardiac muscle. This is evident from a comparison of published reports of ^{3}H-ouabain binding to intact cardiac muscle with data reported here. Advantages of myocytes over whole tissue are the presence of only one cell type, the lack of diffusion barriers and a much reduced

amount of non-specific binding. Estimation of kinetic parameters for ^{3}H-ouabain binding to receptors in Ca^{2+} tolerant myocytes isolated from guinea-pig heart was done to characterize the binding of this cardiac glycoside to its receptor(s) in intact cells. Possible existence of regulatory mechanisms for ^{3}H-ouabain binding unique to intact tissue was then studied by incubating cells with agents known to affect the contractile state of cardiac muscle.

1. Estimation of Non-specific ^{3}H-Ouabain Binding to Guinea-pig Myocytes

Accurate estimation of kinetic parameters for ^{3}H-ouabain binding to Na,K-ATPase requires adequate ligand concentrations and accurate estimation of non-specific binding. Cofactors for the Na,K-ATPase, besides the substrates Na^{+} and K^{+}, are ATP and Mg^{2+}. These ligands are normally maintained at adequate concentrations in viable cells. For Scatchard analysis of binding data, myocytes must be incubated in a range of ^{3}H-ouabain concentrations so that occupancy of 5 to 95% of the binding sites will be described (Burgisser, 1984). Because the sodium-pump is inhibited by ouabain binding to Na,K-ATPase and total inhibition of the sodium-pump destroys the myocytes, equilibrium of the binding reaction with 95% occupancy of binding sites is probably not feasible.

A potentially more serious problem is the necessity for accurate estimates of non-specific binding. Because an effective antagonist for glycoside binding which does not itself inhibit the Na,K-ATPase is not available, an excess of non-labeled drug is included in reaction mixtures when determinations of non-specific binding to isolated enzyme or tissue homogenate preparations are made. Myocytes incubated with concentrations of ouabain sufficient to block specific binding of labeled drug undergo an extreme morphological change within 60 min (Figure 4). Media in which these cells are incubated become yellow, indicating that some cell lysis has occurred. The morphological changes resulting from ouabain toxicity can potentially affect non-specific binding of ^{3}H-ouabain,

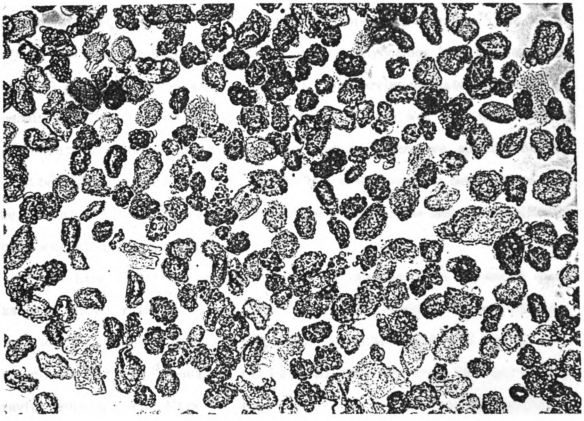

Figure 4. Photograph of myocytes from guinea-pig heart following a 60-min incubation in KHB solution containing 1.8 mM Ca^{2+} and 1.0 mM ouabain. Toxic effects of ouabain at this concentration cause morphological changes in the cells. Lysis of some myocytes was evident with microscopic examination and by a yellow color of the incubation medium containing the cells.

causing it to be under- or overestimated. Another consideration when estimating non-specific binding of ^{3}H-ouabain to intact cells is the potential uptake of ^{3}H-ouabain into the myocytes as a component of non-specific binding. The Na,K-ATPase has been proposed to transport cardiac glycosides into cardiac cells (Dutta et al., 1968; Fricke and Klaus, 1977). Any carrier-mediated influx of ^{3}H-ouabain would be blocked if excess non-labeled ouabain was used when making determinations of non-specific ^{3}H-ouabain binding. Therefore, specific binding would be overestimated. For these reasons it was necessary to substantiate that the amount of ^{3}H-ouabain retained in samples of myocytes and not specifically bound was estimated correctly.

Kinetics of the dissociation of ^{3}H-ouabain from myocytes were examined in an attempt to estimate non-specific binding. Myocytes were incubated in a non-toxic concentration of ^{3}H-ouabain (0.2 μM) for 60 min. Dissociation of bound ^{3}H-ouabain was initiated after collecting the myocytes by gravity sedimentation. Myocytes were resuspended in a 100-fold volume of KHB solution containing an identical concentration of non-labeled ouabain. This suspension was sampled at various time points and bound ligand separated from non-bound ligand by rapid filtration. Dissociation of bound ^{3}H-ouabain from guinea-pig myocytes occurred in two phases (Figure 5). ^{3}H-Ouabain associated with the site from which dissociation was rapid accounting for 84.6±1.1 percent (n=6) of bound drug.

A plot of these data on a semilogarithmic scale produced an upwardly concave curve describing the first sixty minutes of dissociation (Figure 6). The portion of the curve describing dissociation at time points greater than 60 min appeared to become a straight line parallel to the X-axis. The estimates of ^{3}H-ouabain bound to myocytes after greater than 60 min of dissociation were not sufficiently precise to allow the fitting of a straight line and its extrapolation to

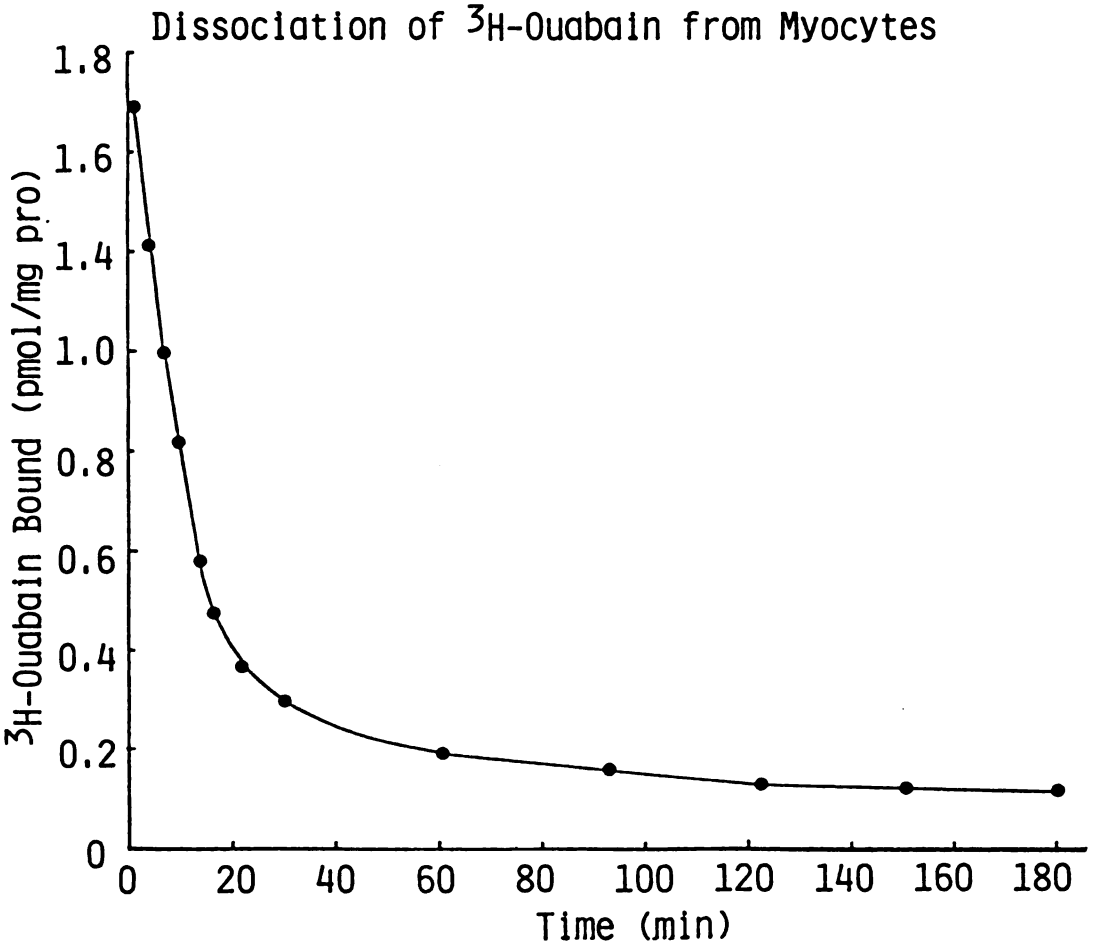

Figure 5. Representative time course for the dissociation of ^{3}H-ouabain from myocytes. Myocytes from guinea-pig heart were incubated in the presence of 0.2 µM ^{3}H-ouabain for 60 min. Cells were collected by gravity sedimentation and the cell pellet suspended in a 100-fold volume of KHB solution at 37^{o}C containing 0.2 µm non-labeled ouabain. The myocyte suspension was sampled by the rapid filtration method at the indicated times following initiation of dissociation. Representative pattern of dissociation from 6 experiments.

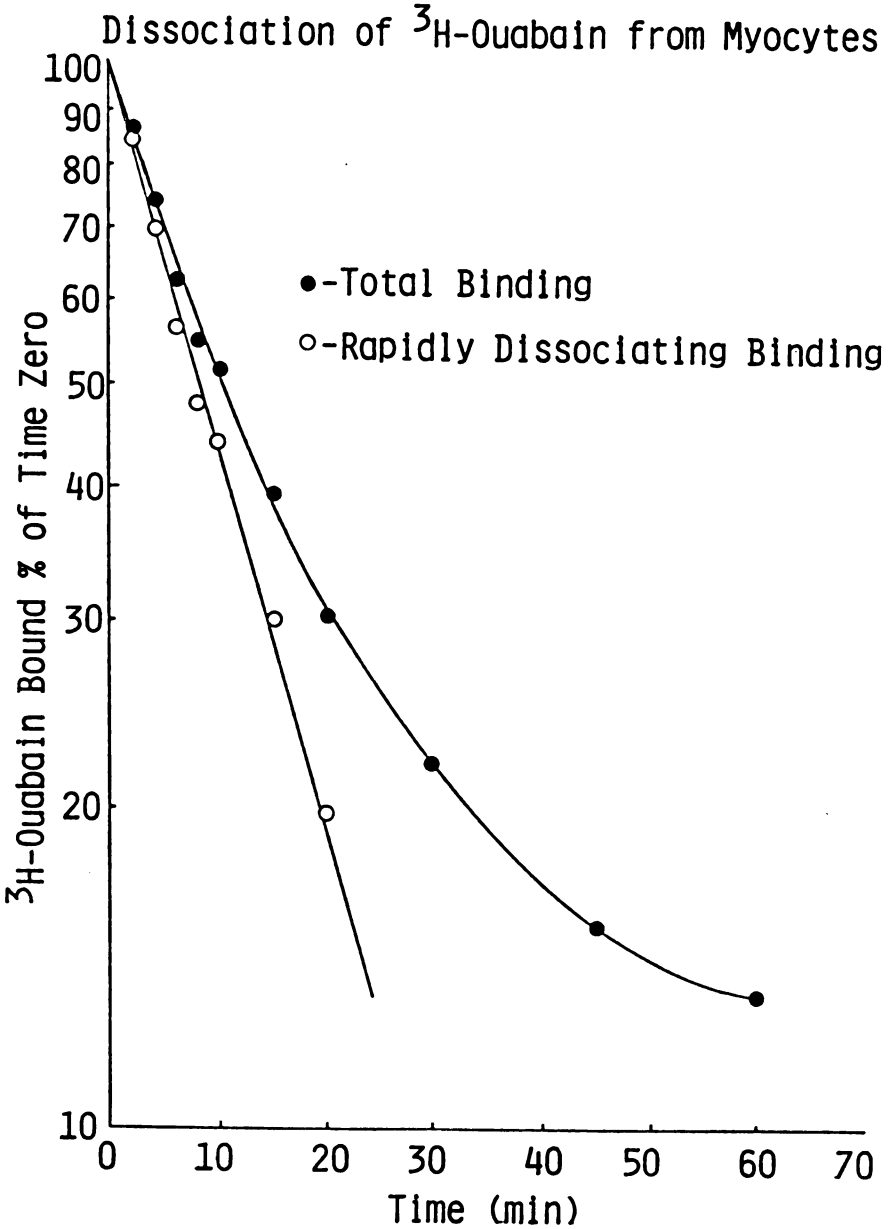

Figure 6. Representative time course for the dissociation of ^{3}H-ouabain from myocytes. Time course of dissociation was determined as described for Figure 5. Data is shown on a logarithmic scale as a function of the amount of ^{3}H-ouabain bound at the initiation of dissociation. ● -total ^{3}H-ouabain bound at indicated time, ○ -rapidly dissociating component of ^{3}H-ouabain bound. Representative pattern is shown. Dissociation rate constant (k^{-1}) of rapidly dissociating component was calculated to be 0.077±.01 min^{-1} ($\bar{X}$ ± SE from 6 determinations) corresponding to a half-life of 9.0±0.7 min. This figure shows data from a representative experiment.

time zero. Despite variations, the curve after 60 min appeared to be reasonably flat and therefore, the 60-min value seems to give an approximation of the X-intercept. Therefore, the amount of ^{3}H-ouabain remaining bound to cells after 60 min of dissociation was used as an estimate for the component of bound ^{3}H-ouabain which is slowly released from myocytes. Subtraction of the amount of ^{3}H-ouabain remaining in the cells after 60 min of dissociation from the amount remaining at each earlier time point produced resultant values which had a linear relationship when plotted on a semilogarithmic scale (Figure 6). This single exponential process was described by a dissociation rate constant (k^{-1}) equal to $0.077 \pm .01$ min^{-1} corresponding to a half-life of 9.0 ± 0.7 min (mean $\pm$ SE of 6 experiments). The rate constant for dissociation of ^{3}H-ouabain from guinea-pig heart Na,K-ATPase is close to this value (Godfraind et al., 1980) suggesting the rapid phase of ^{3}H-ouabain dissociation from myocytes represents the drug bound to the Na,K-ATPase. Using the value of 0.077 min^{-1} as an estimate of the rate of ^{3}H-ouabain dissociation it was calculated that after 60 min of dissociation 99% of drug bound to the rapidly releasing site would have dissociated.

Methods used for estimating non-specific binding were evaluated to determine if they produced comparable results in ^{3}H-ouabain binding experiments using myocytes. Nonspecific binding was estimated either as the ^{3}H-ouabain remaining bound to cells after 60 min of dissociation or as the amount bound in the presence of 1 mM non-labeled ouabain. In this study two methods for separating bound from non-bound ^{3}H-ouabain, namely filtration and centrifugation, were also evaluated.

Estimates of total ^{3}H-ouabain binding were not significantly different when cells were sampled by either filtration or centrifugation (Table 1). The estimates for ^{3}H-ouabain retained by cells after 60 min of dissociation were also

TABLE 1

Estimates of ^{3}H-Ouabain Binding to Myocytes

Sampling Method	N	^{3}H-Ouabain Bound (pmol/mg prot)		
		Total	Ouabain Insensitive (1 mM)	Slowly Dissociating (60 min)
Filtration	8	1.11±0.08	0.058±0.008	0.212±0.024*
Centrifugation	8	1.12±0.05	0.026±0.002*	0.182±0.009*

^{3}Myocytes from guinea-pig heart were incubated in the presence of 0.1 μM ^{3}H-ouabain for 60 min. Total ^{3}H-ouabain bound was determined after separating bound from non-bound ligand by the indicated sampling method. Non-specific binding was estimated by including 1 mM non-labeled ouabain in the incubation medium or by allowing ^{3}H-ouabain to dissociate from the myocytes for 60 min after binding. * Indicates a significant ($p < 0.05$) difference in estimates between sampling methods; * indicates significant difference between ouabain insensitive and slowly dissociating components of ^{3}H-ouabain binding. Significance determined by Student's t-test.

not affected by the method of sampling. Nonspecific binding estimated as ^{3}H-ouabain bound in the presence of 1 mM non-labeled ouabain was significantly higher when the rapid filtration method was used to collect the sample and separate bound from non-bound ligand. More importantly, use of 1 mM ouabain to obtain estimates of non-specific binding of ^{3}H-ouabain to myocytes resulted in values which were significantly lower than those obtained when the dissociation method was used (Table 1). This was true regardless of the method used to collect samples. Because of the extreme morphological change which occurs when myocytes are incubated with a high concentration of ouabain, the estimate of non-specific binding made using the dissociation method are considered to be more accurate. These results indicate that estimates of non-specific binding arrived at by using 1 mM ouabain to block specific binding of ^{3}H-ouabain may cause the amount of drug bound to Na,K-ATPase to be overestimated.

Time courses for ^{3}H-ouabain binding to the rapidly releasing component and the slowly releasing component were examined to determine the optimum incubation time for equilibrium binding studies. Myocytes were incubated in KHB solution containing 50 nM ^{3}H-ouabain for 30 to 120 min. Myocytes were either sampled at those times by filtration or dispersed for dissociation of bound drug and incubated for an additional 60 min before being collected. ^{3}H-ouabain associated with the rapidly releasing site was calculated as the difference between total binding and ^{3}H-ouabain remaining bound after 60 min of dissociation. ^{3}H-Ouabain bound to the fast releasing site increased slightly between 30 and 90 min after which it decreased slightly although values were not significantly different (Figure 7). ^{3}H-Ouabain associated with the slow releasing component increased steadily during the 120 min incubation. These results indicate that a 60-min incubation is adequate for equilibrium binding studies involving guinea-pig myocytes when low concentrations of ^{3}H-ouabain are used.

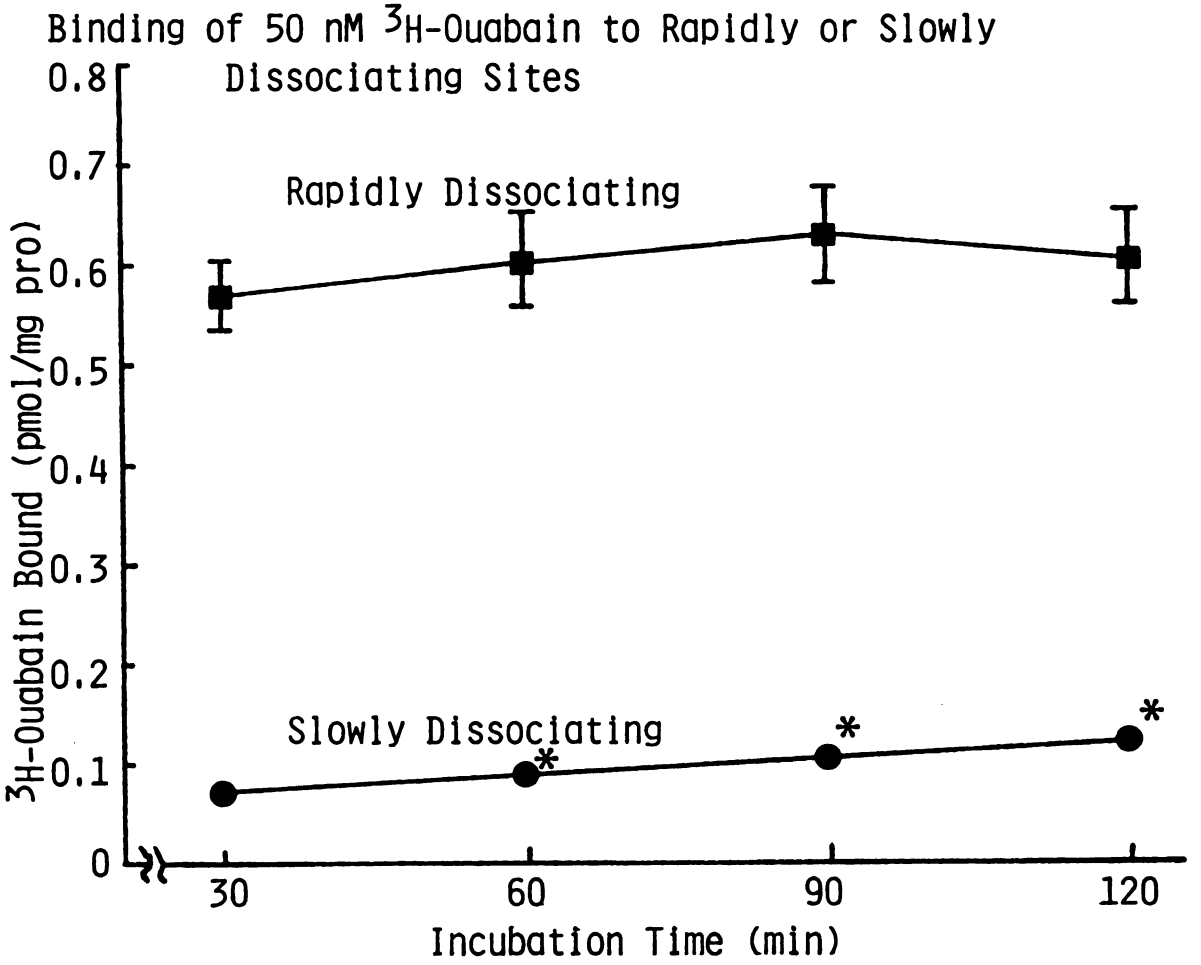

Figure 7. Binding of ^{3}H-ouabain to rapidly and slowly dissociating sites. Myocytes from guinea-pig heart were incubated in the presence of 50 nM ^{3}H-oaubain for the indicated time. Myocytes were sampled by rapid filtration for determination of total ^{3}H-ouabain bound or resuspended to allow bound ^{3}H-ouabain to dissociate for 60 min. ■ Indicates specific ^{3}H-ouabain binding calculated as the difference between total and that remaining after 60 min of dissociation. ● ^{3}H-ouabain remaining bound after 60 min of dissociation. * Indicates a significant ($p < .05$) change in ^{3}H-ouabain bound with time of incubation, n=5. Vertical lines indicate S.E.

2. Estimation of Kinetic Parameters for ^{3}H-Ouabain Binding to Guinea-Pig myocytes

Determination of binding site number and affinity for labeled ligand is commonly accomplished by analysis of displacement of a labeled ligand from specific binding sites by various concentrations of non-labeled ligand (Akera and Cheng, 1977). When this method was used to examine ^{3}H-ouabain binding to isolated myocytes, a linear relationship on logarithmic probability paper between ouabain concentration and percentage displacement of 100 nM ^{3}H-ouabain was seen (Figure 8). From this plot a K_D = 0.57 μM and B_{max} = 10.10 pmol/mg protein were determined. When an identical experiment was performed using homogenates of cells incubated in 1 mM $MgSO_4$, 1 mM Tris PO_4 and 20 mM Tris HCl a K_D = 0.17 μM and a B_{max} = 9.95 pmol/mg protein were determined. The linear relationship on logarithmic probability paper of ouabain concentration versus percentage displacement of labeled ^{3}H-ouabain was taken as an indication that the conditions required for using this procedure were satisfied. These are: a single class of binding sites and no cooperativity in binding. The close agreement in estimates of binding site number for live cells and cell homogenates suggested that all the ^{3}H-ouabain binding to myocytes was to the Na,K-ATPase.

Linear relationships on logarithmic probability paper were not always obtained with this method. For example, when the phorbol ester TPA (phorbol 12-myristate 13-acetate) was present during ^{3}H-ouabain binding the plot describing displacement by non-labeled ouabain was clearly non-linear (Figure 8). TPA stimulates a Ca^{2+}-dependent protein kinase (Nishizuka, 1984). Effects of TPA on ^{3}H-ouabain binding were examined to determine if phosphorylations catalyzed by this kinase affected the affinity of the Na,K-ATPase for ^{3}H-ouabain. The non-linearity shows that the interaction of ^{3}H-ouabain with myocytes is more complex than originally determined. Therefore, with preparations of isolated myocytes, estimates of the dissociation constant (K_D) and binding site number (B_{max}) for

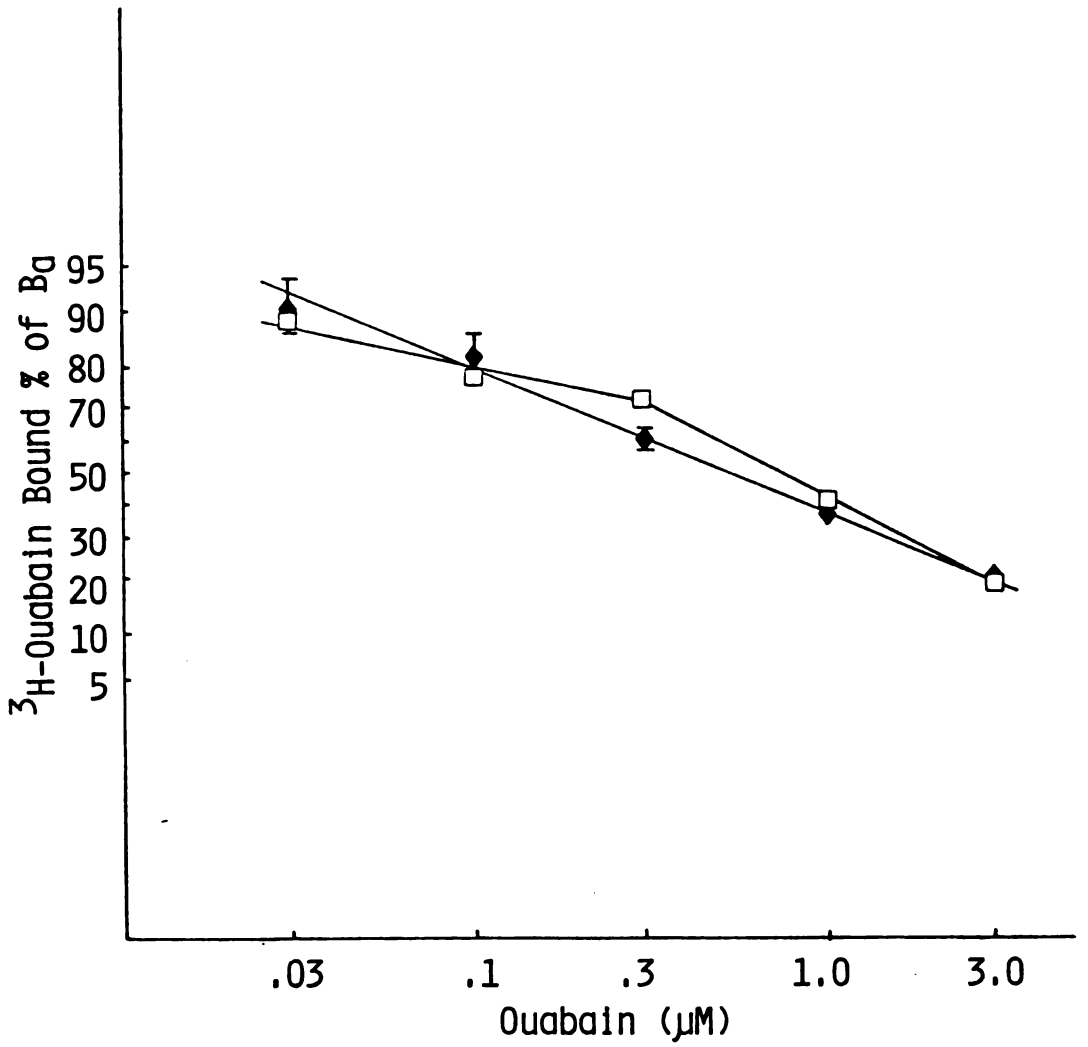

Figure 8. Estimation of kinetic parameters for ^{3}H-ouabain binding by the displacement method. The presence of 100 nM TPA (phorbol 12-myristate 13-acetate) during incubation of myocytes with 100 nM ^{3}H-ouabain caused the logarithmic probit plot describing displacement of ^{3}H-ouabain by non-labeled ouabain to become non-linear. Percentages are calculated on the mean of six experiments. B_a is ^{3}H-ouabain bound in absence of non-labeled ouabain. □-100 mM TPA; ◆-control.

^{3}H-ouabain cannot be obtained with this method and linearity of plots describing displacement of ^{3}H-ouabain from myocytes is insufficient evidence for substantiation of these estimates.

More direct estimates of K_D and B_{max} can be made by determining the specific binding in the presence of various concentrations of labeled ligand and subjecting the data to Scatchard analysis. To utilize Scatchard analysis correctly it is necessary to expose the cells to a range of ligand concentrations so that 5 to 95% of binding sites will be occupied. Because full occupancy of binding sites results in cell death within 60 min, it was necessary to determine the optimum incubation time for a range of ^{3}H-ouabain concentrations. Intracellular Na^+ concentration is expected to be affected by and to affect ^{3}H-ouabain binding. Therefore, the time courses of binding were determined for cells incubated in the absence and presence of 2.0 μM monensin. Monensin, a Na^+ ionophore, is expected to increase the intracellular Na^+ concentration. The Na^+ ionophore was included to augment the toxic effects of ouabain in order to estimate the maximal incubation time myocytes can tolerate in the presence of high concentrations of ouabain.

In the absence of monensin, ^{3}H-ouabain binding to the rapidly dissociating site reached a steady state within 20 min when cells were incubated in 0.5 or 2.0 μM ^{3}H-ouabain but apparently did not reach a steady state within 60 min when cells were incubated in 10 μM ^{3}H-ouabain (Figure 9). Ouabain at a concentration of 0.5 μM has a strong positive inotropic effect on isolated guinea-pig cardiac muscle whereas 2.0 and 10.0 μM ouabain are toxic. Decreased Na^+ influx into quiescent cells as compared to contracting tissue should ameliorate the toxicity of ouabain. Therefore, 2 μM ouabain may not be toxic in the isolated myocyte preparation and failure to achieve steady-state in binding of 10 μM ^{3}H-ouabain within 60 min may reflect the toxicity of this high concentration of

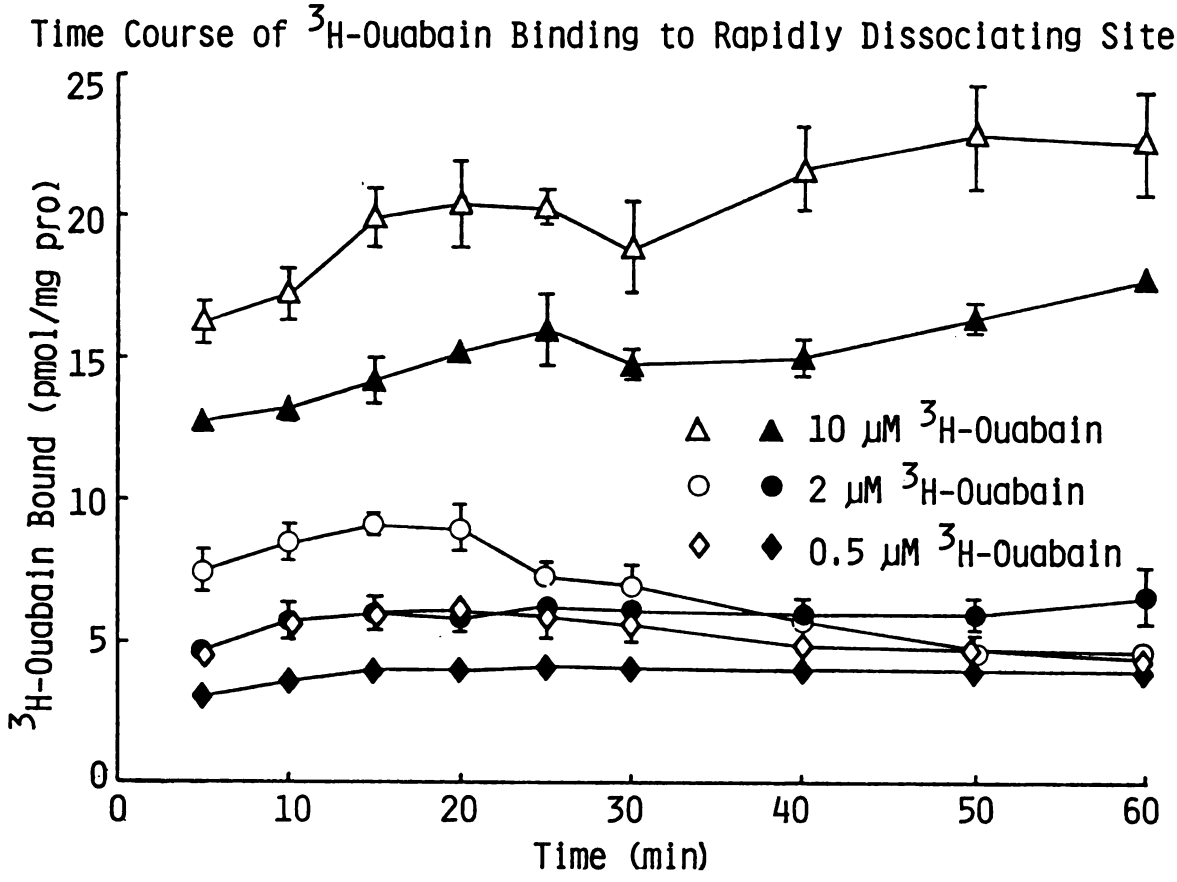

Figure 9. Time course of ^{3}H-ouabain binding to the rapidly dissociating site in myocytes from guinea-pig heart. Myocytes were incubated for indicated times in: △, ▲ 10 μM; *O*, ● 2.0 μM; or ◇, ◆ 0.5 μM ^{3}H-ouabain. Open symbols indicate that 2.0 μM monensin was present during the binding reaction. n=3. Vertical bars indicate SE.

ouabain. Alternatively, the inability to reach equilibrium may indicate a redistribution of Na^+ and K^+ has occurred during the binding which is of sufficient magnitude to effect ^{3}H-ouabain binding. If this is true, 60 min may be insufficient for both binding and ion redistribution to approach steady state.

Monensin at a concentration of 2 μM is not itself toxic to cardiac muscle as judged by a sustained positive inotropic response without contracture in guinea-pig atrial muscle exposed to 1 or 3 μM monensin (Yamamoto et al., 1980). This same concentration of monensin (2 μM) increases the arrhythmogenic actions of digoxin (Kennedy et al., 1983); therefore, it exacerbates the toxicity of cardiac glycosides.

In the presence of 2 μM monensin, binding of ^{3}H-ouabain is increased early in the reaction at each concentration of ^{3}H-ouabain examined. After 20 min of incubation in the presence of both ouabain and monensin, an apparent release of ^{3}H-ouabain from cells occurs. For cells incubated in monensin and 2.0 μM ^{3}H-ouabain, the release is sufficient so that the amount retained by the myocytes is less than the amount bound to cells incubated in 2.0 μM ^{3}H-ouabain alone. In the presence of monensin, however, binding of ^{3}H-ouabain to the rapidly releasing site appears to be at steady state after 60 min of incubation at each concentration of ^{3}H-ouabain examined. These data indicate that high concentrations of ^{3}H-ouabain have effects on myocytes, possibly related to an increase in intracellular Na^+, which cause the time course to equilibrium for binding to deviate from what is expected for a pseudo first-order reaction. These data also indicate that under most conditions, a steady state for the binding reaction is approached after sixty minutes of incubation.

Redistribution of ions, particularly Na^+ and Ca^{2+}, has been shown to be the primary result of Na,K-ATPase inhibition in intact cells. Increases in the intracellular concentrations of Na^+ or Ca^{2+} may be responsible for the complex

behavior of ^{3}H-ouabain binding to myocytes. This could be via a direct action, such as Na^{+} interacting with the Na,K-ATPase, or an indirect action, such as via a Ca^{2+} binding protein or inhibition of ATP synthesis.

To determine if Na^{+} or Ca^{2+} affect binding of cardiac glycosides to the Na,K-ATPase in intact tissue, myocytes were incubated with concentrations of ^{3}H-ouabain ranging from 50 nM to 20 μM and the kinetic parameters for binding were estimated from Scatchard plots of specific binding. Specific binding was estimated as the rapidly dissociating component of ^{3}H-ouabain binding. The slowly dissociating component of bound ^{3}H-ouabain was considered to represent non-Na,K-ATPase binding and, for these experiments, is considered to represent non-specific binding. Four incubation conditions were chosen. First, for a control condition, cells were incubated in KHB solution containing 1.8 mM Ca^{2+}. To augment any effect ^{3}H-ouabain might have via increases in intracellular Na^{+} and Ca^{2+} concentrations myocytes were incubated in KHB solution containing 1.8 mM Ca^{2+} and 2 μM monensin. The third incubation condition was chosen to eliminate effects of Ca^{2+} without affecting the intracellular Na^{+} concentration. For this, cells were incubated in KHB solution containing 0.25 mM EGTA (ethyleneglycol bis(β-aminoethylether)-N,N'-tetraacetic acid) to chelate Ca^{2+}. Finally, to determine if increasing intracellular Na^{+} affected ^{3}H-ouabain binding in the absence of Ca^{2+}, cells were incubated in KHB solution containing 0.25 mM EGTA to eliminate Ca^{2+} and 2.0 μM monensin to increase Na^{+} influx.

Total and non-specific binding of ^{3}H-ouabain to guinea-pig myocytes under the four conditions previously described are shown in Figure 10 for concentrations of ^{3}H-ouabain from 1.0 to 20.0 μM and in Figure 11, on an expanded scale, for concentrations of ^{3}H-ouabain from 50 nM to 3.0 μM. Binding to the slowly dissociating site increases with the concentration of ^{3}H-ouabain in the incubation medium under each incubation condition. There is a significant

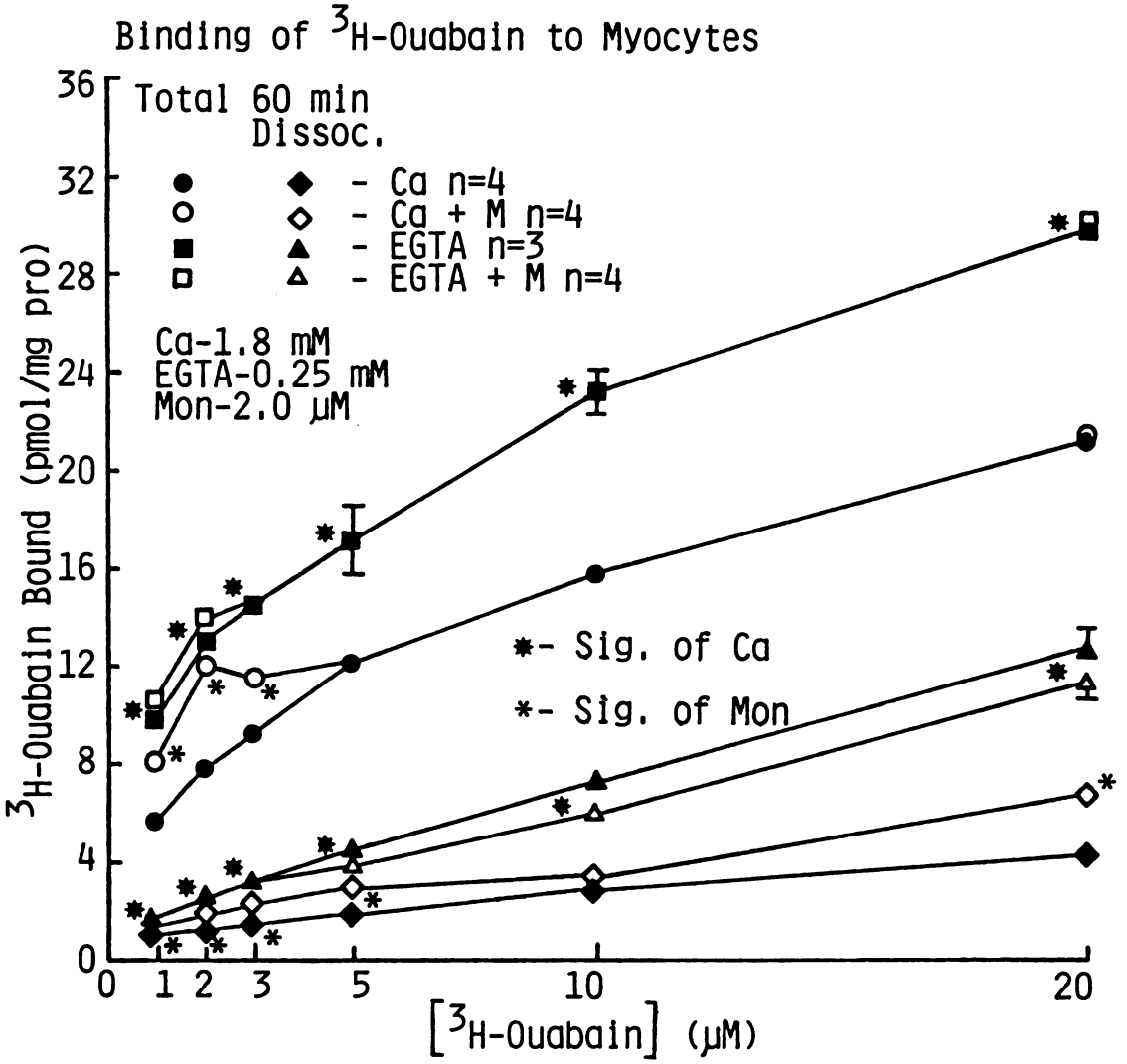

Figure 10. Concentration dependence for ^{3}H-ouabain binding to myocytes. Myocytes from guinea-pig heart were incubated for 60 min in the presence of the indicated concentrations of ^{3}H-ouabain. Symbols represent □, ■, ○, ● total binding or △, ▲, ◇, ◆ ^{3}H-ouabain remaining bound after 60 min of dissociation. Incubation solutions contained: ●, ◆ 1.8 mM Ca^{2+} (n=4); ○, ◇ 1.8 mM Ca^{2+} and 2 µM monensin (n=4); ■, ▲ 0.25 mM EGTA (n=3); □, △ 0.25 mM EGTA and 2 µm monensin (n=4). * Indicates a significant ($p < .05$) effect of Ca^{2+}, * indicates a significant effect of monensin. ^{3}H-ouabain remaining bound after 60 min of dissociation is considered to represent non-specific binding.

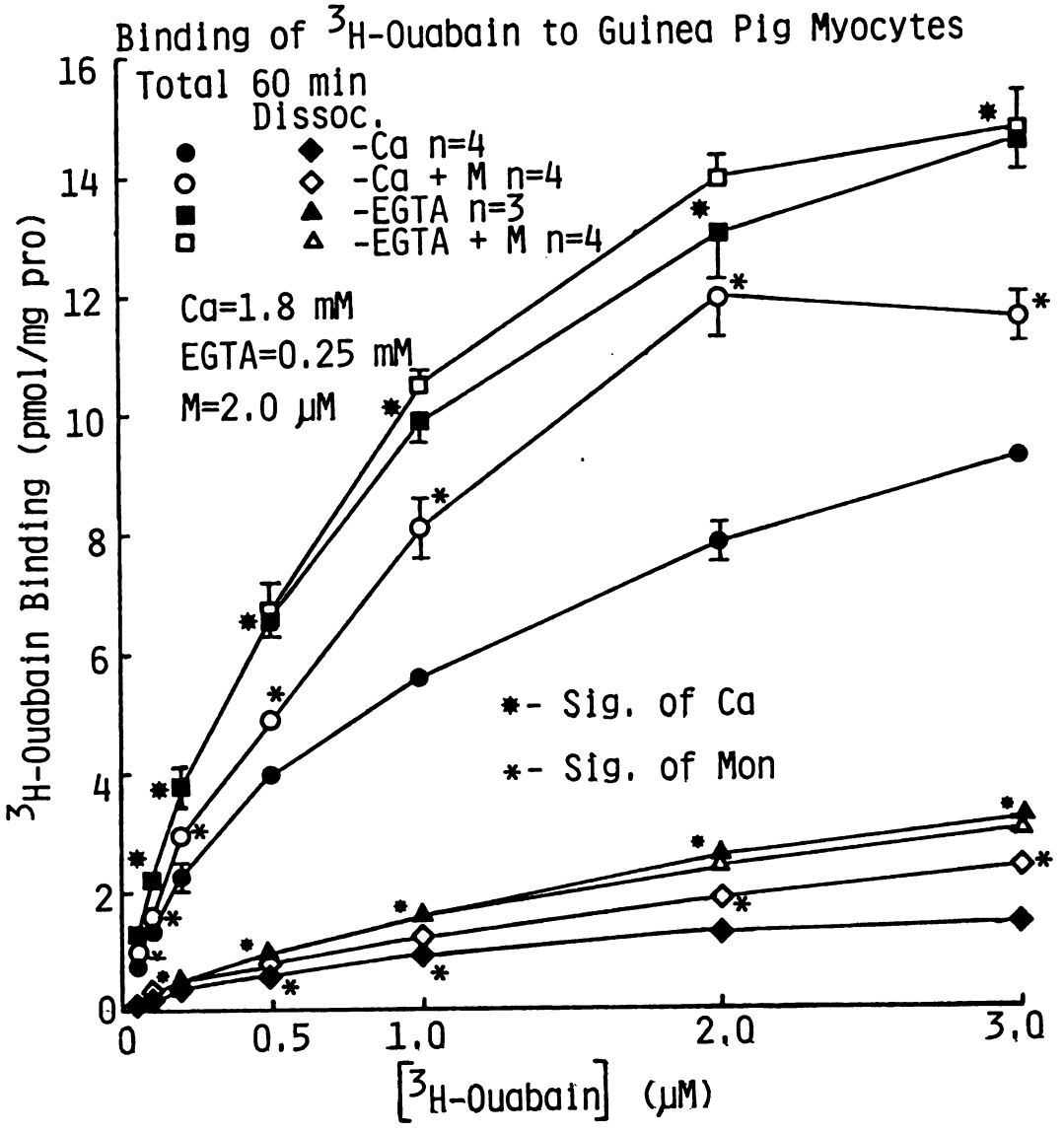

Figure 11. Concentration dependence for ^{3}H-ouabain binding to myocytes. Myocytes from guinea-pig heart were incubated in solution containing 50, 100 or 200 nM ^{3}H-ouabain or the concentrations indicated. Incubations proceeded for 60 min. Symbols represent □ , ■ , ○ , ● total binding or Δ , ▲ , ◇ , ◆ ^{3}H-ouabain remaining bound after 60 min of dissociation. Incubation solutions contained: ● , ◆ 1.8 mM Ca^{2+} (n=4); ○ , ◇ 1.8 mM Ca^{2+} and 2 µm monensin (n=4); ■ , ▲ 0.25 mM EGTA (n=3); □ , Δ 0.25 mM EGTA and 2 µM monensin (n=4). * Indicates a significant ($p < .05$) effect of Ca^{2+}, * indictes a significant effect of monensin. ^{3}H-Ouabain remaining bound after 60 min of dissociation is considered to represent non-specific binding.

effect of monensin to increase non-specific binding when myocytes are incubated in the presence of 1.8 mM Ca^{2+}, but not when they are incubated in the absence of Ca^{2+}. Absence of Ca^{2+} itself also has a significant effect to increase ^{3}H-ouabain binding to the slow releasing site. Whether the presence of 0.25 mM EGTA instead of the absence of Ca^{2+} was necessary for or contributed to this effect was not tested.

Monensin had a significant effect to increase total binding when myocytes were incubated with less than 3 μM ^{3}H-ouabain in the presence of 1.8 mM Ca^{2+}. Monensin had no effect on binding when cells were incubated in ^{3}H-ouabain concentrations greater than 3.0 μM. When incubations were done in the absence of Ca^{2+} (0.25 mM EGTA) monensin did not have any significant effect to increase total ^{3}H-ouabain binding. Under these conditions there was an apparent effect of monensin to stimulate ^{3}H-ouabain binding to cells incubated with the 1.0 and 2.0 μM concentrations of ^{3}H-ouabain, but the increases were not significant (Figure 11).

Specific ^{3}H-ouabain binding to myocytes was calculated as the difference in total ^{3}H-ouabain binding and the ^{3}H-ouabain remaining bound to the myocytes after 60 min of dissociation. Specific binding of ^{3}H-ouabain to myocytes incubated under the four conditions described previously is shown in Figure 12 for concentrations of ^{3}H-ouabain from 1.0 to 20.0 μM and, on an expanded scale in Figure 13 for concentrations of ^{3}H-ouabain from 50 nM to 3.0 μM. Incubating cells in the absence of Ca^{2+} during the binding reaction has a significant effect to increase specific ^{3}H-ouabain binding to myocytes incubated in each concentration of ^{3}H-ouabain examined. Monensin has a significant effect on specific ^{3}H-ouabain binding to myocytes incubated in the presence of Ca^{2+} but not in the absence of Ca^{2+}. In the presence of Ca^{2+} the effect of monensin is to increase ^{3}H-ouabain binding to cells incubated in concentrations of ^{3}H-ouabain

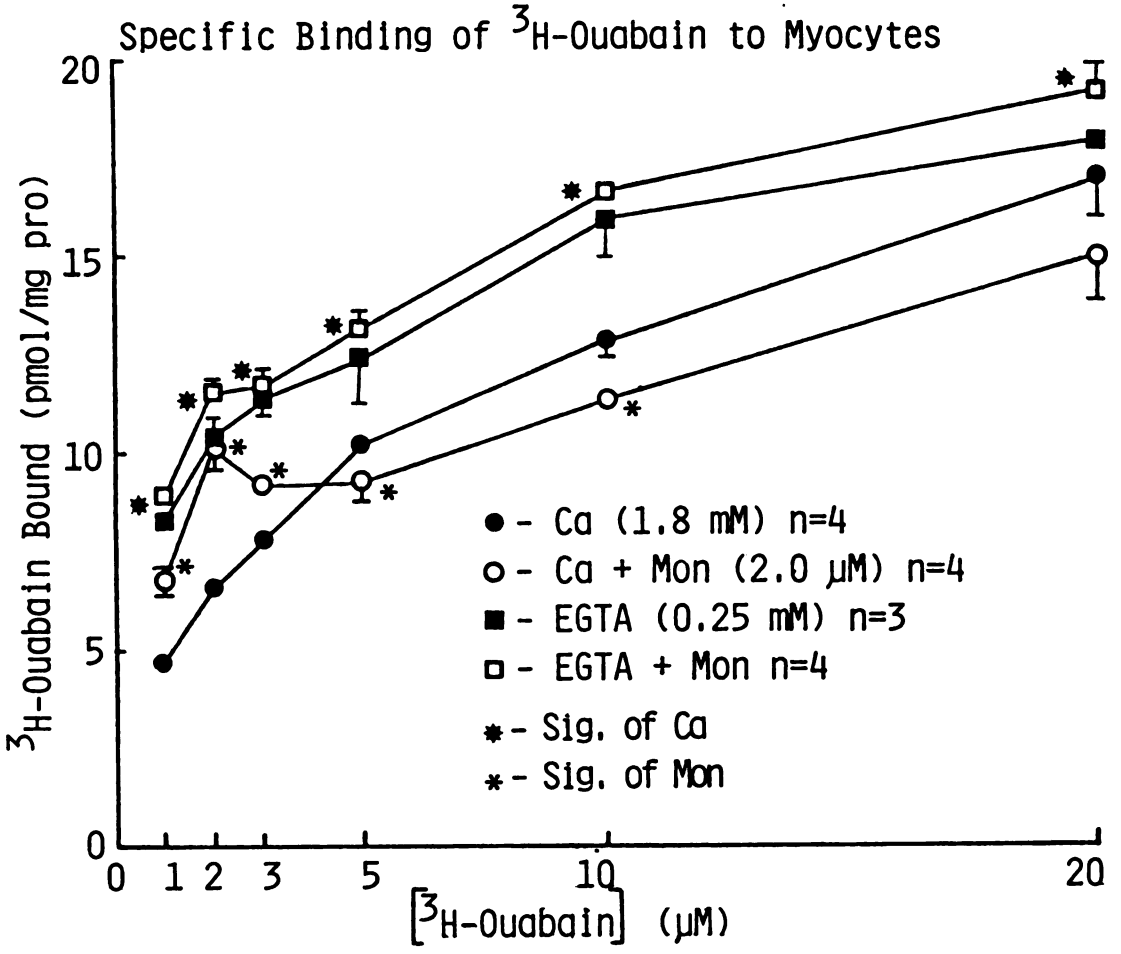

Figure 12. Specific ^{3}H-ouabain binding to myocytes from guinea-pig heart. Myocytes were incubated in the presence of the indicated concentration of ^{3}H-ouabain for 60 min. Specific binding was calculated as the difference between total binding and ^{3}H-ouabain remaining bound after 60 min of dissociation. Incubation solutions contained: ● 1.8 mM Ca^{2+} (n=4); ○ 1.8 mM Ca^{2+} and 2 μM monensin (n=4); ■ 0.25 mM EGTA (n=3); □ 0.25 mM EGTA and 2 μm monensin (n=4). ✱ Indicates a significant ($p < .05$) effect of Ca^{2+}, * indicates a significant effect of monensin.

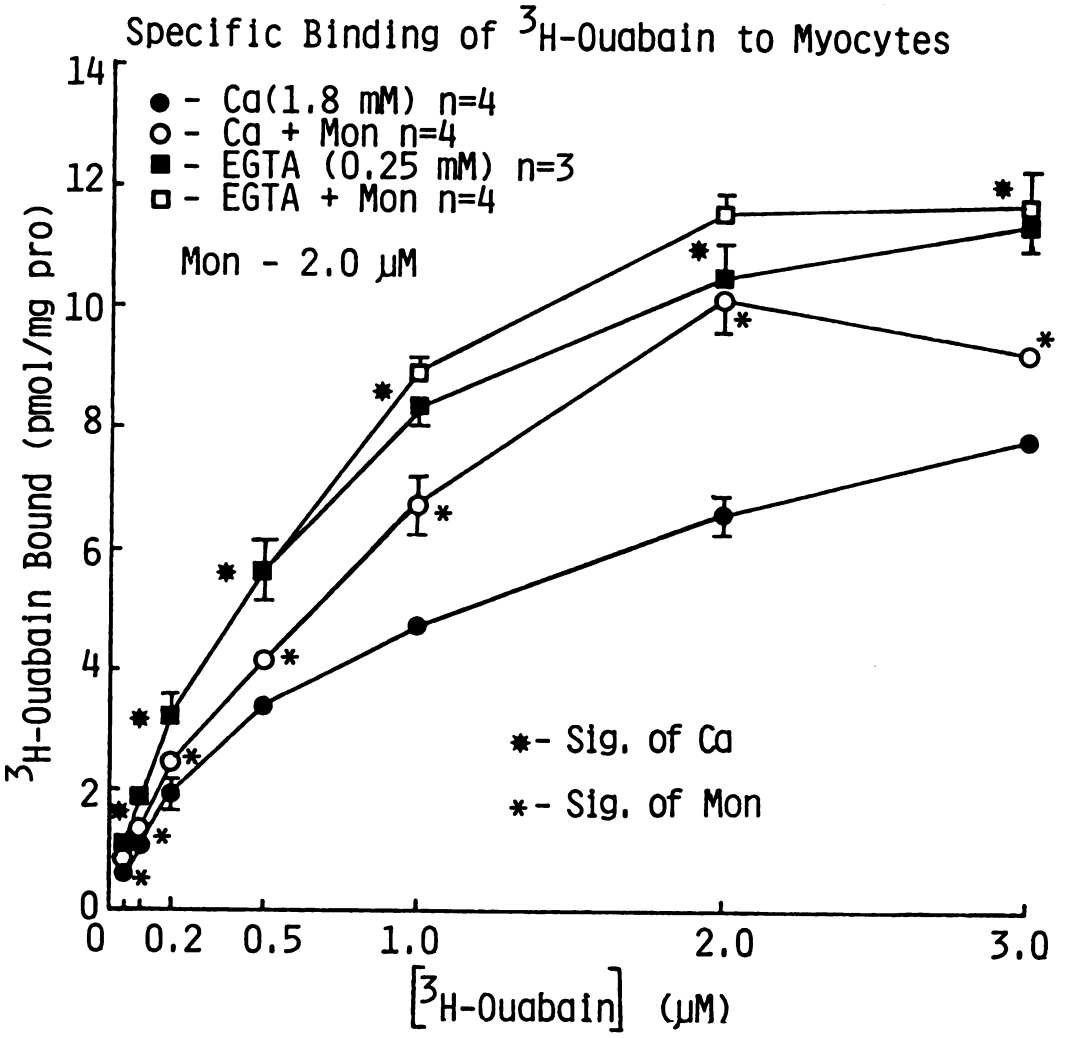

Figure 13. Specific ^{3}H-ouabain binding to myocytes from guinea-pig heart. Myocytes were incubated in solution containing 50, 100 or 200 nM ^{3}H-ouabain or the concentration indicated. Incubations were for 60 min. Specific binding ws calculated as the difference between total binding and ^{3}H-ouabain remaining bound after 60 min of dissociation. Incubation solutions contained: ● 1.8 mM Ca^{2+} (n=4); ○ 1.8 mM Ca^{2+} and 2 µM monensin (n=4); ■ 0.25 mM EGTA (n=3); □ 0.25 mM EGTA and 2 µm monensin (n=4). * Indicates a significant ($p < .05$) effect of Ca^{2+}, * indicates a significant effect of monensin.

less than 3.0 μM but to decrease specific ^{3}H-ouabain binding to cells incubated in concentrations of ^{3}H-ouabain higher than 3.0 μM.

Saturation of specific ^{3}H-ouabain binding sites apparently does not occur under any of the four incubation conditions even when myocytes are incubated in 20.0 μM ^{3}H-ouabain. A plateau in specific ^{3}H-ouabain binding to cells is seen at 2.0 to 3.0 μM concentrations of ^{3}H-ouabain when binding is stimulated by the presence of monensin, or in the absence of Ca^{2+} (Figure 13). This plateau may represent saturation of a high affinity site, probably the Na,K-ATPase.

Scatchard analysis of specific ^{3}H-ouabain binding to guinea-pig myocytes when binding occurs in the presence of 1.8 mM Ca^{2+} is shown in Figure 14. When binding is not stimulated by the presence of monensin, a curved Scatchard plot is produced. For a normal binding reaction, the concave curve shown in Figure 14 could indicate negative cooperativity in binding or that the ^{3}H-ouabain is binding to more than one class of sites which have different affinity. For the cardiac glycosides, binding to Na,K-ATPase in intact cells could be described by this curve if there is positive cooperativity in binding (Herzig et al., 1985a,b). This point shall be addressed more fully in the Discussion section.

The presence of 2 μM monensin during ^{3}H-ouabain binding causes a shift in the curve on Scatchard plots, and appears to increase their degree of curvature. The increased curvature is maintained only for points describing the binding at concentrations of ^{3}H-ouabain up to 2.0 μM. At higher concentrations of ^{3}H-ouabain, a break in the curve is seen then the curve continues upward at 10 and 20 μm ^{3}H-ouabain.

Scatchard plots describing the concentration dependence of ^{3}H-ouabain binding to cells incubated in the absence of Ca^{2+} (0.25 mM EGTA) are shown in Figure 15. Two distinct linear components of this curve are discernable.

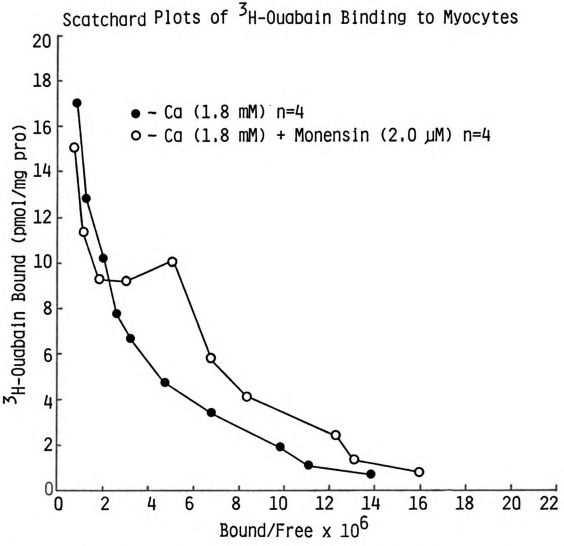

Figure 14. Scatchard plots describing specific ^{3}H-ouabain binding to myocytes from guinea-pig heart. Myocytes were incubated for 60-min in solutions containing concentrations of ^{3}H-ouabain ranging from 50 nM to 20 µm. Incubation solutions contained: ● 1.8 mM Ca^{2+} (n=4) or ○ 1.8 mM Ca^{2+} and 2 µm monensin (n=4).

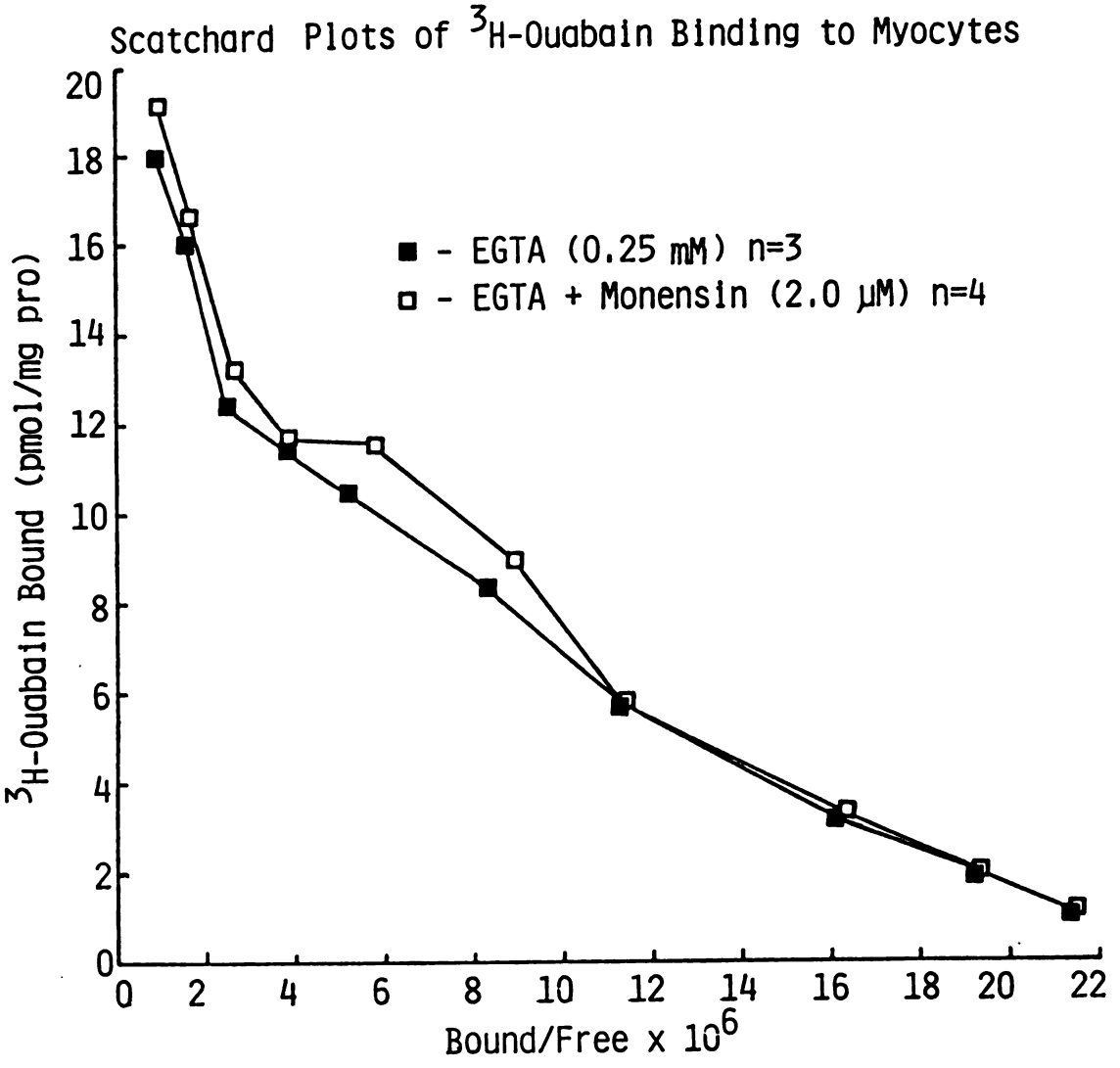

Figure 15. Scatchard plots describing specific ^{3}H-ouabain binding to myocytes from guinea-pig heart. Myocytes were incubated for 60-min in solutions containing concentrations of ^{3}H-ouabain ranging from 50 nM to 20 µM. Incubation solutions contained: ■ 0.25 mM EGTA (n=3) or, □ 0.25 mM EGTA and 2 µM monensin (n=4).

Binding to a high affinity site(s) is described by a single line but a break in this line is accentuated by the curve describing ^{3}H-ouabain binding in the presence of 2 μM monensin. If this break is real, ^{3}H-ouabain binding can be considered to occur at three sites: the highest affinity site with estimted K_D = 0.4 μM and B_{max} = 9.8 pmol/mg protein; the second, K_D = 0.7 μM, B_{max} = 4.3 pmol/mg protein; and the third, K_D = 3.4 μM, B_{max} = 8.4 pmol/mg protein. Monensin would have a modest ability to stimulate ^{3}H-ouabain binding to the site with intermediate affinity but no effect on ^{3}H-ouabain binding to the site with highest or lowest affinity. Binding to the high affinity site(s) probably represents binding to the Na,K-ATPase. If this is true, the break in this portion of the curve may represent binding to different conformations of the Na,K-ATPase. Association and dissociation rate constants for ^{3}H-ouabain binding to Na,K-ATPase are known to be affected by the presence of K^+ (Akera and Brody, 1970). Therefore, the presence of 3.8 mM K^+ in the incubation media during binding could have caused two conformations of the Na,K-ATPase with different association and dissociation rate constants for ^{3}H-oaubain binding to be present. The physiological nature of the low affinity site is unknown. For comparison, Scatchard plots describing ^{3}H-ouabain binding under all four conditions are shown together in Figure 16.

3. Extracellular K^+ and ^{3}H-Ouabain Binding

Potassium ion has a well documented effect to inhibit ^{3}H-ouabain binding to isolated Na,K-ATPase (Akera and Brody, 1970). This inhibition apparently results because K^+ binding to the enzyme causes a conformational change to a form with lower affinity for cardiac glycosides (Akera et al., 1973; Inagaki et al., 1974; Yoda and Yoda, 1986). Normal intracellular K^+ concentration is approximately 140 mM and the extracellular K^+ concentration in our experiments is 3.8 mM. Therefore, redistribution of K^+ down its concentration gradient is expected after inhibition of the sodium-pump. Such a redistribution, with the

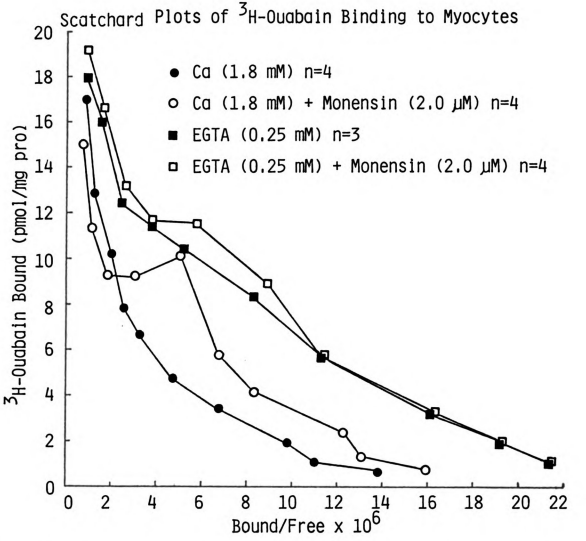

Figure 16. Scatchard plots describing specific ^{3}H-ouabain binding to myocytes from guinea-pig heart. Myocytes were incubated for 60-min in solutions containing concentrations of ^{3}H-ouabain ranging from 50 nM to 20 μm. Incubation solutions contained: ● 1.8 mM Ca^{2+} (n=4) or ○ 0.18 mM Ca^{2+} and 2 μm monensin (n=4); ■ 0.25 mM EGTA (n=3); □ 0.25 mM EGTA and 2 μM monensin (n=4).

extracellular K^+ concentration increasing as the sodium-pump is inhibited, might decrease the apparent affinity of Na,K-ATPase for ^{3}H-ouabain if the increase in extracellular K^+ concentration was of sufficient magnitude. This would be manifested as a negative cooperativity in ^{3}H-ouabain binding and may be responsible, in part or in whole, for the curvature of the Scatchard plots describing ^{3}H-ouabain binding to myocytes in the presence of Ca^{2+}.

To demonstrate that Na,K-ATPase can exist in different binding conformations in intact myocytes, and that extracellular K^+ affects the relative abundance of each conformation, dissociation of bound drug was monitored after binding occurred in nominally K^+-free solution or in KHB solution containing 10 mM KCl (NaCl reduced to maintain osmolarity). A high concentration of ^{3}H-ouabain (2.5 μM) was present during the binding reaction to assure adequate binding of ^{3}H-ouabain to the conformation of Na,K-ATPase with the lower affinity for ouabain. Total ^{3}H-ouabain binding was estimated by sampling cell pellets by the centrifugation method before initiation of dissociation. Dissociation of ^{3}H-ouabain took place in a 200-fold volume of KHB solution containing 2.5 μM non-labeled ouabain and 10 mM K^+. Ouabain and K^+ were each included in the dissociation medium to inhibit rebinding of ^{3}H-ouabain released from the cells. Considerably more ^{3}H-ouabain was bound to cells before initiation of dissociation than after 30 sec of dissociation. Myocytes contained 23.2±0.80 or 12.5±1.1 pmol/mg protein of ^{3}H-ouabain (n=4) before initiation of dissociation when binding had occurred in 0 or 10 mM K^+, respectively. After 30 sec of dissociation cells contained 13.4±0.7 or 7.7±0.2 pmol/mg protein of ^{3}H-ouabain (n=4) for binding in 0 or 10 mM K^+, respectively. Such large differences in estimates of ^{3}H-ouabain bound are unlikely to be artifactual, although this is possible because collection of cells and separation of bound from unbound ligand were by different methods (centrifugation and rapid filtration). Alternatively, the

rapid dissociation of ^{3}H-ouabain may be real and represent binding to the very low affinity site seen on Scatchard plots. Because of the large difference in binding estimated before and 30 sec after initiation of dissociation, the value for ^{3}H-ouabain bound after 30 sec of dissociation was used as time zero for analysis of dissociation.

Release of ^{3}H-ouabain from myocytes was best described by two exponential processes with short half-lives and a third exponential process with a much longer half-life. The slowest phase of dissociation is assumed to represent non-specific binding of ^{3}H-ouabain to myocytes. This phase of release was estimated from the rate of ^{3}H-ouabain release from cells from 60 to 150 min after initiation of dissociation. Half lives for these components of ^{3}H-ouabain binding of 212$\pm$27 and 146$\pm$15 min (n=4) were calculated for cells to which binding had occurred in KHB solution containing 0 or 10 mM K^{+}, respectively. The difference is not significant and therefore, no conclusions can be drawn concerning the effects of extracellular K^{+} on dissociation of ^{3}H-ouabain from non-specific binding sites.

Dissociation of ^{3}H-ouabain from the cells between 60 and 150 min after initiation of dissociation was described by a straight line when data was plotted on a semilogarithmic scale. The best fit line for ^{3}H-ouabain bound to cells after 60 to 150 min of dissociation was determined by the method of least squares and used to estimate the amount of ^{3}H-ouabain associated with the slow releasing component at earlier times of dissociation. Amounts of ^{3}H-ouabain associated with the slow releasing component at times of dissociation less than 60 min were estimated for individual experiments.

Dissociation of ^{3}H-ouabain from rapidly releasing components was analyzed using pooled values from four experiments. Individual values from all experiments were used for calculation of the best fit straight line by least squares

analysis. Values from four experiments were pooled because variation in individual experiments was large. The rapid phase of release of ^{3}H-ouabain was best described by two exponential processes irrespective of binding conditions (Figure 17). Individual values for ^{3}H-ouabain bound to rapidly releasing sites are shown in Figure 17. The best fit straight lines were calculated by least squares analysis of individual values from 14 to 39 minutes of dissociation. When binding had occurred in K^{+}-free solution this component had a half-life of 9.4 min and contained 4.5±0.2 pmol/mg protein of ^{3}H-ouabain (n=4). When binding had occurred in 10 mM K^{+} solution this component had a half-life of 10.8 min and contained 3.7±0.1 pmol/mg protein of ^{3}H-ouabain (n=4). Correlation coefficients of the regression lines were 0.93 and 0.94 for binding in the absence and presence of K^{+}, respectively. Individual values describing the most rapid phase of ^{3}H-ouabain dissociation were calculated by subtracting the estimated content of ^{3}H-ouabain in the slower releasing site at times of dissociation less than 11 min from total ^{3}H-ouabain in the rapidly releasing site. Estimates of ^{3}H-ouabain bound to the slower releasing site were taken from the regression line describing dissociation between 14 and 39 min. The best fit lines determined by least squares analysis of the estimates for ^{3}H-ouabain bound to the fastest releasing site are drawn in Figure 17. When binding had occurred in K^{+}-free solution this component had a half-life of 2.5 min and contained 6.9±0.3 pmol/mg protein of ^{3}H-ouabain (n=4). When binding had occurred in 10 mM K^{+} solution this component had a half-life of 3.5 min and contained 2.9±0.1 pmol/mg protein of ^{3}H-ouabain (n=4). Correlation coefficients of the regression lines were 0.92 and 0.89 for binding in the absence and presence of K^{+}, respectively.

The close agreement in dissociation rate constants for ^{3}H-ouabain bound to cells under these different conditions indicates that ^{3}H-ouabain binds to the same sites under both conditions. In the absence of K^{+} significantly more ^{3}H-

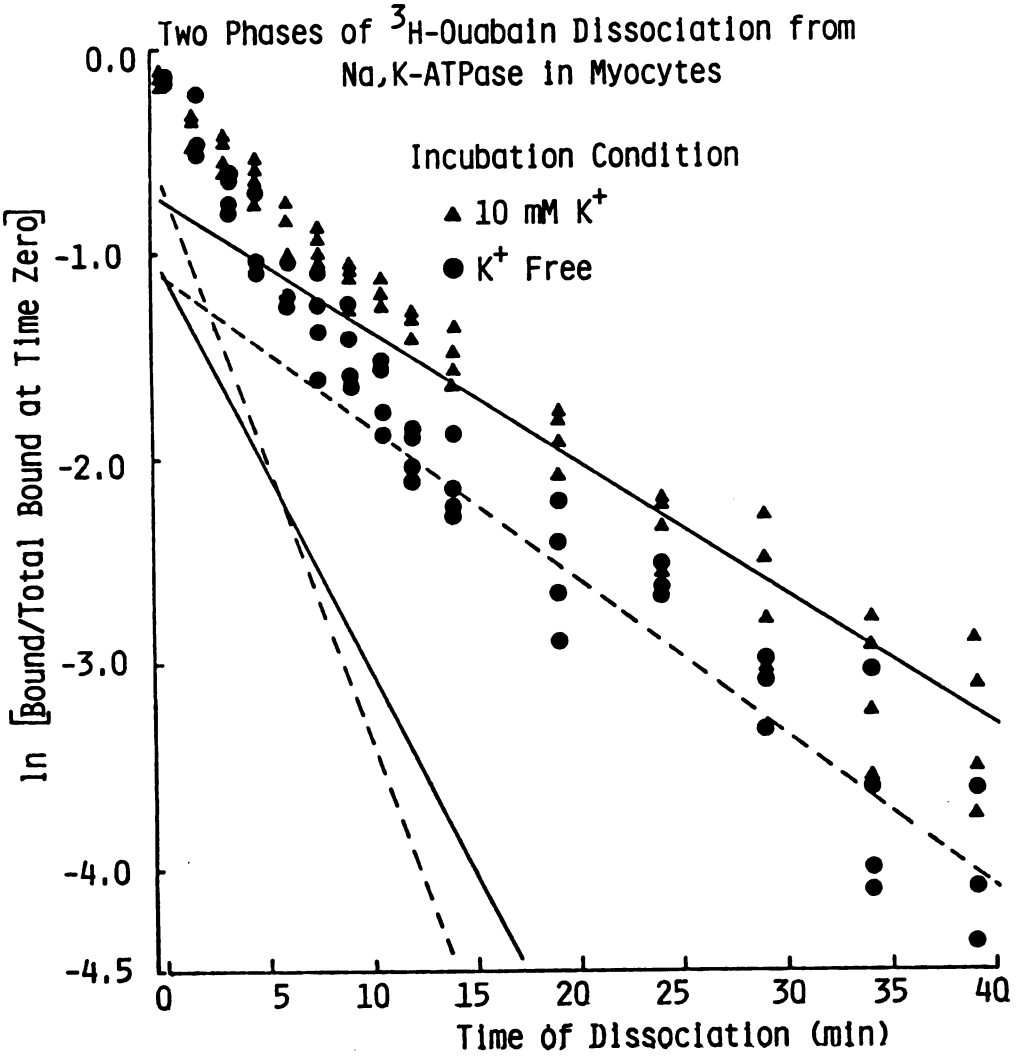

Figure 17. Dissociation of ^{3}H-ouabain from myocytes. Myocytes from guinea-pig heart were incubated in the presence of 2.5 µM ^{3}H-ouabain for 40 min. Cells were collected by gravity sedimentation and resuspended in a 200-fold volume of KHB solution at 37°C containing 2.5 µM non-labeled ouabain and 10 mM KCl for initiation of dissociation. Myocyte suspensions were sampled at various times for 150 min following initiation of dissociation. Regression lines calculated from values normalized to the amount of ^{3}H-ouabain bound at the first time of sampling after initiation of dissociation described linear dissociation with time between 60 and 150 min after initiation of dissociation. Amounts of ^{3}H-ouabain associated with this component were calculated from the regression line and subtracted from all earlier time points. Data plotted are the resultant values from 4 experiments of each type for binding in KHB solution containing ●-0 mM K^+ or ▲-10 mM K^+. Regression lines were calculated from these values and are shown for two components of the rapid phase of dissociation when binding occurred in KHB solution containing --- O mM K^+ or — 10 mM K^+. See Table 2 for calculated values from regression analysis.

ouabain was bound to the cells after 30 sec of dissociation (Table 2). Also, each component to which ^{3}H-ouabain binds, contained significantly greater amounts of ^{3}H-ouabain when binding had occurred in the absence of K^+. This indicates that in the absence of K^+ the Na,K-ATPase remains in a binding conformation for a longer time. These data are in agreement with published reports describing effects of K^+ on the Na,K-ATPase (Akera and Brody, 1970; Post et al., 1972; Inagaki et al., 1974; Lindenmayer et al., 1974).

Although each component contained a greater amount of ^{3}H-ouabain when binding had occurred in the absence of K^+, the greatest increase by far was in ^{3}H-ouabain bound to the site with the fastest dissociation rate constant. This is in agreement with data describing dissociation of ^{3}H-ouabain from isolated Na,K-ATPase from guinea pig heart (Godfraind et al., 1980).

The ability of K^+ to affect the abundance of specific binding sites indicates the fast component of dissociation represents binding to the Na,K-ATPase. More importantly, this indicates it may be possible to alter the apparent affinity of Na,K-ATPase for ouabain by changing the extracellular K^+ concentration. Two mechanisms may be involved; a change in the amount of time the enzyme spends in a binding conformation and a change in the relative abundance of high and low affinity conformations of the enzyme.

The magnitude of change in extracellular K^+ concentration caused by sodium-pump inhibition and the concentration dependence of ouabain to cause K^+ redistribution were examined by incubating myocytes in the absence or presence of ouabain for 60 min, then measuring the K^+ activity in the incubation media. Ouabain concentrations used ranged from 50 nM to 20 μM, as had been used to determine ^{3}H-ouabain binding for Scatchard analysis, and 0.3 mM to determine maximum redistribution of K^+ related to sodium-pump inhibition. Myocyte

TABLE 2

Binding and Dissociation of ^{3}H-Ouabain to Guinea-pig Myocytes Incubated in 2.5 μM ^{3}H-Ouabain for 40 min

Parameter	$[K^+]$	30 sec Dissociation	Non-Specific	Slow Specific Site	Fast Specific Site
^{3}H-Ouabain pmol/mg prot.	0	13.4±0.7	2.0±0.2	4.5±0.2	6.9±0.3
	10	7.7±0.2*	1.0±0.1*	3.7±0.1*	2.9±0.1*
r for regression line	0	---	> .95	0.93	0.92
	10	---	> .89	0.94	0.89
$t_{1/2}$ of dissociation (min)	0	---	212±27	9.4	2.5
	10	---	146±15	10.8	3.5

Dissociation of ^{3}H-ouabain was monitored as described in the text and legend to Figure 17. Values are ^{3}H-ouabain bound to different components as determined by analysis of the dissociation time course and descriptions of each regression line. Non-specific binding was determined by analysis of dissociation between 60 and 150 min after initiation of dissociation. * Indicates a significant ($p < .05$) difference caused by the presence of K^+ during the binding reaction. Analysis of significance was by Student's t-test.

density and incubation conditions were identical to those used in determining ^{3}H-ouabain binding for Scatchard analysis so changes in K^+ concentration should be comparable between these experiments. Total inhibition of the sodium-pump by 0.3 mM ouabain caused an increase in medium K^+ concentration of 0.49±0.06 or 0.52±0.03 mM (n=3) for myocytes incubated in the presence or absence of 2.0 μM monensin, respectively. Potassium ion concentration of medium in which cells had been incubated without ouabain was 4.18 mM, and was not affected by monensin. Therefore, the increase in medium K^+ concentration caused by total sodium-pump inhibition is only 12% of the basal concentration. This relatively modest increase in K^+ is unlikely to be responsible for the non-linearity of Scatchard plots. Further support for this is the shift to the right in the ouabain concentration response curve for release of K^+ which is caused by the presence of 2 μM monensin during the incubation (Figure 18). Monensin would have to shift the ouabain concentration response curve for K^+ release to the left if the increases in K^+ concentration caused by ouabain binding were responsible for the non-linearity of the Scatchard plots. This is deduced because monensin increases the degree of curvature of Scatchard plots describing ^{3}H-ouabain binding to myocytes in the presence of Ca^{2+} (Figure 14), thus indicating that monensin amplifies whatever process is causing the curvature of the Scatchard plots. Since the presence of monensin shifts the ouabain concentration-response curve for release of K^+ to the right, monensin inhibits this effect indicating these two phenomena are not causally related. These results demonstrate that redistribution of K^+ does occur as a result of the exposure of myocytes to high concentrations of ouabain. The magnitude of change in K^+ concentration, however, is probably not sufficient to affect ouabain binding under incubation conditions used in determining ^{3}H-ouabain binding for Scatchard analysis.

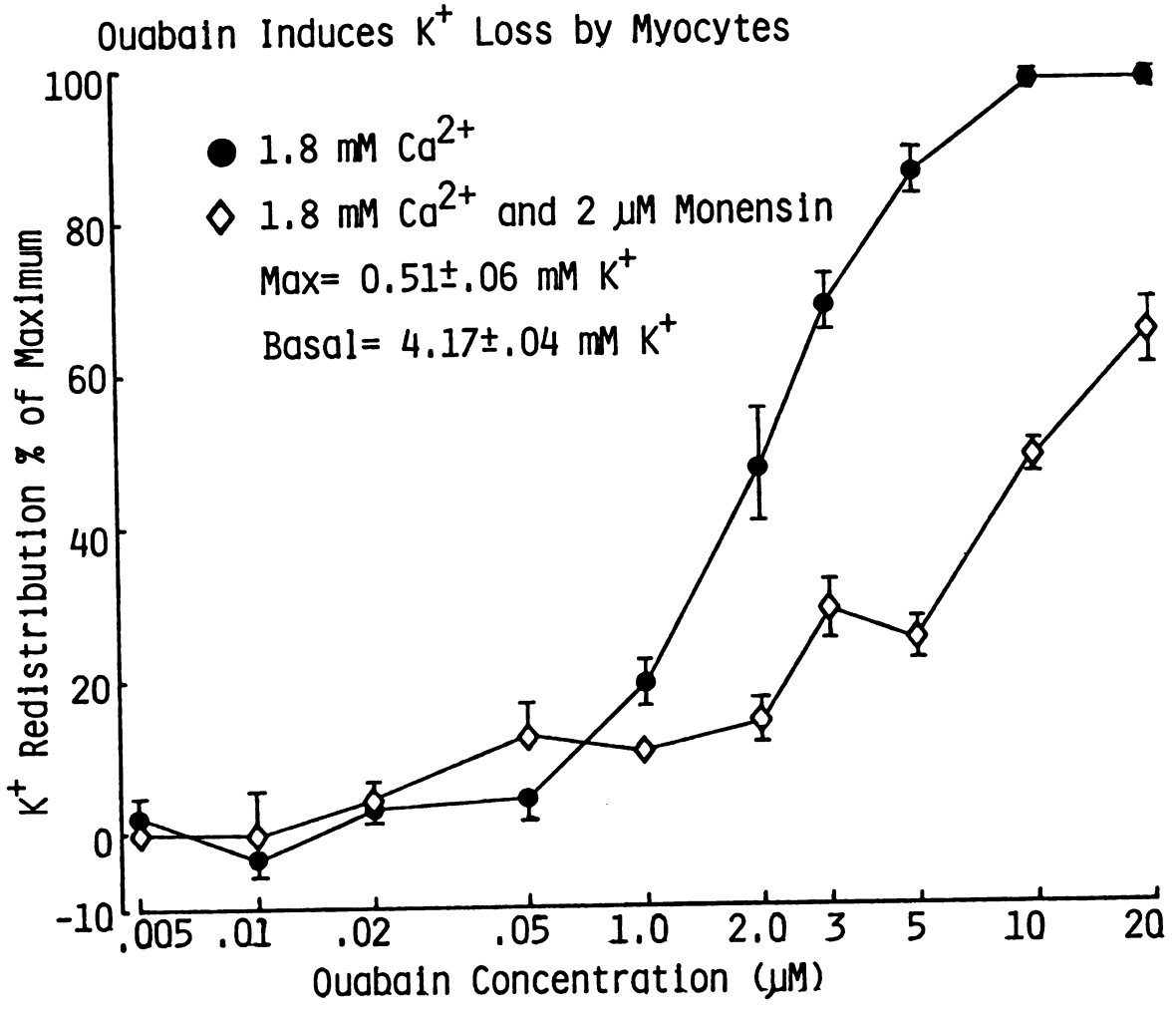

Figure 18. Redistribution of K^+ caused by ouabain. Myocytes from guinea-pig heart were incubated for 60 min in solution containing the indicated concentration of ouabain. Cells were pelleted by centrifugation and supernatants analyzed for K^+ activity (Orion Na^+/K^+ activity analyzer). Values represent the percentage of maximal increase in K^+ activity caused by each concentration of ouabain (n=3). Maximum redistribution of K^+ was assessed by incubating myocytes in 0.3 mM ouabain. Symbols indicate incubation conditions: ● 1.8 mM Ca^{2+}; ◇ 1.8 mM Ca^{2+} and 2 µM monensin. Values represent mean and SE from 3 determinations.

4. Binding of ^{3}H-Ouabain to Myocytes in the Presence of Agents which Alter Intracellular Ca^{2+} Concentration or Stimulate Production of Second Messengers

Stimulation of ^{3}H-ouabain binding caused by chelation of Ca^{2+} with EGTA is consistent with the hypothesis that Ca^{2+} has a direct effect on cardiac glycoside-Na,K-ATPase interactions in intact cardiac muscle. A direct effect is by a mechanism other than that of altering substrate availability, but may involve a Ca^{2+} dependent protein kinase or a Ca^{2+}-binding protein.

Since Ca^{2+} itself at concentrations normally present in cardiac muscle cells, does not inhibit Na,K-ATPase (Tobin et al., 1973; Godfraind et al., 1977), chelation of Ca^{2+} should have no effect to stimulate enzyme activity. Also, it has been shown with isolated Na,K-ATPase, that in the presence of Na^{+} and Mg^{2+}, Ca^{2+} does not have a significant effect to reduce the time the enzyme exists in a binding conformation (Tobin et al., 1973). Direct effects of Ca^{2+} ions themselves on Na,K-ATPase, therefore, cannot be responsible for the stimulation of ^{3}H-ouabain binding to myocytes caused by the absence of Ca^{2+}.

A number of Ca^{2+}-dependent protein kinases and Ca^{2+}-binding proteins exist in the heart. It is possible that a Ca^{2+}-dependent protein present in intact myocytes inhibits ^{3}H-ouabain binding when cells are incubated in millimolar concentrations of Ca^{2+}. Negative cooperativity in ^{3}H-ouabain binding would then be seen as intracellular Ca^{2+} concentration increased in response to enzyme inhibition.

Direct effects of agents known to alter cell handling of Ca^{2+} on ^{3}H-ouabain binding to Na,K-ATPase were examined by incubating myocytes with these agents and 0.5 or 2.5 μM ^{3}H-ouabain in a KHB solution containing 1.0 mM K^{+}. Concentrations of 0.5 and 2.5 μM ^{3}H-ouabain were chosen because they correspond roughly to the concentrations causing 50% or full occupancy, respectively, of high affinity binding sites. The low K^{+} concentration was chosen so that

myocytes would be partially sodium-loaded. Sodium loading and low extracellular K^+ concentration will cause the Na,K-ATPase to exist for a greater time in a binding conformation. Therefore, indirect effects due to Na^+ or K^+ redistribution caused by the agents examined are unlikely to affect ^{3}H-ouabain binding. To maintain cells in a viable condition during incubation in low K^+ solution, Ca^{2+} was reduced to 0.1 mM. Ca^{2+} at a 0.1 mM concentration should still be sufficient to promote any effects caused by Ca^{2+} acting at an intracellular site because normal intracellular Ca^{2+} concentration in resting cardiac cells is less than 1 μM. Entry of Ca^{2+} into myocytes incubated in KHB solution containing 1.0 mM K^+ and 0.1 mM Ca^{2+} was evident by spontaneous contractile activity observed when myocytes were maintained in this solution for greater than 30 min. The source of Ca^{2+} supporting the contractile activity was not identified in these experiments.

Agents examined for direct effects on ^{3}H-ouabain binding were: the phorbol ester TPA (phorbol 12-myristate 13-acetate); the Ca^{2+} ionophore A23187; caffeine; lanthanum (La^{3+}); the β-adrenergic agonist isoproterenol; and the α_1-adrenergic agonist phenylephrine. Effects of TPA were examined because of its ability to stimulate Ca^{2+}-phospholipid-dependent protein kinase (PK-C) (Nishizuka, 1984). This enzyme has been postulated to phosphorylate the Na,K-ATPase of rabbit erythrocytes (Ling and Sapirstein, 1984) and Friend erythroleukemia cells (Ling and Cantley, 1984). Protein kinase C activity has been found in cardiac muscle (Kuo et al., 1980) and phorbol esters have been shown to have specific binding sites on isolated myocytes from rat heart (Limas, 1985). Concentrations of TPA tested were 3.0 and 30 nM. These concentrations of TPA have a modest negative inotropic effect on guinea-pig atrial muscle (unpublished observation) and approximate the K_D reported for binding to receptors in rat heart (3.9 nM). Effects of the Ca^{2+} ionophore, A23187, were examined to determine if increasing the Ca^{2+} concentration, beyond that already present in spontaneously

contracting myocytes affects ^{3}H-ouabain binding. Effects of caffeine were examined because this agent is believed to increase intracellular Ca^{2+} concentration by effects on the sarcoplasmic reticulum (Blayney et al., 1978), and has been shown to increase resting tension in mammalian cardiac muscle when present at millimolar concentrations (Chapman and Leaty, 1975; Eisner and Valdeolmillos, 1984). Also, a related methylxanthine, theophylline, has been shown to inhibit the inotropic response of cardiac muscle to ouabain and to inhibit ^{3}H-ouabain binding (Zavecz, 1986). Effects of La^{3+} were examined because this agent is a potent Ca^{2+} antagonist which is effective in blocking Ca^{2+} movements across sarcolemmal membranes. If the effect of Ca^{2+} on ^{3}H-ouabain binding is mediated by any sarcolemmal Ca^{2+} transport mechanisms such as Na/Ca exchange, La^{3+} should block the effect. Effects of β-adrenergic stimulation by isoproterenol were examined because of this agent's well documented actions to promote phosphorylation of sarcolemmal constituents and increase force of contraction. Effects of α_1-adrenergic stimulation by phenylephrine were studied because α_1 agonists have been shown to stimulate phosphoinositide hydrolysis in cardiac muscle (Brown et al., 1985). Phenylephrine has been shown to be an effective agonist of α_1-adrenergic receptors in cardiac muscle (Bruckner et al., 1984). Phosphoinositide hydrolysis has been shown to result in the release of biologically active polyphosphoinositols and diacylglycerol into cells (Berridge, 1984; Berridge and Irvine, 1984). Phosphoinositols have unknown actions in cardiac muscle. Diacylglycerol is an endogenous stimulus for PK-C activity (Nishizuka, 1984).

Caffeine, at a concentration of 3 mM, significantly inhibited ^{3}H-ouabain binding to myocytes (Table 3). In a separate experiment, this concentration of caffeine also decreased the abundance of rod-shaped cells to 51.0$\pm$1.5% (n=5) from greater than 80% at initiation of incubations. This apparent toxic action of high concentrations of caffeine makes interpretation of these data

TABLE 3

^{3}H-Ouabain Binding to Guinea-pig Myocytes in the Presence of Agents Known to Affect Intracellular Ca^{2+} Concentration or Stimulate Production of Second Messengers

[^{3}H-Ouabain] µM	N	Control	Caffeine 0.1 mM	Caffeine 3.0 mM	La^{3+} 10 µm	La^{3+} 0.1 mM	[Ca^{2+}] mM	[K^{+}] mM
0.5	6	5.52±0.17	5.44±0.27	4.21±0.21*	5.37±0.18	5.58±0.32	0.1	1.0
2.5	6	9.19±0.20	8.93±0.22	6.21±0.53*	8.90±0.35	9.11±0.23	0.1	1.0
		Control	TPA 3 µm	TPA 30 nM	A23187 0.1 µM	A23187 1 µM		
0.5	4	5.99±0.36	5.55±0.33	6.36±0.04	6.07±0.36	6.01±0.44	0.1	1.0
2.5	4	10.57±0.84	10.26±0.26	10.47±0.01	10.59±0.49	10.01±0.22	0.1	1.0

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Table 3 (Continued)

		Control	Ascorbic Acid (1 mM)	ISO 10 nM	ISO 100 nM	PhE 2 μM	PhE 10 μM		
0.5	6	6.47±0.25	6.42±0.30	6.70±0.26	6.78±0.17	6.61±0.21	7.46±0.32	0.1	1.0
2.5	6	9.75±0.47	10.55±0.65	10.9 ±0.30	9.83±0.71	10.23±0.30	10.32±0.35	0.1	1.0

		Control	La^{3+} 0.1 mM	La^{3+} 0.3 mM	A23187 1.0 μM	A23187 3.0 μM	EGTA[a] 0.25 mM		
0.5	4	2.61±0.12	2.70±0.09	2.72±0.12	2.62±0.15	2.63±0.55	4.68±0.31*	1.8	3.8
2.5	4	6.17±0.12	5.91±0.29	5.91±0.33	6.47±0.71	6.26±0.33	9.58±0.40*	1.8	3.8

Myocytes were incubated in the presence of the indicated agent and ^{3}H-ouabain for 60 min. Concentrations of Ca^{2+} and K^{+} are as indicated. Values represent specific ^{3}H-ouabain binding. Decreased ^{3}H-ouabain binding in the presence of 1.8 mM Ca^{2+} and 3.8 mM K^{+} represents a significant effect of these incubation conditions to inhibit ^{3}H-ouabain binding.

[a]Myocytes incubated with EGTA were in Ca^{2+}-free solution.

impossible. All other interventions examined were without significant effect on ^{3}H-ouabain binding (Table 3). In these experiments, effects of the various interventions on ^{3}H-ouabain binding were examined while the cells were in a solution low in K^+ (1 mM K^+) which favors the Na,K-ATPase existing in a binding conformation. Therefore, effects on ^{3}H-ouabain binding would probably result from direct effects of the interventions on the affinity of the Na,K-ATPase for ouabain rather than from indirect effects related to the conformation of the enzyme. The interventions examined should increase (phenylephrine, A23187) or decrease (lanthanum) intracellular Ca^{2+} concentration or stimulate protein kinase activity (isoproterenol, TPA). The inability of these agents to affect ^{3}H-ouabain binding suggests that the mechanism by which Ca^{2+} removal stimulates ^{3}H-ouabain binding to myocytes is indirect.

Stimulation of ^{3}H-ouabain binding by EGTA was re-examined. In the same experiment the effects of La^{3+} and A23187 were also studied again. For this experiment, the K^+ concentration was maintained at 3.8 mM so that cells would be quiescent and intracellular Na^+ concentration low. The concentration of Ca^{2+} was 1.8 mM except in the incubation media with EGTA where free Ca^{2+} concentration was reduced to less than 1 μM. The action of EGTA to stimulate ^{3}H-ouabain binding was seen again in this experiment (Table 3). This was probably not a secondary result of eliminating Na/Ca exchange activity because La^{3+} at the highest concentration tested (0.3 mM) had no effect on ^{3}H-ouabain binding. Also, increasing sarcolemmal permeability to Ca^{2+} with the Ca^{2+} ionophore, A23187, did not affect ^{3}H-ouabain binding. These results once again substantiate that Ca^{2+} has no direct effects on Na,K-ATPase when it is present at concentrations compatible with cell viability.

One further important observation from these experiments is that the Na,K-ATPase of myocytes incubated in solutions containing 1 mM K^+, had an

apparent increase in affinity for ^{3}H-ouabain over that in myocytes incubated in 3.8 mM K^+ (Table 3). Increased affinity of the Na,K-ATPase for ouabain when myocytes are in solution with low K^+ can be attributed to the enzyme existing in the Na^+-induced binding conformation a greater amount of time. This results because K^+ is required for the change to a non-binding conformation. Additionally, because Na,K-ATPase turnover is reduced, intracellular Na^+ concentration should increase and stimulate the change to a Na^+-induced form of the enzyme. These together will result in maintenance of the Na,K-ATPase in a binding conformation. A similar increase in affinity was seen when cells were incubated in KHB solution containing 3.8 mM K^+ and 0.25 m EGTA (Table 3). The increase in affinity cannot be attributed to removal of a Ca^{2+}-dependent inhibition of binding, because when cells were incubated in KHB with 1 mM K^+ and 0.1 mM Ca^{2+}, binding was stimulated even though intracellular Ca^{2+} was elevated as evidenced by spontaneous contraction of the myocytes. To conclude that Ca^{2+} does not directly affect binding, an experiment examining the effect of Ca^{2+} on ^{3}H-ouabain binding to myocyte incubated in medium with a low K^+ concentration must be done. An alternative explanation for the stimulation of ^{3}H-ouabain binding which occurs upon removal of Ca^{2+} is that intracellular Na^+ concentration increases under this incubation condition as it does when myocytes are incubated in low K^+ solutions. This has been demonstrated for myocytes isolated from rat heart (Hohl et al., 1983). These investigators believe that the redistribution of Na^+ in myocytes incubated in Ca^{2+}-free solution is not mediated by ion channels because verapamil and tetrodotoxin have no effect to inhibit the increase in intracellular Na^+ concentration. It is possible that the increase in intracellular Na^+ concentration is caused by a decrease in Na/Ca exchange activity or is due to an increase in sarcolemmal permeability to Na^+ which may occur in the absence of Ca^{2+}. Results of ouabain sensitive $^{86}Rb^+$ uptake studies, however, do not

support the concept that an enhancement of ^{3}H-ouabain binding caused by removing Ca^{2+} results from an increase in intracellular Na^{+} (see below).

C. Sodium-Pump Activity and Capacity Assessed in Myocytes

The physiological representation of Na,K-ATPase is referred to as the sodium-pump (Ku et al., 1974). Active counter-transport of Na^{+} and K^{+} at the expense of ATP is accomplished by the sodium-pump. Therefore, sodium-pump activity can be estimated either by measuring active Na^{+} efflux or K^{+} influx across the sarcolemmal membrane. Most investigators use active uptake of $^{86}Rb^{+}$ as an estimate of sodium pump activity (Akera and Brody, 1985). Rb^{+} substitutes for K^{+} as a substrate for the Na,K-ATPase but slows down the reaction, due to slower dissociation of the enzyme-Rb^{+} complex than of the enzyme-K^{+} complex (Post et al., 1972; Tobin et al., 1973). This decrease in reaction rate is advantageous when the function of the sodium-pump is being examined, because it allows for more accurate estimation of changes in sodium-pump activity (Akera and Brody, 1985). Uptake of $^{86}Rb^{+}$ by the sodium-pump is assessed by measuring $^{86}Rb^{+}$ uptake in the presence and absence of a high concentration of ouabain. Since ouabain is a specific inhibitor of the Na,K-ATPase the ouabain-sensitive $^{86}Rb^{+}$ uptake can be used as an estimate of sodium-pump activity.

Because sodium-pump activity is limited by the intracellular Na^{+} concentration, measurement of this activity when intracellular Na^{+} concentration is low and stable is really an estimate of Na^{+} influx rate (Yamamoto et al., 1979; Akera et al., 1981). Direct effects on the Na,K-ATPase to alter its activity must be examined by determining changes in reserve capacity of the sodium-pump. Reserve capacity of the sodium-pump exists because the enzyme is substrate-limited by the low intracellular Na^{+} concentration. When Na^{+} is accumulating in

cells so that its concentration is increasing, or when the cells are sodium-loaded to the extent that Na^+ concentration is not rate-limiting for Na,K-ATPase activity, the capacity of the sodium pump can be estimated (Akera et al., 1981; Akera and Brody, 1985). Under these conditions, direct effects of agents on function of the Na,K-ATPase can be determined.

Isolated myocytes are a unique preparation of cardiac muscle because extracellular diffusion barriers are eliminated. This is an important consideration when making determinations of sodium-pump capacity by measuring the ouabain-sensitive $^{86}Rb^+$ uptake, because several minutes are generally required for sufficient tracer to diffuse evenly into whole tissues and be taken up into the cells. It is expected that individual ions can cross the sarcolemmal membrane several times during the several minutes it takes to assay $^{86}Rb^+$ uptake in beating cardiac muscle, thus, causing estimates of specific $^{86}Rb^+$ uptake to be low. Another problem is that when intact tissues are sodium-loaded, activity of the sodium-pump may be limited by the availability of the counter-ion ($^{86}Rb^+$). Availability of $^{86}Rb^+$ in intact tissues may be limited by diffusion barriers within the tissue when the sodium-pump is functioning at capacity, also causing estimates of specific $^{86}Rb^+$ uptake to be low. These problems should be minimized in preparations of isolated myocytes.

Additional advantages of the myocyte preparation for assessment of sodium-pump activity are the ease of separating the cells and their content from tracer ions in the incubation medium and the relatively short time needed to change the ionic gradients across the sarcolemmal membrane by changing the incubation medium. When estimating cell content of $^{86}Rb^+$, the centrifugation method was required for separating accumulated from free $^{86}Rb^+$. This was necessary because when myocytes were sampled by rapid filtration, background radioactive material retained on filters frequently exceeded total counts in samples of cells.

The most serious disadvantage of myocyte preparations for estimation of ouabain-sensitive $^{86}Rb^+$ uptake is that high voltages are required to electrically stimulate cells. Threshold voltage for stimulating contractile activity with square wave pulses of 4 msec duration was greater than 40 V when using plate electrodes of pure platinum spaced 1 cm apart. This high threshold for electrical stimulation probably is due to the high electrical resistance of lipid bilayers as compared to KHB solution and is not an indication of poor cell quality. These myocytes did respond to electrical stimulation at normal voltages when impaled directly by the stimulating electrode.[c] High voltage requirements for electrical stimulation of cells precluded this as a mechanism to increase Na^+ influx. Under conditions of the high current flux, electrolysis was visible at the electrode surfaces and cells died rapidly.

1. Activity and Capacity of the Sodium-pump

Activity of the Na,K-ATPase in isolated myocytes from guinea-pig heart was estimated as the difference in uptake of $^{86}Rb^+$ in the absence and presence of 1 mM ouabain. For determination of non-specific $^{86}Rb^+$ uptake myocytes were exposed simultaneously to ouabain and $^{86}Rb^+$. This may cause some overestimation of the non-specific uptake because even with a large excess of ouabain, inhibition of the sodium-pump requires some time. During that time, some $^{86}Rb^+$ will enter the cell via the Na,K-ATPase. The slight overestimation of non-specific $^{86}Rb^+$ uptake which will occur using this procedure is an acceptable error. The alternative method to estimate non-specific $^{86}Rb^+$ uptake is to incubate myocytes with a high concentration of ouabain before exposing the cells to $^{86}Rb^+$. Extended incubation of myocytes in solutions containing 1 mM ouabain causes redistribution of Na^+, K^+, and Ca^{2+} and may alter membrane

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[c]Determination of the excitability of myocytes was done by Robert W. Hadley working in the laboratory of Dr. Joseph R. Hume at MSU.

properties which affect passive uptake of $^{86}Rb^+$. Therefore, exposure to $^{86}Rb^+$ and ouabain was simultaneous when determinations of non-specific $^{86}Rb^+$ uptake were made.

Specific uptake of $^{86}Rb^+$ into quiescent myocytes was linear with respect to time for at least 15 min when ouabain-sensitive $^{86}Rb^+$ uptake was assayed in KHB solution containing 5.0 mM Rb^+ (data not shown). Uptake time was limited to 6 min in all assays of ouabain-sensitive $^{86}Rb^+$ uptake and was reduced to 3 or 2 min when examining the capacity of the sodium-pump. Short incubation times for $^{86}Rb^+$ uptake were used to ensure that specific uptake would be linear with respect to time.

Sodium-pump activity was determined in quiescent cells and in cells exposed to the Na^+ ionophore, monensin. Monensin has been shown to stimulate sodium-pump activity in contracting and quiescent preparations of cardic muscle (Yamamoto et al., 1979). Presumably, this is a result of its ability to increase membrane Na^+ conductance. For this experiment, exposure of cells to monensin was simultaneous with exposure to $^{86}Rb^+$. Monensin had a concentration-dependent effect to increase ouabain-sensitive $^{86}Rb^+$ uptake into myocytes (Figure 19). This effect apparently peaked at 30 μM monensin when uptake was estimated in the absence or presence of 1.8 mM Ca^{2+}. Specific $^{86}Rb^+$ uptake into quiescent cells or cells incubated in low concentrations of monensin was significantly lower when the uptake occurred in the presence of 1.8 mM Ca^{2+} (Figure 19). At higher concentrations of monensin Ca^{2+} did not have a significant effect on ouabain-sensitive $^{86}Rb^+$ uptake. The plateau in the effect of monensin to stimulate $^{86}Rb^+$ uptake into myocytes may indicate that Na^+ influx has been increased to an extent that intracellular Na^+ concentration is not rate-limiting for sodium-pump activity. Alternatively, the ability of monensin to increase Na^+

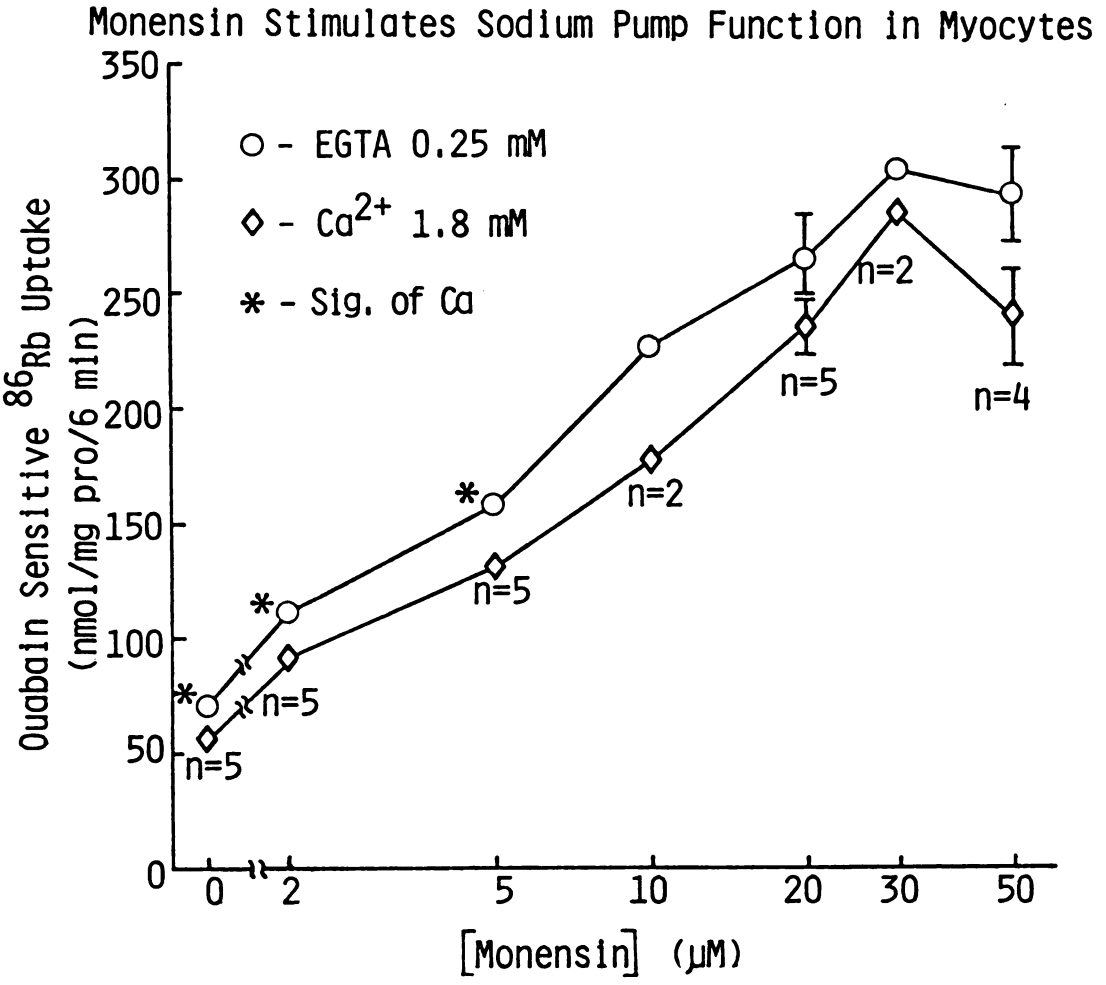

Figure 19. Monensin stimulates sodium-pump activity in myocytes. Myocytes from guinea-pig heart were incubated in the indicated concentrations of monensin during incubation for assessment of ouabain-sensitive $^{86}Rb^{+}$ uptake. Uptake of $^{86}Rb^{+}$ was measured from solutions containing: ◇ 1.8 mM Ca^{2+} or ○ 0.25 mM EGTA. * Indicates a significant ($p < 0.05$) effect of Ca^{2+} on ouabain-sensitive $^{86}Rb^{+}$ uptake. Number of determinations as indicated. Significance determined by Student's t-test.

influx may have plateaued. These possibilities were investigated by preincubating myocytes in monensin for five minutes prior to initiation of $^{86}Rb^+$ uptake.

In the previous experiment (results shown in Figure 19) exposure to monensin and $^{86}Rb^+$ were simultaneous. Therefore, values represent $^{86}Rb^+$ uptake over a period of time when intracellular Na^+ concentration is increasing. It is possible that by the end of the incubation period (6 min) intracellular Na^+ concentration in cells exposed to each concentration of monensin had increased to the extent that the sodium-pump was operating at capacity. The concentration dependence of monensin would, therefore, reflect a more rapid increase in intracellular Na^+ at higher concentrations of monensin. Preincubating myocytes with monensin for five minutes before initiating uptake of $^{86}Rb^+$ and measuring the uptake in KHB solution containing 2 mM $^{86}Rb^+$ caused the maximal effect of monensin to be reached at a lower concentration (Figure 20). The concentration of Rb^+ was decreased to 2 mM from the 5 mM concentration used in previous experiments, so that Na^+ concentration would increase faster and Na^+ would cease to be the limiting substrate sooner. There was no significant difference in $^{86}Rb^+$ uptake for cells exposed to 10, 20, 30 or 50 μM monensin when myocytes were incubated in the absence of Ca^{2+}. A significantly lower ^{86}Rb uptake was observed for myocytes incubated in 10 μM monensin in the presence of 1.8 mM Ca^{2+}, indicating that the effect of monensin was not sufficient to eliminate the sodium-pump reserve capacity at that concentration. The plateau at 20 to 50 μM in the effect of monensin to increase ouabain-sensitive $^{86}Rb^+$ uptake when myocytes are incubated in a solution containing 2 mM Rb^+ suggests that the sodium-pump is working at its capacity under those conditions. It is unlikely that the ability of monensin to increase Na^+ influx had plateaued, because when uptake of $^{86}Rb^+$ was measured from the onset of myocyte exposure to monensin, there

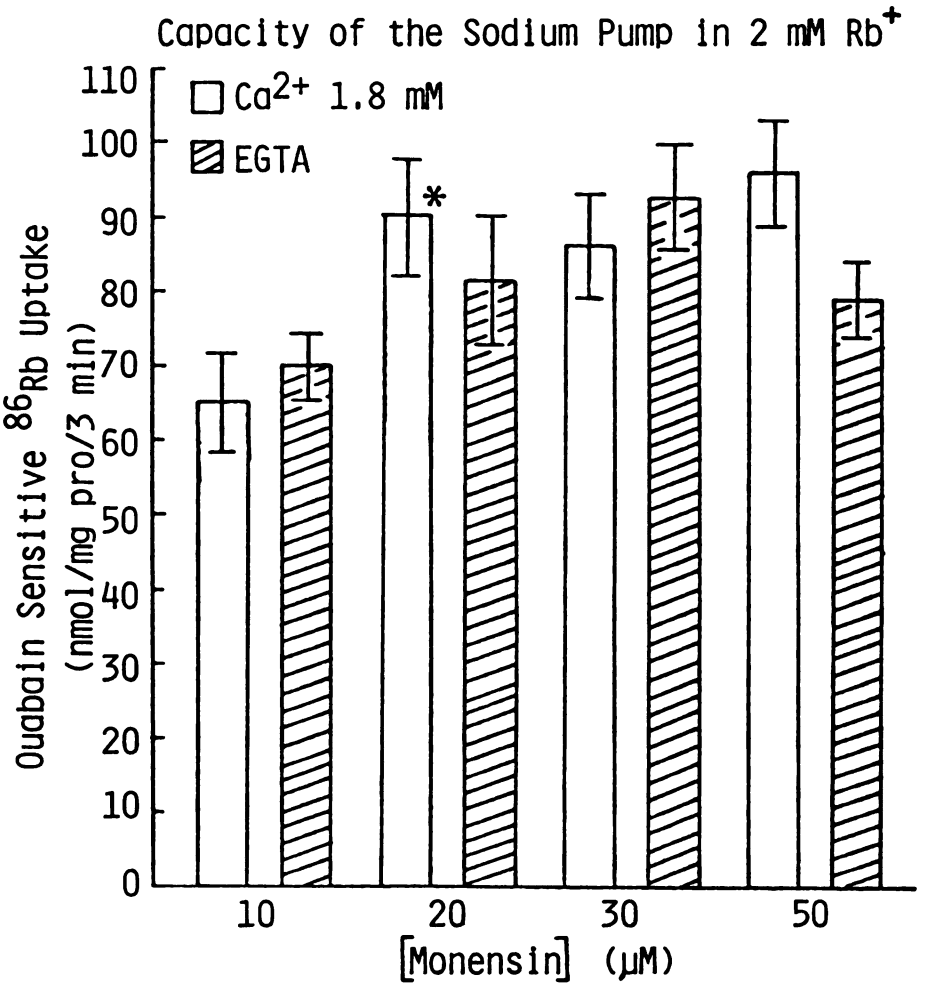

Figure 20. Capacity of the sodium-pump. Ouabain-sensitive $^{86}Rb^{+}$ uptake was measured in myocytes from guinea-pig heart after preincubating the cells for 4 min in the indicated concentrations of monensin. Open bars indicate $^{86}Rb^{+}$ uptake occurred in the presence of 1.8 mM Ca^{2+}, hatched bars indicate $^{86}Rb^{+}$ uptake occurred in solution containing 0.25 mM EGTA. * Indicates a significant ($p < .05$) increase in ouabain-sensitive $^{86}Rb^{+}$ uptake caused by a single step increase in monensin concentration. n=5 for each determination.

were apparent differences (increases) in sodium-pump activity between cells incubated in 10 or 20 and 20 or 30 μM monensin (Figure 19).

The action of Ca^{2+} to decrease ouabain-sensitive $^{86}Rb^{+}$ uptake was statistically significant for quiescent cells or cells incubated in low concentrations of monensin but not for cells incubated in high (> 10 μM) concentrations of monensin (Figures 19 and 20). The concentration dependence for this effect of Ca^{2+} was examined using quiescent cells incubated in the absence of monensin. Concentration of Ca^{2+} was controlled by the presence of 0.25 mM EGTA in the $^{86}Rb^{+}$ uptake media. Ionized Ca^{2+} concentration was measured with a Ca^{2+}-selective electrode and $CaCl_2$ (1.8 M) solution was added to KHB solution containing 0.25 mM EGTA to increase Ca^{2+} activity the desired amount. Cells were incubated in KHB solution containing 1.8 mM Ca^{2+} prior to initiation of $^{86}Rb^{+}$ uptake in solutions with various Ca^{2+} concentrations. Because the myocytes were exposed to high or low concentrations of Ca^{2+} only during the time of $^{86}Rb^{+}$ uptake, effects of extracellular Ca^{2+} on viability of the myocytes should be minimal. Increasing Ca^{2+} concentration from zero to 0.9 mM and from 0.9 to 1.8 mM had a significant effect to inhibit ouabain-sensitive $^{86}Rb^{+}$ uptake (Figure 21). Further increasing Ca^{2+} concentration to 3.6 and 7.2 mM had no additional effect on ouabain-sensitive $^{86}Rb^{+}$ uptake. If Ca^{2+} had toxic effects in the myocytes during the 6-min incubation, a decrease in ouabain sensitive $^{86}Rb^{+}$ uptake is expected at the 3.6 and 7.2 mM concentrations of Ca^{2+}. This, however, was not observed. This indicates that the action of Ca^{2+} to inhibit ouabain-sensitive $^{86}Rb^{+}$ uptake is concentration-dependent but in quiescent myocytes the concentration dependence does not extend to the full range that isolated cardiac muscle preparations are sensitive to extracellular Ca^{2+} concentration. In isolated atrial muscle preparations from guinea-pig heart, increasing Ca^{2+} has a positive inotropic effect even when concentrations of Ca^{2+} exceed 7 mM. This suggests

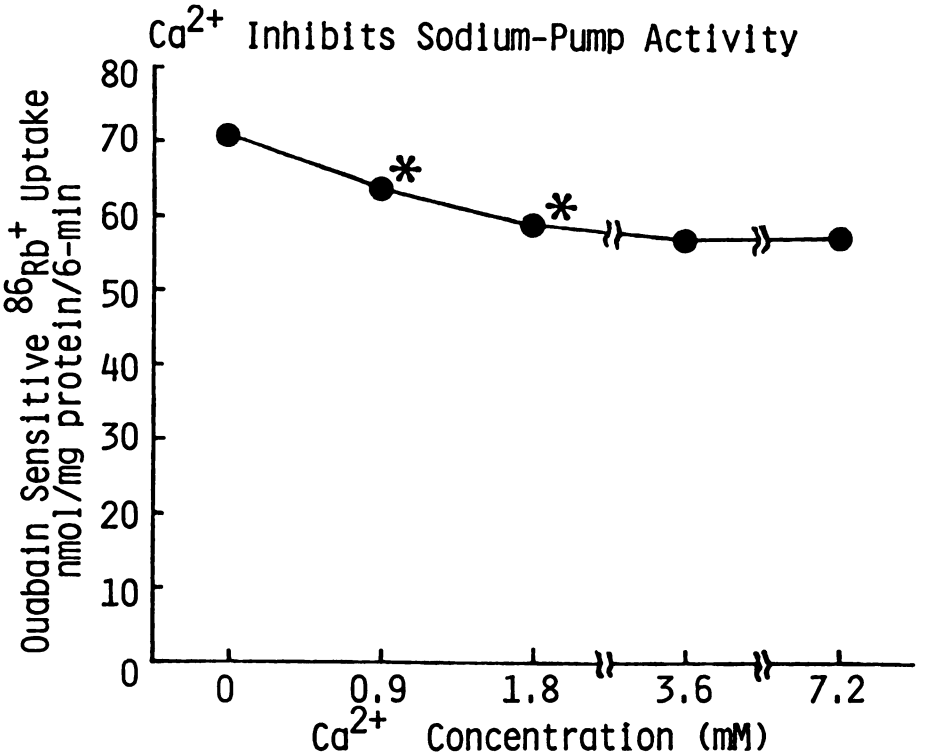

Figure 21. Ouabain-sensitive $^{86}Rb^+$ uptake is inhibited by Ca^{2+} in a concentration-dependent manner. Myocytes from guinea-pig heart were added to incubation media containing the indicated concentrations of Ca^{2+} after a 60-min incubation in KHB solution containing 1.8 mM Ca^{2+}. All solutions contained 5 mM RbCl. Symbols represent: ● ouabain-sensitive $^{86}Rb^+$ uptake. * Indicates a significant ($p < .05$) effect of a one-step increase in Ca^{2+} concentration (n=4).

that the mechanism by which Ca^{2+} increases force of contraction is different from the mechanism by which it inhibits ouabain-sensitive $^{86}Rb^{+}$ uptake. Inhibition of $^{86}Rb^{+}$ uptake by Ca^{2+} could be due to a direct action on the Na,K-ATPase, competition for intracellular Na^{+} via the Na/Ca exchange mechanism, inhibition of Na^{+} entry by occupying membrane cation binding sites, or other mechanisms not considered. The present results cannot distinguish between these possibilities.

Monensin stimulated sodium-pump activity in myocytes. Because monensin can have effects in addition to its effect to increase Na^{+} influx, it was desirable to demonstrate a stimulation of sodium-pump activity by using another intervention to increase intracellular Na^{+} concentration. An alternative method to stimulate sodium-pump activity in myocytes is to load the cells with Na^{+} prior to initiation of $^{86}Rb^{+}$ uptake (Eisner and Lederer, 1979; Akera and Brody, 1985). Myocytes were loaded with Na^{+} by incubating cells in K^{+}- and Rb^{+}-free KHB solution at 37°C. In the absence of a counter-ion to support Na^{+} efflux by the sodium-pump Na^{+} will accumulate in the cells. Incubation of myocytes with K^{+}- and Rb^{+}-free solution was done in a glass column with continuous flow of fresh solution over the cells to prevent accumulation of K^{+} as it leaked from the cells. The Ca^{2+} concentration of KHB solution used to sodium load the myocytes was reduced to 10 μM in an attempt to limit toxic effects of Ca^{2+}. After approximately 30 minutes of perfusion with K^{+}- and Rb^{+}-free solution the myocytes were all spontaneously contracting. The contractions were not discrete, however, but resembled a writhing movement. Upon addition of these sodium-loaded myocytes to KHB solution containing 1.8 mM Ca^{2+} and 3.8 mM K^{+}, the cells began to contract with discrete contractions which included full relaxation when examined microscopically. Contractions continued for several minutes following resuspension of sodium-loaded myocytes in solution containing K^{+} and

Ca^{2+}. The sodium-loading procedure did decrease the percentage of cells which retained a rod-shape in solutions containing Ca^{2+}. This effect was not quantitated, however. Measurement of ouabain-sensitive $^{86}Rb^{+}$ uptake in sodium-loaded myocytes was limited to two minutes in order to minimize toxic effects of Ca^{2+} and to limit the decrease in intracellular Na^{+} concentration which is expected to occur when the sodium-pump is activated.

Incubation of myocytes in K^{+}- and Rb^{+}-free solution exhibited a time-dependent effect to increase the ouabain-sensitive $^{86}Rb^{+}$ uptake measured when cells were exposed to KHB solution containing 5 mM Rb^{+} (Figure 22). Maximum stimulation of $^{86}Rb^{+}$ uptake was achieved by a 45-min incubation of myocytes in K^{+}- and Rb^{+}-free solution. Longer incubation of cells, up to 90 min total time, had no additional effect on sodium-pump activity. The plateau in the effect of time on sodium-loading could be due to the intracellular Na^{+} concentration reaching a plateau or to the sodium-pump operating at capacity. Sodium-pump activity in sodium-loaded cells is significantly inhibited by the presence of Ca^{2+} in the uptake medium, similar to the effect of Ca^{2+} observed in monensin-treated myocytes. The ability of sodium-loading to stimulate sodium-pump activity achieves a plateau with the same time for sodium-loading irregardless of the absence or presence of Ca^{2+}. If the mechanism by which Ca^{2+} inhibits sodium-pump activity is by supporting Na/Ca exchange activity and thereby supporting competition between the Na,K-ATPase and the Na/Ca exchanger for intracellular Na^{+}, then intracellular Na^{+} concentration must have reached a plateau after 45 min of sodium-loading. Alternatively, if the sodium-pump is operating at capacity, Na^{+} could still be accumulating in the cells and Ca^{2+} may act by directly inhibiting the Na,K-ATPase thus causing the capacity of the sodium-pump to be reduced when Ca^{2+} is present (Figure 22).

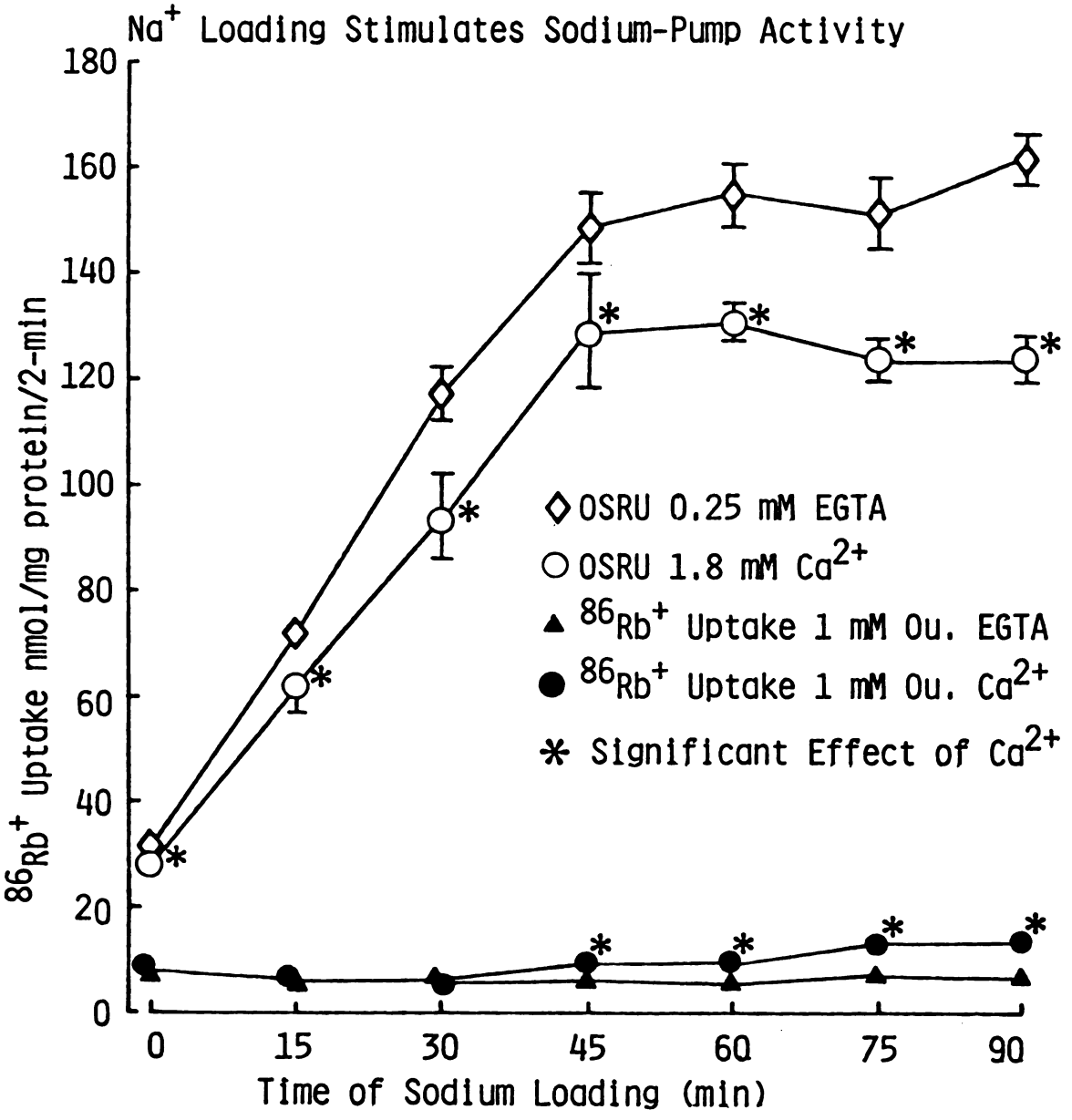

Figure 22. Stimulation of sodium-pump activity by incubation of myocytes in K^+-free, Rb^+-free solution. Myocytes from guinea-pig heart were superfused with K^+-free, Rb^+-free KHB solution containing 10 μM Ca^{2+} for the indicated time. Sodium-pump activity was estimated by the ouabain-sensitive $^{86}Rb^+$ uptake over 2 min when cells were dispersed in solution containing 5 mM Rb^+ with tracer amounts of $^{86}Rb^+$. Open symbols represent ouabain-sensitive $^{86}Rb^+$ uptake. Closed symbols represent $^{86}Rb^+$ uptake in the presence of 1 mM ouabain. Incubation solutions contained ○, ● 1.8 mM Ca^{2+} (n=5) or, ◇, ▲ 0.25 mM EGTA (n=5). * Indicates a significant ($p < .05$) effect of Ca^{2+}.

Non-specific $^{86}Rb^+$ uptake was decreased significantly following 15 min of sodium-loading (Figure 22). This may be related to the Na,K-ATPase being in a high affinity conformation when the cell is sodium-loaded, so that binding of ouabain and inhibition of specific $^{86}Rb^+$ uptake is accelerated. If cells are dispersed into Ca^{2+}-free solution, non-specific $^{86}Rb^+$ uptake is not affected further by increase in the time of sodium-loading. Apparent toxic effects of Ca^{2+} which would compromise the sarcolemmal membrane integrity may have been detected as an increase in non-specific $^{86}Rb^+$ uptake into cells dispersed into KHB solution containing 1.8 mM Ca^{2+} (Figure 22). Non-specific $^{86}Rb^+$ uptake increased significantly when myocytes were exposed to KHB solution devoid of K^+ and Rb^+ for 45 min or longer. Alternatively, this may reflect an increase in Rb^+ conductance of the membrane caused by Ca^{2+} entry via the Na/Ca exchange mechanism and activation of a Ca^{2+}-dependent K^+ channel (Siegelbaum and Tsien, 1980). If the latter explanation is correct, the increase in non-specific $^{86}Rb^+$ uptake after ouabain-sensitive $^{86}Rb^+$ uptake had reached a plateau (Figure 22) may indicate that the Na^+ concentration continues to increase beyond the time required for the maximum increase in ouabain-sensitive $^{86}Rb^+$ uptake. It is unlikely that a difference in cell viability would affect ouabain-sensitive $^{86}Rb^+$ uptake within a two-minute period or that changing permeability of the membrane to Na^+ would affect ouabain sensitive $^{86}Rb^+$ uptake into sodium-loaded cells.

Definitive substantiation that the sodium-pump is working at capacity in sodium-loaded or monensin-treated myocytes would require a demonstration of an increase in sodium-pump activity resulting from an increase in the number of pump units or an increase in the intracellular Na^+ concentration without an increase in the ouabain-sensitive $^{86}Rb^+$ uptake. An attempt was made to increase the number of sodium-pump units per cell by depleting animals of K^+. The number of ^3H-ouabain binding sites in Na,K-ATPase preparations from cardiac

muscle of hypokalemic animals has been shown to be significantly greater than the number of binding sites in Na,K-ATPase preparations from cardiac muscle of animals with normal concentrations of serum K^+ (Erdmann et al., 1971; Bluschke et al., 1976). Specific ^{3}H-ouabain binding site number has also been shown to increase in intact cultured chick heart cells after 48 hours of incubation in a medium containing 1 mM K^+ (Kim et al., 1984) compared to control cultures which were maintained in a medium containing 4 mM K^+. These data suggest that cardiac muscle cells respond to low K^+ concentration by increasing the number of functional sodium-pump units.

Guinea pigs were maintained on a K^+-deficient diet for 14-21 days in order to lower serum K^+ concentration. Blood was collected from two ketamine-anesthetized guinea pigs and serum analyzed for K^+ activity. Serum K^+ activity in the sample from each animal was less than 2 mM, indicating that they had abnormally low serum K^+ concentrations. Myocytes were isolated from control and K^+-deficient animals simultaneously and analyzed for binding site number and affinity for ^{3}H-ouabain (Akera and Cheng, 1977) and for sodium-pump activity in quiescent cells or cells exposed to 5 or 30 μM monensin. There were no apparent morphological differences in myocytes isolated from normal and hypokalemic animals. As detailed earlier, kinetic parameters of ^{3}H-ouabain binding to myocytes cannot be determined by the method used. Therefore, the only reliable binding data are the estimates of total ^{3}H-ouabain binding to the cells incubated in 100 nM ^{3}H-ouabain. These values are 0.73 ± 0.04 and 1.24 ± 0.06 pmol/mg protein for cells from control and K^+-deficient animals, respectively. The increase in ^{3}H-ouabain binding suggests that the binding site number is increased when animals are maintained on a K^+-deficient diet. However, no reliable estimate of non-specific binding was made so this cannot be certain. Estimates of ouabain-sensitive $^{86}Rb^+$ uptake are shown in Figure 23. There was no apparent change

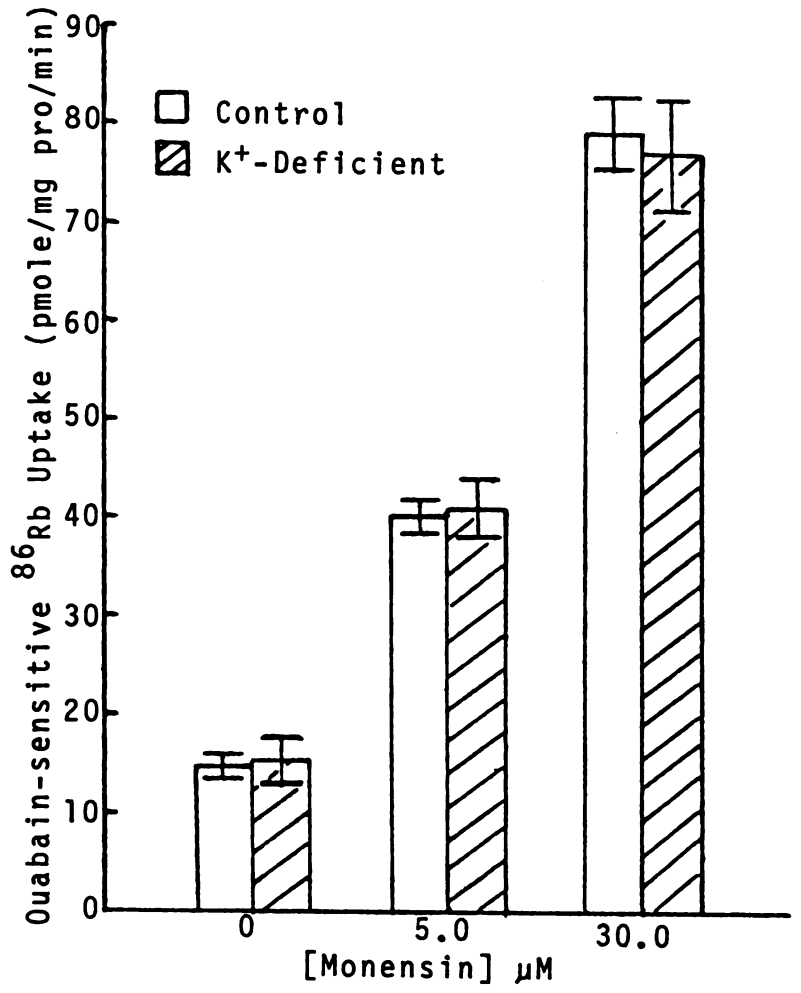

Figure 23. Sodium-pump activity is not increased in myocytes from K^+-deficient animals. Myocytes from hearts of guinea pigs fed a control diet (open bars) or a diet deficient in K^+ (striped bars) were assessed for sodium-pump activity by measuring ouabain-sensitive $^{86}Rb^+$ uptake in the absence of monensin or in the presence of the indicated concentration of monensin. n=7, vertical lines indicate SE.

caused by K^+ deficiency in the ouabain-sensitive $^{86}Rb^+$ uptake of quiescent myocytes or those exposed to 5 or 30 μM monensin. If the apparent change in ^3H-ouabain binding site number corresponds to an actual change in functional sodium-pump number these data suggest that the reserve capacity of the sodium-pump is not expressed in cells exposed to 30 μM monensin. Therefore, even when monensin is present, ouabain sensitive $^{86}Rb^+$ uptake is an estimate of Na^+ influx and is not affected by the number of enzyme units per cell.

2. Reduction of Reserve Capacity by Interventions Increasing Intracellular Na^+ Concentration or Na^+ Influx

Ouabain has a concentration-dependent effect to decrease sodium-pump activity. Because the sodium-pump has a reserve capacity, the inhibition of any given percentage of Na,K-ATPase enzymes is expected to have less effect on sodium-pump activity when Na^+ influx is low than when Na^+ influx is high, or when the cell is Na-loaded (Akera and Brody, 1985). The effect of increased Na^+ influx on ouabain inhibition of sodium-pump activity was examined by incubating myocytes for 60 min in various concentrations of ouabain, then adding cells to media containing the same concentration of ouabain and tracer amounts of $^{86}Rb^+$. Ouabain-sensitive $^{86}Rb^+$ uptake was determined in the absence and presence of 50 μM monensin and in the absence and presence of Ca^{2+}. Monensin increased the ouabain-sensitive $^{86}Rb^+$ uptake 4-fold (Figure 24) and shifted the ouabain concentration response curve to the left (Figure 25) indicating that the reserve capacity of the sodium-pump had been decreased by the augmentation of Na^+ influx. The presence of Ca^{2+} during $^{86}Rb^+$ uptake decreased sodium-pump activity (Figure 24) and also shifted the ouabain concentration response curve to the left. When $^{86}Rb^+$ uptake was monitored in the presence of monensin, Ca^{2+} inhibited sodium-pump activity (Figure 24) but did not change the ouabain concentration response curve. The shift to the left of the ouabain concentration

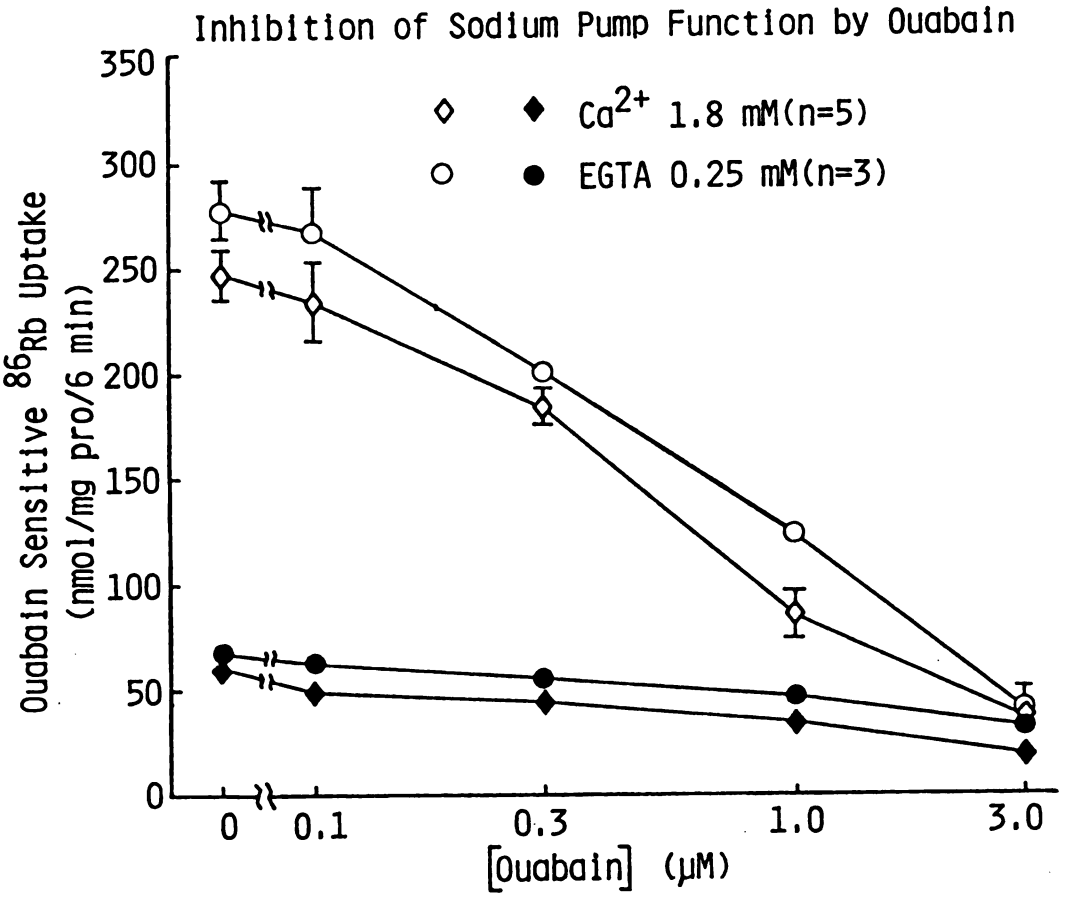

Figure 24. Inhibition of sodium-pump activity by ouabain. Myocytes from guinea-pig heart were incubated in the indicated concentration of ouabain for 60 min. $^{86}Rb^+$ uptake was initiated by adding myocytes to solution containing an identical concentration of ouabain plus tracer amounts of $^{86}Rb^+$. Closed symbols represent values for ouabain-sensitive $^{86}Rb^+$ uptake in the absence of monensin, open symbols in the presence of 50 μM monensin. Incubation media for $^{86}Rb^+$ uptake contained: ◇, ◆ 1.8 mM Ca^{2+} (n=5) or, ○, ● 0.25 mM EGTA (n=3).

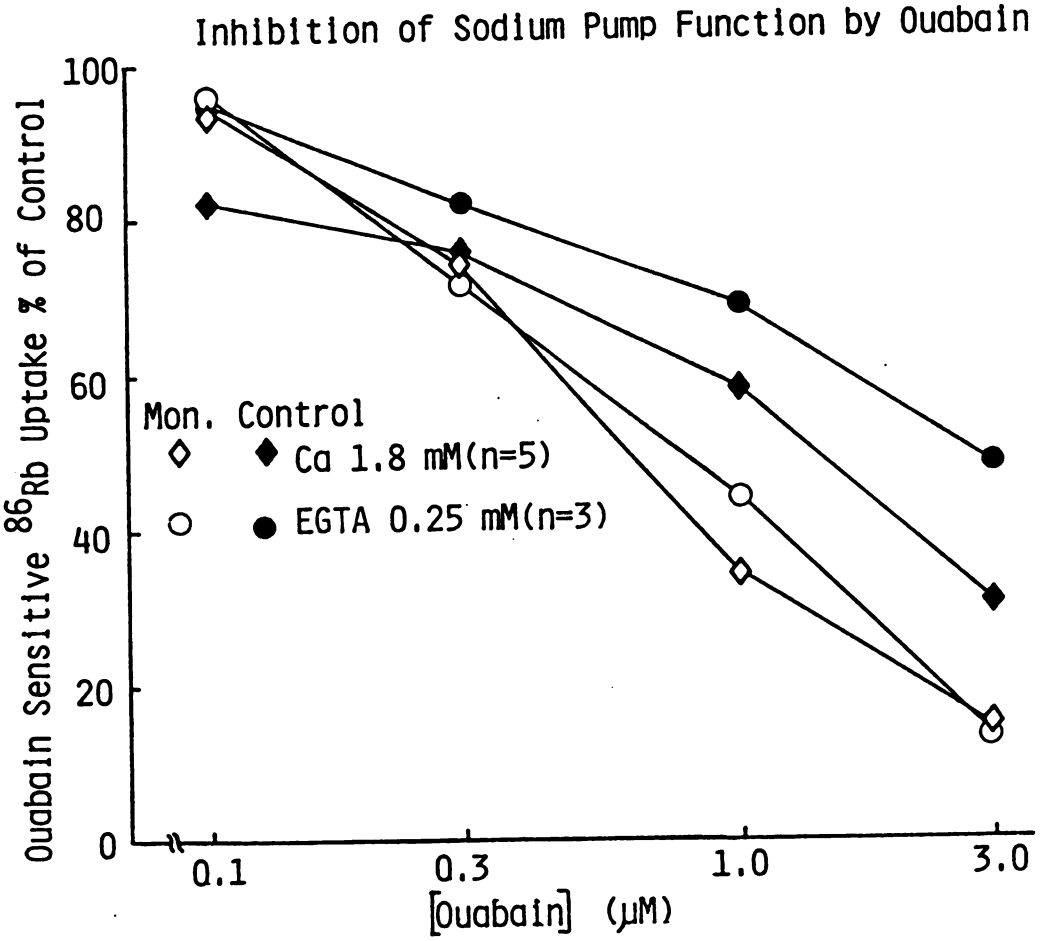

Figure 25. Concentration-response curve for ouabain inhibition of sodium-pump activity. Incubation of myocytes was as described in the legend to Figure 24. Open symbols indicate the presence of 50 μM monensin during $^{86}Rb^{+}$ uptake, closed symbols its absence. Incubation media for $^{86}Rb^{+}$ uptake contained: ◇, ◆ 1.8 mM Ca^{2+} (n=5) or, ○, ● 0.25 mM EGTA (n=3).

response curve caused by the presence of Ca^{2+} during $^{86}Rb^{+}$ uptake in non-treated cells is inconsistent with an effect of Ca^{2+} to inhibit $^{86}Rb^{+}$ uptake by competition for Na^{+} via the Na/Ca exchange mechanism. A shift towards the left could be caused by an increase in ouabain binding or an increase in Na^{+} influx. Exposure to a Ca^{2+}-free solution or one containing monensin lasted for 6 min in experiments to determine ouabain concentration-response curves. Although the absence of Ca^{2+} has been shown to stimulate ^{3}H-ouabain binding to myocytes after a 60-min incubation (Figure 15), the time course for this phenomena was not examined. Monensin significantly increased ^{3}H-ouabain binding after 5 min of exposure (Figure 9). Since the specific binding of ouabain was not examined in this experiment, the shift in the ouabain concentration response curve does not unequivocally demonstrate a decrease in reserve capacity. The shift in the ouabain concentration-response curve caused by the presence of Ca^{2+} is inconsistent with the effect of Ca^{2+} to decrease ^{3}H-ouabain binding but is consistent with a decrease in reserve capacity. The greater shift seen under conditions of increased Na^{+} influx is consistent with both an increase in ^{3}H-ouabain binding and a decrease in the reserve capacity of the sodium-pump caused by monensin.

DISCUSSION

A. Regulation of the Na,K-ATPase and Cardiac Glycoside Binding in Intact Tissue

Positive inotropic effects of the cardiac glycosides on cardiac muscle and the ability of these agents to bind to specific receptor sites on the Na,K-ATPase causing inhibition of the enzyme have each been studied extensively (Lee and Klaus, 1971; Akera and Brody, 1978). Overwhelming evidence exists which supports the hypothesis that inhibition of the Na,K-ATPase is causally linked to both the positive inotropic effect and the toxic effects of the cardiac glycosides. One major question concerning the Na,K-ATPase which is still unanswered is whether or not there are endogenous mechanisms for regulation of the enzyme activity (Anner, 1985). In attempts to answer this question an extensive amount of work has been done, but at this time the only factor known to affect activity of the Na,K-ATPase is substrate availability -- specifically, intracellular Na^+ concentration (Akera and Brody, 1982).

In order to demonstrate that an intervention or regulatory factor has an effect on Na,K-ATPase activity, the enzyme function must be examined under conditions such that the Na^+ concentration is not rate-limiting. This condition probably occurs transiently with each contraction of the heart, but at this time the technology is not available to monitor Na^+ transients; therefore, there is no way to directly monitor this effect in intact tissue.

The isolated myocyte preparation has many features which may facilitate estimation of drug binding and sodium-pump activity. These parameters have

been examined in isolated myocytes from guinea-pig heart and indicate direct effects of agents on Na,K-ATPase activity and cardiac glycoside binding can be studied with this preparation.

B. Myocyte Isolation for Examination of ^{3}H-Ouabain Binding and Sodium-Pump Activity

Myocytes from guinea-pig heart were isolated in high yield and with greater than 80% of all cells retaining normal morphology. These latter cells were quiescent in solutions containing millimolar concentrations of Ca^{2+} and possessed normal characteristics of excitable tissues in terms of membrane electrical properties. The properties of these cells and their stability over time compare favorably with myocyte preparations isolated by other investigators which have been described in the literature (Farmer et al., 1977; Haworth et al., 1980, 1982; Isenberg and Klockner, 1982; Bihler et al., 1984; Bkaily et al., 1984). Differences in isolation procedures which promote isolation of stable cells were not tested directly. Therefore, a comparison of the method for myocyte isolation described in this work with other published methods is not possible. A review and comparison of published procedures for isolation of cardiac myocytes has been published (Farmer et al., 1983).

From the work done in this laboratory, two points seem very clear; first, to isolate myocytes in high yield, hearts must be extremely well digested; second, isolated myocytes are very fragile, and to maintain these cells in viable condition they must be treated gently. The second point may explain the need for good digestion of the tissue; as a well digested tissue requires a minimum of mechanical agitation to dissociate the cells. Collagenase and hyaluronidase were used to digest the connective tissues in the hearts. Collagenase was extremely critical for this process and variation in this enzyme was responsible for most variation in the isolation procedure. Hyaluronidase is not essential for the

digestion, and on one occasion this enzyme was not used. Omission of hyaluronidase did not appear to affect digestion of the tissue or morphology of the cells but this enzyme was included so that there was consistency in the isolation procedure throughout the entire course of these experiments.

The time course of the digestion procedure is well detailed in the Methods section. It seemed important to follow this to as great an extent as possible. The changes in digestion procedure which were used are: increase in time of exposure to enzymes, decrease in time of perfusion with low Ca^{2+} medium before starting recirculation, and decrease in volume of recirculating perfusate. Increasing the time of exposure to enzymes seems an obvious way to enhance digestion. Another consideration, however, is that the enzymes must be in a low Ca^{2+} solution to be effective (increasing Ca^{2+} concentration to even 50 μM in the solution containing enzymes inhibited digestion) and cardiac muscle is subject to a phenomenon termed the "Ca^{2+} paradox" (Baker et al., 1983). The "Ca^{2+} paradox" is a toxic effect of physiologic concentrations of Ca^{2+} (millimolar) after the heart muscle has been exposed to a Ca^{2+}-free medium. This phenomenon may be the reason it is difficult to isolate Ca^{2+}-tolerant myocytes from cardiac muscle. To minimize the extent of the "Ca^{2+} paradox" during isolation, Ca^{2+} (10 μM) was added to all buffers and the amount of time the tissue was perfused with low Ca^{2+} KHB solution was minimized. It is not obvious why reducing the time of perfusion with low Ca^{2+} medium before starting recirculation or reducing the volume of recirculating medium should enhance digestion. These observations were made consistently, however. A possible explanation for the effectiveness of these changes is the release of some factor from the heart during the perfusion with low Ca^{2+} KHB solution which enhances digestion. An observation which is consistent with this is that large hearts were digested more successfully than small hearts. Incorporating these variations into the normal digestion procedure

was not done because insufficient perfusion with low Ca^{2+} media before recirculation was initiated caused visible tissue damage. This was evident from white areas on the heart. Volume of recirculating perfusate was not reduced because smaller volumes of perfusion medium were feared to be inadequate for maintaining the tissue for the required 52 min of perfusion.

Selection of rod-shaped cells was possible because these cells formed loose clusters more readily than rounded cells and because the latter apparently have a greater surface tension. The greater surface tension of rounded cells is a deduction based on the observation that in a droplet of medium containing cells, only rounded cells are on the surface exposed to air whereas both rod-shaped and rounded cells are on the glass surface of the microscope slide. Aggregates of cells have a faster sedimentation rate in the KHB solution than individual cells. This is true irrespective of the shape of the individual cells or the composition of the cell aggregates with regard to cell morphology. The tendency for rod-shaped cells to form aggregates made it possible to elutriate these cells by gravity against a flow of KHB solution. Elutriation of these preparations is an ideal way to prevent the myocytes from forming packed pellets which are relatively inaccessible to the oxygen and nutrients dissolved in solution. This step in the isolation procedure seems to be a significant technical advance for maintaining myocytes in a viable condition after their dissociation. It also significantly increases the percentage of rod-shaped cells in the preparations because single cells are not retained in the column used for the elutriation procedure and rounded cells are less likely to form aggregates.

Final selection for rod-shaped cells was by gravity sedimentation in a test tube. The higher surface tension of the rounded cells made it possible to aspirate media in which myocytes and myocyte aggregates had only partially sedimented and to preferentially remove rounded cells. This was done by aspirating only the

surface of the media from one edge so that a circular flow of medium within the tube was created. Aggregates sedimented more quickly and were less likely to be caught in the current of medium. When they were caught, aggregates were less likely to be aspirated out of solution than the single cells which had higher surface tension.

Significant enhancement of myocyte viability with time by ouabain at a concentration (1 μM) which is toxic to guinea-pig cardiac muscle contracting at a normal frequency was very surprising. This may indicate that energy expenditure to maintain ion gradients is high in the isolated myocytes. No determinations of oxygen consumption by myocytes or the effect of ouabain on oxygen consumption were made, however, so this is only speculation. Alternatively, inhibition of the Na,K-ATPase may stabilize the membrane and promote myocyte viability or 1 μM ouabain may not be toxic because these cells remained quiescent. Beat- or depolarization-dependency of the onset of glycoside action is well established. Toxicity of ouabain at a concentration which caused total inactivation of the sodium-pump (1 mM) is to be expected.

C. Estimation of ^{3}H-Ouabain Binding to Guinea-Pig Cardiac Myocytes

Specific binding of cardiac glycosides to intact cardiac muscle can be estimated directly (Godfraind and Lesne, 1972; Busse et al., 1979; Herzig et al., 1985; Kjelden et al., 1985) or indirectly (Ku et al., 1974; Gelbart and Goldman, 1977; Temma and Akera, 1982). The indirect estimate measures initial velocity of binding of a ^{3}H-glycoside to a sample of tissue homogenate. Initial velocity of binding is proportional to the free receptor concentration (Gelbart and Goldman, 1977), therefore, the number of receptors which had non-labeled glycoside bound can be estimated. Determinations of receptor occupancy by this method can provide information about the relative number of free receptors, but are not

useful to obtain data for estimation of kinetic parameters of binding. Direct measurement of ^{3}H-ouabain or ^{3}H-digoxin binding to intact muscle has the disadvantage of high non-specific binding. Whether it is estimated by including an excess of non-labeled ouabain (Godfraind and Lesne, 1972) or by estimating incorporation into tissue components by monitoring dissociation after binding (Kjelden et al., 1985) the non-specific binding accounts for approximately 50% of total binding. High non-specific binding makes accurate determination of specific binding difficult. Intact tissues have the additional disadvantage of containing many cell types; therefore, the percentage of binding to receptors which are on muscle cells cannot be determined. Despite these problems the concentration dependence of ^{3}H-ouabain binding has been assessed in guinea pig atrial (Busse et al., 1979; Herzig et al., 1985) and ventricular muscle (Kjelden et al., 1985). Data from these studies are generally consistent with the results of the ^{3}H-ouabain binding studies utilizing guinea-pig myocytes. These results will be considered in more detail when the concentration dependence of ^{3}H-ouabain binding to guinea-pig myocytes is discussed.

Binding of ^{3}H-ouabain to isolated cardiac muscle cells in culture (Friedman et al., 1980; Werden et al., 1983; Kim et al., 1984) and to dissociated myocytes from adult animals (Onji and Liu, 1981; Adams et al., 1982) have also been reported. These investigators find a single class of high affinity binding site in cells derived from embryonic chick ventricle (Werden et al., 1983; Kim et al., 1984), a single class of high affinity binding site in cells from rat heart (Friedman et al., 1980; Adams et al., 1982), and two classes of high affinity binding sites for ^{3}H-ouabain in myocytes from dog heart (Onji and Liu, 1980).

In these reports of ^{3}H-ouabain binding to isolated myocytes or cultured cells no difficulty was reported in determining non-specific binding. Each experimental design utilized an excess (millimolar) concentration of non-labeled ouabain to

block specific binding of ^{3}H-ouabain when determinations of non-specific binding were made. Toxic effects of these saturating concentrations of ouabain were not reported but certainly must have occurred. Toxicity of ouabain may have not been observed by these investigators because Ca^{2+} was not present in the reaction mixture (Friedman et al., 1980; Onji and Liu, 1981; Werden et al., 1983) or was present at a low (50 μM) concentration (Kim et al., 1984). A higher (0.5 mM) concentration of Ca^{2+} was present during the incubation of myocytes with ouabain described by Adams and coworkers (1982) but the incubation time was short (30 min) and the cells examined were from rat heart which contains mainly the sodium-pump which has low affinity for ^{3}H-ouabain (Akera et al., 1979; Adams et al., 1982). In experiments with myocytes from guinea-pig heart, exposure to millimolar concentrations of ouabain did not cause spontaneous contractile activity in KHB solution containing 1.8 mM Ca^{2+} until cells had been incubated with ouabain for 10 to 15 min. Time required to induce contracture of these myocytes by ouabain varied widely within preparations, but most cells retained a rod-shape for at least 30 min. Therefore, the toxic reactions of myocytes to ouabain are slow to develop and may not be significant with short incubations. Binding reactions were allowed to proceed for 60 or 240 min in experiments reported by Onji and Liu (1981) or Werden et al. (1983), respectively. Toxic effects of ouabain may not have developed even with these longer incubation times due to the absence of Ca^{2+}. Alternatively, adverse reactions to ouabain may have been overlooked.

Data from experiments with guinea-pig myocytes clearly demonstrate that myocytes are not stable when incubated in a combination of ouabain and Ca^{2+} if each is present at a millimolar concentration. The effect of 1 mM ouabain to decrease non-specific binding to myocytes incubated with 0.2 μM ^{3}H-ouabain is very clear. Whether the toxicity of ouabain in the presence of Ca^{2+} is responsible

for the decrease in non-specific binding is not certain. The possibility exists that ouabain decreases estimates of non-specific binding by blocking a putative uptake process for ^{3}H-ouabain. If it exists, this uptake may proceed as a transport of glycoside by the Na,K-ATPase (Dutta et al., 1968; Park and Vincenzi, 1975; Fricke and Klaus, 1977) or as an internalization of the glycoside-enzyme complex (Pollack et al., 1981; Cook et al., 1982). Internalization of the ouabain-receptor complex is proposed to occur as part of the process of Na,K-ATPase turnover and glycoside binding is proposed to increase the rate of Na,K-ATPase turnover (Aiton et al., 1981). The evidence for internalization of the ouabain-receptor complex comes from experiments with HeLa cells, however, and therefore may not be applicable to mammalian cardiac myocytes.

Transport of cardiac glycosides into cardiac muscle cells has been proposed to occur and the intracellular injection of glycosides has been reported to increase velocity of shortening in isolated myocytes (Isenberg, 1984); thus, suggesting an intracellular site exists for cardiac glycoside binding. Transport of cardiac glycosides, however, has not been demonstrated in cardiac muscle. Localization of ^{3}H-ouabain in the microsomal fraction of cardiac muscle homogenates prepared from tissue exposed to ^{3}H-ouabain prior to homogenization and fractionation suggested to some investigators that the glycoside was transported into the cells and then bound to sites in the sarcoplasmic reticulum (Dutta et al., 1968). This was taken as evidence that the glycosides had specific binding sites on the sarcoplasmic reticulum. Because it is virtually impossible to isolate either sarcolemmal or sarcoplasmic reticulum vesicles which are not contaminated to some extent by the other fraction, the conclusion that ^{3}H-ouabain has an intracellular binding site is not justified from these studies. Association of ^{3}H-glycosides with cardiac muscle which is affected by the Na^{+} concentration (Fricke and Klaus, 1977) or the species of glycoside used (Godfraind and Lesne, 1972; Park

and Vincenzi, 1975) was used as evidence that the Na,K-ATPase transports cardiac glycosides into muscle cells. The basis for this conclusion, however, is not obvious as similar changes in tissue concentrations of glycoside would be expected if specific binding of cardiac glycosides to extracellular sites is affected by the Na^+ concentration or the species of the glycoside. Since both of these are well documented to affect ^{3}H-glycoside binding to Na,K-ATPase, the available evidence does not indicate that the Na,K-ATPase transports glycosides into cardiac muscle.

Neither mechanism (toxicity or blocking an uptake process), by which non-specific binding of ^{3}H-ouabain would be decreased in the presence of high concentrations of non-labeled ouabain is supported by evidence published by other investigators. Nevertheless, the experimental design used appears sound and sufficient to detect the reported effect of millimolar ouabain concentrations to reduce estimates of non-specific ^{3}H-ouabain binding. Therefore, it must be concluded that including excess non-labeled ouabain in order to make determinations of non-specific ^{3}H-ouabain binding to guinea-pig myocytes in the presence of Ca^{2+} is a method subject to large errors.

Measurement of bound ^{3}H-ouabain before and after partial dissociation at 0^oC has been used to estimate specific and non-specific binding to guinea-pig skeletal and cardiac muscle (Kjeldsen et al., 1985). In these experiments specific binding is quantitated as the slow phase of release. Dissociation of ^{3}H-ouabain from guinea-pig myocytes at 37^oC occurred in fast and slow phases. The fast phase had two components, the relative abundance of each being dependent on the presence of K^+ during binding, thus indicating that this represents binding to Na,K-ATPase. The dissociation rate constant calculated for ^{3}H-ouabain bound to the rapidly releasing site in guinea pig myocytes is similar to the value reported for the dissociation rate constant of ^{3}H-ouabain bound to the Na,K-ATPase from

guinea pig heart (Godfraind et al., 1980), further supporting the hypothesis that the fast phase of ^{3}H-ouabain release from guinea-pig myocytes represents ^{3}H-ouabain bound to the Na,K-ATPase. The slow phase is assumed to represent non-ATPase binding and to be non-specific. A half-life for release of ^{3}H-ouabain from non-specific binding sites was not determined in initial experiments due to the large relative errors in sampling and counting and the small amount of ^{3}H-ouabain associated with this site. Therefore, ^{3}H-ouabain remaining bound after 60 min of dissociation at 37^{o}C in a 100-fold volume of KHB solution containing 13.8 mM K^{+} and with a concentration of non-labeled ouabain equal to the concentration of ^{3}H-ouabain with which the myocytes had been incubated, was used as an estimate of non-specific binding.

It is unreasonable to expect that non-specific binding is irreversible. In fact, at 0^{o}C non-specifically bound ^{3}H-ouabain is released more rapidly than is the specifically bound ligand (Kjeldsen et al., 1985). In experiments examining the effect of K^{+} on specific ^{3}H-ouabain binding, dissociation of ^{3}H-ouabain was monitored after binding had proceeded for 40 min in the presence of 2.5 μM ^{3}H-ouabain and either 0 or 10 mM K^{+}. With this high concentration of ^{3}H-ouabain present during the binding reaction, sufficient ligand was associated with the slowly releasing site so that its rate of dissociation could be estimated. Half lives of approximately 212 and 146 min were calculated for dissociation of non-specifically bound ^{3}H-ouabain, when binding had occurred in the absence or presence of 10 mM K^{+}, respectively. These values are not significantly different, therefore, no conclusions can be drawn concerning the effects of K^{+} on the dissociation of ^{3}H-ouabain from non-specific binding sites. If the faster rate of release is the more accurate estimate of dissociation from this site, then after 60 min 25% of non-specifically bound ^{3}H-ouabain would be released from the

myocytes. Therefore, if non-specific binding accounted for 20% of total ^{3}H-ouabain binding, an overestimation of approximately 7% for specific binding would occur because too small a value for non-specific binding would be subtracted. Even with this systematic error to underestimate non-specific binding, the dissociation method produced estimates for this component of binding which were more than three times as great as estimates made by including 1 mM ouabain in the incubation mixture to block specific binding.

It is unlikely that such a large difference is due to overestimating the non-specific binding by the dissociation method. Overestimation of the amount of ^{3}H-ouabain associated with the slow-releasing site could occur due to allowing inadequate time for dissociation or insufficient dilution of ^{3}H-ouabain during dissociation. Because dissociation occurred in KHB solution containing the same concentration of non-labeled ouabain as the binding incubation solution had contained ^{3}H-ouabain, no change in specific binding should occur and ^{3}H-ouabain should occupy 1% of those sites when equilibrium is re-established. Therefore, the maximum overestimate of non-specific binding due to inadequate dilution is 1% of the specific binding. Rebinding of ^{3}H-ouabain would cause the dissociation half-life to be overestimated rather than underestimated, so it is unlikely that insufficient time had been allowed for dissociation. The largest estimate of half-life for ^{3}H-ouabain dissociation from specific binding sites was 10.8 min. If this is an accurate estimate then 2% of specific binding sites which had ^{3}H-ouabain bound at the initiation of dissociation would have ^{3}H-ouabain bound after 60 min of dissociation. Errors due to inadequate dilution and insufficient time of dissociation should be roughly additive when both are small. Therefore, the maximum overestimate of non-specific binding by this method should be 3% of the specific binding.

From the available information concerning cardiac glycoside binding to myocytes from guinea-pig heart in the presence of Ca^{2+}, it is apparent that the most accurate estimates of specific binding can be obtained by using the dissociation method to estimate non-specific binding. Estimating the non-specific binding of ^{3}H-ouabain as the amount of ^{3}H-ligand remaining bound to the myocytes after 60 min of dissociation in a 100-fold volume of KHB solution is subject to systematic errors which can cause non-specific binding to be underestimated by as much as 25% of the non-specific component of binding or overestimated by as much as 3% of the specific component of binding. As the relative amount of non-specific binding increases, the underestimation of non-specific binding can become a serious problem. Therefore, the dissociation method may not be adequate to determine non-specific binding when that binding accounts for greater than 20% of the total binding.

Estimation of kinetic parameters for drug receptor interactions is generally accomplished by analysis of displacement from specific binding sites of ^{3}H-ligand present at a constant concentration in the incubation media, caused by the presence of various concentrations of non-labeled ligand. Alternatively, specific binding can be determined when receptors are incubated with various concentrations of ^{3}H-ligand. In the first instance displacement of ^{3}H-ligand by non-labeled ligand can be analyzed according to the method of Akera and Cheng (1977). These authors are careful to point out that this method for estimating kinetic parameters can only be used when there is a single class of binding site and where there is no cooperativity in drug binding. When this method was used to examine ^{3}H-ouabain binding to guinea-pig myocytes in the presence of Ca^{2+} and K^{+}, the data appeared to confirm that ^{3}H-ouabain binds to a single class of receptors and that there is no cooperativity. This, however, is apparently not true. The first evidence indicating this was the non-linearity of the logarithmic probit plots

describing displacement of 100 nM ^{3}H-ouabain by non-labeled ouabain when binding to guinea-pig myocytes occurred in the presence of TPA. Later experiments using Scatchard analysis to estimate kinetic parameters for ^{3}H-ouabain binding indicated that there may be more than one binding site with different affinity for ^{3}H-ouabain in guinea-pig myocytes and/or that there may be cooperativity in binding. These data are consistent with findings from experiments examining ^{3}H-ouabain binding to isolated Na,K-ATPase from guinea-pig heart in the presence of K^{+}. Under these conditions ^{3}H-ouabain binds to conformations of the enzyme which have different affinities for the glycoside (Godfraind et al., 1980). These data are also consistent with the theoretical considerations concerning glycoside binding to Na,K-ATPase in intact tissue which predict cooperativity in binding (Herzig et al., 1985a,b). Thus, experimental evidence and theoretical considerations both indicate that the method of displacement cannot accurately estimate kinetic parameters of cardiac glycoside binding to Na,K-ATPase in intact myocytes when binding occurs in the presence of Ca^{2+} and K^{+}. It may be possible, however, to use this method if Ca^{2+} is not present, or if K^{+} is present in very low concentrations. Under these conditions cooperativity in binding has not been demonstrated and a single class of binding site may predominate.

Estimates of kinetic parameters for ^{3}H-ouabain binding to high affinity sites in myocytes as determined by the displacement method have been published by Adams and coworkers (1982). Binding reactions to myocytes from rat heart occurred in an incubation solution containing 3.8 mM K^{+} and 0.5 mM Ca^{2+}. Therefore, cooperativity in binding is expected. However, Na,K-ATPase from rat cardiac muscle is unusual because two forms of the enzyme exist and only about 15% of the total binding sites have a high affinity for ouabain (Adams et al., 1982). Because so few sites have a high affinity for ^{3}H-ouabain, it may be

possible to inactivate those pump sites without significant positive cooperativity resulting from the increased intracellular Na^+ concentration. Thus, determination of ^{3}H-ouabain binding to cardiac myocytes from rat may not be subject to the same limitations that exist for guinea-pig myocytes. When a single class of binding sites are present, or when two classes with a similar affinity are present, such as with guinea-pig myocytes, and binding occurs in the presence of Ca^{2+} and K^+, the displacement method is not adequate for estimation of kinetic parameters for ^{3}H-ouabain binding.

Scatchard analysis of ^{3}H-ouabain binding to specific binding sites is a more direct method for estimation of kinetic parameters than is the method of displacement. Guinea-pig myocytes when incubated in the presence of 1.8 mM Ca^{2+} and various concentrations of ^{3}H-ouabain, bind the ^{3}H-ouabain in a manner such that Scatchard plots describing the specific binding are non-linear. Other investigators have found binding to cultured chick heart cells is described by linear Scatchard plots (Werden et al., 1983, 1984; Kim et al., 1984). Binding to dog cardiac myocytes, however, also is described by non-linear plots (Onji and Liu, 1981). Linear Scatchard plots were interpreted to indicate the presence of a single class of binding sites in cultured chick heart cells. Non-linear Scatchard plots were interpreted to indicate the presence of two classes of binding sites in myocytes from dog heart; these sites were shown to be interconvertible by the addition of K^+, and therefore probably represent different conformations of Na,K-ATPase. The relationship of these findings to the curved Scatchard plot describing ^{3}H-ouabain binding to guinea-pig myocytes is uncertain because in each of the studies ^{3}H-ouabain binding to cells had occurred in the absence of Ca^{2+} and in low K^+ or K^+-free solution. The presence of Ca^{2+} is a major factor in non-linearity of Scatchard lots describing ^{3}H-ouabain binding to guinea-pig myocytes. This is not the only factor, however, because when binding occurs in the absence

of Ca^{2+}, the Scatchard plot has at least two components, representing high and low affinity binding sites.

Experiments describing binding of ^{3}H-ouabain to isolated myocytes in the presence of Ca^{2+} at millimolar concentrations have not been previously reported. Similar experiments examining ^{3}H-ouabain binding to intact atrial muscle from guinea-pig heart in the presence of Ca^{2+} and K^+ (Herzig et al., 1985) or ventricular muscle from guinea-pig heart (Kjeldsen et al., 1985) in the absence of Ca^{2+} and K^+ have been published. The Scatchard plot describing binding to atrial muscle is non-linear and the authors suggest this indicates positive cooperativity in ^{3}H-ouabain binding (Herzig et al., 1985a,b). The highest concentration of ^{3}H-ouabain used in these experiments with guinea-pig atrial muscle was 1.0 μM so the presence of the very low affinity site seen in preparations of guinea-pig ventricular myocytes in this study cannot be confirmed. Binding of ^{3}H-ouabain to guinea-pig ventricular muscle has been examined in a system using Mg^{2+} and vanadate (VO_4^{3-}) to support binding (Kjeldsen et al., 1985). The Scatchard plot describing ^{3}H-ouabain binding in this system was non-linear when binding occurred in ^{3}H-ouabain concentrations of less than 5.0 μM. Higher concentrations of ^{3}H-ouabain (10.0 and 20.0 μM) did not promote greater specific binding. This may indicate that Mg^{2+} and VO_4^{3-} cannot support ^{3}H-ouabain binding to the very low affinity binding site. Alternatively, the Na,K-ATPase may have low affinity for the glycoside if it has a low affinity for phosphate. This would result in a lower fraction of the enzyme existing in a phosphorylated (binding) form, and therefore, a low glycoside binding rate. Vanadate has a higher affinity for phosphate binding sites on Na,K-ATPase than phosphate itself does (Wallick et al., 1979). Vanadate would, therefore, increase the apparent affinity of the enzyme for ouabain. If this occurs, then all specific ^{3}H-ouabain binding sites may be saturated by a 5 μM concentration of ^{3}H-ouabain when the binding is supported by vanadate. Lack of

a very low affinity binding site in intact guinea-pig ventricular muscle (Kjeldsen et al., 1985) may also mean that identification of this site in guinea-pig myocytes is due to an artifact. Each experimental design described above used a dissociation system to estimate non-specific ^{3}H-ouabain binding. Authenticity of the low-affinity site for ^{3}H-ouabain binding to myocytes shall be discussed later in this section.

Non-linear Scatchard plots describing ^{3}H-ouabain binding to high affinity sites in guinea-pig cardiac muscle have been produced by three independent investigators using three different preparations (Herzig et al., 1985b; Kjeldsen et al., 1985; present results). Possible explanations of the curvature include: multiple independent binding sites with different affinity; negative cooperativity in ^{3}H-ouabain binding; positive cooperativity in ^{3}H-ouabain binding; a combination of cooperativity and binding sites with different affinity.

Multiple independent binding sites with different kinetic parameters for ^{3}H-ouabain binding are to be expected for the Na,K-ATPase of guinea-pig cardiac muscle (Godfraind et al., 1980), and Na,K-ATPase in general (Inagaki et al., 1974; Choi and Akera, 1977; Wellsmith and Lindenmayer, 1980; Yoda and Yoda, 1986). The presence of K^{+} is a key determinant of the relative abundance of each type of binding site (Choi and Akera, 1977) and can cause one conformation of the enzyme to predominate over the other by its presence (Hansen, 1976) or its absence (Godfraind et al., 1980) as shown by the conditions under which binding produces linear Scatchard plots. The different effect of K^{+} reported by these investigators may reflect different sources of the enzyme or different ligand conditions to support binding. Different affinities of Na,K-ATPase for ^{3}H-ouabain are believed to reflect binding of the glycoside to different conformations of the enzyme which exist transiently as intermediates in the reaction sequence (Yoda and Yoda, 1986). The existence of two conformations of Na,K-ATPase, in intact guinea-pig

myocytes, which can bind ^{3}H-ouabain was confirmed by showing the relative abundance of specific binding sites for ^{3}H-oubain was changed by the presence or absence of 10 mM K^+.

Non-linear Scatchard plots describing binding to Na,K-ATPase of guinea-pig myocytes cannot be explained solely on the basis of multiple binding sites whose relative abundance is affected by K^+ concentration. Another factor may be redistribution of K^+ subsequent to ouabain binding. This causes K^+ in the incubation media to increase in a manner dependent on ouabain concentration. The effect of K^+ to inhibit ^{3}H-ouabain binding to myocytes suggest there may be some negative cooperativity in binding caused by such a redistribution. It is impossible to know what a Scatchard plot, describing ^{3}H-ouabain binding to the Na,K-ATPase in myocytes, would look like if this occurred. If K^+ was the only ion redistributed, then an upwardly concave curve which would become linear after complete redistribution of K^+ had occurred, is expected on a Scatchard plot. However, Na^+ must also be redistributed in myocytes when K^+ is redistributed. Therefore, an increase in intracellular Na^+ concentration will increase apparent affinity of the Na,K-ATPase for ^{3}H-ouabain at the same time that the increase in extracellular K^+ concentration decreases apparent affinity of the enzyme. The shape of the curve on the Scatchard plot will be determined by the relative amount of change in the Na,K-ATPase affinity for ^{3}H-ouabain that redistribution of each ion can cause. The increase in K^+ concentration with full sodium-pump inhibition was only 12% of the initial K^+ concentration under conditions of the incubation for Scatchard analysis. This corresponds to an absolute change in K^+ concentration of approximately 0.5 mM and is unlikely to significantly increase the turnover rate of the enzyme to a conformation which does not bind ^{3}H-ouabain. The change in intracellular Na^+ concentration is expected to be at least a 10-fold increase, therefore, the effects of Na^+ redistribution are expected to be

predominant. Also, the presence of monensin shifted the concentration response curve for K^+ release from myocytes incubated with ouabain toward the right, indicating a desensitization to the action of ouabain. This is inconsistent with the effect of monensin to increase the degree of curvature of the Scatchard plot if K^+ redistribution and negative cooperativity contribute to the non-linearity. Furthermore, redistribution of K^+ in a manner dependent on the concentration of ouabain occurs in the absence as well as in the presence of Ca^{2+}. Since Scatchard plots describing ^{3}H-ouabain binding to high affinity sites in the absence of Ca^{2+} are relatively linear, a redistribution of K^+ is probably not contributing to the non-linearity of Scatchard plots describing binding in the presence of Ca^{2+}.

The action of monensin to shift concentration response curves for K^+ redistribution by ouabain to the right is surprising and warrants further investigation. Monensin stimulates binding by ^{3}H-ouabain, therefore, the sodium-pump should experience a greater degree of inhibition in the presence of monensin than in its absence. Also, monensin is not entirely specific for Na^+; therefore, some K^+ should be carried out of the cell by this ionophore. One feasible mechanism by which monensin may cause the shift being discussed is to increase the driving force for K^+ accumulation by the Na,K-ATPase. An increase in Na,K-ATPase turnover is also consistent with the increased curvature of the Scatchard plot if a positive cooperativity caused by an increase in intracellular Na^+ concentration is responsible for the non-linearity.

The presence of two binding conformations of the Na,K-ATPase may also contribute to the non-linearity of Scatchard plots describing ^{3}H-ouabain binding to a high affinity site(s). Even when binding occurs in the absence of Ca^{2+} the high affinity portion of the Scatchard plot is not really linear. This non-linearity is further accentuated by the presence of monensin during binding. Monensin does not have a significant effect to increase ^{3}H-ouabain binding to cells incubated in

the absence of Ca^{2+} but it seems to change the shape of the Scatchard plot in the middle portion of the curve. The interpretation that this is due to the presence of two binding sites is highly speculative and may not be justified due to insensitivity of this method of analysis for discerning small differences in receptor affinity. Nevertheless, the subtle change in the shape of the Scatchard plot describing ^{3}H-ouabain binding to guinea-pig myocytes caused by monensin when Ca^{2+} is absent does support the possibility that Na,K-ATPase exists in two high affinity conformations under these conditions.

In the presence of Ca^{2+}, monensin stimulates binding of ^{3}H-ouabain to myocytes and increases the degree of curvature of the Scatchard plot describing this binding. Monensin is presumed to do this by increasing Na^+ influx. This mechanism of action is consistent with the effect of increasing Na^+ influx to stimulate ^{3}H-ouabain binding in intact cardiac muscle (Yamamoto et al., 1980; Temma and Akera, 1982). Binding of ^{3}H-ouabain to myocytes is not significantly affected by monensin when binding occurs in the absence of Ca^{2+}. This suggests that each of these interventions increase binding of ^{3}H-ouabain to the high affinity site by the same mechanism, namely an increase in Na^+ influx. This interpretation, however, is not consistent with the rightward shift in the ouabain concentration response curve caused by Ca^{2+} removal (Figure 25). If the hypothesis that both the presence of monensin and the removal of Ca^{2+} stimulate ^{3}H-ouabain binding by causing an increase in intracellular Na^+ concentration is correct, then either Ca^{2+} has a direct effect to decrease the reserve capacity of the sodium-pump or the data indicating a rightward shift in the ouabain concentration response curve as a result of Ca^{2+} removal are inaccurate. An effect of Ca^{2+} to increase the permeability of the sarcolemma of cardiac myocytes from rat heart to Na^+ has been reported (Hohl et al., 1983), and is consistent with data showing Na^+ activity in sheep Purkinje fibers increases

approximately 50% when there is a 10-fold decrease in extracellular Ca^{2+} concentration over the range of Ca^{2+} concentrations from 0.2 to 16 mM (Ellis, 1977; Deitmer and Ellis, 1978). Elimination of the upwardly concave non-linearity of Scatchard plots by increasing intracellular Na^{+} concentration is consistent with positive cooperativity in cardiac glycoside binding to Na,K-ATPase in intact cardiac muscle as proposed by Lullman and coworkers (Herzig et al., 1985a,b). The reason monensin does not promote linearity of Scatchard plots itself may be because it does not raise the intracellular Na^{+} concentration significantly until the reserve capacity of the sodium pump is eliminated. Removal of Ca^{2+}, on the other hand may cause intracellular Na^{+} to increase even though the reserve capacity of the sodium-pump is not eliminated. How this is possible is not immediately obvious but this may indicate that Ca^{2+} has some effect to regulate the activity of the sodium-pump. This possibility is supported by the data indicating that removal of Ca^{2+} causes a rightward shift in the ouabain concentration response curve for sodium-pump inhibition.

Positive cooperativity in binding generally is described by convex Scatchard plots as shown in Figure 26. Binding of cardiac glycosides to Na,K-ATPase in intact tissue is expected to exhibit positive cooperativity (Herzig et al., 1985a,b). However, the cooperativity in binding will be by a different mechanism than normally seen. As the sodium-pump reserve capacity is reduced by digitalis binding, the turnover of the remaining Na,K-ATPase enzymes will increase. That is, the number of times an individual enzyme will catalyze the transfer of Na^{+} and K^{+} in any given unit of time will be increased. This means the amount of time the individual enzyme exists in a binding configuration will increase. The number of binding sites, therefore, effectively increases without any change in k^{1} or k^{-1} for individual receptors. This is much different from the "normal" type of cooperativity which results from partial occupancy of a population of receptors affecting

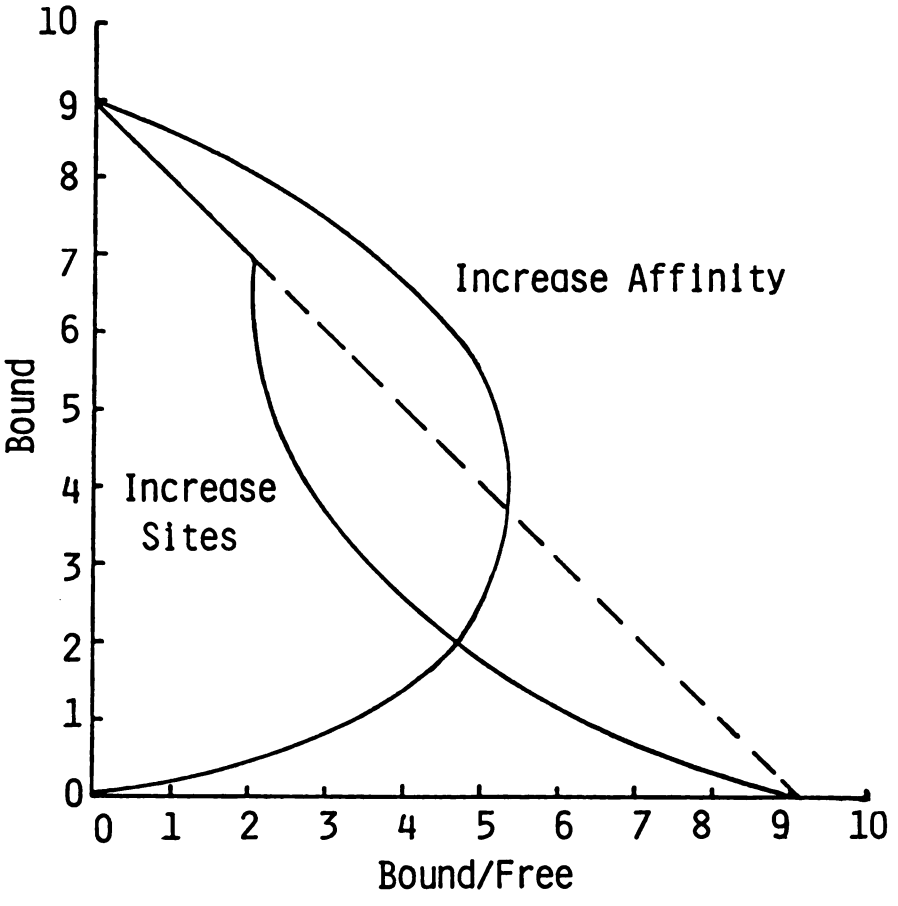

Figure 26. Scatchard plots describing binding to one population of receptors without cooperativity in binding ---, or —— positive cooperativity resulting from increased affinity for the ligand, or with positive cooperativity resulting from unmasking of binding site.

the affinity of the remaining unoccupied receptors for a ligand. Positive cooperativity caused by an "unmasking" of receptor sites should be described by a Scatchard plot in which the curve is below the normal straight line because the low intracellular Na^+ concentration reduces the amount of time the enzyme exists in a binding conformation, and therefore, reduces the "net" binding rate. When intracellular Na^+ concentration has increased to an extent that all the receptors are in a binding conformation, the plots should be superimposable.

If the absence of Ca^{2+} causes an unmasking of ^{3}H-ouabain binding sites in this manner then the linear Scatchard plot describing the binding could be considered an accurate description of the ouabain-enzyme interaction under conditions of maximum time for enzyme existence in a binding conformation. The unmasking may be an effect of increased intracellular Na^+ concentration or some direct effect of Ca^{2+} or a Ca^{2+}-binding protein to alter affinity of the enzyme. Binding in the presence of Ca^{2+} would then be a situation where the binding site number is effectively increased by ^{3}H-ouabain binding, resulting in positive cooperativity. The effective increase in binding site number is not an increase in total binding sites or pump units but merely indicates the enzymes which are present exist for a greater amount of time in a Na^+-induced binding conformation as more ouabain is specifically bound. This is because there is an increase in intracellular Na^+ concentration caused by ^{3}H-ouabain binding. Increased curvature of Scatchard plots when binding occurs in the presence of Ca^{2+} and monensin indicates that monensin simply amplifies the unmasking effect of ouabain. With this amplification the curves describing ^{3}H-ouabain binding to myocytes in a system where there is positive cooperativity and one where there is no cooperativity will be superimposed at a lower ^{3}H-ouabain concentration. Scatchard plots describing ^{3}H-ouabain binding to high affinity sites on guinea-pig myocytes are in good agreement with this model of positive cooperativity in the binding of the

cardiac glycosides. Deviation of the experimental data from the model is not serious, considering the error involved in estimating non-specific binding and the potential contribution of multiple binding sites and changes in conformation which can affect ^{3}H-ouabain binding.

Authenticity of the low affinity binding site for ^{3}H-ouabain is questionable. This site was not shown to be saturable and may represent an artifact of the method used to estimate non-specific binding. At concentrations of ^{3}H-ouabain in excess of 3.0 μM, non-specific binding was greater than 20% of the specific binding and at the highest concentration of ^{3}H-ouabain used (20 μM) non-specific binding was estimated to account for 37% of total binding when binding occurred in the absence of Ca^{2+}.

Accuracy of estimates for non-specific binding is a serious concern during interpretation of data describing ^{3}H-ouabain binding to myocytes incubated in high concentrations of ^{3}H-ouabain. As discussed earlier, non-specific binding can be underestimated when the amount of ^{3}H-ouabain remaining bound after 60 min of dissociation is used as an estimate of non-specific binding. Binding of ^{3}H-ouabain to a low affinity site in intact, guinea-pig ventricular muscle was not detected when binding was supported by Mg^{2+} and VO_4^{3-}. It is possible these ligands cannot support binding to low affinity forms of the Na,K-ATPase, or that vanadate increases the affinity of low affinity binding sites. Alternatively, the low affinity binding site detected in guinea-pig myocytes may be artifactual.

Support for the authenticity of the low affinity binding site for ^{3}H-ouabain is the increase in ^{3}H-ouabain bound when myocytes are incubated in the absence of K^{+}. Total binding to myocytes following a 40-min incubation with 2.5 μM ^{3}H-ouabain in the absence of K^{+} was 23.2±0.7 pmol/mg protein. After 30 sec of dissociation at 37°C these myocytes contained 13.4±0.7 pmol/mg protein of ^{3}H-ouabain. This rapid dissociation may represent release of ^{3}H-ouabain bound to the

low affinity site described in Scatchard plots. Additional experiments would be necessary to substantiate this point.

Even though the data support the hypothesis that there is positive cooperativity in ^{3}H-ouabain binding to guinea-pig myocytes in the presence of Ca^{2+} which is caused by changes in intracellular Na^{+} concentration, it is possible that Ca^{2+} has a direct effect on ^{3}H-ouabain binding which causes Scatchard plots to be non-linear. In preparations of isolated Na,K-ATPase, Ca^{2+} inhibits enzyme activity by competition with Na^{+} (Tobin et al., 1973). This inhibition, however, should not be significant at intracellular Ca^{2+} concentrations present in quiescent cardiac muscle because the IC_{50} for Ca^{2+} inhibition of Na,K-ATPase activity is approximately 0.5 mM (Tobin et al., 1973; Godfraind et al., 1977; Beauge and Campos, 1983; Powis et al., 1983). The possibility that Ca^{2+} acts through a Ca^{2+}-binding protein to affect the affinity of Na,K-ATPase for ouabain has been discussed in the Introduction section of this work.

Direct actions of intracellular Ca^{2+} apparently do not increase or decrease affinity of Na,K-ATPase for ^{3}H-ouabain in guinea-pig myocytes. This is indicated by the fact that the Ca^{2+} ionophore, A23187, does not affect ^{3}H-ouabain binding when cells are incubated in solution containing 1.8 mM Ca^{2+} and 3.8 mM K^{+}. Under these conditions, intracellular Ca^{2+} concentration is low as judged by myocytes being quiescent. A23187 should increase intracellular Ca^{2+} concentration under these conditions. Furthermore, when cells are incubated in solution containing 1 mM K^{+} and 0.1 mM Ca^{2+}, the myocytes contract spontaneously, indicating that the intracellular Ca^{2+} concentration is increased. Yet under these incubation conditions, ^{3}H-ouabain binding is stimulated. It is possible that binding of ^{3}H-ouabain in solutions containing 1 mM K^{+} and 0.1 mM Ca^{2+} is increased because of the effect of the low K^{+} concentration to promote the enzyme to exist in a Na^{+}-induced binding conformation, and that Ca^{2+} actually is inhibiting

binding. This possibility was not tested directly, but if this was the case, ^{3}H-ouabain binding should have been stimulated by lanthanum, which blocks Ca^{2+} entry. Because lanthanum had no effect on ^{3}H-ouabain binding, the available evidence suggests that Ca^{2+} does not have an effect on cardiac glycoside binding.

Actions of Ca^{2+}-dependent protein kinases or other Ca^{2+}-binding proteins also are without effect to increase or decrease affinity of the Na,K-ATPase for ^{3}H-ouabain. This is deduced because when intracellular Ca^{2+} concentration is elevated by incubating myocytes in solution with 1 mM K^+, there are no effects on ^{3}H-ouabain binding by agents which increase (A23187) or decrease (lanthanum) intracellular Ca^{2+} concentration, or by agents which promote phosphorylation of sarcolemmal constituents (isoproterenol, TPA).

Because no direct action of Ca^{2+} or a Ca^{2+}-dependent protein kinase can be detected, the effect of EGTA to stimulate ^{3}H-ouabain binding must be attributed to an indirect effect of Ca^{2+} removal.

Data from experiments examining ^{3}H-ouabain binding to guinea-pig myocytes indicate that there are two or three classes of binding sites for cardiac glycosides corresponding to different conformations of the Na,K-ATPase. The total number of high affinity ^{3}H-ouabain binding sites in guinea-pig cardiac myocytes was estimated to be approximately 14 pmol/mg protein. This estimate was made from analysis of the Scatchard plot describing ^{3}H-oaubain binding to the myocytes incubated in the absence of Ca^{2+}. Fourteen pmol/mg protein corresponds to approximately 40 million ^{3}H-ouabain binding sites per cell. These sites have an affinity (K_D) for ^{3}H-ouabain of 0.4-0.7 μM in the absence of Ca^{2+} and a range of apparent affinity from 0.15-1.9 μM in the presence of Ca^{2+}. Estimates of affinity (K_D) for ^{3}H-ouabain were made by analysis of Scatchard plots. Analysis of the curve describing ^{3}H-ouabain binding in the presence of Ca^{2+} was done by drawing tangents to the curve where it was most linear (K_D

estimate of 0.15 μM) and prior to the linear portion of the curve which describes binding to the very low affinity site (K_D estimate of 1.9 μM). There is good evidence for positive cooperativity in ^{3}H-ouabain binding which is observed only when binding occurs in the presence of Ca^{2+}. Direct effects of Ca^{2+}, either by the ion itself or via a Ca^{2+}-binding protein, do not appear to affect ^{3}H-ouabain binding to guinea-pig myocytes.

D. Estimation of Sodium-Pump Activity and Capacity in Guinea-pig Cardiac Myocytes

Measuring ouabain-sensitive $^{86}Rb^+$ or $^{42}K^+$ uptake is a well established method for estimating sodium-pump activity (Akera and Brody, 1985). Experiments with intact cardiac muscle have established that unless Na^+ is accumulating in the tissue, or has accumulated to the extent that Na^+ is not the rate-limiting substrate for Na,K-ATPase activation, then sodium-pump activity is determined by Na^+ influx rate or intracellular Na^+ concentration (Yamamoto et al., 1979; Akera et al., 1981). If sodium-pump activity is determined by substrate availability, it will be impossible to detect a direct effect of any intervention on the Na,K-ATPase by monitoring sodium-pump activity. Therefore, interventions which may affect the time course of the sodium transient, an early event in contraction of cardiac muscle (Akera and Brody, 1978), by affecting performance of the Na,K-ATPase during the Na^+ transient, a time at which enzyme activity is not limited by Na^+ concentration, cannot be detected by measuring effects on sodium-pump activity but only by measuring effects on sodium-pump capacity (Akera and Brody, 1985).

Sodium-pump activity in intact cardiac muscle is stimulated by interventions which increase Na^+ influx (Yamamoto et al., 1979; Yamamoto et al., 1980; Akera et al., 1981). Theoretically, increasing Na^+ influx to a large degree, will eliminate the reserve capacity of the sodium-pump so that the capacity of the

pump can be estimated (Akera and Brody, 1985). This has been previously attempted with intact muscle preparations and shifts in the ouabain concentration response curve were detected (Yamamoto et al., 1980).

Direct comparison of the magnitude of $^{86}Rb^{+}$ fluxes between myocytes and intact tissue would require estimates of total cell surface area for each preparation and estimates of the relative abundance of muscle to non-muscle cells as well as the flux into each type of cell for the intact tissue. These estimates are not available, therefore, only relative changes in ion fluxes caused by similar interventions can be compared. The most effective means for increasing ouabain-sensitive $^{86}Rb^{+}$ uptake into intact muscle preparations is to electrically stimulate those preparations (Yamamoto et al., 1979; Akera et al., 1981). The Na^{+} ionophore, monensin, also causes an increase in ouabain-sensitive $^{86}Rb^{+}$ uptake into intact muscle preparations, but the magnitude of the increase is only about 25% of that achieved with electrical stimulation (Yamamoto et al., 1979). Thus, the largest increase in ouabain-sensitive $^{86}Rb^{+}$ uptake into intact tissue which has been reported, is caused by electrical stimulation and corresponds to an increase in sodium-pump activity of approximately 200% above that which is observed in quiescent tissue (Yamamoto et al., 1979; Akera et al., 1981).

Stimulated increases in ouabain-sensitive $^{86}Rb^{+}$ uptake, relative to the rate observed in quiescent preparations of each type, are much larger in myocytes than the values reported for intact tissue. Technical problems with electrical stimulation of myocytes precluded use of this method to enhance Na^{+} influx and therefore, sodium-pump activity. However, the maximum relative increase with monensin or with sodium-loading was approximately 400% or twice that which was achieved with electrical stimulation of intact tissue. Similar attempts to maximally stimulate sodium-pump activity in cultured embryonic cells or dissociated myocytes from hearts of mature animals have not been reported.

The effectiveness of monensin to stimulate ouabain-sensitive $^{86}Rb^+$ uptake in guinea-pig myocytes is distinctly different from its modest ability to do so in intact muscle and the limited effective concentration range of monensin (< 2 μM) in intact muscle preparations (Yamamoto et al., 1979). This limited effective concentration range may be due to the longer incubation and uptake period used in intact muscle (generally 30 min) which usually allow toxic effects of high concentrations of monensin to be seen. The relatively modest effect of monensin to stimulate sodium-pump activity (maximum increase is 50%) in intact tissues may be due to diffusion barriers which limit ion fluxes. It is unlikely that the isolated myocytes have a lower basal Na^+ influx rate than that which exists for the cells in intact tissue (a factor which would cause the relative increase to be larger than the absolute increase). Therefore, the ability to stimulate ouabain-sensitive $^{86}Rb^+$ uptake in myocytes beyond what has been achieved with intact tissue probably represents a real increase in sodium-pump activity. The most likely cause for this is the elimination of diffusion barriers which can limit the movement of $^{86}Rb^+$ in intact tissue.

The plateau in ouabain-sensitive $^{86}Rb^+$ uptake into myocytes ultimately achieved at high concentrations of monensin or with long periods of sodium-loading suggests that intracellular Na^+ has increased to the degree that it is no longer rate-limiting for sodium-pump activity. The fact that each of these interventions produce a maximum effect of approximately 400% over basal values to increase sodium-pump activity, also suggests that reserve capacity has been eliminated by each intervention. This must be established by an independent method, however, such as measuring an increase in cellular Na^+ load without a concomitant increase in ouabain-sensitive $^{86}Rb^+$ uptake.

The effect of Ca^{2+} to inhibit ouabain-sensitive $^{86}Rb^+$ uptake has not been previously reported. This effect of Ca^{2+} was significant in quiescent myocytes,

myocytes subjected to all degrees of sodium-loading, and cells exposed to low or moderate concentrations of monensin; but not for myocytes exposed to high concentrations of monensin. Possible mechanisms for this effect of Ca^{2+} to inhibit sodium-pump activity are: a direct action on the Na,K-ATPase; competition for intracellular Na^+ via the Na/Ca exchange mechanism, or altered membrane permeability to Na^+ (Hohl et al., 1983). Because the ouabain-sensitive $^{86}Rb^+$ uptake is an estimate of Na^+ influx unless the sodium-pump is operating at capacity (Akera et al., 1981; Akera and Brody, 1985), the effect of Ca^{2+} to decrease sodium-pump activity in quiescent myocytes and those subjected to moderate concentrations of monensin or short periods of Na^+-loading must be due to either an altered sarcolemmal permeability or competition between the Na,K-ATPase and the Na/Ca exchanger for intracellular Na^+. Sarcolemmal permeability to Na^+ should not affect sodium-pump activity when the myocytes are maximally sodium-loaded. Therefore, the effect of Ca^{2+} to inhibit sodium-pump activity in sodium-loaded myocytes is inconsistent with this explanation for the action of Ca^{2+}. If, however, the intracellular Na^+ concentration continues to increase with time as myocytes are sodium-loaded, then competition for intracellular Na^+ via the Na/Ca exchange cannot explain why the plateau in ouabain-sensitive $^{86}Rb^+$ uptake occurs at the same time for sodium-loading but at a lower value for activity of the sodium-pump when the $^{86}Rb^+$ uptake is monitored in solution containing Ca^{2+}. Thus, each explanation for the effect of Ca^{2+} on sodium-pump activity is inconsistent with some aspect of the data. It is possible that each of the three mechanisms is functioning under some of the conditions examined. Further experiments are required in order to draw firm conclusions concerning the mechanism by which Ca^{2+} decreases ouabain-sensitive $^{86}Rb^+$ uptake.

Concentration-dependent inhibition of sodium-pump activity by ouabain has been demonstrated in intact tissues (Yamamoto et al., 1980), isolated myocytes (Silver and Houser, 1985) and cultured cardiac cells (McCall, 1979; Werden et al., 1983; Kazazoglou et al., 1983). It has also been demonstrated, in intact cardiac muscle, that the ouabain concentration response curve is shifted to the left by interventions which increase Na^+ influx and sodium-pump activity (Yamamoto et al., 1980). These data are in good agreement with the shift to the left caused by monensin of the ouabain concentration response curves for inhibition of sodium-pump activity. These data from intact tissue and myocytes have been interpreted to indicate that augmented Na^+ influx decreases the reserve capacity of the sodium-pump. As pointed out by Yamamoto and coworkers (1980), however, an increase in ouabain binding caused by the increased Na^+ influx (Temma and Akera, 1982; Kennedy et al., 1983) can be at least partially responsible for the shift to the left of this curve.

SUMMARY AND CONCLUSIONS

Preparations of Ca^{2+}-tolerant myocytes can be isolated from hearts of adult animals after digesting connective tissue in the hearts with collagenase and hyaluronidase. These preparations are composed of a mixed population of viable and non-viable myocytes from which the living myocytes can be selected to greater than 80% purity by elutriation and gravity sedimentation.

Binding of ^{3}H-ouabain for the estimation of kinetic parameters of binding can be examined using these myocytes. Accurate estimation of non-specific binding to myocytes requires that a dissociation method be used if binding occurs in the presence of Ca^{2+} and K^+. This is in contrast to experiments utilizing isolated enzyme or tissue homogenate preparations where non-specific binding can be estimated using an excess of non-labeled drug.

^{3}H-ouabain binding to myocytes in the presence of Ca^{2+} and K^+ at millimolar concentrations is described by non-linear Scatchard plots. This indicates that there is more than one binding site for ^{3}H-ouabain or that there is cooperativity in binding. Each of these aspects of ^{3}H-ouabain binding preclude use of the displacement method for estimating kinetic parameters of ^{3}H-ouabain binding. In the absence of Ca^{2+}, ^{3}H-ouabain binds to guinea-pig myocytes in such a manner that two distinct linear components of binding can be discerned on Scatchard plots; a high affinity site with a K_D of 0.5 μM and a B_{max} equal to 14.0 pmol/mg protein and a low affinity site with a K_D of 3.4 μM and a B_{max} equal to 8.4 pmol/mg protein. The high affinity site probably represents the Na,K-ATPase as evidenced by the ability of K^+, when present during the binding reaction at a

concentration of 10 mM, to decrease incorporation of ^{3}H-ouabain into this site. The nature of the low affinity site is uncertain and it is possible that this represents an artifact of the method used to estimate non-specific binding.

Non-linearity of Scatchard plots describing ^{3}H-ouabain binding to the high affinity site in guinea-pig myocytes when binding occurs in the presence of Ca^{2+} and K^{+}, is probably caused by positive cooperativity in binding under these incubation conditions. The cooperativity results from an inactivation of sodium-pump units by ^{3}H-ouabain binding. This causes an increase in intracellular Na^{+} concentration which is a key determinant promoting ^{3}H-ouabain binding. Positive cooperativity by this mechanism is described by Scatchard plots which are upwardly concave. The possibilities that multiple binding sites or negative cooperativity associated with K^{+} redistribution were contributing to the non-linearity of the Scatchard plots describing ^{3}H-ouabain binding to myocytes were examined. Each of these phenomena may contribute toward the non-linearity, however, neither can have a large influence because when binding occurs in the absence of Ca^{2+}, Scatchard plots are linear but these phenomena still occur. The possibility that intracellular Ca^{2+} itself or a Ca^{2+}-dependent process inhibited ^{3}H-ouabain binding was examined by determining if agents which increase Ca^{2+} influx (A23187) decrease Ca^{2+} influx (lanthanum) or stimulate a protein kinase (isoproterenol and TPA) affect ^{3}H-ouabain binding. None of these agents had a significant effect on ^{3}H-ouabain binding, therefore, a direct effect of Ca^{2+} or a Ca^{2+}-dependent process on the Na,K-ATPase to affect cardiac glycoside binding appears to be unlikely.

Sodium-pump activity can be readily measured in preparations of myocytes using ouabain-sensitive $^{86}Rb^{+}$ uptake as an index of pump activity. Sodium-pump activity is stimulated by the presence of monensin in a concentration-dependent manner with maximum uptake exceeding basal uptake by 400%. This degree of

stimulation is also observed following incubation of myocytes in a K^+-free Rb^+-free solution containing 10 μM Ca^{2+} for 45 min or longer. The high ouabain-sensitive $^{86}Rb^+$ uptake seen when myocytes are exposed to high concentrations of monensin or long periods of sodium-loading may represent the capacity of the sodium-pump in intact cells. The shift to the left of the concentration response curve for sodium-pump inhibition by ouabain which is caused by monensin represents a decrease in the reserve capacity of the sodium-pump.

The presence of Ca^{2+} in incubation media during determination of ouabain-sensitive $^{86}Rb^+$ uptake inhibits both basal and stimulated uptake. The mechanism for this inhibition is unknown.

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