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

David J. Mogul, Helge H. Rasmussen, Donald H. Singer, and Robert E. Ten Eick

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 \( \mu \text{M} \)) 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 \( \mu \text{M} \) and 64.5 \( \mu \text{M} \). This is consistent with previously reported \(^3\text{H}-\text{ouabain} \) 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...
电压-钳位技术

杆状的静息肌细胞在使用整个细胞电压钳位后被电位钳位。

结果

膜电流被要求在膜电位中保持膜电流

电压-钳位脉冲被应用到

通过一个Ag-AgCl电极对-KCl甘油桥。

在细胞外溶液中，电压钳位的

当细胞被首先通过DHO-自由的

在实验中，我们已经证明了在细胞外溶液中的

钠-可刺激的

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FIGURE 1. Holding current ($I_H$) recorded over 23 minutes from an isolated guinea pig ventricular myocyte voltage clamped at $-40$ mV. Upper and lower traces are contiguous in time. Cell was twice exposed to 100 µM dihydroouabain (DHO) in the external solution during the periods indicated by the solid horizontal lines. Double diagonal lines in the upper and lower traces represent discontinuities in the record of 3 and 2 minutes, respectively. These two segments were removed because of a long washout in the first interval and long second DHO exposure time. After steady state in DHO was achieved, DHO was washed out and $I_H$ recovered towards the original current level shown by the dashed line. Vertical spikes in the traces are 10 msec, 10 mV hyperpolarizing pulses used to assess electrode resistance.

For this analysis, the difference current was assumed to be proportional to the concentration of the DHO/Na-K pump complex. 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 ($K_D=2.34$ µM and $V_{\text{max}}=0.456$ µA/µF, $\lambda=1$). 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 ($K_D=2.34$ µM and $V_{\text{max}}=0.559$ µA/µF, $\lambda=0.44$). 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 —90 by 1 mM Ba\(^{2+}\) and that may be DHO-sensitive. In our experiments, we assumed that the effect of DHO on membrane holding current is mediated solely by an inhibition of the Na-K pump current. The experimental conditions were chosen to assure the validity of this assumption. Holding potential was —40 mV. At this potential, Na\(^+\) current is expected to be totally inactivated.\(^{15}\) If any appreciable low threshold Ca\(^{2+}\) current is present, it too should be almost completely inactivated.\(^{16}\) In addition, because the voltage threshold for steady-state voltage-dependent activation of the high-threshold (L-channel type) calcium 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.\(^{17}\) 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.\(^{18}\) In addition, the delayed outward-rectifying K\(^+\) current is not expected to be activated at —40 mV.\(^{19}\) 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.

In the previous experiment, while [K\(^+\)]\(_o\) was set to 0 mM to remove Na-K pump current, [K\(^+\)]\(_o\) =0 mM also removes the inward-rectifying K\(^+\) current. 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\(^+\)]\(_o\). However, this could also produce a level of inward-rectifying K\(^+\) current that is insufficiently blocked by 1 mM Ba\(^{2+}\) and that may be DHO-sensitive. Therefore, the effect of DHO on membrane conductance was assessed using the same internal solution as before, but [K\(^+\)]\(_o\) in the external solution was 5.4 mM. The current-voltage relations (Figure 4) were determined by measuring membrane current at the end of 70-msec voltage steps from a holding potential of —40 mV to potentials ranging from —100 to —20 mV before and during exposure to 1,000 \(\mu\)M DHO. Lines were fit by linear regression to current-voltage data from cells in control and DHO-containing external solutions. No significant difference was found between the slopes as in the previous experiment indicating that the glycoside had no effect on steady-state membrane conductance at a holding potential of —40 mV. This finding agrees with earlier reports for intact guinea pig myocardium\(^{5}\) and sheep Purkinje fibers.\(^{14}\) The voltage-dependence of the DHO-sensitive current was also derived from the mean current-voltage curves obtained before and during exposure to 1,000 \(\mu\)M DHO and \(I_{DF}\) was plotted as a function of the membrane voltage. The result (inset, Figure 4) indicates that \(I_{DF}\) was not affected by the membrane voltage over the range of —100 to —20 mV.

**Discussion**

In our experiments, we assumed that the effect of DHO on membrane holding current is mediated solely by an inhibition of the Na-K pump current. The experimental conditions were chosen to assure the validity of this assumption. Holding potential was —40 mV. At this potential, Na\(^+\) current is expected to be totally inactivated.\(^{15}\) If any appreciable low threshold Ca\(^{2+}\) current is present, it too should be almost completely inactivated.\(^{16}\) In addition, because the voltage threshold for steady-state voltage-dependent activation of the high-threshold (L-channel type) calcium 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.
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DHO Inhibits Na-K Pump at Two Sites

**FIGURE 4.** Effect of dihydroouabain (DHO) on membrane conductance when [K+]o=5.4 mM. Holding potential=—40 mV. [Na+]i=30 mM. [Ba2+]o=1 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 μM 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).

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. 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+] 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. In that study, however, measurements were made under non-steady-state conditions. Intracellular sodium concentration was not controlled and possibly varied greatly from one 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_{[Na]}^*. Therefore, it is reasonable to assume that I_{[Na]}^* 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 3H-ouabain to guinea pig cardiac tissue. In contrast, however, a previous study on the effect of DHO on electrogenic Na-K pump current in guinea pig ventricular tissue reported that the concentration-inhibition relation can be fit by a linear Scatchard plot suggesting that functionally significant binding occurs at sites with a single affinity (K_D=46 μM) only. Similarly, despite findings of apparent binding of cardiac glycosides at both high- and low-affinity sites in canine cardiac tissue, 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 μM) is associated with Na-K pump inhibition.
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 electrogentic 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 $I_{DIF}$ suggests that the Na-K pump current may be essentially voltage-sensitive between $-100$ and $-20$ mV when defined under our particular experimental conditions. This finding is somewhat different from that reported by Gadsby et al. 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. 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, 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, 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 $I_{DIF}$. Despite this possibility the $I_{DIF}$ versus voltage curve is remarkably linear and voltage-independent. This result resembles findings in *Xenopus* oocytes reported by Eisner et al., which suggest that under conditions with intracellular sodium activity at a relatively normal level, strophantin-sensitive current also was voltage-insensitive. Their results 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. 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 electrogentic 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.

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