Intracellular Sodium Affects Ouabain Interaction with the Na/K Pump in Cultured Chick Cardiac Myocytes

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ABSTRACT   Whether a given dose of ouabain will produce inotropic or toxic effects depends on factors that affect the apparent affinity ($K_{0.5}$) of the Na/K pump for ouabain. To accurately resolve these factors, especially the effect of intracellular Na concentration (Na_i), we have applied three complementary techniques for measuring the $K_{0.5}$ for ouabain in cultured embryonic chick cardiac myocytes. Under control conditions with 5.4 mM K_o, the value of the $K_{0.5}$ for ouabain was 20.6 ± 1.2, 12.3 ± 1.7, and 6.6 ± 0.4 μM, measured by voltage-clamp, Na-selective microelectrode, and equilibrium [^{3}H]ouabain-binding techniques, respectively. A significant difference in the three techniques was the time of exposure to ouabain (30 s–30 min). Since increased duration of exposure to ouabain would increase Na_i, monensin was used to raise Na_i to investigate what effect Na_i might have on the apparent affinity of block by ouabain. Monensin enhanced the rise in Na content induced by 1 μM ouabain. In the presence of 1 μM [^{3}H]ouabain, total binding was found to be a saturating function of Na content. Using the voltage-clamp method, we found that the value of the $K_{0.5}$ for ouabain was lowered by nearly an order of magnitude in the presence of 3 μM monensin to 2.4 ± 0.2 μM and the magnitude of the Na/K pump current was increased about threefold. Modeling the Na/K pump as a cyclic sequence of states with a single state having high affinity for ouabain shows that changes in Na_i alone are sufficient to cause a 10-fold change in $K_{0.5}$. These results suggest that Na_i reduces the value of the apparent affinity of the Na/K pump for ouabain in 5.4 mM K_o by increasing its turnover rate, thus increasing the availability of the conformation of the Na/K pump that binds ouabain with high affinity.

INTRODUCTION

Cardiac glycosides interact with the Na/K pump to produce profound changes in the physiological behavior of cardiac cells (Gadsby, 1984; Lee, 1985; Glitsch and Krahn, 1986). Of particular interest is the interaction of cardiac glycosides (e.g.,

ouabain) with the Na/K pump that produces both inotropic and toxic effects (i.e., Werdan et al., 1985). Generally, an inotropic response is produced by low concentrations of ouabain while toxic effects result from higher ouabain concentrations. However, both effects can be produced by a given concentration of ouabain depending on the activity of the preparation. Thus, several investigators have suggested that heart cell activity, e.g., mechanical (Moran, 1967), electrical (Bentfeld et al., 1977), and chemical (Akera et al., 1977), might contribute to the apparent differences in the sensitivity of the preparation to ouabain. In a recent review, Lee (1985) indicated that the inotropic effects of ouabain result from a block of the Na/K pump leading to a rise in Na_i, which then leads to a rise in Ca_i, probably via Na/Ca exchange. The toxic effects of ouabain occur as the ouabain occupancy of the receptor is increased to the extent that Na_i rises to significantly reduce the Na gradient and thus interfere with the Na-dependent regulatory mechanisms.

Because of these pharmacological effects it is important to know what factors affect ouabain binding (for review, see Heller, 1987). To investigate the effects that physiological activity of cardiac myocytes might have on the interaction of ouabain with the Na/K pump, the apparent affinity of the Na/K pump for ouabain has been measured in several cardiac preparations. Ouabain binds specifically to two classes of sites in cultured rat heart myocytes but the physiological role of these sites is still unclear (Friedman et al., 1980; Werdan et al., 1984b; Herzig and Mohr, 1984, 1985; Lutz et al., 1987). The dissociation constant (K_D) of the high affinity site was estimated to be between 32 and 89 nM, while the K_D of the low affinity site was between 2.3 and 7.1 μM. Herzig and Mohr (1985) suggested that the high affinity sites are associated with the inotropic effects of ouabain and ion transport; however, Werdan et al. (1984b) found that binding of ouabain only to the low affinity sites produced the noted physiological effects. In guinea pig cardiac muscle (Daut and Rudel, 1982; Herzig and Mohr, 1984) and cultured embryonic chick cardiac myocytes (Kim et al., 1984; Werdan et al., 1984a, 1985; Lobaugh and Lieberman, 1987), a single class of ouabain-binding sites has been found (but see Kazazoglou et al., 1983). Values for the K_D for ouabain binding ranged from 0.13 to 1.5 μM in guinea pig and from 0.15 to 6.6 μM in chick. Binding to this site was associated with the inotropic effects of ouabain (Biedert et al., 1979; Kim et al., 1984; Werdan et al., 1984b, 1985).

This wide range of values for ouabain affinity could be due to different experimental conditions, such as the ionic composition of the assay solution. At least part of the variability in the reported values of ouabain affinity is due to the known competitive interaction of external K concentration (K_o) with ouabain (Herzig and Mohr, 1984; Kim et al., 1984; Lobaugh and Lieberman, 1987). Physiological levels of K_o can increase the value of the apparent K_D for ouabain by an order of magnitude or more (Lobaugh and Lieberman, 1987). At the high concentrations of ouabain required to measure specific binding in normal K_o solutions, nonspecific ouabain binding becomes larger than specific binding. For this reason most ouabain binding studies have been done in low K_o solution.

Binding of cardiac glycosides in vitro to the enzyme equivalent of the Na/K pump, Na/K-ATPase, is enhanced by Na in the presence of Mg and ATP (Matsui and Schwartz, 1968; Schwartz et al., 1975). Therefore, it is reasonable to hypothe-

size that elevating intracellular sodium (Na_i) will enhance ouabain binding in intact preparations. While this has been suggested by several investigators, the supporting evidence in cardiac muscle relies mainly on changes in inotropic and toxic effects of the drug (Akera et al., 1977; Bentfeld et al., 1977; Busse et al., 1979) rather than direct measurements of ouabain binding or Na/K pump activity. In secretory epithelium, Hootman and Ernst (1988) have shown that epinephrine and carbachol increase the apparent affinity of ouabain presumably by opening Ca channels, which causes an increased Na influx via Ca-activated Na channels.

We have studied the apparent affinity ($K_{0.5}$) of the Na/K pump for ouabain at physiological K_o and evaluated the effect that Na_i may have on $K_{0.5}$ by applying three techniques to cultured chick cardiac myocytes: (a) voltage clamp, (b) Na-selective microelectrodes (Na-SME), and (c) equilibrium [^{3}H]ouabain binding. The use of these three techniques allows us to evaluate the methodologies for measuring ouabain binding as well as the physiological parameters that affect ouabain binding. We have found that the three methods yield different estimates for the $K_{0.5}$ for ouabain under control conditions with physiological K_o (5.4 mM). The data suggest that elevations of Na_i can account for the differences in $K_{0.5}$ and can decrease the value of the $K_{0.5}$ by an order of magnitude. Since the Na/K pump exists in several conformations, $K_{0.5}$ is not necessarily constant and is influenced by any factors that alter the distribution of the Na/K pump molecules among the various conformations. The fact that $K_{0.5}$ is not a constant is the subject of this paper. A preliminary report of some of these results has appeared (Stimers et al., 1986).

METHODS

Tissue Culture

The techniques used to culture chick heart muscle cell aggregates were described in the previous paper (Stimers et al., 1990). In addition to aggregates, we made use of the polystrand preparation (Horres et al., 1977) and confluent mass cultures (Lobaugh and Lieberman, 1987). Briefly, polystrand preparations were made by directing the growth of cardiac myocytes around a 20-μm-diameter nylon monofilament that was wound on a U-shaped silver wire. The cells were incubated at 37°C in a 95% air-5% CO_2 atmosphere for 3 d, at which time we observed the formation of spontaneously beating strands of muscle around the nylon cores. Confluent mass cultures of contracting cardiac myocytes were formed by culturing 1 × 10^6 cells/2 ml of culture medium in 35-mm culture dishes (Falcon 3001; Becton Dickinson and Co., Oxnard, CA) for 3 d.

Solutions

Control perfusate for the voltage-clamp experiments was a modified Hanks solution (H/TBSS) with the following composition in millimolar: 144 Na, 5.4 K, 0.8 Mg, 2.7 Ca, 156 Cl, 5.6 HEPES, 4.2 Trizma Base, and 5.6 dextrose (pH 7.4 ± 0.1). Control perfusate for the Na-SME experiments was a modified Earle's solution (MEBSS) containing in millimolar: 143 Na, 5.4 K, 0.8 Mg, 2.7 Ca, 127 Cl, 0.8 PO_4, 0.8 SO_4, 26 HCO_3, and 5.6 dextrose (pH 7.35 ± 0.05 in 5% CO_2-95% air). In the Na-SME experiments using monensin, H/TBSS was used, but it was modified to reduce Cl to 150 mM and 2.7 mM SO_4 was added. The MEBSS used in the equilibrium [^{3}H]ouabain-binding experiments was slightly different from that described above; they contained in millimolar: 145 Na, 5.4 K, 0.8 Mg, 1.8 Ca, 129 Cl, 0.8 PO_4, 0.8 SO_4,

26 HCO_3, and 5.6 dextrose. Altered K solutions were made by equimolar substitution with Na. Low Na solutions were made by equimolar substitution with tetramethylammonium. Ba and ouabain were added to solutions as indicated in the results. Monensin was dissolved in ethanol to make a 4- or 6-mM stock solution and was added to other solutions to the desired final concentration of 1–30 μM (ethanol concentration between 0.05% and 1.0%). All experiments were done at 37°C.

Voltage Clamp

The switching single-electrode voltage-clamp technique used for this study was described in the previous paper (Stimers et al., 1990).

Na-selective Microelectrode

The method used for measuring intracellular Na activity (a_{Na}^i) with Na-SME was recently described (Liu et al., 1987). Briefly, a 10-μl drop of liquid neutral carrier (Na-cocktail; Fluka Chemical Co., Hauppauge, NY) was back-filled into a clean, silanized micropipette, followed by internal filling with 0.1 M NaCl. Simultaneous measurement of the intracellular potential from a Na-SME and a conventional 3 M KCl–filled microelectrode allows the calculation of a_{Na}^i using the following equation:

$$a_{Na}^i = 10^{(V_d - E_o)/S} - K_{NaK}(a_K^i)$$ (1)

where V_d is the difference potential between the Na-SME and the voltage microelectrode; E_o and S are the standard potential and the slope determined from the calibration of the Na-SME in a series of single electrolyte solutions; K_{NaK} (0.023 ± 0.002, n = 21) is the potentiometric selectivity coefficient of the Na-SME for K relative to Na as determined from biionic calibration solutions; a_K^i is the intracellular K activity, which is 103 ± 1.3 mM (n = 57) (Liu et al., 1987). Correction for intracellular Ca activity (a_{Ca}^i) was not done because the selectivity coefficient for Ca relative to Na (K_{NaCa}) is 1.0 ± 0.1 (n = 21). Assuming a_{Ca}^i is 100 nM, contamination of a_{Na}^i would be ~0.3 mM.

Equilibrium [³H]Ouabain Binding

Equilibrium [³H]ouabain-binding experiments were carried out on confluent mass cultures as previously described (Lobaugh and Lieberman, 1987). Briefly, cells were preincubated for 20 min in MEBSS with or without monensin. The bathing solution was then replaced with the same solution plus [³H]ouabain. After a 30-min incubation period, cells were rinsed with ice-cold MEBSS to remove unbound [³H]ouabain and trypsinized to form a cell suspension. Aliquots were assayed for radioactivity and total cell protein.

Nonspecific [³H]ouabain binding is defined as the amount of [³H]ouabain bound in the presence of an excess of unlabeled ouabain (2 mM). Nonspecific binding was subtracted from binding in the absence of unlabeled ouabain (total [³H]ouabain bound) to obtain values for specific [³H]ouabain bound.

Sodium Content

Cell Na content was measured by atomic absorption spectrophotometry (model 460; Perkin-Elmer, Norwalk, CT) along with standards containing 5–50 μM Na as previously described (Murphy et al., 1983).

Analysis

Assuming that there is a single class of ouabain-binding sites and ouabain binds reversibly to the Na/K pump at a single binding site per Na/K pump, the fraction of sites bound (B/B_{max})

can be expressed as:

$$\frac{B}{B_{max}} = \frac{C_{ouab}}{C_{ouab} + K_{0.5}} \tag{2}$$

where B is the number of sites bound with ouabain, B_{max} is the maximum number of binding sites, C_{ouab} is the concentration of ouabain, and $K_{0.5}$ is the apparent affinity of the Na/K pump for ouabain and represents the concentration of ouabain, which binds 50% of the sites. If ouabain were binding to a receptor that had only a single state or conformation then this equation would be a complete description of its binding and $K_{0.5}$ would be a constant equivalent to the true affintity of the binding site for ouabain (see Discussion).

Assuming the block of the Na/K pump current (I_p) is directly proportional to the number of Na/K pump sites bound with ouabain, then the fraction of sites bound is equal to the fraction of I_p blocked (I_{p-o}) by each concentration of ouabain:

$$\frac{B}{B_{max}} = \frac{I_{p-o}}{I_{p-max}} = \frac{C_{ouab}}{C_{ouab} + K_{0.5}} \tag{3}$$

where I_{p-max} is I_p blocked by a maximal concentration of ouabain (1 mM). Similarly, assuming that: (a) the passive Na influx is constant, (b) Na_i is not significantly buffered, and (c) Na entering the cell equilibrates throughout the cytoplasm; then the block of the Na/K pump by ouabain should produce a proportional change in the initial rate of increase in a_{Na}^i ($\Delta[a_{Na}^i]$) and:

$$\frac{B}{B_{max}} = \frac{\Delta(a_{Na}^i)}{\Delta(a_{Na-max}^i)} = \frac{C_{ouab}}{C_{ouab} + K_{0.5}} \tag{4}$$

where $\Delta(a_{Na-max}^i)$ is the maximal initial rate of change in a_{Na}^i on exposure to 1 mM ouabain. $\Delta(a_{Na}^i)$ was estimated as the change in a_{Na}^i in 30 s of exposure to ouabain.

When specified in the text, $K_{0.5}$ was fit to the data using a nonlinear-regression computer program (Pennzyme) that uses Simplex and Fletcher-Powell algorithms to estimate values of fitted parameters that minimize the weighted error sum of squares (Schremmer et al., 1984). Statistical significance was evaluated using a paired t test. Data are expressed as mean ± SEM (n = number of individual determinations).

RESULTS

Concentration Response Curve: $K_{0.5}$ for Ouabain

Ouabain sensitivity of Na/K pump current. Measurement of the Na/K pump current (I_p) in voltage-clamped aggregates of cultured chick cardiac myocytes was described in the previous paper (Stimers et al., 1990). Briefly, in voltage-clamped aggregates bathed in control H/TBSS, inhibition of the Na/K pump, either by K-free H/TBSS perfusion or addition of 1 mM ouabain to the bath, caused the holding current (I_h) to become more negative, demonstrating the existence of a steady-state outward I_p. Ouabain sensitivity of I_p was determined by applying various concentrations of ouabain (10^{-8}–10^{-3}M) to an aggregate voltage clamped to -70 mV. Fig. 1 shows typical inward deflections of I_h obtained from a single preparation exposed to the indicated concentrations of ouabain. As expected from the well known concentration dependence of the association rate of ouabain, the kinetics of binding slowed as the concentration of ouabain decreased. Because of the slow association rate at low ouabain concentrations, in other experiments of this type, as

much as 4 min of exposure to ouabain was needed to allow I_h to reach a steady level. Furthermore, due to the slow kinetics and small signals at low concentrations of ouabain we did not attempt to determine if lower concentrations of ouabain might stimulate Na/K pump activity (for review see Lee, 1985).

The changes in membrane current at concentrations of ouabain >1 μM were fast and of sufficient magnitude to be clearly resolved from possible slow drifts in I_h. These current deflections were measured with respect to the value of I_h just before applying ouabain and referred to current changes measured from the same preparation exposed to 1 mM ouabain. Fig. 2 A summarizes the data obtained from all preparations in which currents were successfully obtained in the presence of 1 mM ouabain and at least one other concentration of ouabain. It is possible that a very slow association rate would result in a current change too slow to detect with this method and could result in a slight decrease in apparent affinity. For this reason, in
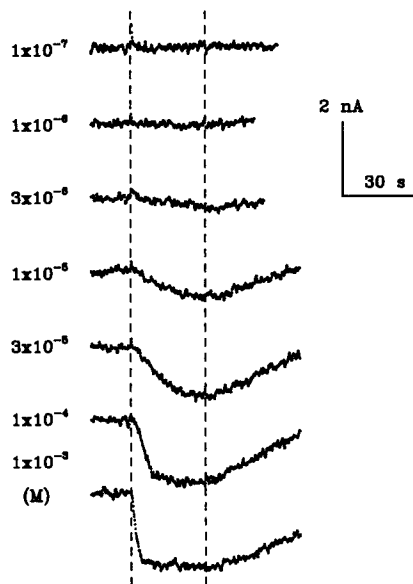

FIGURE 1.   Ouabain sensitivity of I_p. The current traces are taken from a continuous record of I_h from a single aggregate voltage clamped at −70 mV and bathed in H/TBSS + 1 mM Ba. During the time between the vertical dashed lines, ouabain was added to the bath (concentration of ouabain indicated next to each trace). Relative to the top trace, the time of each was 0, 5.5, 11, 17, 22.5, 29, and 39 min.

fitting the $K_{0.5}$ to these data (Eq. 3), the data points were weighted by a constant error of 10%, except for the value at 1 mM ouabain which has no error due to normalization. The best fit of Eq. 1 to the I_p data has a $K_{0.5}$ of 20.6 ± 1.2 μM (Fig. 2 A, *solid line*).

Na-SME estimate of ouabain binding. Using a similar protocol, the effect of ouabain on a_{Na}^i was measured with two intracellular microelectrodes, a Na-sensitive and a membrane potential reference electrode (not voltage clamp). Fig. 3 shows a typical set of records obtained for the change in a_{Na}^i in response to the indicated concentration of ouabain. The initial rate of change of a_{Na}^i was estimated as the change in a_{Na}^i in the first 30 s after ouabain application ($\Delta(a_{Na}^i)$). As with the pump current data, $\Delta(a_{Na}^i)$ was normalized by dividing by $\Delta(a_{Na-max}^i)$ measured in the same preparation. The results are summarized in Fig. 2 B. The best fit of Eq. 1 to the data gives a $K_{0.5}$ of 12.3 ± 1.7 μM.
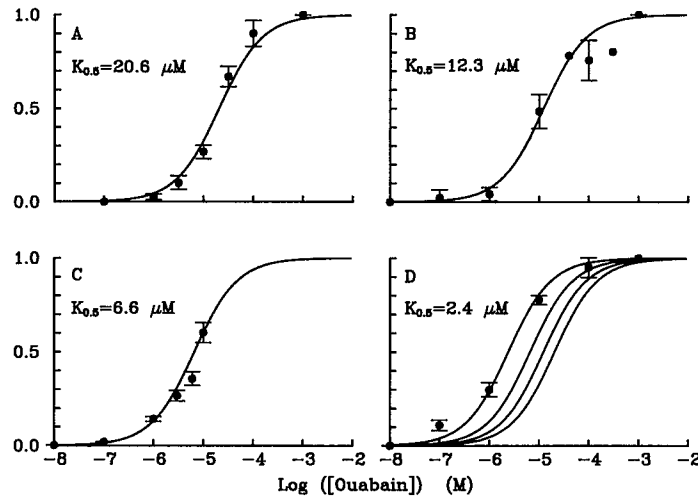
FIGURE 2. Dose-response curves for ouabain. Dose-response curves for ouabain were measured under control conditions in the presence of 5.4 mM K_o by voltage clamp (A), Na-SME (B), and equilibrium [^{3}H]ouabain binding (C), or in the presence of 3 μM monensin by voltage clamp (D). Data are plotted as mean ± SEM ($n = 3$–12), except in B, where the points at 40 and 300 μM ouabain are single observations. The best fit of Eq. 2 to the data is shown as the solid curve through the data. In D, the fitted curves in A–C are reproduced (three rightmost curves) for comparison with the data and fit to the monensin data.

Equilibrium [^{3}H]ouabain binding. Equilibrium [^{3}H]ouabain binding was measured in confluent mass cultures of cardiac myocytes exposed to concentrations of [^{3}H]ouabain between 10 nM and 10 μM (Lobaugh and Liebermen, 1987). Maximal specific binding (B_{max}) was previously determined to be 11.7 ± 0.6 pmol/mg protein in confluent mass cultures (Lobaugh and Lieberman, 1987). Specific binding in 5.4 mM K_o was normalized to this value (Fig. 2 C). The best fit of Eq. 1 to the data yields an estimate for the $K_{0.5}$ of 6.6 ± 0.4 μM.
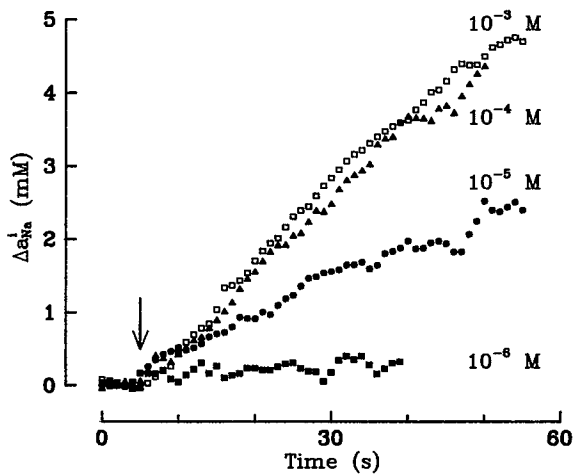

FIGURE 3. Ouabain sensitivity of $\Delta(a^i_{Na})$. a^i_{Na} was recorded from a polystrand preparation. At the arrow ouabain was added to the bath at the concentration indicated next to each trace in the range of 1 μM to 1 mM.

The value for $K_{0.5}$ determined by [^{3}H]ouabain binding is significantly ($P = 0.01$) different from that obtained by Na-SME experiments, which is significantly ($P = 0.01$) different from that obtained by voltage clamp. The discrepancy in the data might be explained by careful examination of the experimental conditions, namely the time of exposure to ouabain. In the equilibrium [^{3}H]ouabain-binding experiments, the preparations were exposed to ouabain for 30 min, whereas the voltage-clamp experiments used a 30-s exposure in most cases and up to 4 min at the very lowest concentrations of ouabain ($\leq 10^{-6}$ M). During these short exposures, Na_i could only increase a small amount (maximal $\Delta(a_{Na}^i) = 3$ mM/30 s; maximal rate of increase in Na_i calculated from resting $I_p = 4$ mM/30 s; Stimers et al., 1990); however, during the 30-min exposures to [^{3}H]ouabain, Na_i rose 2.5–3-fold (15–20 mM) at the higher concentrations of ouabain (Lobaugh and Lieberman, 1987). The Na-SME experimental data was based on a 30-s exposure to ouabain, but the absence of voltage clamp could also affect the results. Application of a high concentration of ouabain increased beating frequency, which would decrease the driving force for Na influx. Therefore the maximum $\Delta(a_{Na}^i)$ may be underestimated with increasing ouabain concentration, thus causing a lower estimate for the value of $K_{0.5}$. When the first 60 s of data were used to calculate $\Delta(a_{Na}^i)$, a $K_{0.5}$ of 9.2 ± 1.0 μM was also calculated. This finding suggests that the value of the apparent ouabain-binding affinity is reduced by elevated Na_i.

Increasing Na_i with Monensin

The effect of Na_i on the $K_{0.5}$ for ouabain was evaluated in preparations treated with monensin, a Na/H exchange ionophore (Pressman, 1976). We selected monensin because it would raise Na_i without having a direct effect on membrane conductance, which is important because of the limited current passing ability of the switching voltage clamp. Monensin, which has a Na/K selectivity ratio of 16 (Antonenko and Yaguzhinsky, 1988), was previously used successfully to raise Na_i in a variety of experimental situations (Mendoza et al., 1980; Temma and Akera, 1982; Hume and Uehara, 1986; Barcenas-Ruiz et al., 1987; Haber et al., 1987). As a Na/H exchange ionophore, monensin might affect the $K_{0.5}$ for ouabain via a change in intracellular pH. Preliminary experiments using a pH-SME have shown that monensin produces a transient alkalinization that recovers in 5–10 min, at which time the new steady-state pH is ~0.1 pH units alkaline compared with control (data not shown). This small change in pH induced by monensin probably has little effect on the results because the activity of Na/K ATPase isolated from cultured chick cardiac myocytes is relatively insensitive to pH in the range of 7.0–8.0 (Sperelakis and Lee, 1971).

The effective range of monensin concentrations was determined by measuring its effect on steady-state Na content in confluent mass cultures of cardiac myocytes (Fig. 4 A). Na content increased approximately linearly with the log of monensin concentration between 1 and 6 μM. The abrupt increase in Na content that occurred between 6 and 10 μM monensin suggests that the Na transport mechanisms are unable to cope with the excess Na influx induced by 10 μM monensin. Evidence that the Na/K pump is unable to fully compensate for the increased influx is seen when comparing the effect of ouabain on Na content in the presence or absence of monensin (Fig. 4 B). When confluent mass cultures were exposed to 1

μM ouabain for 10 min, the rise in Na$_i$ was greatly enhanced by the presence of monensin. Fig. 4 *B* shows that exposure of confluent mass cultures to 1 or 3 μM monensin for 20 or 30 min caused a rise in Na content above control levels (*filled circles*). Addition of 1 μM ouabain to the control solution caused no change in Na content in 10 min; however, a 20-min preincubation with 1 or 3 μM monensin followed by exposure to 1 μM ouabain led to an increase in Na content (*open circles*).

In polystrand preparations, exposure to 6 μM monensin for 6 min caused an increase in a^i_{Na} from 6 to ~23 mM, reaching a new steady level (Fig. 5 *A*). Monensin also hyperpolarized the membrane potential by 5–10 mV, presumably because of increased Na/K pump activity caused by elevating a^i_{Na}. In aggregates that were volt-
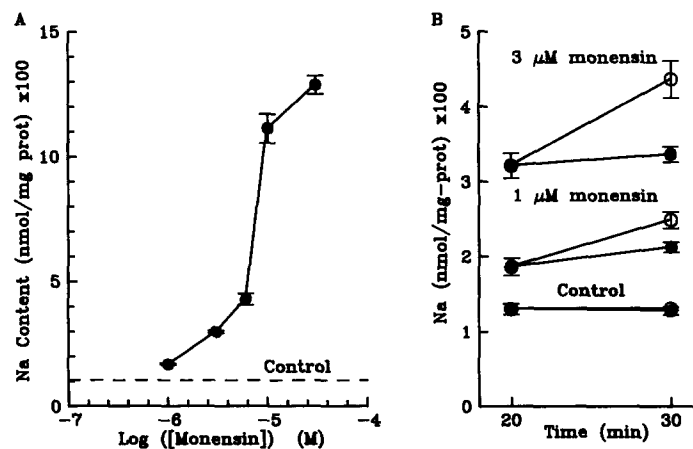

FIGURE 4. Monensin effects on Na content and reserve capacity. (*A*) The dose effect of monensin on Na content in mass cultures was determined and the data are plotted as mean ± SEM (*n* = 3). (*B*) Control values of Na content (*filled circles*) in mass cultures were determined after 20 and 30 min without monensin (*control*) or in the presence of 1 or 3 μM monensin. As shown in *A*, monensin causes a dose-dependent increase in Na content. After 20 min in control or monensin, 1 μM ouabain was added to the bathing solution and Na content was measured after 10 min (*open circles*). While control cultures (without monensin) showed no increase in Na content after 10 min in ouabain, the monensin-treated preparations, when exposed to ouabain, showed a marked increase in Na content above that found in cultures exposed to the same concentration of monensin alone. Data are plotted as mean ± SEM (*n* = 3–4).

age clamped at −70 mV for several minutes in the presence of 6 μM monensin, we observed the development of a sustained contracture. An explanation for this observation may be that as Na$_i$ increases, the reversal potential for Na/Ca exchange becomes more negative than the holding potential so that the exchanger reverses direction and brings sufficient Ca into the cell to cause a contracture. To counter this effect we used 3 μM monensin and a holding potential of −80 mV in the subsequent voltage-clamp experiments. In the presence of 3 μM monensin for 3–5 min, the membrane current reached a new steady level (Fig. 5 *B*) and no contracture was observed. In the presence of monensin the data suggest that Na$_i$ achieves a new
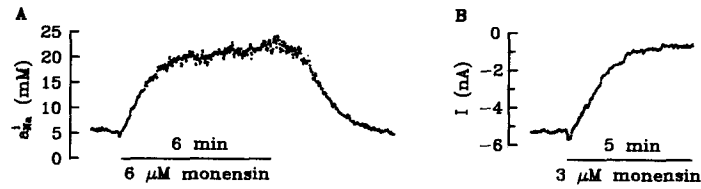
FIGURE 5.   Monensin effects on a^i_{Na} and I_h. (A) a^i_{Na} was recorded from a polystrand exposed to H/TBSS. During the 6-min interval indicated by the bar below the data, 6 μM monensin was added to the perfusing solution. Monensin caused a reversible increase in a^i_{Na} from ~5 to 22 mM. (B) I_h was recorded from an aggregate voltage clamped at −80 mV and exposed to H/TBSS. During the 5-min interval indicated by the bar below the data, 3 μM monensin was added to the bathing solution. An outward current of ~5 nA was generated on exposure to monensin.

steady-state level. The generation of an outward current is consistent with the hyperpolarization seen in the Na-SME experiments.

Effect of Na_i on Total $[^3H]Ouabain$ Binding

The effect of Na_i on total $[^3H]$ouabain binding was determined either by lowering extracellular Na (Na_o) or exposing confluent mass cultures to 1–10 μM monensin (Fig. 6). Equilibrium ouabain binding was measured using a subsaturating concentration of $[^3H]$ouabain (1 μM), so that Na_i-induced changes in apparent affinity would appear as relatively large changes in the amount of ouabain bound. In the presence of monensin and 1 μM $[^3H]$ouabain, total binding was relatively stable between 10 and 50 min, although apparent nonspecific binding (measured by the addition of 2 mM unlabeled ouabain) increased linearly over this same period of time, surpassing total binding in 30 min. This linear increase in apparent nonspecific $[^3H]$ouabain binding must be an artifact resulting from the combined presence of monensin and a high concentration of unlabeled ouabain (2 mM), as opposed to the low concentration of $[^3H]$ouabain (1 μM) present when total binding was measured. In the absence of monensin, nonspecific ouabain binding was 0.82 pmol/mg protein at 1 μM $[^3H]$ouabain and a linear function of $[^3H]$ouabain concentration
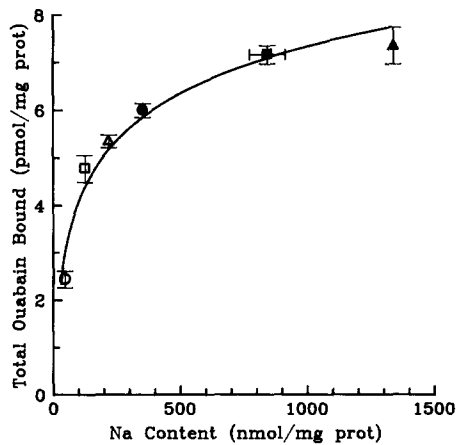

FIGURE 6.   Na content effects on total $[^3H]$ouabain binding. Mass cultures were exposed to 30 mM Na_o (*open circle*) or 143 mM Na_o plus 0 (*open square*), 1 (*open triangle*), 3 (*filled circle*), 6 (*filled square*), or 10 (*filled triangle*) μM monensin, which caused an increase in Na content and total $[^3H]$ouabain binding. Data are plotted as mean ± SEM (n = 3–4) for both Na content and $[^3H]$ouabain bound. Error bars not shown for Na content are within the symbols. The smooth curve through the data was drawn by eye.

between 0.1 and 10 μM (Lobaugh and Lieberman, 1987). Because of this apparent discrepancy between total binding and nonspecific binding, Fig. 6 shows total rather than specific [³H]ouabain binding. Total [³H]ouabain binding increased threefold as Na content increased between 50 and 800 nmol/mg protein. The saturating relationship between Na_i and total [³H]ouabain bound suggests that actual nonspecific binding is either constant (as it is in the absence of monensin) or also a saturating function of Na content. In either case, the shape of this curve would not be grossly altered by a correction for nonspecific binding; only the steepness and/or the magnitude of the change would be decreased. Since 1 μM [³H]ouabain is a nonsaturating concentration, this increase in [³H]ouabain bound probably reflects an increase in the apparent ouabain affinity of the Na/K pump.

Effect of Monensin on Ouabain-sensitive Current

The shift in $K_{0.5}$ induced by Na_i was determined by measuring the effect of ouabain on Na/K pump current in the presence of 3 μM monensin (Fig. 7). As expected,
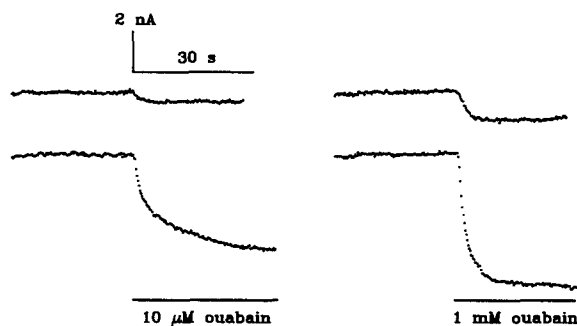

FIGURE 7. Monensin effects on I_p block by ouabain. These current traces are portions of a continuous record of I_h from an aggregate voltage clamped at -80 mV and bathed in H/TBSS. This figure shows the effect of addition of 10 μM (*left two traces*) and 1 mM ouabain (*right two traces*) in the absence (*upper traces*) or presence (*lower traces*) of 3 μM monensin. Using the upper-left trace as time 0, the upper-right, lower-right, and lower-left traces started at 5, 45, and 65 min, respectively.

monensin greatly increased the magnitude of the Na/K pump current. In a single aggregate two concentrations of ouabain were applied, with and without monensin, to verify that the shift in $K_{0.5}$ for ouabain was not the result of differences between cultures or the 10-mV increase in holding potential. The upper pair of traces in Fig. 7 shows the response of an aggregate to 10 μM and 1 mM ouabain without monensin. The ratio of the current changes in this aggregate shows that 30% of the Na/K pump current was blocked by 10 μM ouabain, which agrees with the data presented above (Fig. 2 A). However, in the presence of 3 μM monensin nearly 70% of the Na/K pump current was blocked by 10 μM ouabain (Fig. 7, lower pair of traces). In four experiments monensin increased the fraction of I_p blocked by 10 μM ouabain from 37.5 $\pm$ 1.4% to 77.8 $\pm$ 2.2%. Fig. 2 D summarizes the data for all ouabain concentrations used. The leftmost curve was calculated as the best fit of Eq. 2 to the data from preparations exposed to 3 μM monensin and normalized ($K_{0.5} = 2.4 \pm 0.2$

μM). The fitted curves in Fig. 2, A–C are reproduced in Fig. 2 D for comparison with the data obtained in monensin.

DISCUSSION

Evaluation of Techniques Used to Measure $K_{0.5}$

Under "control" conditions, the measured apparent ouabain affinity of the Na/K pump differs by a factor of 3–4 when measured either by equilibrium [³H]ouabain binding, changes in a_{Na}^i measured by a Na-SME, or changes in I_p measured by voltage clamp. First, consider the differences between the three techniques used to measure the $K_{0.5}$ of the Na/K pump for ouabain.

Equilibrium [³H]ouabain binding is a direct measure of ouabain interaction with the individual Na/K pump molecules and so theoretically represents the best technique for measuring this interaction. In practice however, the results must be carefully interpreted because nonspecific binding of ouabain becomes relatively large at ouabain concentrations greater than the $K_{0.5}$, and because maintenance of steady-state ion balance for long periods of time (30 min) is difficult at high concentrations of ouabain due to interactions with other Na-dependent transport mechanisms. However, the sensitivity of the equilibrium [³H]ouabain-binding technique at low concentrations of ouabain provided us with the opportunity to evaluate the possible existence of an ouabain-binding site with a higher affinity such as that reported in mammalian cardiac preparations (Wellsmith and Lindenmayer, 1980; Brown and Erdmann, 1983; Werdan et al., 1984b). Contrary to these findings, but in agreement with other studies on cultured chick cardiac myocytes, no evidence was found for a second higher affinity binding site (Kim et al., 1984; Werdan et al., 1984a; Lobaugh and Lieberman, 1987).

The Na-SME technique is sensitive to small changes in a_{Na}^i and has the added advantage that the measurements can be made rapidly (seconds). Another advantage is that changes in a_{Na}^i after application of ouabain are a direct consequence of changes in Na/K pump activity, and so is not sensitive to nonspecific ouabain binding. Assuming that (a) steady-state Na/K pump activity balances the resting Na influx with an equal and opposite Na efflux, (b) Na influx is constant during the measurement, and (c) ouabain only inhibits the Na/K pump, then the $\Delta(a_{Na}^i)$ immediately following Na/K pump blockade would be directly proportional to the number of Na/K pump sites blocked. As noted in the Results, application of ouabain can increase beating frequency, resulting in the depolarization of the average membrane potential in cardiac myocytes and a reduction in the passive Na influx. In Purkinje fibers, it has been shown that the decrease in resting a_{Na}^i on depolarization is most likely due to the reduction in driving force (Eisner et al., 1981). Though the effect is small, it could account for the difference between the Na-SME estimate of $K_{0.5}$ and that obtained from voltage-clamp experiments.

Assuming that the coupling ratio remains constant, the voltage-clamp technique can be used to measure the $K_{0.5}$ for ouabain directly because the method measures the transmembrane current, I_p, that is generated by Na/K pump activity. Like the Na-SME, the measurement is rapid and insensitive to nonspecific ouabain binding. A potential problem with this technique is that it is first necessary to establish that

the measured current is due to Na/K pump activity. In the preceding paper we showed that the measured current has the properties expected for the Na/K pump and is quantitatively accurate in measuring several parameters of the Na/K pump (Stimers et al., 1990). Another possible error in the I_p measurements is the separation of relatively small changes in I_h on exposure to low concentrations of ouabain from random fluctuations of I_h. Small changes in I_h can occur for many different reasons (such as changes in the resistance of the leak pathway around the electrode) and can then complicate the interpretation of results from experiments at low concentrations of ouabain. This problem was minimized by using concentrations of ouabain >1 μM to analyze the $K_{0.5}$ and by requiring that I_h return to the control level after the removal of ouabain. At higher concentrations of ouabain little interference of baseline drift was encountered because the signal was quite large and rapid. The potential contribution of drift was further reduced by averaging results from several different preparations. This method has been shown to be effective in quantifying cardiac glycoside interaction with the Na/K pump (Daut and Rudel, 1982; Cohen et al., 1987). However, these studies used dihydroouabain to analyze Na/K pump activity in adult cardiac preparations of guinea pig ventricular muscle (Daut and Rudel, 1982) and canine Purkinje myocytes (Cohen et al., 1987) so that a direct comparison of the results with those of the present study is not possible. Nevertheless, qualitatively similar results were obtained in each case.

Na$_i$ and the Apparent Ouabain-binding Affinity

In cultured chick cardiac myocytes, monensin caused an increase in Na$_i$ in a concentration-dependent manner. In frog atrial cells, 30 μM monensin was used to elevate Na$_i$ to ~20 mM (Hume and Uehara, 1986). In guinea pig heart, Na$_i$ was elevated to unspecified levels using either 10 μM (Brody et al., 1984) or 30 μM monensin (Barcenas-Ruiz et al., 1987). In contrast with these studies, we found that concentrations of monensin >6 μM result in an increase in Na content that, when converted to concentration, approached extracellular levels. This suggests that the Na-transporting mechanisms of the cardiac myocyte are unable to cope with the increased Na influx. The higher concentrations of monensin used by others may reflect species differences; however, since no other group reported on the concentration dependence of monensin, the explanation of the dose difference is unresolved.

In voltage-clamp experiments we have shown that the monensin-induced elevation in Na$_i$ decreased the value of $K_{0.5}$ for ouabain by an order of magnitude (Fig. 2 D). Previous studies have suggested that elevation of Na$_i$ can decrease the value of $K_{0.5}$ for ouabain. Brody and his colleagues (for review, see Brody et al., 1984) attempted to correlate interventions that enhance Na influx with inotropic and toxic effects of ouabain and with the rate of ouabain binding. Based on observations that stimulation at high frequencies, as well as the presence of monensin, batrachotoxin, or grayanotoxin I, all enhance the rate of ouabain binding to the Na/K pump, Brody et al. (1984) concluded that the rate of ouabain binding may be increased by either the rate of Na influx or the Na$_i$ availability to the Na/K pump. Bentfeld et al. (1977) showed that increasing the rate of stimulation of guinea pig atria from 1 to 5 Hz in the presence of 0.4 μM ouabain increased the rate of ouabain binding and augmented the inotropic response, with toxic effects being observed at the higher

stimulation frequencies. Ebner and Siegl (1986) also reported that ouabain binding increased with stimulation frequency but concluded that the parallel increase in inotropy was due to Na_i saturation of the Na/K pump rather than the increased binding. These results are consistent with our finding that ouabain binding is increased at elevated Na_i. Recent experiments using Na-SME to directly measure a_{Na}^i, under various conditions, have shown that the inotropic effects of ouabain are mediated by an increase in a_{Na}^i (for review, see Lee, 1985).

The enhancement of ouabain binding by Na has long since been established in isolated Na/K ATPase preparations (Matsui and Schwartz, 1968). In a recent review, Hansen (1984) discussed the molecular basis for the Na enhancement of ouabain binding. Ouabain has been shown to bind with high affinity to the conformation of the Na/K ATPase that is favored by Mg, ATP, and Na. In an intact preparation these substrates most probably bind to the inside of the membrane to produce the high affinity ouabain binding conformation. If this is the case then increasing Na_i should increase apparent ouabain affinity.

Herzig et al. (1985) presented a model for guinea pig atria that could account for the increase in apparent ouabain affinity as Na_i increases. They presented the concept of a modified binding site density in which Na_i increases the probability that a given Na/K pump is in the conformation to bind ouabain with high affinity. This model has also been used to explain later work in guinea pig ventricle (Herzig et al., 1988) and isolated guinea pig myocytes (Stemmer and Akera, 1988). We have tested this concept on our voltage-clamp data using the state model of Chapman et al. (1983) modified to include high affinity ouabain binding to state E_2-P (Cantley, 1981; Hootman and Ernst, 1988) as follows:
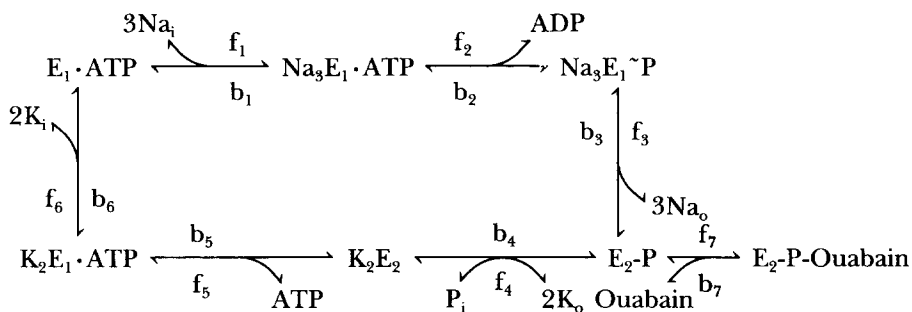
The parameter values of the model were identical to those of Chapman et al. (1983) except that ion concentrations (Liu et al., 1987) and pump site density (total[ATPase]) (Lobaugh and Lieberman, 1987) were changed to values measured in the chick heart (Table I). The dissociation rate constant (b_7) was set equal to that measured kinetically and the association rate constant (f_7) was calculated to give the measured ouabain affinity in K-free solution, 0.5 μM (Lobaugh and Lieberman, 1987). The model was implemented using SCoP and SCoP-Fit (National Biomedical Simulation Resource Center, Duke University Medical Center, Durham, NC). In fitting the model parameters to the data, the only free parameter was Na_i. We found that for the four values of $K_{0.5}$ measured in this report, 20.6, 12.3, 6.6, and 2.4 μM, the model predicts that Na_i should be 7.1, 9.1, 12.4, and 25.4 mM, respectively. For the case of the Na-SME experiments ($K_{0.5}$ = 12.3 μM, predicted Na_i = 9.1 mM),

measured Na_i was 8.4 mM (Liu et al., 1987). In equilibrium [^{3}H]ouabain-binding experiments, calculations from Na content measurements show that Na_i increases from ~10 mM without ouabain to 26 mM in 10 μM ouabain (Lobaugh and Lieberman, 1987). This range spans the predicted value of 12.4 mM and may explain the relatively poor fit of Eq. 2 to the binding data (Fig. 2 C). While we have not measured Na_i in a voltage-clamped preparation, the predicted values of Na_i (control = 7.1 mM, monensin treated = 25.4 mM) are reasonable considering the values of Na_i measured in non–voltage-clamped polystrand preparations without (8.4 mM, Liu et al., 1987) and with (30 mM, Fig. 5 A, activity coefficient = 0.74) 6 μM monensin. Note that even though the $K_{0.5}$ is calculated from a complex multistate model, the binding curves appear as first order, being superimposable on the curves in Fig.

TABLE I

Parameter Values

Parameter	Value	Units
Total[ATPase]	1.66×10^{-13}	mol/cm^2
Na_i	8.4	mM
Na_o	144	mM
K_i	140	mM
K_o	5.4	mM
[ATP]	4.99	mM
[P_i]	4.95	mM
[ADP]	0.06	mM
Temperature	310	K
f_1	2.5×10^{11}	$/M^3/s$
b_1	10^5	/s
f_2	10^4	/s
b_2	10^5	/M/s
f_3	172	/s
b_3	1.72×10^4	$/M^3/s$
f_4	1.5×10^7	$/M^2/s$
b_4	2×10^5	/M/s
f_5	2×10^6	/M/s
b_5	30	/s
f_6	1.15×10^4	/s
b_6	6×10^8	$/M^2/s$
f_7	1.9×10^4	/M/s
b_7	9.5×10^{-3}	/s

2. The model also predicts that as Na_i increases there is an increase in turnover rate and the distribution of Na/K pump sites among the states changes from being primarily in the state $K_2E_2 \cdot ATP$ to being split between K_2E_2 and $Na_3E_1 \sim P$. The high levels of K_o and Na_o prevent accumulation in state E_2-P; however, the presence of ouabain shifts the equilibrium to the bound state. Using monensin to increase Na_i and consequently the magnitude of the Na/K pump current more than threefold (Fig. 7), the Na/K pump spends a larger fraction of its time in the high affinity conformation, increasing the apparent ouabain-binding affinity (decreasing the value of $K_{0.5}$). Thus, our data support the modified binding site density hypothesis of Herzig et al. (1985) and show that increasing Na_i also increases the turnover rate and apparent affinity.

Saturation of the Na/K Pump by Na$_i$

In addition to increasing apparent ouabain affinity, increasing Na$_i$ stimulates Na/K pump activity, approaching saturation of the Na/K pump by Na$_i$, i.e., reducing the reserve capacity of the Na/K pump. The reduced reserve capacity lowers the amount of Na/K pump inhibition necessary to produce toxic effects (Brody et al., 1984). Using quiescent guinea pig atria, Brody et al. (1984) reported that 10 μM monensin enhanced ouabain binding; they attributed this finding to a monensin-stimulated increase in Na influx. They also showed that increasing concentrations of monensin (1–10 μM) decreased the ouabain concentration needed to induce arrhythmias. They suggested that the monensin-induced increase in Na influx decreases the reserve capacity of the Na/K pump, rendering cardiac muscle more susceptible to cardiac glycosides, i.e., under these conditions cardiac muscle is less able to maintain its ion balance. These results, though determined under conditions very different from those of the present study, are consistent with our Na-content (Fig. 4 B) and ouabain-binding (Fig. 6) data. In addition, the voltage-clamp experiments show that monensin increases the maximal ouabain-sensitive current magnitude (Fig. 7), which would be equivalent to a reduced reserve capacity of the Na/K pump. Na activity and contractile force measurements have shown that the magnitude of increase in both parameters produced by a given concentration of cardiac glycoside is augmented by raising the initial a^i_{Na} (for review, see Lee, 1985). These findings support the hypothesis that the reserve capacity of the Na/K pump can make cardiac muscle more resistant to ouabain toxicity.

In summary, the variability in the measurement of the $K_{0.5}$ of the Na/K pump for ouabain reported here and present in the literature can be explained by environmental conditions (e.g., Na$_i$, K$_o$, ATP, etc.) that influence the distribution of the Na/K pumps among the various conformations in the steady state. Thus, even though there is a single high affinity ouabain-binding site with a constant K_D, the apparent affinity is variable because it depends on the transitional rate constants between all of the states of the Na/K pump, some of which include the concentrations of the various substrates. We have shown that elevating Na$_i$ can increase apparent ouabain-binding affinity by at least an order of magnitude in the presence of normal extracellular K (5.4 mM). These results should help to explain the discrepancies in the literature about the inotropic and toxic effects of ouabain.

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