Inhibition of Na-K Pump Current in Guinea Pig Ventricular Myocytes by Dihydroouabain Occurs at High- and Low-Affinity Sites

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Binding of cardiac glycosides to the Na\(^+\),K\(^+\) -dependent ATPase has been shown to occur at both high- and low-affinity sites. However, recent reports suggest that glycoside-induced inhibition of electrogenic Na-K pump current occurs with simple first-order binding kinetics at relatively low-affinity sites. This implies that high-affinity binding sites have little to do with Na-K pump inhibition during exposure to cardiac glycosides. To better understand the role of the high-affinity site, we investigated the concentration dependence of \(I_{\text{pump}}\), inhibition by dihydroouabain (DHO) in guinea pig ventricular myocytes through use of wide-pore patch pipettes to "fix" internal Na\(^+\) activity at -30 mM and to voltage clamp at -40 mV (\(T=34^\circ\) C). DHO was found to have no effect on membrane conductance at a holding potential of -40 mV. Holding current was monitored and the difference between steady-state holding current before and during external exposure to nine concentrations (range, 0.01-1,000 \(\mu\)M) of DHO was measured and normalized to cellular membrane capacitance. The concentration dependence of the inhibition of Na-K pump current was biphasic and well fitted to a two-binding site model with inhibitory \(K_D\) values of 0.05 \(\mu\)M and 64.5 \(\mu\)M. This is consistent with previously reported \(^3\)H-ouabain binding studies in guinea pig myocardium. These findings indicate that the electrogenic properties of the Na-K pump can be inhibited by glycoside binding to both high- and low-affinity sites. (Circulation Research 1989;64:1063-1069)

Cardiac glycosides can bind to the sarcolemmal Na-K pump at both high- and low-affinity sites in a variety of species.\(^1\)-\(^4\) However, the relation between glycoside concentration and inhibition of electrogenic Na-K pump current has been reported to be well described assuming simple first-order kinetics for the interaction between the glycoside and sites with a relatively low affinity.\(^5\)-\(^6\) This implies that Na-K pump inhibition does not involve binding at the sites with the higher affinity. Because binding of cardiac glycosides to this site at therapeutically relevant concentrations has been reported to be associated with the positive inotropic action of the glycosides,\(^7\)-\(^8\) such findings suggest that inhibition of the electrogenic Na-K pump is not causally related to the clinically relevant inotropic action.

To better understand the role of glycoside binding to high-affinity sites in the inhibition of the electrogenic Na-K pump, we determined the effect of dihydroouabain (DHO) on the Na-K pump current generated by single, isolated mammalian cardiac cells. DHO was used because its rapid onset of action and rapid washout permitted verification that any observed effect on the pump current was specifically due to the action of the cardiac glycoside. The relation between the concentration of DHO and inhibition of pump current was biphasic and well described by a model that assumes two pump binding sites. This finding strongly suggests that binding of cardiac glycosides at high- as well as at low-affinity sites can inhibit electrogenic Na-K pumping in the myocardium.

**Materials and Methods**

**Preparation of Myocytes**

Isolated guinea pig ventricular myocytes were prepared using a modification of the method described by Mitra and Morad.\(^9\) Adult guinea pigs (250-300 g) of either sex were killed by cervical

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dislocation. The chest cavity was quickly exposed, and the aorta was cannulated in situ. The heart was then excised, hung on a 0.75 m-high Langendorff column, and perfused retrogradely at constant pressure through the aorta and coronary arteries. The perfusate column was jacketed, and a recirculating octanol-paraffin oil mixture was perfused through the jacket via a heat exchanger to maintain solution temperatures at 36°C. Two high-intensity incandescent lamps were used to maintain the heart surface at a warm temperature during the perfusion.

The solutions used for cell isolation were bubbled with 100% O2. For the first 5 minutes, nominally Ca2+-free Tyrode's solution (mM: NaCl 135, KCl 5.4, MgCl2 1, NaH2PO4 0.33, HEPES 10, pH set to 7.3) was perfused through the heart to remove residual blood. The heart was then perfused to weaken the intercellular connective tissue by recirculating 25 ml enzyme solution (Ca2+-free Tyrode plus [mg%] collagenase Sigma Type I 150, protease Sigma Type XIV 20.6, albumin 100) for 7.5 minutes. Finally, 100 ml KB solution10 (mM: KCl 25, HEPES 10, KOH 115.9, glutamate 80, taurine 10, oxalic acid 14, KH2PO4 10, glucose 11, EGTA 0.5, with albumin 100 mg%, pH set to 7.4) replaced the enzyme-containing solution and the first 50 ml of the circulating KB solution were discarded.

The heart was then cut down from the perfusion apparatus and placed in the final 50 ml of KB solution. The apical half of the left and right ventricles was excised, miniced with scissors (to help disperse the cells), and filtered through a 210-µm nylon mesh to harvest dispersed cells. The harvested cells were centrifuged gently for 15-20 seconds, the supernatant was aspirated, and the pellet was resuspended in KB solution. Only rod-shaped quiescent cells were used. Voltage-Clamp Technique

Rod-shaped quiescent myocytes were voltage-clamped using the whole-cell patch configuration.11 Gigaohm seals were obtained with wide-tipped patch micropipettes (pipette resistance—1.5 MΩ). Suction pipettes were made from glass capillary tubes (1.1 mm i.d.; lead-free borosilicate glass; Garner Glass, Claremont, California) by use of a Narishige vertical puller (Narishige, Tokyo, Japan). Current recordings were filtered with a low-pass 11.4 kHz single-pole filter, sampled at 10 kHz, and recorded digitally using a PDP 11/73 (Digital Equipment, Maynard, Massachusetts).

Inverted voltage-clamp pulses were applied to the bath through an Ag-AgCl pellet—KCl agar bridge. Junction potentials were nullified in intracellular solution by application of an equal and opposite polarity potential. The pipette potential was maintained at the ground level. Total series resistance produced by the pipette tip and cell interior was partially compensated (typically 65% was compensated) before the membrane current was recorded by feeding back a fraction of the original signal into the command pulse. Uncompensated series resistances were 0.8±0.3 MΩ. The maximal currents elicited during determination of membrane current-voltage relations never exceeded 1.0 nA. Therefore, the maximal voltage errors should be less than 1 mV. Experiments were performed at 34°C (±1°C).

Experimental Solutions

For experiments in which the effect of DHO on membrane conductance was investigated, the following external solution was used (mM): NaCl 130, CaCl2 1.8, MgCl2 1.2, glucose 11.1, HEPES 5.5, BaCl2 1, TMA • Cl + KCl 15.2, pH adjusted to 7.4 at 22°C with ~2.5 mM NaOH. For experiments investigating the concentration-inhibition effect of DHO on Na-K pump current, control data were obtained in an external solution containing (mM) NaCl 140, KCl 5.4, CaCl2 1.8, MgCl2 1.2, BaCl2 1, glucose 11.1, HEPES 5.5, pH adjusted to 7.4 at 22°C with ~2.5 mM NaOH, that was rapidly perfused through the cell bath. Glycose-containing external solution was prepared by dissolving DHO in the control solution. Each cell was exposed to only one concentration of DHO. The micropipette solution in all experiments contained K-glutamate 70, Na-glutamate 20, TMA • Cl 50, K2HPO4 1, NaCl 10, EGTA 5, HEPES 5, Mg(ATP) 2, pH adjusted to 7.2 at 22°C with ~5 mM KOH. Because Na-K pump current depends on intracellular Na+ ([Na+]i), it is critical that [Na+]j be well controlled, especially if the level of [Na+]j chosen for experiments is close to the K50 value for Na+-stimulation of the pump. Theoretical and experimental analyses of ionic diffusion within a whole-cell, voltage-clamped myocyte under experimental conditions similar to those described in this study12,13 have demonstrated that steady-state [Na+]j at intracellular pump sites ([Na+]jps) is well regulated by [Na+]j in the pipette ([Na+]jps) even when [Na+]jps=50 mM. Therefore, [Na+]jps can be assumed to closely approximate [Na+]jps=30 mM in the steady state regardless of the level of DHO-sensitive pump current.

Results

Membrane current required to hold membrane potential at ~40 mV during two exposures to 100 µM DHO is shown in Figure 1. Differences in the steady-state holding current (I_D1F) were measured when cells were first superfused with the DHO-free external solution followed by DHO-containing solution. The I_D1F for each of 35 cells was normalized for cellular membrane capacitance to account for cell-to-cell differences in myocyte membrane surface area.

The log concentration-inhibition relation for the data is shown in Figure 2. The means of the normalized difference currents obtained during exposure to nine different concentrations of DHO are plotted. These data are also fitted to Equations 1
and 2 (see below) describing the models for one and two binding sites, respectively:

\[
V = \frac{V_{\text{max1}} [\text{DHO}] + V_{\text{max2}} [\text{DHO}]}{K_{\text{D1}} + [\text{DHO}] + K_{\text{D2}} + [\text{DHO}]}
\]

where \( V \) is the difference current, \( K_D \) is the dissociation constant, and \( V_{\text{max}} \) is the maximally available DHO-sensitive difference current of the glycoside-binding site(s), respectively. The exponent in Equation 1, \( \lambda \), is the Hill coefficient.

For this analysis, the difference current was assumed to be proportional to the concentration of the DHO/Na-K pump complex.5 Estimates of \( K_D \) and \( V_{\text{max}} \) were made using a nonlinear regression technique (BINKIN2, PROPHET Computer System; National Institutes of Health, Bethesda, Maryland). The curve depicted by the dashed line in Figure 4 plots the best-fitting theoretical dose-response relation for a one-binding site model in which no cooperativity between binding sites is assumed (\( K_D = 2.34 \mu M \) and \( V_{\text{max}} = 0.456 \mu A/\mu F, \lambda = 1 \)). Analysis of the data with the Hill equation gave a Hill coefficient equal to 0.44, indicating that a one-binding-site model would be better fit if negative cooperativity between the sites was assumed. This model is depicted by the dotted line in which the maximal difference current is assumed to equal that predicted by the two-binding-site model (\( K_D = 2.34 \mu M \) and \( V_{\text{max}} = 0.559 \mu A/\mu F, \lambda = 0.44 \)). The curve depicted by the solid line plots the best-fitting theoretical relation for a two-
Therefore, the effect of DHO on membrane conductance was assessed using the same internal solution by 1 mM Ba\(^{2+}\) and that may be DHO-sensitive.

In the experiments in which the Na-K pump needs to be active to test the effect of DHO on the Na-K pump, it is necessary to have a nonzero \([K^+]_o\). A nonzero \([K^+]_o\) is expected to be strongly buffered by the 5 mM EGTA in the internal solution. Furthermore, to eliminate from consideration. The threshold voltage for activation of the transient outward current is positive to -40 mV, neither sodium current nor calcium current should be expected to contribute to the steady-state membrane current at -40 mV.

The several components of \(K^+\) current were also eliminated from consideration. The threshold voltage for activation of the transient outward current is positive to -40 mV and is not expected to contribute any steady-state current. The inward-rectifying \(K^+\) current and delayed outward-rectifying \(K^+\) current should be blocked by addition of 1 mM Ba\(^{2+}\) to the extracellular solution. In addition, the delayed outward-rectifying \(K^+\) current is not expected to be activated at -40 mV. Therefore, differences between membrane holding currents recorded in control and DHO-containing solutions should contain virtually no contribution from these channel currents. To test this idea, membrane conductance was assessed and found to be unchanged by exposure to DHO.

The possibility that some portion of the DHO-induced change in holding current involved a change in the electrogenic Na-Ca exchange should be remote because the steady-state intracellular Ca\(^{2+}\) activity is expected to be strongly buffered by the 5 mM EGTA in the internal solution. Furthermore, to
Mogul et al  DHO Inhibits Na-K Pump at Two Sites  1067

Figure 4. Effect of dihydroouabain (DHO) on membrane conductance when \([K^+]_o=5.4\,\text{mM. Holding potential}=\text{—40 mV.} [Na^+]_i=30\,\text{mM.} [Ba^{2+}]_o=1\,\text{mM.} Current measured at end of 70-msec voltage steps to potentials from —100 to —20 mV before (A) and during (O) exposure to 1,000 \mu\text{M DHO. Points are connected by straight lines. Slopes of lines fit by linear regression to data from four experiments were not statistically different (see text). Inset: Plot of current-voltage relation for the DHO-sensitive current. Points represent means (n=4).}

date, there is no evidence that cardiac glycosides have any direct effect on Na-Ca exchange in cardiac cells. Thus, any contribution of electrogenic Na-Ca exchange to the membrane current during control should be identical to that during exposure to DHO and hence should not contribute to \(I_{\text{def}}\). Therefore, it is reasonable to assume that \(I_{\text{def}}\) was composed solely of DHO-sensitive Na-K pump current.

The biphasic concentration-inhibition relation (Figure 2) agrees with published reports of the existence of two different binding affinities for \(^3\)H-ouabain to guinea pig cardiac tissue.\(^{1,20,21}\) In contrast, however, a previous study on the effect of DHO on electrogenic Na-K pump current in guinea pig ventricular tissue\(^4\) reported that the concentration-inhibition relation can be fitted by a linear Scatchard plot suggesting that functionally significant binding occurs at sites with a single affinity \((K_D=46\,\mu\text{M})\) only. Similarly, despite findings of apparent binding of cardiac glycosides at both high- and low-affinity sites in canine cardiac tissue,\(^2,8\) inhibition by DHO of electrogenic pump current reported in isolated canine Purkinje myocytes suggests that only binding at a single affinity site \((K_D=3.7\,\mu\text{M})\) is associated with Na-K pump inhibition.\(^6\)

One possible reason for the difference between the previously reported results and those reported here is that in the previous studies the intracellular ionic composition was not controlled. It is, therefore, possible that the intracellular sodium concentration increased during Na-K pump blockade.\(^6\) Such an increase in internal \([Na^+]\) may have offset a fraction of pump blockade by stimulating additional Na-K pump current, thus partially obscuring the concentration dependence of the DHO-induced inhibition of the Na-K pump (i.e., the measured inhibition of pump current may not have been proportional to the number of pump sites inhibited by binding of glycoside molecules). The whole-cell, patch-clamp technique used for this study should have eliminated such problems by clamping intracellular \([Na^+]\)^\(^{11,12}\) and thus facilitated the demonstration of moderate degrees of Na-K pump inhibition associated with binding of DHO to high-affinity sites.

Inhibition of electrogenic Na-K pumping attributed to binding of a cardiac steroid to high-affinity sites has also been reported in experiments on human cardiac tissue.\(^22\) In that study, however, measurements were made under non-steady-state conditions. Intracellular sodium concentration was not controlled and possibly varied greatly from one
experiment to the next at the time when the Na-K pump was evaluated. As the authors indicated, use of a Na-K pump induced hyperpolarization during electrogenic extrusion of an intracellular sodium load as an index of pump activity rather than direct measurement of pump current made quantitative interpretation of the data difficult. Therefore, those results cannot be easily extrapolated to cardiac cells under steady-state conditions.

The current-voltage relation for $I_{\text{DIF}}$ suggests that the Na-K pump current may be essentially voltage-insensitive between $-100$ and $-20$ mV when defined under our particular experimental conditions. This finding is somewhat different from that reported by Gadsby et al.\(^1\) Their report indicated that the ouabain-sensitive current (derived from the membrane currents recorded before and during exposure to ouabain) exhibited a monotonic voltage dependence with a nonzero slope. The reason for the difference between their results and the present results is not apparent. It may be a result of differences in experimental conditions. In the case of our results, the lack of voltage dependence of Na-K pump current may have been due to incomplete isolation of the sodium pump current at voltage-clamp pulses negative or positive to $-40$ mV. A sodium window current in the picoampere range would be expected to flow at membrane voltages between $-60$ and $-70$ when steady-state holding potential is $-40$ mV.\(^15\) Similarly, a calcium current is expected at voltages positive to $-30$ mV. While it has been shown that Ba\(^{2+}\)-induced block of potassium currents exhibits voltage dependence,\(^24\) this will not influence the results obtained with membrane potential clamped at $-40$ mV. Because 1 mM Ba\(^{2+}\) is expected to completely block the inward-rectifying K\(^+\) current in the voltage range between $-100$ and 0 mV,\(^25,26\) it also should not introduce artifact into the DHO-sensitive current voltage relation. However, if these components of the total membrane current are slightly altered by DHO, they would contribute to $I_{\text{DIF}}$. Despite this possibility the $I_{\text{DIF}}$ versus voltage curve is remarkably linear and voltage-independent. This result resembles findings in *Xenopus* oocytes reported by Eisner et al.,\(^27\) which suggest that under conditions with intracellular sodium activity at a relatively normal level, strophanthine-sensitive current also was voltage-insensitive. Their results\(^27\) also suggested that for the sodium pump current elicited in oocytes to exhibit voltage dependence, a rather large sustained sodium load must exist. If there is a lower limit to what intracellular sodium concentration must be for the sodium pump current to exhibit substantial voltage dependence, it seems to exceed 30 mM under our experimental conditions. While there may still be much to decipher about the nature of the voltage dependence of the sodium pump current, we have avoided problems that it could precipitate by holding voltage constant during the determination of the DHO-concentration dependence of the DHO-sensitive current.

The presence of two binding sites for DHO suggests that two forms of the Na-K pump may coexist in the sarcolemma of each guinea pig cardiocyte. The genetic expression of two functionally distinct forms of the Na,K-ATPase, each with a different ouabain affinity, has been identified in mouse fibroblasts.\(^29\) Alternatively, the prospect that only a single type of Na,K-ATPase exists in guinea pig myocardium possessing both high- and low-affinity glycoside binding sites would require that high-affinity binding does not cause complete pump inhibition in order to be consistent with our finding of biphasic inhibition of Na-K pump current. This requirement is necessary because complete pump inhibition by high-affinity binding would preclude any effect of low-affinity glycoside binding on Na-K pump current.

By using a method for which it can reasonably be assumed that inhibition of pump current is proportional to the fraction of Na-K pump units binding DHO, the present study is the first direct evidence to support the idea that binding of cardiac glycosides at high-affinity sites is associated with inhibition of electrogenic Na-K pumping in intact cardiac cells under steady-state conditions. This finding may have important implications for our understanding of the mechanism of action of the cardiac glycosides because it is binding at high-affinity sites that appears to be associated best with their therapeutically relevant positive inotropic action.\(^4,7,20,29\)

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Mogul et al  DHO Inhibits Na-K Pump at Two Sites 1069

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