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 $0.46 \pm 0.07$ (n = 5) to $1.34 \pm 0.08 \mu A/cm^2$ (n = 13) with a $K_{0.5}$ for $Na_i$ of 17.4 mM and decreased the $K_{0.5}$ for ouabain from $18.5 \pm 1.8$ (n = 4) to $3.1 \pm 0.4 \mu M$ (n = 3). Similarly, varying $K_o$ between 0.3 and 10.8 mM ($Na_i = 24$ mM) increased $I_p$ from $0.13 \pm 0.01$ (n = 5) to $0.90 \pm 0.05 \mu A/cm^2$ (n = 5) with a $K_{0.5}$ for $K_o$ of 1.94 mM and increased $K_{0.5}$ for ouabain from $0.56 \pm 0.14$ (n = 3–6) to 10.0 ± 1.1 $\mu M$ (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 and K. 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., 1989a). 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 Aker (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, lowers the value of the apparent affinity ($K_{0.5}$) of the Na/K pump for ouabain (Stimers et al., 1990c). 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 and K 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 \( \text{Na}^+ \) 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 \( \text{K}^+ \) dependence of ouabain binding.

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

**METHODS**

**Model**

The interpretation of measured \( K_{50} \) values is model dependent. A Michaelis-Menten type of model would require that the \( K_{50} \) 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., 1990c; see also Chapman et al., 1983) is shown below:

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\begin{align*}
3\text{Na}^+ \rightarrow & \text{E}_1 \text{ATP} \rightarrow \text{Na}_3\text{E}_1\text{ATP} \rightarrow \text{ADP} \\
2\text{K}^+ \rightarrow & \text{E}_2\text{ATP} \rightarrow \text{K}_2\text{E}_2\text{ATP} \rightarrow \text{P} \\
\text{Ouab} \rightarrow & \text{E}_2\text{P} \rightarrow \text{E}_2\text{P-Ouab}
\end{align*}
\]

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 (\( \text{E}_2\text{P} \), the state following \( \text{Na}^+ \) release to the extracellular solution and preceding \( \text{K}^+ \) 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 \( K_{50} \) on the ionic environment. Fig. 1 shows the prediction this model makes about the effect of \( \text{Na}^+ \) 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 \( \text{Na}^+ \). 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., 1990c). 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 \( 1 \times 10^6 \) 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
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 $Na_i$. 

**Table 1**

| Parameter          | Old values | Fitted values | Units |
|--------------------|------------|---------------|-------|
| Total[ATPase]      | 0.166 pmol/cm² |               |       |
| $Na_k$             | 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 s⁻¹    | 320 s⁻¹       | /mM/s |
| $b_1$              | 54 × 10⁴ s⁻¹ |               |       |
| $f_2$              | 100 s⁻¹    |               | /mM/s |
| $b_2$              | 172 s⁻¹    |               |       |
| $b_3$              | 1.72 × 10⁻⁵ /mM/s |       |       |
| $f_4$              | 15 s⁻¹     | 23 s⁻¹        | /mM/s |
| $b_4$              | 200 s⁻¹    | 20 s⁻¹        | /mM/s |
| $f_5$              | 2.000 /mM/s |               |       |
| $b_5$              | 30 s⁻¹     |               |       |
| $f_6$              | 1.15 × 10⁹ /mM/s |       |       |
| $b_6$              | 600 s⁻¹    |               | /μM/s |
| $f_7$              | 9.5 × 10⁻⁵ /s |              |       |
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<sub>a</sub> 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<sub>H</sub>) in control experiments. All experiments were done at 37°C.

**Switching Voltage-Clamp Technique**

As previously described (Stimmers et al., 1990b), 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., 1990c). 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\textsubscript{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\textsubscript{Na} using the following equation:

$$a_{\text{Na}} = 10^{\frac{V_d - E_0}{S}} - K_{\text{Na}}(a_K)$$

where $V_d$ is the difference potential between the Na-SME and the voltage microelectrode, $E_0$ and $S$ are the standard potential and the slope determined from the calibration of the Na-SME in a series of single electrolyte solutions, $K_{\text{Na}}$ 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 $a_K$ is the intracellular K activity which is 103 ± 1.3 mM (n = 57) (Liu et al., 1987). The values obtained for each electrode for $K_{\text{Na}}$ 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\textsubscript{Ca}) was not done since the selectivity coefficient for Ca relative to Na ($K_{\text{Ca,Na}}$) is 1.0 ± 0.1 (n = 21). Assuming a\textsubscript{Ca} is 100 nM, contamination of the measurement of a\textsubscript{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\textsubscript{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 was varied by replacing K, 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\textsuperscript{2} (Mathias et al., 1981).

Using protocols similar to those previously described for the switching voltage clamp (Stimers et al., 1990c), we have isolated a current using the WCPC technique identifiable as Na/K pump current (I\textsubscript{p}), and show that this current, as expected, is sensitive to K\textsubscript{o}, Na\textsubscript{o}, 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., 1990b, 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.

**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., 1990c). 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 ($I_{p-o}$) 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 $I_{p-o}$:

$$K_0.5 = \frac{C_{ouab}(I_{p\text{-max}}-I_{p-o})}{I_{p-o}}$$

where $C_{ouab}$ is the ouabain concentration. This method has the advantage that $I_{p\text{-max}}$ and $I_{p-o}$ 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 $I_{p-o}$ 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

$a_{\text{Na}}$ 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_+ + 1 mM Ba) and decreased to 2.3 μM with 3 μM monensin added to raise Na_+ (Stimers et al., 1990c). In those experiments, while we were able to show that monensin increased Na_+ with respect to the control level, we were not able to quantify Na_+. Using a cyclic model to represent the Na/K pump, we predicted that Na_+ 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 $a_{\text{Na}}$ was simultaneously measured with a Na-SME. Fig. 4 shows a continuous record of $a_{\text{Na}}$ 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 $a_{\text{Na}}$, followed by a return to near control levels. Addition of 1 mM Ba (indicated by the horizontal bar) resulted in a decrease in $a_{\text{Na}}$ from 7.1 ± 0.7 to 5.8 ± 1.2 mM (n = 3). This corresponds to a decrease in Na_+ from 9.7 ± 0.9 to 7.9 ± 1.6 mM (converted from $a_{\text{Na}}$ with an activity coefficient of 0.735). At this time we have no explanation for this decrease in Na_+ but note it as a consistent finding. Addition of 3 μM monensin increased Na_+ to 24.1 ± 0.4 mM (n = 3). These results are in excellent agreement with our previous predictions for Na_+ 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 $I-V$ relation 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., 1989a; Nakao and Gadsby, 1989) and sheep Purkinje myocytes (Glitsch et al., 1989a). Since Na/K pump inhibition may cause changes in K-sensitive currents other than $I_p$ (e.g., $I_{K1}$ and $I_i$; see Stimers et al., 1990b) the magnitude of K-sensitive currents was measured by removing $K_o$ in the presence of 1 mM ouabain (Fig. 5, open circles). Note that no K-sensitive current is present under these conditions.

Na Dependence of $K_o$ for Ouabain

To show that the apparent affinity for ouabain is influenced by Na, the concentration of Na in the pipette solution was varied between 6 and 51 mM by substitution with K. Our ability to control Na by altering pipette Na will be discussed below with the Na 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 \( \text{Na}_i \) stimulated \( I_p \) as expected. The fraction of \( I_p \) inhibited by 10 \( \mu \text{M} \) ouabain (\( A \) and \( C \)) shows that \( K_{0.5} \) was 7.6 \( \mu \text{M} \) and 4 \( \mu \text{M} \) for 14 and 51 mM \( \text{Na}_i \), respectively. Data from all such experiments are summarized in Fig. 7A 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 \( \text{Na}_i \) and \( K_{0.5} \) (Stimers et al., 1990c).

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 \( \mu \text{M} \), \( I_p \) measured with 1 mM ouabain is 98% of

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

\( K_n \) 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 \( K_n \). Similar experiments to those described above for \( \text{Na}_i \) were done to measure the \( K_n \) dependence of \( K_{0.5} \) for ouabain. \( \text{Na}_i \) was kept constant at 24 mM to compare these results with those obtained in equilibrium \(^3\text{H}\)ouabain binding experiments (Lobaugh and Lieberman, 1987) at 0 and 0.5 mM \( K_n \). While \( \text{Na}_i \) was not precisely known in those experiments (content measurements show \( \text{Na}_i \) 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 $a_{Na}$. (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 $[^3H]Ouabain$ 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 $[^3H]Ouabain$ 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 $N_a$ and $K_o$. $N_a$ dependence of $I_p$ was measured as the change in $I_p$ 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 $N_a$ 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 $N_a$ was omitted from the pipette there was no measurable $I_p$. This suggests that pipette $N_a$ is a reasonable estimate of $N_a$ (see Discussion). If these data are fit by the Hill equation, the estimate for $K_{0.5}$ for $N_a$ is 17.4 mM and the Hill coefficient is 2.1.

$K_o$ dependence was determined as previously described (Stimers et al., 1990b). Briefly, myocytes were whole-cell patch clamped ($N_a = 24$ 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 $N_a$ 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 $N_a$ 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., 1990c) 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 $N_a$ 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 $N_a$ 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 $N_a$ 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**

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., 1989a; 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 μA/cm$^2$, and a resting pump current in 9 mM Na$_i$ of 0.4 μA/cm$^2$ (Stimers et al., 1990b), 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, 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 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., 1989b). 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., 1990c). 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 elusively after decades of study. The Na/K pump sequentially binds and transports Na out of the cell and then K 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 sites and the two K sites were allowed to be independent and nonidentical, with more free parameters. However, the important conclusion remains that Na and K 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 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, 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, 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, and Ko 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, and the enhancement of ouabain binding by Na,. Herzig et al. (1985) had to make the assumption that Ko was at saturating levels in their analysis. With high Ko and Na, 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 Ko is also limiting, which is the case for most equilibrium binding experiments (e.g., Lobaugh and Lieberman, 1987). Similarly, the effects of Ko on ouabain binding are explained in terms of competition between Ko and ouabain for the Na/K pump without regard to the effect either might have on Na,. When considering the entire Na/K pump cycle, it is clear that Na, Ko, 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 Ko decreases the probability that the Na/K pump will be in the E-P conformation. Increasing Na, results in an increase in Na,E, ~ P which is in rapid equilibrium with E-P, thus favoring ouabain binding. Thus the simple concept of state distribution is sufficient to explain both Na, stimulation and Ko 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, and low Ko), 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, and Ko**

Fig. 7, C and D, shows, as expected, that I P depends on both Na, and Ko. 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 $\text{Na}$, the $K_{0.5}$ is 17.4 mM and $n$ (Hill coefficient) is 2.1. For $\text{K}$, the $K_{0.5}$ is 1.94 mM and $n$ is 1.63 (fits not shown). With respect to $\text{K}$, these estimates are close to our previous values for these parameters (Lieberman et al., 1982; Lobaugh et al., 1987; Stimers et al., 1990b). In contrast to earlier reports in cardiac muscle (for review see Stimers et al., 1990b; cf., Nakao and Gadsby, 1989), a recent report has also found $\text{K}$ activation of $I_p$ to be sigmoidal with $n = 1.94$ (Glitsch et al., 1989b). $\text{Na}$ 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., 1989a; Nakao and Gadsby, 1989). Our value of 2.1 for the Hill coefficient of $\text{Na}$ 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 $\text{K}$ and $\text{Na}$ 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 $\text{K}$ and $\text{Na}$ 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 $\text{K}$ and $\text{Na}$ may reduce the apparent cooperativity of $\text{Na}$ in these experiments (Sejersted et al., 1988); and (c) our control of $\text{Na}$ at low levels of $\text{Na}$ may not have been sufficient to accurately determine the shape of the $\text{Na}$ versus $I_p$ curve (Mogul et al., 1989b).

**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 $\text{Na}$ and $\text{K}$. 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., 1989a; Mogul et al., 1989a; Nakao and Gadsby, 1989). Decreasing $\text{Na}$ appears to make $I_p$ more voltage dependent, while high levels of $\text{Na}$ tend to flatten the $I-V$ relation (Glitsch et al., 1989a). 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 $\text{Na}/\text{K}$ pump for ouabain is variable. $K_o$ and $\text{Na}$ strongly influence the apparent affinity, causing at least a 40-fold variation in its value. Considering the $\text{Na}/\text{K}$ pump as a complex sequence of states leads to the conclusion that $K_o$ inhibition and $\text{Na}$ stimulation of ouabain binding can be explained by a change in the probability that the $\text{Na}/\text{K}$ pump will be in the conformation that binds ouabain with high affinity. Other ligands, such as $\text{Na}_o$, $K_o$, ATP, etc. may also produce effects on apparent affinity as evidenced by reported changes in cooperativity (Sejersted et al., 1988).
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