Apparent Affinity of the Na/K Pump for Ouabain in Cultured Chick Cardiac Myocytes

Effects of Na_i and K_o

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ABSTRACT The measured apparent affinity ($K_{0.5}$) of the Na/K pump for ouabain has been reported to vary over a wide range. In a previous report we found that changing Na_i could alter apparent affinity by at least an order of magnitude and that the model presented predicted this variability. To increase our understanding of this variability, isolated cells or two- to three-cell clusters of cardiac myocytes from 11-d embryonic chick were used to measure the effects of Na_i and K_o on the $K_{0.5}$ of the Na/K pump for ouabain. Myocytes were whole-cell patch clamped and Na/K pump current (I_p) was measured in preparations exposed to a Ca-free modified Hank's solution (HBSS) that contained 1 mM Ba, 10 mM Cs, and 0.1 mM Cd. Under these conditions there are no K_o-sensitive currents other than I_p because removal of K_o in the presence of ouabain had no effect on the current–voltage (*I-V*) relation. The *I-V* relation for I_p showed that in the presence of 5.4 mM K_o and 51 mM Na_i, I_p has a slight voltage dependence, decreasing ~30% from 0 to −130 mV. Increasing Na_i in the patch pipette from 6 to 51 mM (K_o = 5.4 mM) caused I_p to increase from 0.46 ± 0.07 (n = 5) to 1.34 ± 0.08 μA/cm² (n = 13) with a $K_{0.5}$ for Na_i of 17.4 mM and decreased the $K_{0.5}$ for ouabain from 18.5 ± 1.8 (n = 4) to 3.1 ± 0.4 μM (n = 3). Similarly, varying K_o between 0.3 and 10.8 mM (Na_i = 24 mM) increased I_p from 0.13 ± 0.01 (n = 5) to 0.90 ± 0.05 μA/cm² (n = 5) with a $K_{0.5}$ for K_o of 1.94 mM and increased $K_{0.5}$ for ouabain from 0.56 ± 0.14 (n = 3–6) to 10.0 ± 1.1 μM (n = 6). All of these changes are predicted by the model presented. A qualitative explanation of these results is that Na_i and K_o interact with the Na/K pump to shift the steady-state distribution of the Na/K pump molecules among the

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kinetic states. This shift in state distribution alters the probability that the Na/K pump will be in the conformation that binds ouabain with high affinity, thus altering the apparent affinity. In intact cells, the measured apparent affinity represents a combination of all the rate constants in the model and does not equate to simple first-order binding kinetics. The results show that the apparent affinity for ouabain is variable (0.5–20 μM) and depends on Na_i and K_o. Although this range is large, the data can be completely accounted for by the model presented.

INTRODUCTION

Reported values for the apparent affinity ($K_{0.5}$) of ouabain binding to the Na/K pump in cardiac muscle preparations vary widely. Values of $K_{0.5}$ in isolated cardiac myocytes range from 0.13 to 65 μM in guinea pig, 0.15 to 20.6 μM in embryonic chick, and 0.032 to 0.089 μM for the high affinity and 2.3 to 7.1 μM for the low affinity site in rat (for review, see Heller, 1987). Recent data in isolated guinea pig ventricular myocytes suggest that dihydroouabain binds to two sites ($K_{0.5}$ = 0.05 and 64.5 μM; Mogul et al., 1989*a*). It is well known that the external K concentration (K_o) inhibits ouabain binding; therefore, at least part of the variability in the reported values of ouabain affinity is due to K_o. Physiological levels of K_o can increase the value of the $K_{0.5}$ for ouabain by at least an order of magnitude (Lobaugh and Lieberman, 1987). This effect has been attributed to a competitive or antagonistic interaction between K_o and ouabain, or to K_o and ouabain competing for the same conformation of the Na/K pump (for review, see Schwartz et al., 1975).

Other factors have also been proposed to influence the $K_{0.5}$ for ouabain. Brody et al. (1984) reported that increasing the Na influx rate and beating frequency caused an increase in the amount of Na load for the pump and thus enhanced glycoside binding. Herzig et al. (1985) suggested that these effects can be explained by an increase in the turnover rate of the Na/K pump making the high affinity binding site for ouabain available more frequently, thus lowering the value of the $K_{0.5}$. This effect was described as a "positive cooperativity" for ouabain binding. Stemmer and Akera (1988) also supported this concept of positive cooperativity and stated that it is a novel type of cooperativity. In their paper they were able to simulate the alteration in binding affinity as the sum of two separate affinities with the lower affinity (larger value of $K_{0.5}$) dominating at low glycoside concentrations and the higher affinity dominating at high glycoside concentrations. In a previous study we presented evidence that increasing Na_i lowers the value of the apparent affinity ($K_{0.5}$) of the Na/K pump for ouabain (Stimers et al., 1990*c*). These data were explained by a cyclic model which predicts that changes in the distribution of pumps among the various states of the model of the Na/K pump would result in >10-fold changes in apparent affinity.

In this study we test the predictions of this model and present new evidence on the effect Na_i and K_o have on the apparent affinity of the Na/K pump for ouabain using combined switching voltage clamp and Na-selective microelectrode (Na-SME) techniques as well as whole-cell patch-clamp methods. We provide further evidence to support the idea that variations in the apparent ouabain affinity produced by the ionic environment are caused by a shift in the steady-state distribution of the Na/K pump molecules among the conformational states. Experimentally we have verified

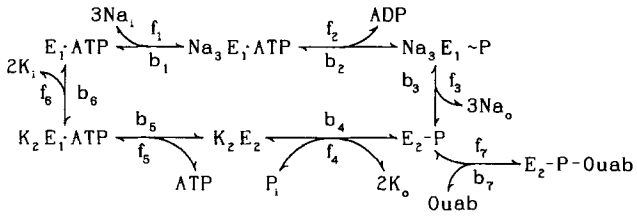
our previous predictions about the Na_i dependence of ouabain binding and extended those observations over a wider range. We have also combined new data in this paper with previously published results on the K_o dependence of ouabain binding.

Preliminary reports of some of these results have appeared (Stimers et al., 1989, 1990*a*).

METHODS

Model

The interpretation of measured $K_{0.5}$ values is model dependent. A Michaelis-Menten type of model would require that the $K_{0.5}$ for ouabain be a constant value regardless of the ionic environment. The model that we have previously used to interpret our results (Stimers et al., 1990*c*; see also Chapman et al., 1983) is shown below:



Briefly, the model represents the Na/K pump as a cyclic sequence of reversible reactions between six conformational states. Ouabain binds with high affinity to one of these states (E_2-P, the state following Na release to the extracellular solution and preceding K_o binding), generating a seventh state. Values for the parameters of the model are given in Table I. This model makes a very different prediction about the dependence of $K_{0.5}$ on the ionic environment. Fig. 1 shows the prediction this model makes about the effect of Na_i on the apparent affinity of the Na/K pump for ouabain. Since this is an experimentally testable prediction from a model that was developed to explain other properties of the Na/K pump current (Chapman et al., 1983), we decided to further test this prediction by experimentally manipulating Na_i. Other predictions of this model are discussed below.

Simulations and parameter fits of the model were made using SCoP and SCoPFit, respectively (National Biomedical Simulation Resource Center, Duke University Medical Center, Durham, NC). In the fittings only f_1, b_1, f_4, and b_4 were allowed to vary from the values previously published (Chapman et al., 1983; Stimers et al., 1990*c*). The steady-state solution of the simultaneous differential equations describing this model was obtained by the method of Gaussian elimination and back substitution into the matrix of coefficients. SCoPFit uses the "principal axis" method to systematically search the parameter space for values that minimize the sum of the squares of the residuals (least-squares method).

Tissue Culture

11-d-old chick embryo hearts were disaggregated by a series of incubations in 0.05% (wt/vol) trypsin (GIBCO Laboratories, Grand Island, NY) as previously described (Jacob et al., 1987). The resultant cell suspension was incubated at 1×10^6 cells/3 ml of culture medium (60% medium 199, 5% fetal bovine serum, and 2% chick embryo extract with a base of Earle's solution) in 60-mm tissue culture dishes (Falcon 3002; Becton Dickinson Co., Oxnard, CA). After 3–4 d in culture, spontaneously contracting (1–2 Hz) cardiac cell aggregates formed and

TABLE I

Parameter Values for the Na/K Pump Model

Parameter	Old values	Fitted values	Units
Total[ATPase]	0.166		pmol/cm^2
Na_i	8.4		mM
Na_o	145		mM
K_i	140		mM
K_o	5.4		mM
[ATP]	1.9		mM
[P_i]	0.1		mM
[ADP]	0.1		mM
Temperature	310		°K
f_1	250	320	/mM^3/s
b_1	10^5	54×10^3	/s
f_2	10^4		/s
b_2	100		/mM^3/s
f_3	172		/s
b_3	1.72×10^{-5}		/mM^3/s
f_4	15	23	/mM^2/s
b_4	200	20	/mM/s
f_5	2,000		/mM/s
b_5	30		/s
f_6	1.15×10^4		/2
b_6	600		/mM^2/s
f_7	1.9×10^{-2}		/μM/s
b_7	9.5×10^{-3}		/s

attached in 50–75-μm-diam holes that were made in a thin coat of 1% agar on the bottom of the culture dishes (Ebihara et al., 1980). Aggregates were used on days 3–10 in culture for experiments that involved simultaneous use of switching voltage clamp (SVC) and Na-SME techniques. For the whole-cell patch-clamp technique, myocyte suspensions were cultured in untreated 60-mm petri dishes (Falcon 1007; Becton Dickinson Co.) for 24–36 h. Adherent individual cells or two- to three-cell clusters of contracting myocytes were then whole-cell patch clamped.

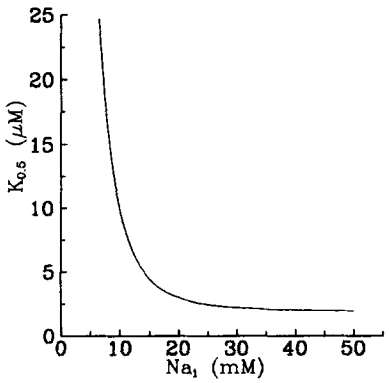


FIGURE 1. Simulation of $K_{0.5}$ versus Na_i. From the model presented, $K_{0.5}$ for ouabain was calculated as a function of Na_i and the results are plotted here. This illustrates that the model predicts that $K_{0.5}$ should increase dramatically as Na_i decreases and that the apparent affinity for ouabain is very sensitive to Na_i near resting levels of Na_i.

Solutions

Control perfusate was a modified Hanks solution (H/TBSS) with the following composition (in mM): 144 Na, 5.4 K, 0.8 Mg, 1 Ca, 153 Cl, 5.6 HEPES, 4.2 Trizma base, and 5.6 dextrose (pH = 7.4). In some experiments the HEPES/TRIS buffer was replaced with 10 mM HEPES (HBSS). Altered K_o solutions were made by equimolar substitution with Na. Ba, Cs, Cd, and ouabain were added to solutions as indicated in the Results. Ca-free HBSS with 1 mM Ba, 10 mM Cs, and 0.1 mM Cd added was used in these experiments to reduce the current through inward rectifier, pacemaker, Ca, and delayed rectifier channels, and to stabilize the voltage clamp by linearizing and flattening the current–voltage (*I-V*) relationship (Fig. 2). Monensin was dissolved in ethanol to make a 4- or 6-mM stock solution that was diluted into the final solution at a concentration of 3 μM (ethanol concentration ≤0.1%). This concentration of ethanol added to control solution had no effect on holding current (I_h) in control experiments. All experiments were done at 37°C.

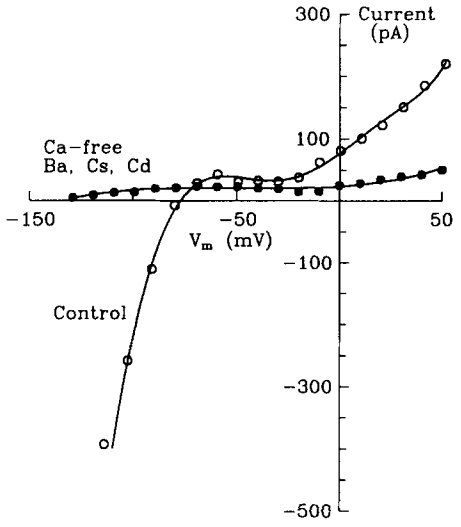


FIGURE 2. Effect of Ca-free, Ba, Cs, and Cd on the *I-V* relation of cardiac myocytes. This graph shows that the standard solution used to isolate I_p greatly reduces other membrane currents present in control HBSS (*open circles*). The pipette solution contained 51 mM Na. Superfusion of this preparation with Ca-free HBSS with 1 mM Ba, 10 mM Cs, and 0.1 mM Cd (*filled circles*) linearized the *I-V* relation by a substantial block of the inward rectifier, delayed rectifier, pacemaker, and Ca currents. The data are plotted as the current present 300 ms after a pulse from the holding potential of −70 mV to the indicated potential. Membrane capacitance was 31 pF.

Switching Voltage-Clamp Technique

As previously described (Stimers et al., 1990*b*), a single microelectrode SVC amplifier (model 8100; Dagan Corp., Minneapolis, MN) was used to perform the voltage clamp in combined voltage clamp and Na-SME experiments. Briefly, low resistance microelectrodes (<10 MΩ) made from Omega dot borosilicate glass (1.0 mm o.d. × 0.75 mm i.d.) were used to impale cardiac myocytes. Since a single electrode is used to pass current and measure membrane potential, a low resistance was necessary to provide an adequate frequency response and sufficient current to the membrane. A switching frequency of 1 kHz was used in all experiments. The experiments were done at a holding potential of −80 mV for comparison with our previous results (Stimers et al., 1990*c*). Both membrane potential and current were filtered at 300 Hz and continuously recorded on videotape using a digital acquisition and storage system (Unitrade Inc., Philadelphia, PA). Portions of the data were later transferred to

an IBM PC/AT microcomputer using an Axolab acquisition system (Axon Instruments, Inc., Burlingame, CA) for analysis.

Na-selective Microelectrode Technique

The method used for measuring intracellular Na activity (a^i_{Na}) with a Na-SME was recently described (Liu et al., 1987). The method was modified in these studies to use a concentric double-barrel microelectrode. Briefly, a 10-μl drop of liquid neutral carrier (Na-cocktail; Fluka Chemical, Hauppauge, NY) was back-filled into the inner barrel of a clean, silanized micropipette, followed by internal filling with 0.1 M NaCl. The outer reference barrel was used as a conventional 3 M KCl-filled microelectrode. Simultaneous measurement of the intracellular potential from a Na-SME and a conventional 3 M KCl-filled microelectrode allows the calculation of a^i_{Na} using the following equation:

$$a^i_{Na} = 10^{(V_d - E_o)/S} - K_{NaK}(a^i_K) \quad (1)$$

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} is the potentiometric selectivity coefficient of the Na-SME for K relative to Na, determined in 100 mM NaCl and 100 mM KCl (acceptable values were <0.02) and a^i_K is the intracellular K activity which is 103 ± 1.3 mM (n = 57) (Liu et al., 1987). The values obtained for each electrode for K_{NaK} are compatible with the value of 0.023 ± 0.002 (n = 21) obtained from biionic (Na and K) calibration solutions (Liu et al., 1987). Correction for intracellular Ca activity (a^i_{Ca}) was not done since the selectivity coefficient for Ca relative to Na (K_{NaCa}) is 1.0 ± 0.1 (n = 21). Assuming a^i_{Ca} is 100 nM, contamination of the measurement of a^i_{Na} would be ~0.3 mM. The presence of Ba, Cs, Cd, and monensin did not interfere with the Na-SME response. The maximum diastolic membrane potential and a^i_{Na} measured by concentric double-barreled Na-SME were −73.9 ± 0.6 mV and 7.4 ± 0.3 mM (n = 7), respectively. These values are comparable to those previously measured with single-barreled Na-SME and a separate reference electrode (Liu et al., 1987).

Whole-Cell Patch-Clamp Technique

Isolated cells or two- to three-cell clusters of cardiac myocytes attached to petri dishes were whole-cell patch clamped (WCPC) by conventional techniques (Hamill et al., 1981) using a Dagan 9600 patch-clamp amplifier (Dagan Corp.). Fire polished borosilicate glass (7052; Corning Glass Works, Corning, NY) patch electrodes with tip diameters of 1–2 μM were filled with an "intracellular" solution containing (in mM): 150 K, 14 Na, 2 Mg, 0.1 Ca, 120 aspartate (ASP), 40 Cl, 1.1 EGTA, 2 ATP, and 10 HEPES (pH 7.2). Na_i was varied by replacing K_i. Seal resistances were in the range of 3–20 GΩ. Both capacity compensation and series resistance compensation were adjusted to reduce associated artifacts. The continuously recorded current was low-pass filtered at 300–3,000 Hz (AP Circuit Corp., New York, NY) and stored on videotape for later analysis, and in some cases was also directly acquired by a PC/AT computer using the Axolab acquisition system. Currents are shown using the standard convention of inward current being negative. To normalize measured membrane currents to membrane area, capacity current transients in response to a 10-mV hyperpolarizing pulse were recorded with high time resolution (20–50 μs/point). These transients were integrated to give total membrane capacitance. Capacitance was converted to membrane area by dividing by the specific membrane capacitance of 1.3 μF/cm^2 (Mathias et al., 1981).

Using protocols similar to those previously described for the switching voltage clamp (Stimers et al., 1990*c*), we have isolated a current using the WCPC technique identifiable as Na/K pump current (I_p), and show that this current, as expected, is sensitive to K_o, Na_i, and ouabain.

Furthermore, if ATP or Na is omitted from the pipette solution, I_p is completely inhibited. For simplicity of presentation the current will be referred to as I_p throughout the paper. We believe that this is justified due to its similarity to I_p measured previously (Stimers et al., 1990*b, c*). I_p is defined as the change in holding current induced by application of ouabain. In all cases, before measuring ouabain-sensitive current, a stable baseline holding current was obtained and reversibility was tested by allowing the holding current to recover when ouabain was washed out. An example of the protocol used is shown in Fig. 3. This figure shows a stable holding current for >20 min and four separate applications of ouabain illustrating both the reversibility and reproducibility of the measurements.

To measure the voltage dependence of Na/K pump current (I_p), pulses from the holding potential (−70 mV) to potentials between −150 and +50 mV were applied for 300 ms in both control (Ca-free HBSS + 1 mM Ba + 10 mM Cs + 0.1 mM Cd) and ouabain-containing solutions. I_p was determined by subtracting the current measured at the end of the pulse in ouabain-containing solution from the corresponding current measured in control solution.

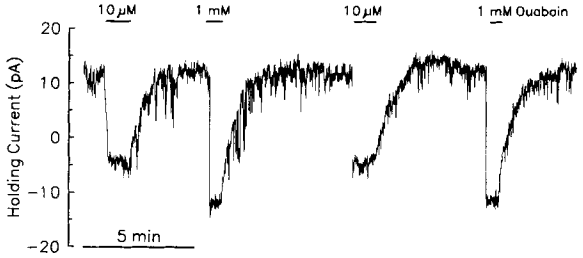


FIGURE 3. Ouabain-sensitive current. A continuous record of holding current from a single cardiac myocyte is illustrated. The cell was bathed in Ca-free HBSS with 1 mM Ba, 10 mM Cs, and 0.1 mM Cd. Pipette solution contained 51 mM Na. This record shows the reversibility and repeatability in measuring the ouabain-sensitive current. The bars above the record indicate when ouabain was applied and the concentration used.

Analysis

We have previously demonstrated under a wide range of ionic conditions that the dose–response curve for ouabain is well described by the Michaelis-Menten relation (Stimers et al., 1990*c*). In those experiments voltage-clamped aggregates of cardiac myocytes were briefly exposed to concentrations of ouabain between 0.1 μM and 1 mM. These data were normalized and parameters for maximum I_p ($I_{p\text{-}max}$) and $K_{0.5}$ were fit to the data by nonlinear regression. In all cases the data were well fit by a unimolecular reaction of ouabain to a single binding site. Given this fact, the $K_{0.5}$ for ouabain was estimated in the WCPC experiments by assuming that the fraction of I_p inhibited by a given dose of ouabain ($I_{p\text{-}o}$) followed the Michaelis-Menten relation. From this relation it is possible to calculate $K_{0.5}$ by measuring in a single preparation $I_{p\text{-}max}$ and $I_{p\text{-}o}$:

$$K_{0.5} = \frac{C_{ouab}(I_{p\text{-}max} - I_{p\text{-}o})}{I_{p\text{-}o}} \tag{2}$$

where C_{ouab} is the ouabain concentration. This method has the advantage that $I_{p\text{-}max}$ and $I_{p\text{-}o}$ are measured in each preparation independently and so each preparation is its own control for experimental conditions. Whenever possible, multiple determinations of $I_{p\text{-}max}$ and $I_{p\text{-}o}$ were

made in each cell and the results were averaged. The greatest sensitivity in these experiments is obtained when the dose of ouabain used is as close as possible to the actual $K_{0.5}$. Our choice of 10 μM ouabain resulted in all the measurements being within a factor of 2–3 of the calculated $K_{0.5}$. Data are pooled between preparations and are expressed as mean ± SEM.

RESULTS

a^i_{Na} under Voltage Clamp

Previously, using the SVC technique, we reported that the $K_{0.5}$ for ouabain was 20.6 μM under control conditions (5.4 mM K_o + 1 mM Ba) and decreased to 2.3 μM with 3 μM monensin added to raise Na_i (Stimers et al., 1990*c*). In those experiments, while we were able to show that monensin increased Na_i with respect to the control level, we were not able to quantify Na_i. Using a cyclic model to represent the Na/K

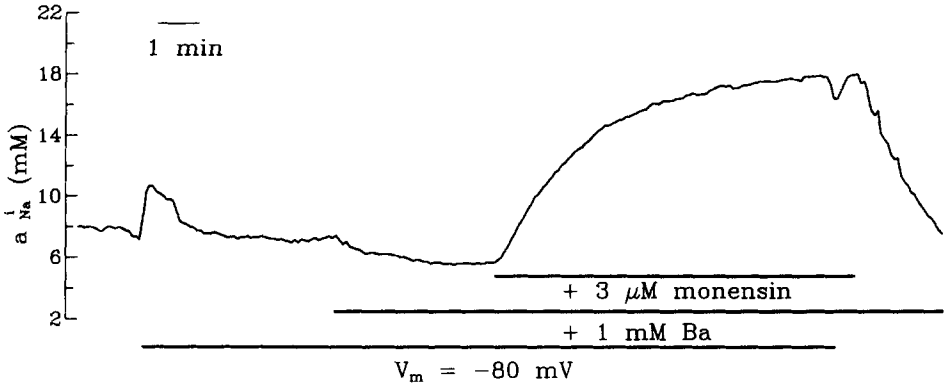


FIGURE 4. a^i_{Na} in an aggregate of cardiac myocytes. Shown is a continuous record of a^i_{Na} which was voltage clamped (SVC) at −80 mV for the period indicated by the lower bar. After the aggregate was voltage clamped there was a transient increase in a^i_{Na} which was reproducible but quite variable in magnitude and time course in comparison with other preparations. Addition of 1 mM Ba resulted in a decrease in a^i_{Na} from ~7 mM to 5.5. Addition of 3 μM monensin increased a^i_{Na} to ~18 mM. This pattern was reproduced in three preparations.

pump, we predicted that Na_i would be 7.1 and 25.4 mM for control and monensin-treated preparations, respectively. To test this prediction, preparations were voltage clamped at −80 mV and a^i_{Na} was simultaneously measured with a Na-SME. Fig. 4 shows a continuous record of a^i_{Na} from one such aggregate. At the time indicated by the lower bar, the preparation was voltage clamped at −80 mV. There is a transient increase in a^i_{Na} followed by a return to near control levels. Addition of 1 mM Ba (indicated by the horizontal bar) resulted in a decrease in a^i_{Na} from 7.1 ± 0.7 to 5.8 ± 1.2 mM (n = 3). This corresponds to a decrease in Na_i from 9.7 ± 0.9 to 7.9 ± 1.6 mM (converted from a^i_{Na} with an activity coefficient of 0.735). At this time we have no explanation for this decrease in Na_i but note it as a consistent finding. Addition of 3 μM monensin increased Na_i to 24.1 ± 0.4 mM (n = 3). These results are in excellent agreement with our previous predictions for Na_i under these conditions.

Measuring I_p with the WCPC technique

Fig. 3 shows the procedure used to measure I_p. Myocytes voltage clamped at −70 mV were bathed in Ca-free HBSS with 1 mM Ba, 10 mM Cs, and 0.1 mM Cd added to reduce membrane currents unrelated to I_p as shown by the *I-V* relations in Fig 2. The internal (pipette) solution contained 51 mM Na to maximally stimulate I_p (Nakao and Gadsby, 1989). Application of 1 mM ouabain caused an immediate inward shift of the holding current (I_h) due to inhibition of outward I_p (see Fig. 3). In similar experiments, when ATP or Na was omitted from the pipette solution, addition of ouabain had no effect on I_h. The *I-V* relationship for I_p with 2 mM ATP was determined in three preparations (Fig. 5, filled circles) and noted to be similar to that published for guinea pig cardiac myocytes (Gadsby and Nakao, 1989; Mogul et al., 1989*a*; Nakao and Gadsby, 1989) and sheep Purkinje myocytes (Glitsch et al., 1989*a*). Since Na/K pump inhibition may cause changes in K_o-sensitive currents other than I_p (e.g., I_{K1} and I_f; see Stimers et al., 1990*b*) the magnitude of K_o-sensitive currents was measured by removing K_o in the presence of 1 mM ouabain (Fig. 5, open circles). Note that no K_o-sensitive current is present under these conditions.

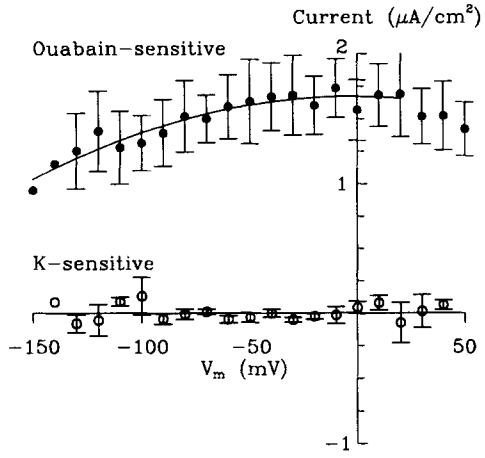


FIGURE 5. Voltage dependence of I_p and K_o-sensitive currents. WCPC of one to three cardiac cell clusters in Ca-free HBSS with 1 mM Ba, 10 mM Cs, and 0.1 mM Cd added (51 mM Na_i) revealed a ouabain-sensitive current (I_p, *filled circles*) that was slightly voltage dependent in the hyperpolarized direction (~30% reduction in I_p between 0 and −130 mV). Shown is the difference in current measured 300 ms after stepping the membrane potential from a holding potential of −70 mV to the indicated potential in the presence and absence of 1 mM ouabain. The K_o-sensitive current (*open circles*) is shown to be negligible under these conditions. This was determined in the presence of 1 mM ouabain by measuring the difference in current measured 300 ms after a pulse from −70 mV to the indicated potential in the presence or absence of 5.4 mM K_o. Data are plotted as mean and SEM (n = 3). The points at −140 and −150 mV represent single observations.

Na_i Dependence of $K_{0.5}$ for Ouabain

To show that the apparent affinity for ouabain is influenced by Na_i, the concentration of Na in the pipette solution was varied between 6 and 51 mM by substitution with K. Our ability to control Na_i by altering pipette Na will be discussed below with the Na_i dependence of I_p. Fig. 6 shows results from two preparations voltage clamped at −70 mV and internally perfused with 14 mM (*A* and *B*) or 51 mM (*C* and *D*) Na. Current

records were normalized to membrane area. Comparing the magnitude of $I_{p\text{-}max}$ (1 mM ouabain; *B* and *D*), it is clear that raising Na_i stimulated I_p as expected. The fraction of I_p inhibited by 10 μM ouabain (*A* and *C*) shows that $K_{0.5}$ was 7.6 μM and 4 μM for 14 and 51 mM Na_i, respectively. Data from all such experiments are summarized in Fig. 7 *A* as mean ± SEM (filled circles). This figure also includes the two points obtained from the combined SVC and Na-SME experiments (open circles) and Na-SME data from non–voltage clamped preparations (open triangle). As predicted, there is a steep relationship between Na_i and $K_{0.5}$ (Stimers et al., 1990*c*).

Since $K_{0.5}$ is variable in these experiments, it is necessary to determine how the magnitude of I_p measured with 1 mM ouabain compares with $I_{p\text{-}max}$. Using Eq. 1 we calculate that for $K_{0.5}$ as high as 20 μM, I_p measured with 1 mM ouabain is 98% of

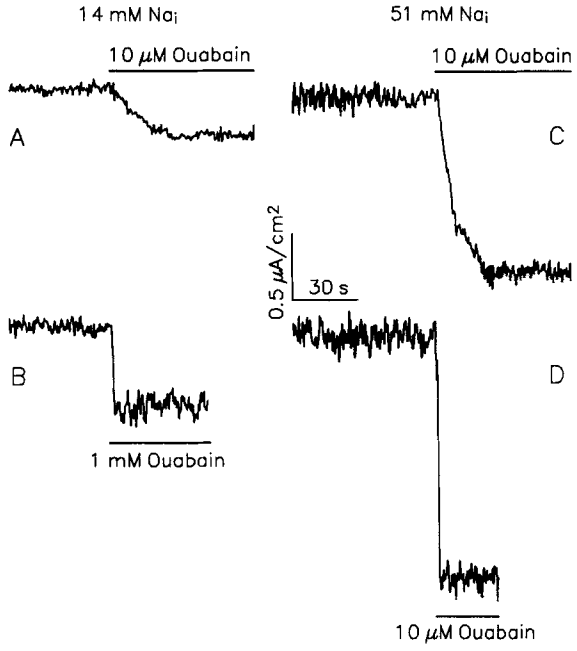


FIGURE 6. Ouabain sensitivity and Na dependence of I_p and $K_{0.5}$ for ouabain. Increasing Na_i (pipette concentration) from 14 mM (*A* and *B*) to 51 mM (*C* and *D*) increases both the magnitude of I_p about twofold (*B* and *D*) and the fraction of I_p blocked by 10 μM ouabain (*A* and *C*). Assuming a single binding site model, the $K_{0.5}$ calculated from these data is 7.6 and 4 μM for 14 and 51 mM Na_i, respectively. The traces labeled *A* and *B* are from one preparation (membrane capacitance = 26 pF), while those labeled *C* and *D* are from another preparation (25 pF). The data are normalized to membrane area for comparison.

$I_{p\text{-}max}$. This underestimation of $I_{p\text{-}max}$ would result in a maximum of 3% underestimation of $K_{0.5}$. Since this error is less than the standard errors for the data, this is not a significant error.

K_o Dependence of $K_{0.5}$ for Ouabain

The methods used to obtain the results in this paper were validated by confirming the well-known inhibition of ouabain binding by increasing K_o. Similar experiments to those described above for Na_i were done to measure the K_o dependence of $K_{0.5}$ for ouabain. Na_i was kept constant at 24 mM to compare these results with those obtained in equilibrium [^{3}H]ouabain binding experiments (Lobaugh and Lieberman, 1987) at 0 and 0.5 mM K_o. While Na_i was not precisely known in those experiments (content measurements show Na_i was 20–30 mM), the results are comparable to the

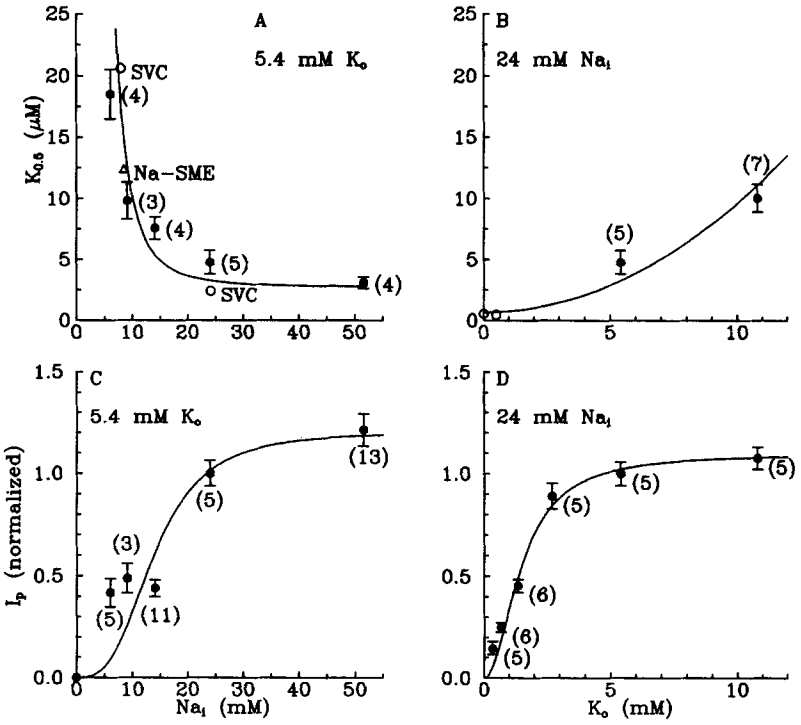


FIGURE 7. Dependence of $K_{0.5}$ of ouabain and I_p magnitude on Na_i and K_o. (*A*) The Na_i dependence of the $K_{0.5}$ for ouabain was measured in 5.4 mM K_o. This panel includes data from WCPC (*filled circles*), SVC (*open circles*), and non–voltage-clamped preparations (*open triangle*, Na-SME). For WCPC, Na_i represents pipette Na concentration, while for the others (SVC and Na-SME) Na_i was calculated from the measured a^i_{Na}. (*B*) The K_o dependence of $K_{0.5}$ was measured with 24 mM Na_i in the patch pipette (*filled circles*). The two open circles were taken from previously published results from equilibrium [^{3}H]ouabain binding experiments (Lobaugh and Lieberman, 1987). The dependence of I_p magnitude on Na_i (*C*) and K_o (*D*) was measured with 5.4 mM K_o and 24 mM Na_i, respectively. For comparative purposes, the data in both panels were normalized to the current magnitude in 5.4 mM K_o and 24 mM Na_i (the only common condition between the panels). These data were all obtained from WCPC experiments. Data are shown as mean and SEM. The number in parentheses next to each data point is the number of observations. The solid curves through the data are the best-fit lines generated from the model described in the text.

present experiments because in this range of Na_i, $K_{0.5}$ is nearly constant (Fig. 7 *A*). In the present WCPC experiments, K_o was either 5.4 or 10.8 mM (Fig. 7 *B*, filled circles). The open circles in Fig. 7 *B* represent previous measurements in equilibrium [^{3}H]ouabain binding experiments (Lobaugh and Lieberman, 1987). As expected, K_o increased the value of $K_{0.5}$ for ouabain.

Dependence of I_p on Na_i and K_o

To verify that the model parameters chosen (see below) provide realistic estimates of I_p while explaining the effects of Na_i and K_o on the $K_{0.5}$ of the Na/K pump for

ouabain, parallel experiments were done to measure the dependence of I_p magnitude on Na_i and K_o. Na_i dependence of I_p was measured as the change in I_h produced by 1 mM ouabain (Fig. 6, *B* and *D*). These data were normalized to I_p measured in 5.4 mM K_o and 24 mM Na_i and are summarized in Fig. 7 *C*. This point was chosen for normalization since it is the only condition in common between all four panels in Fig. 7. Note that if Na was omitted from the pipette there was no measurable I_p. This suggests that pipette Na is a reasonable estimate of Na_i (see Discussion). If these data are fit by the Hill equation, the estimate for $K_{0.5}$ for Na_i is 17.4 mM and the Hill coefficient is 2.1.

K_o dependence was determined as previously described (Stimers et al., 1990*b*). Briefly, myocytes were whole-cell patch clamped (Na_i = 24 mM) at −70 mV and superfused with K-free, Ca-free HBSS with 1 mM Ba and 0.1 mM Cd added; no Cs was used in these experiments since Cs would significantly activate the Na/K pump at low levels of K_o (see below). When I_h stabilized (about 5 min), preparations were repeatedly exposed to solutions with K_o between 0.34 and 10.8 mM for 30 s with at least 90 s in K-free between tests. I_p was measured as the change in I_h produced by K_o. Since we previously showed that under these conditions there are no K_o-sensitive currents other than I_p (Fig. 5), this should be an accurate estimate of I_p. Data were normalized as described for Fig. 7 *C* and summarized in Fig. 7 *D*. Fitting the Hill equation to these data gives a $K_{0.5}$ for K_o of 1.94 mM and a Hill coefficient of 1.63. The dependence of I_p on Na_i and K_o is in the expected direction, with increases in either ion stimulating I_p.

Na/K Pump Model

The data in Fig. 7 *A* show that the prediction of this model of the Na/K pump about the Na_i dependence of the apparent affinity for ouabain (Fig. 3) is verified. To provide an optimal fit of the model predictions to the experimental data, a single set of parameters was simultaneously fit to the data in all four panels of Fig. 7. The parameters for the model were chosen to be identical to those previously published (Chapman et al., 1983; Stimers et al., 1990*c*) with the exception that four of the rate constants (f_1, b_1, f_4, and b_4) were allowed to vary to produce the best fit of the model to the data. These rate constants were chosen to be fit because these are the rate constants for the binding of Na_i and K_o. The fitted parameters were optimized using a least-squares fitting procedure (see Methods). Table I gives the best-fit values for those rate constants and the solid curves in Fig. 7 are the best fits to the data. While the fit to the data is not perfect it is important to remember that all the data in Fig. 7 were fit simultaneously and that the shapes of the curves are constrained more by the form of the model than by the parameter values (see Discussion).

DISCUSSION

The results in this paper have shown that the apparent affinity for ouabain is variable and depends on Na_i and K_o. By changing the ionic conditions, we have measured apparent affinities from 0.5 to 20 μM. Even though this range is large (40-fold), the data can be completely explained by the model presented. The results suggest that Na_i decreased the value of $K_{0.5}$ by increasing the relative abundance of the conforma-

tion that binds ouabain with high affinity, while K_o increased the value of $K_{0.5}$ by favoring conformations that do not bind ouabain. These results are in contrast to the finding that ouabain binding to partially purified Na/K-ATPase preparations is independent of Na concentration between 1 and 8 mM (Berlin et al., 1986). However, our results do agree with earlier reports in isolated Na/K-ATPase where ouabain binding was found to depend on Na concentration over a wide range (Siegel and Josephson, 1972; Lindenmayer and Schwartz, 1973).

Control of Na_i

While we are unable to claim precise control of Na_i by changing Na in the patch electrode, several lines of evidence suggest that Na_i is being controlled to a significant degree. The preparations used were small and nearly spherical, ~15–25 μm in diameter, so diffusion from the pipette tip throughout the intracellular compartment should be relatively rapid. The cells were voltage clamped at −70 mV so Na influx through Na channels would be minimal. Also, influx or efflux of Na via the Na/Ca exchange should be minimal since the experiments were done in Ca-free solution outside and EGTA-buffered solution inside. In the absence of Na in the pipette, there is no measurable ouabain-sensitive current. Raising pipette Na results in a Na-dependent increase in I_p which saturates at high levels and is half-maximal between 10 and 15 mM Na. This result is consistent with other published values in mammalian cardiac myocytes (Sejersted et al., 1988; Glitsch et al., 1989*a;* Nakao and Gadsby, 1989). Changing pipette Na causes a significant change in apparent ouabain affinity. Finally, there is good agreement between the WCPC data and that obtained with the Na-SME. Taken as a whole, these results suggest that we are controlling Na_i to a significant degree and that pipette Na is a good indicator of Na_i in these experiments.

Recently, Mathias et al. (1990) published a model that predicts the effect of Na/K pump activity on the control of Na_i by a patch electrode. In their case of a mammalian ventricular myocyte, which is a relatively large cell, with a maximum flux of Na through the Na/K pump of 8.9 fmol/s, they showed that there was a significant deviation of Na_i from pipette Na due to the resistance of the patch electrode. We have applied their model to our data for chick myocytes having an average membrane area of 2,000 μm^2, a maximum pump current of 1.74 $\mu A/cm^2$, and a resting pump current in 9 mM Na_i of 0.4 $\mu A/cm^2$ (Stimers et al., 1990*b*), which corresponds to a Na flux of 1.1 and 0.25 fmol/s, respectively. First of all, this approximately eightfold decrease in maximum flux will decrease the difference between pipette Na and Na_i eightfold in their model with all other factors being equal. The actual improvement in control of Na_i also depends on the series resistance of the patch electrode. In our experiments, the series resistance was always <20 MΩ, often much less. If we assume a series resistance of 20 MΩ, we calculate that Na_i would differ from pipette Na by <2 mM for Na_i between 5 and 15 mM, and by 8.7 and 13.6 mM for pipette Na of 24 and 51 mM, respectively. Looking at the data in Fig. 7 *A*, it is clear that these worst case deviations in Na_i would not change the results at high Na_i and that correcting the data for low Na_i (moving the points toward the resting concentration of Na_i by <2 mM) would give the data a better fit to the simulated curve. Therefore, the conclusion remains that Na_i has a strong effect on

the apparent affinity of the Na/K pump for ouabain and that we have reasonable control of Na_i under these conditions.

Cs Activation of I_p

The presence of 10 mM Cs in the extracellular solution might influence the measurements of I_p since 10 mM Cs will activate the Na/K pump ($K_{0.5}$ for Cs is 3.2 mM; Glitsch et al., 1989*b*). In most of the experiments described above, K_o was 5.4 or 10.8 mM, which is well above the $K_{0.5}$ of 1–2 mM for K_o activation of the Na/K pump and is nearly a saturating concentration of K_o (Stimers et al., 1990*c*). In those experiments Cs had a negligible effect on I_p, since the presence and absence of 10 mM Cs had no effect on the measurement of the ouabain-sensitive current. However, when K_o was varied between 0.3 and 10.8 mM to test the effect of K_o on I_p magnitude (Fig. 7 *D*), 10 mM Cs may have an appreciable effect on the results. Therefore, in those experiments Cs was omitted from the extracellular solutions to eliminate that problem.

Model of the Na/K Pump and Ouabain Binding

The Na/K pump is a structurally and kinetically complex molecule; consequently, its detailed mechanism is still elusive after decades of study. The Na/K pump sequentially binds and transports Na_i out of the cell and then K_o into the cell. While the exact number of transitions or states of the enzyme involved in this cycle is questionable, the model presented here probably represents an underestimation of the true number of states. Cardiac glycosides reportedly bind with high affinity to either a single conformation of the Na/K pump or just a few (Cantley, 1981; Herzig et al., 1985; Hootman and Ernst, 1988). Despite this kinetic complexity, Michaelis-Menten kinetics and terminology, developed to interpret single transition kinetic schemes, have persistently been used in describing ouabain binding to the Na/K pump. Some of this has been justified since equilibrium binding does appear first order and in isolated enzyme systems the conditions can be adjusted such that single transitions can be studied in relative isolation. However, when studying the Na/K pump in intact cells, certain aspects of the data compel us to consider a more complete model of the Na/K pump. We do not argue that the model presented here is the only one that would explain these results, but rather that simpler models are probably inappropriate. Our data would almost certainly be better fit if the three Na_i sites and the two K_o sites were allowed to be independent and nonidentical, with more free parameters. However, the important conclusion remains that Na_i and K_o have profound effects on $K_{0.5}$.

Interpretation of the interaction of ouabain with the Na/K pump in intact preparations is difficult because the properties of the whole system may alter the conditions under which the study is being conducted. Herzig et al. (1985, 1988) have discussed the effect of Na/K pump turnover rate on ouabain binding affinity. They suggested that with partial Na/K pump inhibition, the rise in Na_i increases the turnover rate of the uninhibited pumps, making the high affinity conformation available a larger fraction of the time and increasing the apparent affinity. While presenting a model that clearly recognizes the need to consider the complex kinetics of the Na/K pump, they continue to interpret the data with only a limited

modification of Michaelis-Menten kinetics. They proposed the inclusion of a proportionality constant in the apparent affinity to account for the effects of turnover rate. Stemmer and Akera (1988) elaborated on Herzig's theory by examining ouabain binding in isolated myocytes and found binding to be positive cooperative based on a nonlinear Scatchard plot analysis. They argue that this is not the usual positive cooperativity since there is only a single ouabain binding site on the Na/K pump, but rather reflects an interaction between Na_i accumulation due to Na/K pump inhibition and ouabain binding. The important contribution of these studies has been the idea that ouabain binding to the Na/K pump will enhance its own binding by increasing Na_i. These studies are completely consistent with our results.

A simple single site binding (i.e., Michaelis-Menten) analysis is inadequate to interpret the effect of Na_i and K_o on the apparent affinity for ouabain. By considering the entire reaction sequence, a single mechanism can be shown to account for both the inhibition of ouabain binding by K_o and the enhancement of ouabain binding by Na_i. Herzig et al. (1985) had to make the assumption that K_o was at saturating levels in their analysis. With high K_o and Na_i limiting, they concluded that turnover rate of the Na/K pump was the regulating factor for ouabain binding. While correct under those conditions, the model is not applicable to conditions where K_o is also limiting, which is the case for most equilibrium binding experiments (e.g., Lobaugh and Lieberman, 1987). Similarly, the effects of K_o on ouabain binding are explained in terms of competition between K_o and ouabain for the Na/K pump without regard to the effect either might have on Na_i. When considering the entire Na/K pump cycle, it is clear that Na_i, K_o, and ouabain are altering the distribution of Na/K pump molecules among the various kinetic states. In other words, these ligands alter the probability that the Na/K pump will be in a particular conformation. Simulations of the model show that increasing K_o decreases the probability that the Na/K pump will be in the E_2-P conformation. Increasing Na_i results in an increase in $Na_3E_1 \sim P$ which is in rapid equilibrium with E_2-P, thus favoring ouabain binding. Thus the simple concept of state distribution is sufficient to explain both Na_i stimulation and K_o inhibition of ouabain binding to the Na/K pump.

An important point to remember when considering multistate models is that transitions between any two states cannot usually be studied experimentally. When the molecule is free to evolve into any given state, the experimentally observable time constants are complex functions of the rate constants for all the transitions (e.g., Stimers et al., 1987). Therefore, in intact cell preparations under physiological conditions, it is erroneous to assume that on and off rates for ouabain binding represent the on and off rate constants for the transition between free and bound Na/K pump. However, if the enzyme is restricted to a single conformation by appropriate conditions (i.e., high Na_i and low K_o), then the on and off rates do approximate the transition rate constants. This may be part of the reason that kinetic estimates for ouabain binding usually differ from equilibrium measurements (see Lobaugh and Lieberman, 1987).

Na/K Pump Current Dependence on Na_i and K_o

Fig. 7, *C* and *D*, shows, as expected, that I_p depends on both Na_i and K_o. The lines drawn in Fig. 7, *C* and *D*, represent the best fit of the model to the data. If, however,

the Hill equation (Hill, 1910) was used, we would calculate that for Na_i the $K_{0.5}$ is 17.4 mM and n (Hill coefficient) is 2.1. For K_o the $K_{0.5}$ is 1.94 mM and n is 1.63 (fits not shown). With respect to K_o these estimates are close to our previous values for these parameters (Lieberman et al., 1982; Lobaugh et al., 1987; Stimers et al., 1990*b*). In contrast to earlier reports in cardiac muscle (for review see Stimers et al., 1990*b;* cf., Nakao and Gadsby, 1989), a recent report has also found K_o activation of I_p to be sigmoidal with n = 1.94 (Glitsch et al., 1989*b*). Na_i activation of I_p has been reported to be linear (Eisner et al., 1984), hyperbolic (Borlinghaus and Apell, 1988), and sigmoid, with Hill coefficients between 1.4 and 2.8 (Philipson and Nishimoto, 1983; Sejersted et al., 1988; Glitsch et al., 1989*a;* Nakao and Gadsby, 1989). Our value of 2.1 for the Hill coefficient of Na_i falls within this range but is considerably less than the value of 3 assumed in the model. The curves in Fig. 7 show that the model produces a good fit to the K_o and Na_i versus $K_{0.5}$ data (*A* and *B*); however, the fit is not as good to the I_p data (*C* and *D*). Several possibilities might explain this: (*a*) the individual binding sites for K_o and Na_i might not be identical, therefore making the assumption of identical binding sites in this model an over simplification (Borlinghaus and Apell, 1988); (*b*) the interaction between K_i and Na_i may reduce the apparent cooperativity of Na_i in these experiments (Sejersted et al., 1988); and (*c*) our control of Na_i at low levels of Na_i may not have been sufficient to accurately determine the shape of the Na_i versus I_p curve (Mogul et al., 1989*b*).

Voltage Dependence of I_p

In characterizing I_p in these preparations we measured the voltage dependence of I_p under conditions that strongly favor the forward mode of transport, high Na_i and K_o. As a result, while some voltage dependence was detected, it was slight over the voltage range studied. This is in agreement with other studies on voltage dependence of I_p in mammalian isolated cardiac myocytes (Gadsby and Nakao, 1989; Glitsch et al., 1989*a;* Mogul et al., 1989*a*; Nakao and Gadsby, 1989). Decreasing Na_i appears to make I_p more voltage dependent, while high levels of Na_i tend to flatten the *I-V* relation (Glitsch et al., 1989*a*). Nakao and Gadsby (1989) have shown this to be a shift in the *I-V* relation but were unable to quantitatively relate this shift to that expected from the change in electrochemical driving force. If we assume a linear *I-V* relation for I_p in our experiments, we can extrapolate a reversal of −240 mV (Fig. 5), which is well positive of the calculated reversal potential of −360 mV (De Weer et al., 1988). However, since the *I-V* relation for I_p is probably nonlinear (Gadsby and Nakao, 1989), this difference is probably much less than it would appear from our data. Further experiments are needed to clarify these quantitative discrepancies between experimental and theoretical results.

In summary, the apparent affinity of the Na/K pump for ouabain is variable. K_o and Na_i strongly influence the apparent affinity, causing at least a 40-fold variation in its value. Considering the Na/K pump as a complex sequence of states leads to the conclusion that K_o inhibition and Na_i stimulation of ouabain binding can be explained by a change in the probability that the Na/K pump will be in the conformation that binds ouabain with high affinity. Other ligands, such as Na_o, K_i, ATP, etc. may also produce effects on apparent affinity as evidenced by reported changes in cooperativity (Sejersted et al., 1988).

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REFERENCES

Berlin, J. R., T. Akera, and T. M. Brody. 1986. Lack of pharmacodynamic interactions between quinidine and digoxin in isolated atrial muscle of guinea pig heart. *Journal of Pharmacology and Experimental Therapeutics.* 238:632–641.

Borlinghaus, R., and H.-J. Apell. 1988. Current transients generated by the Na^+/K^+-ATPase after an ATP concentration jump: dependence on sodium and ATP concentration. *Biochimica et Biophysica Acta.* 939:197–206.

Brody, T. M., K. Temma, R. H. Kennedy, and T. Akera. 1984. The dual effect of sodium ion on the digitalis-sodium pump interaction. *European Heart Journal.* 5:291–296.

Cantley, L. C. 1981. Structure and mechanism of the (Na,K)-ATPase. *Current Topics in Bioenergetics.* 11:201–237.

Chapman, J. B., E. A. Johnson, and J. M. Kootsey. 1983. Electrical and biochemical properties of an enzyme model of the sodium pump. *Journal of Membrane Biology.* 74:139–153.

De Weer, P., D. C. Gadsby, and R. F. Rakowski. 1988. Voltage dependence of the Na-K pump. *Annual Review of Physiology.* 50:225–241.

Ebihara, L., N. Shigeto, M. Lieberman, and E. A. Johnson. 1980. The initial inward current in spherical clusters of chick embryonic heart cells. *Journal of General Physiology.* 75:437–456.

Eisner, D. A., W. J. Lederer, and R. D. Vaughan-Jones. 1984. The electrogenic Na pump in mammalian cardiac muscle. *In* Electrogenic Transport: Fundamental Principles and Physiological Implications. M. P. Blaustein and M. Lieberman, editors. Raven Press, New York. 193–213.

Gadsby, D. C., and M. Nakao. 1989. Steady-state current-voltage relationship of the Na/K pump in guinea pig ventricular myocytes. *Journal of General Physiology.* 94:511–537.

Glitsch, H. G., T. Krahn, and H. Pusch. 1989*a*. The dependence of sodium pump current on internal Na concentration and membrane potential in cardioballs from sheep Purkinje fibres. *Pflügers Archiv.* 414:52–58.

Glitsch, H. G., T. Krahn, and F. Verdonck. 1989*b*. Activation of the Na pump current by external K and Cs ions in cardioballs from sheep Purkinje fibres. *Pflügers Archiv.* 414:99–101.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high resolution current recording from cells and cell free membrane patches. *Pflügers Archiv.* 391:85–100.

Heller, M. 1987. Interactions and modes of action of cardiac glycosides with cultured heart cells. *In* The Heart Cell in Culture. Vol. III. A. Pinson, editor. CRC Press, Inc., Boca Raton, FL. 1–17.

Herzig, S., H. Lullmann, and K. Mohr. 1985. On the cooperativity of ouabain-binding to intact myocardium. *Journal of Molecular and Cellular Cardiology.* 17:1095–1104.

Herzig, S., H. Lullmann, K. Mohr, and R. Schmitz. 1988. Interpretation of [^{3}H]ouabain binding in guinea-pig ventricular myocardium in relation to sodium pump activity. *Journal of Physiology.* 396:105–120.

Hill, A. V. 1910. A new mathematical treatment of changes of ionic concentration in muscle and nerve under the action of electric currents, with a theory as to their mode of excitation. *Journal of Physiology.* 40:190–224.

Hootman, S. R., and S. A. Ernst. 1988. Estimation of Na,K-pump numbers and turnover in intact cells with [^{3}H]ouabain. *Methods in Enzymology.* 156:213–229.

Jacob, R., M. Lieberman, E. Murphy, and D. R. Piwnica-Worms. 1987. Effect of sodium-potassium pump inhibition and low sodium on membrane potential in cultured embryonic chick heart cells. *Journal of Physiology.* 387:549–566.

Lieberman, M., C. R. Horres, J. F. Aiton, N. Shigeto, and D. M. Wheeler. 1982. Developmental aspects of cardiac excitation: active transport. *In* Normal and Abnormal Conduction in the Heart. A. Paes de Carvalho, B. F. Hoffman, and M. Lieberman, editors. Futura Publishing Co., Mount Kisco. 313–326.

Lindenmayer, G. E., and A. Schwartz. 1973. Nature of the transport ATPase glycoside complex. IV. Evidence that sodium and potassium competition for a common site modulates the rate of glycoside interaction. *Journal of Biological Chemistry.* 248:1291–1300.

Liu, S., R. Jacob, D. R. Piwnica-Worms, and M. Lieberman. 1987. (Na + K + 2Cl) cotransport in cultured embryonic chick heart cells. *American Journal of Physiology.* 253:C721–C730.

Lobaugh, L. A., and M. Lieberman. 1987. Na/K pump site density and ouabain binding affinity in cultured chick heart cells. *American Journal of Physiology.* 253:C731–C743.

Lobaugh, L. A., S. Liu, and M. Lieberman. 1987. Na/K pump function in cultured embryonic chick heart cells. *In* Heart Function and Metabolism. N. S. Dhalla, G. N. Pierce, and R. E. Beamish, editors. Martinus Nijhoff, Boston. 181–190.

Mathias, R. T., I. S. Cohen, and C. Oliva. 1990. Limitations of the whole cell patch clamp technique in the control of intracellular concentrations. *Biophysical Journal.* 58:759–770.

Mathias, R. T., L. Ebihara, M. Lieberman, and E. A. Johnson. 1981. Linear electrical properties of passive and active currents in spherical heart cell clusters. *Biophysical Journal.* 36:221–242.

Mogul, D. J., H. H. Rasmussen, D. H. Singer, and R. E. Ten Eick. 1989*a*. Inhibition of Na-K pump current in guinea pig ventricular myocytes by dihydroouabain occurs at high- and low-affinity sites. *Circulation Research.* 64:1063–1069.

Mogul, D. J., D. H. Singer, and R. E. Ten Eick. 1989*b*. Ionic diffusion in voltage-clamped isolated cardiac myocytes. Implications for Na,K-pump studies. *Biophysical Journal.* 56:565–577.

Nakao, M., and D. C. Gadsby. 1989. [Na] and [K] dependence of the Na/K pump current–voltage relationship in guinea pig ventricular myocytes. *Journal of General Physiology.* 94:539–565.

Philipson, K. D., and A. Y. Nishimoto. 1983. ATP-dependent Na^+ transport in cardiac sarcolemmal vesicles. *Biochimica et Biophysica Acta.* 733:133–141.

Schwartz, A., G. E. Lindenmayer, and J. C. Allen. 1975. The sodium-potassium adenosine triphosphatase: pharmacological, physiological and biochemical aspects. *Pharmacological Reviews.* 27:3–134.

Sejersted, O. M., J. A. Wasserstrom, and H. A. Fozzard. 1988. Na,K pump stimulation by intracellular Na in isolated, intact sheep cardiac Purkinje fibers. *Journal of General Physiology.* 91:445–466.

Siegel, G. J., and L. Josephson. 1972. Ouabain reaction with microsomal (sodium + potassium)-activated adenosinetriphosphatase: characteristics of substrate and ion dependencies. *European Journal of Biochemistry.* 25:323–335.

Stemmer, P., and T. Akera. 1988. Sodium-pump activity and its inhibition by extracellular calcium in cardiac myocytes of guinea pigs. *Biochimica et Biophysica Acta.* 940:188–196.

Stimers, J. R., F. Bezanilla, and R. E. Taylor. 1987. Sodium channel gating currents. Origin of the rising phase. *Journal of General Physiology.* 89:521–540.

Stimers, J. R., S. Liu, and M. Lieberman. 1990*a*. Apparent affinity of the Na/K pump for ouabain in embryonic chick cardiac myocytes. *FASEB Journal.* 4:A295.

Stimers, J. R., S. Liu, L. A. Lobaugh, and M. Lieberman. 1989. $[Na]_i$ and $[K]_o$ determine apparent affinity of the Na/K pump for ouabain in cardiac myocytes. *Journal of General Physiology.* 94:15*a*. (Abstr.)

Stimers, J. R., N. Shigeto, and M. Lieberman. 1990*b*. Na/K pump current in aggregates of cultured chick cardiac myocytes. *Journal of General Physiology*. 95:61–76.

Stimers, J. R., N. Shigeto, L. A. Lobaugh, S. Liu, and M. Lieberman. 1990*c*. Intracellular sodium affects ouabain interaction with the Na/K pump in cultured chick cardiac myocytes. *Journal of General Physiology*. 95:77–95.