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Effects of Ca^{2+} on the sodium pump observed in cardiac myocytes isolated from guinea pigs

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It is presently unknown whether Ca^{2+} plays a role in the physiological control of Na^+/K^+-ATPAse or sodium pump activity. Because the enzyme is exposed to markedly different intra- and extracellular Ca^{2+} concentrations, tissue homogenates or purified enzyme preparations may not provide pertinent information regarding this question. Therefore, the effects of Ca^{2+} on the sodium pump were examined with studies of [^{3}H]ouabain binding and $^{86}Rb^+$ uptake using viable myocytes isolated from guinea-pig heart and apparently maintaining ion gradients. In the presence of K^+, a reduction of the extracellular Ca^{2+} increased specific [^{3}H]ouabain binding observed at apparent binding equilibria: a half-maximal stimulation was observed when extracellular Ca^{2+} was lowered to about 50 μM. The change in [^{3}H]ouabain binding was caused by a change in the number of binding sites accessible by ouabain instead of a change in their affinity for the glycoside. Ouabain-sensitive $^{86}Rb^+$ uptake was increased by a reduction of extracellular Ca^{2+} concentration. Benzocaine in concentrations reported to reduce the rate of Na^+ influx failed to influence the inhibitory effect of Ca^{2+} on glycoside binding. When [^{3}H]ouabain binding was at equilibrium, the addition of Ca^{2+} decreased and that of EGTA increased the glycoside binding. Mn^{2+}, which does not penetrate the cell membrane, had effects similar to Ca^{2+}. In the absence of K^+, cells lose their tolerance to Ca^{2+}. Reducing Ca^{2+} concentration prevented the loss of rod-shaped cells but failed to affect specific [^{3}H]ouabain binding observed in the absence of K^+. These results indicate that a large change in extracellular Ca^{2+} directly affects the sodium pump in cardiac myocytes isolated from guinea pigs.

Introduction

Intracellular Na^+ and extracellular K^+ regulate the interaction between cardiac glycosides and the sodium pump in intact cells by modulating pump turnover [1]. Glycoside binding in intact cells, however, appears to be regulated by other factors in addition to pump turnover. For example, enhancement of glycoside binding to Na^+/K^+-ATPase in intact skeletal muscle and adipocytes caused by catecholamines or insulin [2] does not appear to depend entirely on changes in intracellular Na^+ concentration or transmembrane K^+ flux. Whether turnover of Na^+/K^+-ATPase, and therefore glycoside binding to the enzyme, is affected by Ca^{2+} in intact cardiac muscle is an important and unanswered question.

Approx. 0.1 mM Ca^{2+} is required for a significant inhibition of isolated Na^+/K^+-ATPase. This concentration of Ca^{2+} stimulates phosphoenzyme formation from ATP in the absence of Mg^{2+}, apparently by substituting for Mg^{2+}, but inhibits the conversion of the phosphoenzyme from the ADP-sensitive to the K^+-sensitive form, and thereby blocks turnover of the enzyme [3]. More recently, Ca^{2+} has been shown to be capable of occupying the monovalent cation-binding center for K^+ in a medium containing no monovalent inorganic cations [4]. The concentration of Ca^{2+} used in the latter study was 0.4–2 mM. It is doubtful that these inhibitory effects of Ca^{2+} on isolated Na^+/K^+-ATPase occur when the enzyme is in an intact cell with a physiological extracellular medium. If the inhibitory effect of Ca^{2+} observed with isolated enzyme preparations represents

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; KHB, Krebs-Henseleit bicarbonate; Na^+/K^+-ATPase, Mg^{2+}-dependent ($Na^+ + K^+$)-activated ATP phosphohydrolase.

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an effect at a site accessible by extracellular Ca^{2+}, then the sodium pump would be inhibited at all times. Because the concentrations of Ca^{2+} needed to inhibit the isolated enzyme cannot be achieved in the cytoplasm of functioning myocardial cells, the general feeling has been that Ca^{2+} does not affect Na^+/K^+-ATPase activity in intact cells.

Yingst and his associates [5–8], however, have shown that the sodium pump in intact erythrocytes is sensitive to intracellular Ca^{2+} at physiological concentrations. This is dependent on the presence of a protein factor (not calmodulin) which increases Ca^{2+}-sensitivity of Na^+/K^+-ATPase. The possible presence of protein factors that modulate Na^+/K^+-ATPase has also been suggested by Lilievre and his associates [9]. These investigators reported that Ca^{2+} has a marked effect on the affinity of Na^+/K^+-ATPase for the cardiac glycosides under certain conditions [9–11]. Recently, Huang and Askari [12,13] have demonstrated the presence of high-affinity Ca^{2+} binding sites on the purified enzyme in addition to low-affinity sites which apparently correspond to Mg^{2+} binding sites. These findings raise a possibility that Ca^{2+} may act directly on the Na^+/K^+-ATPase at intracellular sites. Thus, Na^+/K^+-ATPase activity in intact cells might be modulated by Ca^{2+}. Meldgaard et al. [14] have reported that low extracellular Ca^{2+} stimulates [^{3}H]ouabain binding and increases the dissociation rate constant for the glycoside-Na^+/K^+-ATPase complex in cultured myocytes obtained from neonatal rat heart, indicating that Ca^{2+} affects Na^+/K^+-ATPase independent of changes in intracellular Na^+. Therefore, effects of Ca^{2+} on sodium pump activity and [^{3}H]ouabain binding were examined using myocytes isolated from guinea-pig heart.

Materials and Methods

Isolation of myocytes. Guinea pigs of either sex weighing approx. 400 g (albino, Mdh-A strain) were obtained from the Michigan Department of Public Health, Lansing, MI. The animals were stunned by a sharp blow to the head and their hearts were rapidly removed. Myocytes were isolated from ventricular muscle by digestion with collagenase and hyaluronidase in a modified Krebs-Henseleit bicarbonate (KHB) buffer solution containing a low concentration (10 μM) of $CaCl_2$ as described previously [15]. The composition of modified KHB buffer solution was 118 mM NaCl/27.1 mM $NaHCO_3$/2.8 mM KCl/1 mM KH_2PO_4/1.2 mM $MgSO_4$ with an indicated concentration of $CaCl_2$, 2.5 mM sodium pyruvate and 10 mM dextrose. The solution was saturated with a 95% O_2/5% CO_2 gas mixture yielding a pH value of 7.4 at 37°C. Dispersed myocytes were separated from tissue debris by filtration through nylon mesh and preparations were enriched in viable cells by elutriation and gravity sedimentation [15].

Preincubation in Ca^{2+} or Mn^{2+} solution. Isolated myocytes were loaded into a vertical hollow glass column and superfused at 37°C with KHB buffer solution containing the indicated concentration of $CaCl_2$ or $MnCl_2$. The flow of a solution from the bottom of the column to the top at the rate of 3 ml/min matched the sedimentation rate of viable myocytes, thereby maintaining the cells suspended in the column. Zero Ca^{2+} solution contained 20 μM EGTA.

[^{3}H]Ouabain binding to myocytes. After a 60 min superfusion in the columns, aliquots of myocyte suspension were transferred into test tubes and incubated at 37°C in the presence of the indicated concentration of [^{3}H]ouabain (protein concentration, 0.5–1.0 mg/ml). After a 40 min incubation, the cell suspension was filtered through a Whatman GF/D filter to collect myocytes. The filter was treated with a tissue solubilizer (Protosol, New England Nuclear, Boston, MA) and counted for bound [^{3}H]ouabain. A 100 μl aliquot of the supernatant solution was taken and the concentration of free [^{3}H]ouabain was estimated. Nonspecific binding (nonsaturable uptake) was determined by performing the above binding study in the presence of 0.1 mM unlabeled ouabain. This value was subtracted from the value observed in the absence of unlabeled ouabain to calculate specific [^{3}H]ouabain binding. Radioactivity was estimated by using a liquid scintillation spectrometer. Counting efficiency (approx 30%) was monitored by the external standard channel ratio method.

Benzocaine. Effects of benzocaine on [^{3}H]ouabain binding were examined following a preincubation of myocytes for 20 min at 37°C in KHB buffer solution containing 0.3 mM benzocaine. This concentration of benzocaine has been shown to cause a significant reduction of the Na^+ influx rate in isolated guinea-pig heart [16]. Following preincubation with 0.3 mM benzocaine, myocytes were superfused with a solution containing benzocaine and the indicated concentration of Ca^{2+}. [^{3}H]ouabain binding to these cells was then assayed in the presence of 0.3 mM benzocaine.

The time-course of Ca^{2+} effects on [^{3}H]ouabain binding. Myocytes were incubated at 37°C for 40 min with 500 nM [^{3}H]ouabain in the presence of 3.8 mM K^+ and 0.2 mM $CaCl_2$. Subsequently, a small amount of concentrated $CaCl_2$ or EGTA solution was added to the medium yielding a final concentration of 1.8 mM $CaCl_2$ or 1 mM EGTA. Aliquots of myocyte suspension were removed at the indicated time and assayed for bound [^{3}H]ouabain.

^{86}Rb uptake studies. After a 60 min superfusion with a solution containing the indicated concentration of $CaCl_2$, a small amount of RbCl solution containing tracer amounts of ^{86}RbCl was added to the myocyte suspension. The final concentration of RbCl was 1 mM. For determination of ouabain-insensitive $^{86}Rb^+$ uptake, 1 mM ouabain was added with the tracer $^{86}Rb^+$. This

value was subtracted from the value observed in the absence of ouabain to calculate specific $^{86}Rb^+$ uptake. Following a 10 min incubation with $^{86}Rb^+$, myocytes were separated from the incubation medium by centrifugation at 1500 r.p.m. through a layer of 0.24 M sucrose at 0°C. Although preparations were centrifuged for 3 min, passage of cells through the sucrose layer took less than 25 s. This time-period is apparently sufficient for surface-bound $^{86}Rb^+$ to be removed from the cells; however, preliminary studies on the time-course of the release of $^{86}Rb^+$ taken up into the cells indicate that the release of $^{86}Rb^+$ from myocytes in a medium containing no cations was negligible during this time-period. Radioactivity was estimated using a gamma scintillation spectrometer. An aliquot of the supernatant solution was assayed for specific activity of $^{86}Rb^+$.

The activity of the sodium pump optimally stimulated by intracellular Na^+, and therefore independent of the rate of Na^+ influx, was estimated by incubating myocytes in the presence of the Na^+ ionophore, monensin [17]. After superfusion with a solution containing the indicated concentration of $CaCl_2$, a small amount of monensin solution was added to the myocyte suspension, yielding a final concentration of 50 μM. This concentration of monensin has been shown to maximally stimulate ouabain-sensitive $^{86}Rb^+$ uptake in a physiological solution observed in similar preparations [18]. The mixture was incubated for 3 min and then the $^{86}RbCl$ solution was added. Myocytes were collected after an additional 3 min incubation with $^{86}Rb^+$.

[^{3}H]ouabain binding in a K^+-free solution. After isolation, myocytes were superfused in a vertical column as above with a K^+-free KHB buffer solution containing 0.01 mM $CaCl_2$. After a 20 min superfusion, an aliquot of myocyte suspension was added to a pre-warmed K^+-free KHB buffer solution containing 1 μM [^{3}H]ouabain and the indicated concentration of either $CaCl_2$ or EGTA. The final concentration of Ca^{2+} was either 0, 0.05, 0.2 or 1.8 mM. The concentration of EGTA in the medium that did not contain Ca^{2+} was 0.25 mM. After a 40 min incubation, bound [^{3}H]ouabain and the percentage of viable (rod-shaped) cells were examined. Control experiments were performed in the same manner, except that 3.8 mM K^+ was present in all solutions.

Miscellaneous. Protein concentration was assayed by the method of Bradford [19] using Coomassie brilliant blue G-250. The binding site concentration (B_{max}) and the dissociation constant (K_d) were estimated using the LIGAND program [20] adapted to IBM-PC microcomputers [21]. The number of cells and percentage of rod-shaped cells were estimated using a hemocytometer. [^{3}H]ouabain (generally labeled, spec. act. 20.0 Ci/mmol) and $^{86}RbCl$ were purchased from New England Nuclear, Boston, MA. Monensin was obtained from Sigma Chemical Company, St. Louis, MO.

Results

Effect of extracellular Ca^{2+} concentration on [^{3}H]ouabain binding

Following the addition of 0.02–1 μM [^{3}H]ouabain to the incubation medium, glycoside binding to guinea-pig myocytes reached an apparent plateau within 30 min as reported earlier [22]. A reduction in the Ca^{2+} concentration increased saturable glycoside binding observed at equilibrium at each [^{3}H]ouabain concentration examined (Fig. 1A). Such an increase in [^{3}H]ouabain binding may result from either an increase in the number of available binding sites or an increase in affinity of those sites for the glycoside. To distinguish between these two possibilities, binding data were kinetically analyzed. Eadie-Hofstee plots of [^{3}H]ouabain binding to myocytes in the absence or presence of 1.8 mM $CaCl_2$ indicate that the elimination of Ca^{2+} from the incubation medium increased the number of binding sites available to ouabain (Fig. 1B and Table I). Changes in the apparent affinity of the binding sites for the glycoside were minimal. The presence of low-affinity glycoside binding sites in guinea-pig cardiac myocytes was not examined in this study because myocytes did

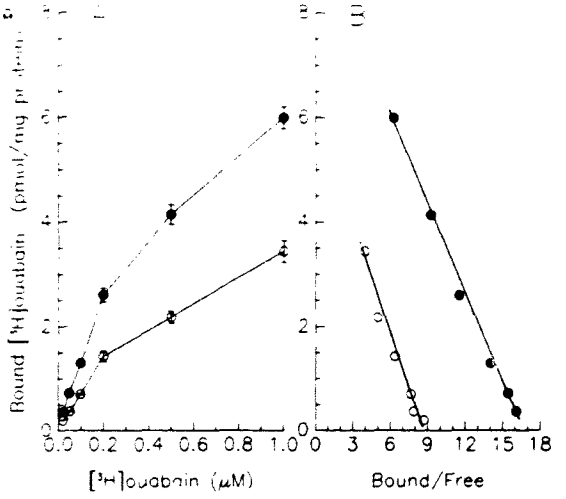

Fig. 1. Effects of Ca^{2+} on [^{3}H]ouabain binding to myocytes. Panel A; specific (saturable) [^{3}H]ouabain binding to myocytes observed in the presence of 1.8 mM $CaCl_2$ (○) or in the absence of $CaCl_2$ and the presence of 20 μM EGTA (●). Myocytes obtained from guinea-pig heart were incubated with the indicated concentration of [^{3}H]ouabain in a modified KHB buffer solution at 37°C for 40 min, and then bound [^{3}H]ouabain was estimated. Nonsaturable binding observed in the presence of excess unlabeled ouabain was subtracted. Each value represents the mean of four experiments. Vertical lines indicate S.E. Panel B: Eadie-Hofstee plots of the same data.

TABLE I

Concentrations of high-affinity ouabain binding sites and their affinity for ouabain observed in the presence and absence of Ca^{2+}

Maximal binding (B_{max}) and apparent dissociation constant (K_d) were calculated from the data shown in Fig. 1 using the LIGAND program.

Ca^{2+}	N	B_{max} (pmol/mg protein)	K_d (nM)
None [a]	4	9.27 ± 0.31	550
1.8 mM	4	5.96 ± 0.38	692

[a] EGTA (20 μM) was added to a solution containing no $CaCl_2$.

not tolerate high concentrations of ouabain. Observed results, therefore, represent glycoside binding only to high-affinity binding sites.

The concentration-dependence of the Ca^{2+} effect was examined by estimating [^{3}H]ouabain binding to myocytes incubated in a solution containing 0.1 μM [^{3}H]ouabain and various concentrations of $CaCl_2$. This low concentration of [^{3}H]ouabain was chosen in order to minimize nonspecific binding. Results of the above study indicate that Ca^{2+} alters the number of [^{3}H] ouabain binding sites with minimal effect on their affinity for the glycoside. Therefore, changes in [^{3}H]ouabain binding at any concentration should parallel the Ca^{2+}-induced changes in the number of ouabain binding sites. Calcium caused a concentration-dependent inhibition of [^{3}H]ouabain binding (Fig. 2). The effect of Ca^{2+} appears to be biphasic. As $CaCl_2$ concentration was decreased from 1.8 mM, [^{3}H]ouabain binding increased

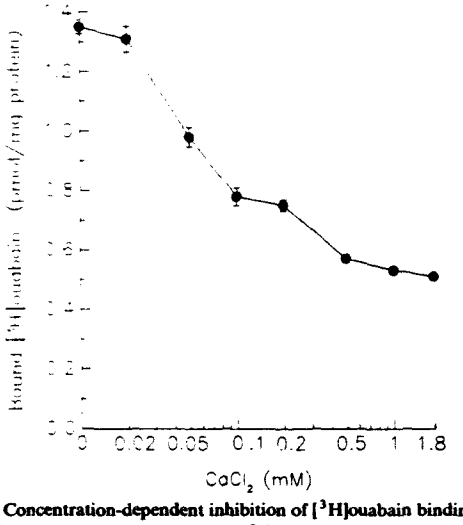

Fig. 2. Concentration-dependent inhibition of [^{3}H]ouabain binding by Ca^{2+}. Myocytes were incubated at 37 °C in a solution containing 100 nM [^{3}H]ouabain and the indicated concentration of $CaCl_2$. After a 40 min incubation, bound [^{3}H]ouabain was estimated. Nonsaturable binding observed in the presence of excess unlabeled ouabain was subtracted. Each value represents the mean of five experiments. Vertical lines indicate S.E.

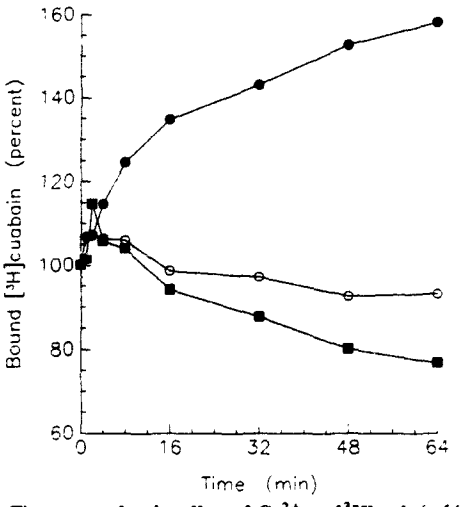

Fig. 3. Time-course for the effect of Ca^{2+} on [^{3}H]ouabain binding. After a 40 min incubation at 37 °C with 0.5 μM [^{3}H]ouabain and 0.2 mM $CaCl_2$, the concentration of Ca^{2+} in the medium was increased or decreased without changing the concentration of [^{3}H]ouabain. Subsequent changes in [^{3}H]ouabain binding were examined. The value for bound [^{3}H]ouabain observed immediately before the change in Ca^{2+} concentration was set as 100%. (○) control preparations in which $CaCl_2$ concentration was unchanged (0.2 mM); (■) $CaCl_2$ concentration was increased to 1.8 mM at time zero; (●) EGTA (final concentration, 1 mM) was added at time zero. Each point represents the mean of three experiments.

reaching an apparent plateau at 0.1–0.2 mM $CaCl_2$ with a half-maximal effect observed at about 0.5 mM. An additional large increase in saturable [^{3}H]ouabain binding was observed as the $CaCl_2$ concentration was reduced below 0.1 mM, with a half-maximal effect observed at about 50 μM $CaCl_2$. These results indicate that an increase in extracellular Ca^{2+} ion concentration inhibits [^{3}H]ouabain binding to isolated adult myocytes and that there may be more than one mechanism for the observed inhibition.

The time-course of the Ca^{2+} effect

The time-course of changes in [^{3}H]ouabain binding following an abrupt change in Ca^{2+} concentration was examined. Myocytes preincubated for 40 min in a medium containing 0.5 μM [^{3}H]ouabain and 0.2 mM $CaCl_2$ were dispersed into a solution containing 1.8 mM Ca^{2+} or sufficient EGTA to eliminate free Ca^{2+}, and subsequent changes in [^{3}H]ouabain binding were monitored. In control preparations in which $CaCl_2$ concentration was maintained at 0.2 mM, the amount of bound [^{3}H]ouabain was relatively stable after a 40 min incubation (Fig. 3). An increase in $CaCl_2$ concentration to 1.8 mM caused a gradual decrease in [^{3}H]ouabain binding over the 60 min observation period. After the addition of EGTA, [^{3}H]ouabain binding increased significantly, indicating that the inhibitory effect of Ca^{2+}

is reversible. Changes in glycoside binding were gradual and the binding failed to reach an apparent steady-state within 60 min.

Effects of Ca^{2+} on sodium pump activity

Sodium pump activity was estimated as the ouabain-sensitive $^{86}Rb^+$ uptake without or with Na^+ loading of myocytes using monensin. Without Na^+ loading, intracellular Na^+ is the limiting factor for sodium pump activity, and ouabain-sensitive $^{86}Rb^+$ uptake is equivalent to the Na^+ influx rate [1]. We have shown previously that extracellular Ca^{2+} exerts a concentration-dependent effect to decrease ouabain-sensitive $^{86}Rb^+$ uptake in quiescent myocytes [18]. These results were confirmed (Fig. 4), indicating that the Na^+ influx rate is higher when Ca^{2+} concentration is low. This effect of Ca^{2+} occurred between 0.2 and 0.5 mM $CaCl_2$ and was monophasic (Fig. 4).

We have shown previously that 50 μM monensin caused the maximal stimulation of ouabain-sensitive $^{86}Rb^+$ uptake observed in isolated myocytes, and that this value can be used as an estimate for capability of the sodium pump under a given condition unaffected by the rate of Na^+ influx [18]. This value increased as the concentration of Ca^{2+} was increased to 50 μM and then decreased at concentrations above 50 μM (Fig. 4). A half-maximal decrease in ouabain-sensitive $^{86}Rb^+$ uptake was observed at a $CaCl_2$ concentration of 0.5 mM. These results indicate that Ca^{2+} has dual effects on the

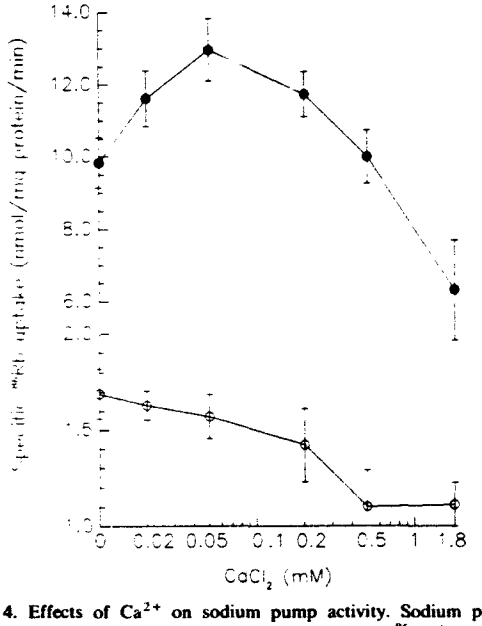

Fig. 4. Effects of Ca^{2+} on sodium pump activity. Sodium pump activity was estimated from the ouabain-sensitive $^{86}Rb^+$ uptake. Myocytes were incubated at 37°C for 10 min with 1 mM $^{86}RbCl$ in the absence (○) or presence (●) of 50 μM monensin. Each point represents the mean of eight experiments. Vertical lines indicate S.E.

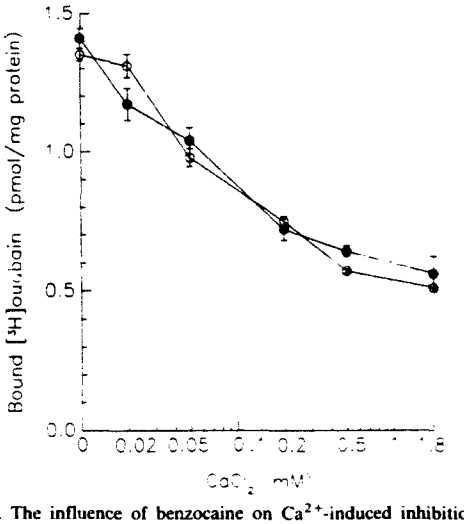

Fig. 5. The influence of benzocaine on Ca^{2+}-induced inhibition of [^{3}H]ouabain binding. Myocyte preparations were pretreated with 0.3 mM benzocaine to reduce Na^+ influx, and then incubated with 100 nM [^{3}H]ouabain and the indicated concentration of Ca^{2+} at 37°C for 40 min (●). Benzocaine was present during the pretreatment and also during the incubation with [^{3}H]ouabain. Control preparations were treated in a similar manner but benzocaine was omitted (○). Each point represents the mean of five experiments. Vertical lines indicate S.E.

capacity of the sodium pump and that the inhibitory effect of Ca^{2+} on sodium pump activity is not solely dependent on the turnover rate of the pump and, therefore, is unlikely to be secondary to changes in Na^+ influx or intracellular Na^+ concentration.

The influence of benzocaine on Ca^{2+}-induced modulation of the sodium pump

Myocyte preparations were treated with 0.3 mM benzocaine to reduce Na^+ influx [16] and then incubated with 100 nM [^{3}H]ouabain. Benzocaine alone did not alter [^{3}H]ouabain binding to myocytes, confirming our earlier results obtained with multicellular preparations [16]. Moreover, benzocaine failed to modify the effect of Ca^{2+} on [^{3}H]ouabain binding (Fig. 5), supporting the concept that the effect of Ca^{2+} is not entirely dependent on changes in Na^+ influx rate.

Comparative effects of Mn^{2+} and Ca^{2+} on [^{3}H]ouabain binding

The effects of Ca^{2+} were compared with those of Mn^{2+}, a divalent cation which does not enter the cells. Myocytes were incubated with 100 nM [^{3}H]ouabain in a medium containing the indicated concentration of either $MnCl_2$ or $CaCl_2$. A phosphate-free KHB buffer solution was used in this study in order to prevent the precipitation of Mn^{2+}. Glycoside binding was reduced by the addition of either Ca^{2+} or Mn^{2+} to the medium (Fig. 6). The inhibitory effects and concentration-de-

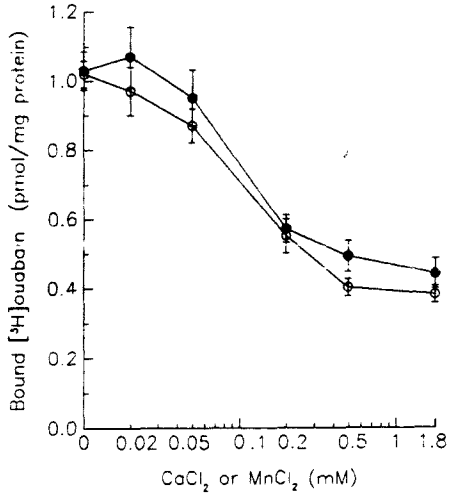

Fig. 6. Comparative effects of Mn^{2+} and Ca^{2+} on [^{3}H]ouabain binding. Myocytes were incubated with 100 nM [^{3}H]ouabain at 37°C for 40 min in a medium containing the indicated concentration of either $MnCl_2$ (●) or $CaCl_2$ (○). A phosphate-free KHB buffer solution was used in this study to prevent the precipitation of Mn^{2+}. Each point represents the mean of three experiments. Vertical lines indicate S.E.

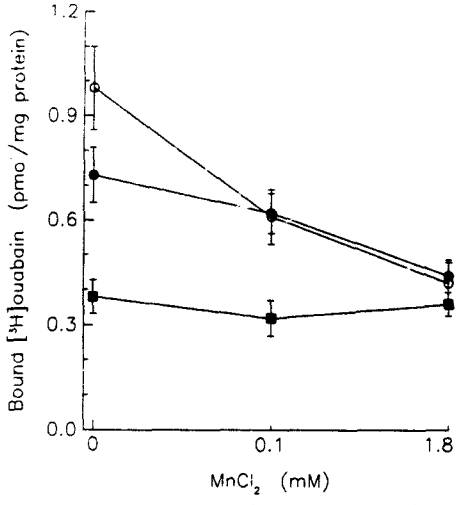

Fig. 7. The combined effect of Mn^{2+} and Ca^{2+} on [^{3}H]ouabain binding. [^{3}H]ouabain binding was assayed as in Fig. 1 in the presence of Ca^{2+} and/or Mn^{2+}. The indicated concentration of $MnCl_2$ was added to the medium in the absence of Ca^{2+} (○), or in the presence of 0.1 (●) or 1.8 mM $CaCl_2$ (■). Each point represents the mean of three to six experiments. Vertical lines indicate S.E.

pendence of these two divalent cations on [^{3}H]ouabain binding were equivalent.

The combined effect of Mn^{2+} and Ca^{2+} on [^{3}H]ouabain binding was examined in order to test if these cations are interchangeable with respect to their effects on [^{3}H]ouabain binding. In the absence of Ca^{2+}, [^{3}H]ouabain binding was reduced as the concentration of $MnCl_2$ was increased from 0 to 0.1 and then to 1.8 mM (Fig. 7). In the presence of 0.1 mM Ca^{2+}, however, the effect of Mn^{2+} was smaller. In the presence of maximally effective concentration (1.8 mM) of Ca^{2+}, Mn^{2+} failed to cause a significant decrease in glycoside binding. These results indicate that the effects of Ca^{2+} and Mn^{2+} are additive, and they are interchangeable with respect to their inhibitory effect on [^{3}H]ouabain binding.

The effect of Ca^{2+} on [^{3}H]ouabain binding in a K^+-free KHB buffer solution

Myocytes were incubated in a modified KHB buffer solution containing the indicated concentration of $CaCl_2$ without or with 3.8 mM K^+ for 40 min in the presence of 1 μM [^{3}H]ouabain and then bound ouabain was estimated. The presence of K^+ reduced glycoside binding regardless of Ca^{2+} concentration (Fig. 8A). In the presence of K^+, addition of Ca^{2+} caused a further decrease in glycoside binding. In the absence of K^+, the effect of Ca^{2+} to reduce glycoside binding was minor.

In a K^+-free KHB buffer solution, myocytes became rounded at the end of a 40 min incubation period when the solution contained 1.8 mM $CaCl_2$; however, the cells maintained their rod shape in a K^+-free medium when the Ca^{2+} concentration was low (Fig. 8B). This

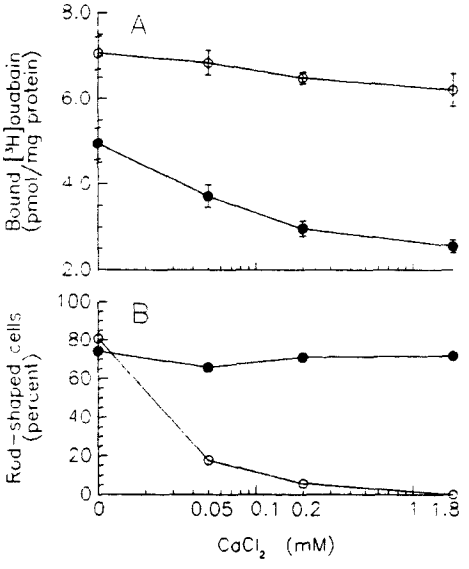

Fig. 8. The effect of Ca^{2+} on [^{3}H]ouabain binding and myocyte viability observed in the absence or presence of K^+. Myocytes were incubated in a modified KHB buffer solution containing the indicated concentration of $CaCl_2$ without (○) or with 3.8 mM K^+ (●) at 37°C for 40 min in the presence of 1 μM [^{3}H]ouabain. Panel A: specific [^{3}H]ouabain binding observed after a 40 min incubation at 37°C. Panel B: percent of rod-shaped (viable) myocytes. Each point represents the mean of four experiments. Vertical lines indicate S.E.

change in myocyte morphology, from rod shape to rounded, was not associated with changes in [^{3}H]ouabain binding (Fig. 8A). These results indicate that changes in glycoside binding are not secondary to changes in the shape or viability of myocytes.

Discussion

A decrease in extracellular Ca^{2+} concentration from 1.3 to 0.1 mM has been reported to cause an increase in binding of a low concentration (10 nM) of [^{3}H]ouabain observed with cultured cardiac myocytes obtained from neonatal rats [15]. The present results demonstrate that changes in extracellular Ca^{2+} concentration cause significant changes in sodium pump activity as estimated from $^{86}Rb^+$ uptake and also [^{3}H]ouabain binding in viable cardiac myocytes obtained from guinea-pig heart and suspended in a physiological solution containing K^+. It should be noted that changes in glycoside binding was observed with concentrations of [^{3}H]ouabain that have been shown to produce positive inotropic effects in this species. Because [^{3}H]ouabain binding to Na^+/K^+-ATPase requires phosphorylation and a conformational change of the enzyme molecule from the K^+-induced- to the Na^+-induced form [1], these results appear to indicate that turnover of the sodium pump in intact myocytes is influenced by changes in the extracellular Ca^{2+} concentration.

It has been shown that the cytoplasmic membrane of myocytes becomes permeable to several cations after an incubation in a medium containing a low concentration of Ca^{2+} [23–25]. Reduction of Ca^{2+} also causes ultrastructural changes [26–28] and increases membrane fluidity [29,30], which in turn may enhance the turnover of Na^+/K^+-ATPase [31,32].

An increase in ouabain-sensitive $^{86}Rb^+$ uptake by non-sodium loaded myocytes indicates that the Na^+ influx rate was increased when the extracellular $CaCl_2$ concentration was reduced from 1.8 to 0.2 mM. This is because the rate of Na^+ extrusion by the sodium pump is equal to the rate of Na^+ influx in myocytes maintaining ionic equilibria. Ouabain sensitive $^{86}Rb^+$ uptake observed without Na^+ loading, therefore, represents the rate of Na^+ influx [1]. Increased [^{3}H]ouabain binding to quiescent myocytes caused by a reduction in the extracellular Ca^{2+} concentration is expected from an increase in the Na^+ influx rate or the intracellular Na^+ concentration. It should be noted, however, that the number of available binding sites (apparent B_{max}) is greatly increased by Ca^{2+} removal and that the apparent affinity (observed K_d value) of the binding sites for [^{3}H]ouabain is increased only slightly. Increases in intracellular Na^+ concentration should increase the affinity rather than the binding-site concentration [15,33].

It should be pointed out that [^{3}H]ouabain binding observed with a 40 min incubation is at apparent equilibrium.. Our previous study [22] indicates that glycoside binding to similar myocyte preparations reaches a plateau at about 30 min in the absence of Ca^{2+}. In the presence of 0.2 mM $CaCl_2$, a similar time-course of [^{3}H]ouabain binding has been observed and no additional increase in glycoside binding was observed during a 64 min period following a 40 min incubation (Fig. 3). Because the largest change in glycoside binding was observed between 0 and 0.2 mM $CaCl_2$, the possibility that Ca^{2+} delays glycoside binding and that the binding reaction failed to reach equilibrium in the presence of Ca^{2+}, resulting in underestimation of the observed B_{max} value, can be excluded.

It has been shown previously that an increase in the rate of Na^+ influx increases [^{3}H]ouabain binding [34–36], whereas a decrease in the rate of Na^+ influx from the normal value does not reduce glycoside binding [16]. Benzocaine, in concentrations similar to that used in the present study, has been shown to decrease sodium current [37], sodium influx rate [38] and intracellular sodium ion concentration [39,40]; however, it failed to antagonize the effect of low extracellular Ca^{2+} concentrations to increase [^{3}H]ouabain binding. The finding that benzocaine failed to affect [^{3}H]ouabain binding when the Na^+ influx was not enhanced is consistent with the known behavior of this agent [16]. Benzocaine, however, should antagonize stimulation of glycoside binding caused by a low extracellular Ca^{2+} concentration if the stimulation is caused by an increase in the rate of Na^+ influx. This was not the case, indicating that the effects of Ca^{2+} removal are not mediated by changes in Na^+ influx. The failure of benzocaine, a potent membrane stabilizer, to antagonize the effect of Ca^{2+} removal on [^{3}H]ouabain binding also indicates that the effect of low concentration Ca^{2+} does not result from the simple destabilization of the sarcolemma.

In further support of the concept that the effect of Ca^{2+} is not simply mediated by changes in the Na^+ influx rate or intracellular Na^+ concentration, the effect of the Na^+ ionophore, monensin, to stimulate ouabain-sensitive $^{86}Rb^+$ uptake is much greater than that of Ca^{2+} removal (Fig. 4). In previous studies, we have shown that the incubation of myocytes in Ca^{2+}-free media has a larger effect than monensin to increase [^{3}H]ouabain binding and that monensin has no effect on the binding in the absence of Ca^{2+} [15]. The rate of $^{86}Rb^+$ uptake by isolated myocytes increases as monensin concentration is increased and reaches a plateau at 50 μM [18]. The rate of $^{86}Rb^+$ uptake observed in the presence of monensin in the present study, therefore, may be regarded as sodium pump activity that is maximally stimulated by intracellular Na^+. Such stimulation, however, is limited under the constraints of its environment in intact myocytes, because it does not apparently represent the true capacity corresponding to

the maximal turnover of Na^+/K^+-ATPase observed with isolated enzyme preparations or sodium pump activity estimated under the ideal condition. Nevertheless, the present results show that Ca^{2+} removal maximizes [^{3}H]ouabain binding without eliminating sodium pump reserve capacity. These findings further support the conclusion that the effect of Ca^{2+} on [^{3}H]ouabain binding are not dependent on changes in Na^+ influx rate.

Moreover, Ca^{2+} concentrations that caused changes in Na^+ influx rate, as estimated from ouabain-sensitive $^{86}Rb^+$ uptake in myocytes that are not Na^+-loaded, are different from those which caused the largest increase in [^{3}H]ouabain binding. The former effect was observed at about 0.5 mM $CaCl_2$, whereas the latter occurred at about 50 μM Ca^{2+}. There was a small increase in [^{3}H]ouabain binding when the concentration of Ca^{2+} was reduced from 1 to 0.2 mM, i.e., in the range similar to that which caused an increase in $^{86}Rb^+$ uptake; however, the increase in [^{3}H]ouabain binding observed in this range of Ca^{2+} concentrations was relatively minor and is expected from the increase in sodium pump turnover. It should be noted that the two curves in fig. 4 representing specific $^{86}Rb^+$ uptake observed in the presence and absence of monensin are not parallel. The finding that a larger increase in [^{3}H]ouabain binding was observed at about 50 μM $CaCl_2$ indicates that an increase in intracellular Na^+ is not the primary mechanism responsible for increased [^{3}H]ouabain binding observed in solutions of low concentration Ca^{2+}.

Reduction of the extracellular K^+ concentration increased glycoside binding. This result is anticipated because K^+ is known to inhibit glycoside binding to Na^+/K^+-ATPase by reducing the time during which the enzyme molecule is in a Na^+-induced conformation that preferentially binds the glycoside [1]. In the absence of K^+ with millimolar Ca^{2+} present, myocytes rapidly lost their viability. After a few minutes, all cells had a rounded appearance. This consequence of K^+ removal was abolished by lowering the extracellular Ca^{2+} concentration, indicating that low concentration K^+ inhibits turnover of the sodium pump and thereby causes Ca^{2+} overload via an increase in intracellular Na^+ and ensuing modification of the Na^+/Ca^{2+} exchange reaction. Surprisingly, the change in the appearance of myocytes had a relatively minor effect on [^{3}H]ouabain binding.

The time-course for changes in glycoside binding following an abrupt change in the extracellular Ca^{2+} concentration is relatively slow and [^{3}H]ouabain binding does not reach a new steady-state within 60 min. In contrast, glycoside binding to cells pre-equilibrated in a solution with a fixed concentration of Ca^{2+} reached a steady-state within 40 min. These results seem to indicate that the extracellular Ca^{2+} does not have free access to the site of action. The time-course for changes in [^{3}H]ouabain binding after an abrupt change in Ca^{2+} is slower than the time-course for the glycoside binding to, or release from, Na^+/K^+-ATPase [15,22].

The precise mechanism by which extracellular Ca^{2+} in the range of 50 μM modulates [^{3}H]ouabain binding is unknown. That this effect is mediated by changes in the bulk intracellular Ca^{2+} may be ruled out even though the access of Ca^{2+} to the site of action is a slow process. This is because the effect is shared by Mn^{2+}, which does not easily penetrate the sarcolemma [41]. Moreover, Mn^{2+} had an additive effect with a low concentration of Ca^{2+} but does not affect the maximal decrease in [^{3}H]ouabain binding observed with a high concentration of Ca^{2+}, indicating that these two cations act by the same mechanism. Experiments with Ca^{2+} ionophores appear to further support this hypothesis; however, definite conclusions could not be reached because myocytes incubated with the Ca^{2+} ionophore, A23187, in a solution containing millimolar Ca^{2+} rapidly lost their viability and all cells became rounded within a few minutes (data not shown).

It should be noted that a reduction of extracellular Ca^{2+} concentration from 100 to 20 μM caused a marked increase in [^{3}H]ouabain binding but failed to increase ouabain-sensitive $^{86}Rb^+$ uptake in Na^+-loaded myocytes. These findings, namely a stimulation of [^{3}H]ouabain binding and a simultaneous reduction in the capacity of the sodium pump, may indicate that the low extracellular Ca^{2+} increases the fraction of Na^+/K^+-ATPase molecules taking the Na^+-induced conformation but inhibits the subsequent step of enzyme turnover. This hypothesis is consistent with the finding that low concentration Ca^{2+} failed to cause a marked change in [^{3}H]ouabain binding when the extracellular K^+ concentration is low and the rate of the conformational transition from the Na^+-induced form to the K^+-induced form is already markedly reduced.

Extracellular Ca^{2+} apparently affects the Na^+/K^+-ATPase by two mechanisms, one occurs at a concentration of approx. 50 μM and the other at about 500 μM. The capacity of the sodium pump and the rate of Na^+ influx were influenced at about 500 μM $CaCl_2$, whereas a marked effect on [^{3}H]ouabain binding was observed at 50 μM. The relatively minor effect of Ca^{2+} on [^{3}H]ouabain binding observed at 500 μM probably results from an increase in Na^+ influx. Alternatively, these two actions might correspond to Ca^{2+} binding at the high- and low-affinity Ca^{2+} binding sites reported by Huang and Askari [12,13,42]. It is also possible that all or a part of the effect of Ca^{2+} on Na^+/K^+-ATPase might be indirect and mediated by changes in membrane lipids associated with Na^+/K^+-ATPase.

The mechanism by which Ca^{2+} reduces the B_{max} value for [^{3}H]ouabain binding is unknown. It should be noted that only binding sites with high affinity are observed in the present protocol. Changes in Ca^{2+}

concentration should not alter the number of Na^+/K^+-ATPase molecules in myocytes. Instead, a part of enzyme population may be altered such that they have extremely low affinity for the glycoside. Alternatively, Ca^{2+} may cause sodium pump units to be 'occluded' or 'internalized' and may therefore make them inaccessible by [^{3}H]ouabain under the condition of the present binding study.

In conclusion the number of apparent [^{3}H]ouabain binding sites, the rate of Na^+ influx and the capacity of the sodium pump were increased by a reduction of the extracellular Ca^{2+}. These effects of Ca^{2+} seem to result from the action of Ca^{2+} acting at the sites also accessible by extracellular Mn^{2+} and not by intracellular bulk Ca^{2+}.

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