Amiodarone Inhibits the Na\(^+\)-K\(^+\) Pump in Rabbit Cardiac Myocytes after Acute and Chronic Treatment\(^1\)

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ABSTRACT

Amiodarone has been shown to affect cell membrane physicochemical properties, and it may produce a state of cellular hypothyroidism. Because the sarcolemmal Na\(^+\)-K\(^+\) pump is sensitive to changes in cell membrane properties and thyroid status, we examined whether amiodarone affected Na\(^+\)-K\(^+\) pump function. We measured Na\(^+\)-K\(^+\) pump current (I_{p}) using the whole-cell patch-clamp technique in single ventricular myocytes isolated from rabbits. Chronic treatment with oral amiodarone for 4 weeks reduced I_{p} when myocytes were dialyzed with patch-pipettes containing either 10 mM Na\(^+\) or 80 mM Na\(^+\). In myocytes from untreated rabbits, acute exposure to amiodarone in vitro reduced I_{p} when patch pipettes contained 10 mM Na\(^+\) but had no effect on I_{p} at 80 mM Na\(^+\). Amiodarone had no effect on the voltage dependence of the pump or the affinity of the pump for extracellular K\(^+\) either after chronic treatment or during acute exposure. We conclude that chronic amiodarone treatment reduces overall Na\(^+\)-K\(^+\) pump capacity in cardiac ventricular myocytes. In contrast, acute exposure of myocytes to amiodarone reduces the apparent Na\(^+\) affinity of the Na\(^+\)-K\(^+\) pump. An amiodarone-induced inhibition of the hyperpolarizing Na\(^+\)-K\(^+\) pump current may contribute to the action potential prolongation observed during treatment with this drug.

The mechanism of action of the widely used antiarrhythmic agent amiodarone is thought to involve use-dependent block of Na\(^+\), Ca\(^2+\), and K\(^+\) channels (see Nattel et al., 1992, for review). However, when studying electrophysiological effects of amiodarone, the Na\(^+\)-K\(^+\) pump should also be considered. The pump directly, or indirectly through the operation of secondary active ion transport mechanisms, maintains the transmembrane ionic gradients. These gradients in turn drive the channel currents that are modulated by amiodarone. In addition, the electrogenic Na\(^+\)-K\(^+\) pump current, arising from the 3Na\(^+\):2K\(^+\) exchange ratio, contributes to repolarization of the cardiac action potential (Gadsby et al., 1985).

Treatment with amiodarone influences physicochemical properties of the cell membrane, including fluidity and cholesterol content (Chatelain et al., 1986). Because the Na\(^+\)-K\(^+\) pump is affected by such changes in membrane properties (Cornelius, 1991), it seems reasonable to speculate that amiodarone may alter pump function. Amiodarone might also alter pump function through an alternative mechanism. This drug is believed to induce a state of cellular hypothyroidism with chronic use (Patterson et al., 1986; Singh et al., 1983). Thyroid status in turn is an important determinant of synthesis of Na\(^+\)-K\(^+\) pump units (Doohan et al., 1995; Kjeldsen et al., 1986).

Several previous studies using isolated myocardial membrane or microsomal preparations (Broekhuysen et al., 1972; Dzimir and Almotrefi, 1991) have suggested that acute exposure to amiodarone decreases Na\(^+\)-K\(^+\)/ATPase activity, the enzymatic equivalent of the Na\(^+\)-K\(^+\) pump. Although demonstrating that amiodarone has the potential to affect pump activity, the physiological relevance of such studies is unclear. The drug concentrations used were much higher than those encountered clinically, and the studies were performed using K\(^+\) and Na\(^+\) concentrations expected to saturate binding sites rather than at physiologically relevant concentrations. In addition, Na\(^+\)-K\(^+\)/ATPase studies require the pump molecule to be isolated from the native lipid membrane and purified. As the membrane lipid environment

ABBREVIATIONS: I_{p}, Na\(^+\)-K\(^+\) pump current; QTc, corrected QT interval; T_{a}, thyroxine; T_{b}, triiodothyronine; HEPES, N-2-hydroxyethylpiperazine-N\(^-\)2-ethanesulfonic acid; EGTA, ethylene glycol-bis(\(\beta\)-aminethy ether)-N,N,N\(^-\)N\(^-\)tetraacetic acid; TMA, tetramethylammonium; a_{i}, intracellular Na\(^+\) activity; [Na]_{pip}, pipette Na\(^+\) concentration; [K]_{o}, extracellular K\(^+\) concentration; K_{0.5}, concentration for half-maximal pump activation; V_{m}, membrane potential; I_{p}, pump current-voltage relationship.
modulates pump activity (Cornelius, 1991), it is unclear how these results relate to the activity of in situ pumps.

In the present study, we examined the effects of both acute and chronic amiodarone exposure on the Na\(^+\)-K\(^+\) pump in intact cardiac myocytes. We used the whole-cell patch-clamp technique to measure \(I_p\). This approach allows independent control of ligands for the Na\(^+\)-K\(^+\) pump on both sides of the intact native membrane, as well as control of membrane potential, variables that are important determinants of pump function.

Methods

Treatment protocols. Male New Zealand White rabbits, weighing 2.5 to 3.0 kg, were used in the study. Amiodarone was supplied in its hydrochloride powder form. In experiments examining the effects of chronic treatment, gelatin capsules containing appropriate quantities of amiodarone were made up at two weekly intervals. A single daily amiodarone capsule was administered to rabbits orally in a dose of 80 mg/kg/day for 4 weeks. This protocol was based on a previous study in rabbits demonstrating clinically relevant changes in electrophysiological and electrocardiographic parameters with 50 to 100 mg/kg oral amiodarone daily (Kodama et al., 1992). Control rabbits received empty gelatin capsules in the same manner.

A surface electrocardiogram was obtained before treatment and at the end of the 4-week treatment protocol to assess the effects of amiodarone on the QT interval. This parameter has been shown to correlate well with myocardial concentrations of amiodarone (Debbas et al., 1984). Bazett’s formula was used to correct the QT interval (QT\(_c\)) for heart rate (QT/\(\sqrt{RR}\) interval). The ECG electrodes were placed on the shaved precordium of conscious rabbits in positions that provided ECG deflections of adequate amplitude to make accurate determinations of QT interval. Recordings were performed using a single-channel ECG machine (Nihon Kohden Cardiofax, model SC-513E) at a paper speed of 50 mm/sec. Recordings were obtained from at least two lead orientations. Blood was taken from a marginal ear vein at base line and after 4 weeks of treatment to assess the effects of amiodarone on thyroid function. Serum total \(T_4\) and \(T_3\) were measured quantitatively using the Ciba Corning Autoassayed Chemiluminescence System (ACS:180 system, Ciba Corning Diagnostics Corp, Medfield, MA). The serum amiodarone level was determined in the blood sample collected after 4-week treatment. The level was measured using an HPLC method adapted from Law et al. (1984).

In experiments examining the acute effects of amiodarone on the Na\(^+\)-K\(^+\) pump, myocytes isolated from untreated rabbits were exposed to the drug in vitro. A 1 mM stock solution of amiodarone in 100% ethanol was prepared and used within 2 weeks. On the day of experimentation, the stock solution was diluted 100-fold in solutions used to superfuse the myocytes; thus, the superfusates had a nominal amiodarone concentration of 10 \(\mu\)M in 1% ethanol, but these solutions appeared to be supersaturated. We therefore measured amiodarone content in the superfusate using HPLC. The mean final amiodarone concentration in the superfusate was 0.61 ± 0.13 \(\mu\)M (\(n = 6\); range, 0.30–1.20 \(\mu\)M). Control myocytes in these experiments were exposed to superfusates containing 1% ethanol only.

Measurement of \(I_p\). Single ventricular myocytes were isolated as described previously (Hool et al., 1995). After isolation, myocytes were stored at room temperature until used for experimentation. Myocytes were used on the day of isolation only, and pump currents were measured 2 to 10 hours after the heart was excised. Myocytes were voltage clamped with wide-tipped (4–5 \(\mu\m\)) borosilicate glass pipettes made as described previously (Whalley et al., 1993). In experiments measuring \(I_p\) at a fixed membrane potential of about 40 mV, pipettes were filled with a solution containing (in mM) 70 potassium glutamate, 1 KH\(_2\)PO\(_4\), 5 HEPES, 5 EGTA, 2 MgATP and sodium glutamate plus TMA-Cl 90. The Na\(^+\) concentration in the solution was either 10 mM, which is near the physiological intracellular level, or 80 mM, a level expected to nearly saturate intracellular pump sites. The solution was titrated to pH 7.05 ± 0.01 at 35°C with 1 M KOH. In experiments designed to examine the relationship between \(I_p\) and membrane voltage pipettes were filled with a solution containing (in mM) 10 sodium glutamate, 1 KH\(_2\)PO\(_4\), 5 HEPES, 5 EGTA, 2 MgATP, 60 TMA-Cl, 20 tetraethylammonium chloride, 70 CsCl and 50 aspartic acid. To examine the \(I_p\)-Vm relationship at a high intracellular Na\(^+\) level, the solution was similar except that sodium glutamate was 80 mM, CsCl was 65 mM, aspartic acid was 45 mM, and TMA-Cl was omitted. The solution was titrated to pH 7.05 ± 0.01 at 35°C with 1 M HCl. When filled with the above solutions, patch pipettes had resistances of 0.8 to 1.2 MΩ.

Myocytes were suspended in a tissue bath mounted on an inverted microscope. They were superfused with modified Tyrode’s solution that contained (in mM) 140 NaCl, 5.6 KCl, 2.16 CaCl\(_2\), 1 MgCl\(_2\), 0.44 NaH\(_2\)PO\(_4\), 10 glucose and 10 HEPES. It was titrated to pH 7.4 at 35°C with 1 M NaOH. This solution was used while the whole-cell configuration was established and cell membrane capacitance was measured. In most experiments, the superfusate was then changed to a solution that was similar except that it was nominally Ca\(^++\)-free and in addition contained 2 mM BaCl\(_2\) and 0.2 mM CdCl\(_2\). Superfusates that were Ca\(^++\)-free and contained CdCl\(_2\) were used to prevent Ca\(^++\) overloading of myocytes when cells were patch-clamped with pipettes containing high Na\(^+\) concentrations. In experiments examining the effect of extracellular K\(^+\) on pump activity, we varied the K\(^+\) concentration of this Ca\(^++\)-free superfusate between 0 and 116 mM. TMA-Cl was used to maintain constant osmolality in these experiments.

Dose of ouabain. A previous study in noncardiac tissue suggested that amiodarone may competitively inhibit ouabain binding to the Na\(^+\)-K\(^+\) pump (Prasada Rao et al., 1986). To ensure that the concentration of ouabain used in experimental protocols (100 \(\mu\)M) was sufficient to completely block the Na\(^+\)-K\(^+\) pump in the presence of amiodarone, we performed a series of preliminary experiments on control myocytes and myocytes exposed to amiodarone in vitro. We found that after the pump had been inhibited by 100 \(\mu\)M ouabain, there was no additional shift in holding current on increasing the ouabain concentration to 500 \(\mu\)M, indicating that 100 \(\mu\)M ouabain caused complete pump inhibition. Unless otherwise indicated, \(I_p\) is identified by the shift in holding current induced by exposure of myocytes to 100 \(\mu\)M ouabain (Sigma Chemical, St. Louis, MO).

Membrane currents were recorded using the continuous single-electrode voltage-clamp mode of an Axoclamp-2A amplifier and Axopatch or pCLAMP software (Axon Instruments, Foster City, CA). Voltage-clamp protocols were generated with pCLAMP. Details of the experimental protocols used to measure membrane capacitance and \(I_p\) and details of the electronic recording system have been described previously (Whalley et al., 1993). \(I_p\) is reported normalized for membrane capacitance unless otherwise indicated.

Statistics. Results are expressed as mean ± S.E.M. Statistical comparisons are made using Student’s t test for paired or unpaired observations. Dunnnett’s test was used when the same control group was used for more than one comparison. P < .05 is regarded as significant in all comparisons. Nonlinear regression was used to fit the Hill equation to data.

Results

Effects of chronic amiodarone on ECG and thyroid function. Amiodarone is known to prolong the QT interval on the surface ECG and affects thyroid hormone levels by inhibiting peripheral conversion of \(T_2\) to \(T_3\). The effects of chronic treatment on thyroid function, heart rate, and QTc are summarized in table 1. There was no significant difference in any of these parameters between the control and treated groups of rabbits at base line. Amiodarone treatment
TABLE 1
Effects of chronic amiodarone treatment on thyroid function, heart rate and QTc.

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th></th>
<th>Amiodarone</th>
<th></th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>Base line</td>
<td>4 weeks</td>
<td></td>
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<tr>
<td>T3 (nmol/liter)</td>
<td>1.9 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>NS</td>
<td></td>
<td></td>
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<tr>
<td>T4 (nmol/liter)</td>
<td>61.4 ± 4.4</td>
<td>66.8 ± 5.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate</td>
<td>226 ± 10</td>
<td>227 ± 15</td>
<td>NS</td>
<td></td>
<td></td>
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<tr>
<td>QTc, (sec)</td>
<td>0.28 ± 0.003</td>
<td>0.27 ± 0.003</td>
<td>&lt;.007</td>
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<sup>a</sup> Level of significance for differences in variables at 4 weeks compared with base line in each group of rabbits.
<sup>b</sup> Level of significance for differences between control rabbits and amiodarone-treated rabbits after 4-week treatment.

Amiodarone treatment causes a reduction in overall Na<sup>+</sup> uptake.

The treatment protocol was well tolerated. One rabbit lost weight during the treatment period and was found to have a toxic serum amiodarone level of 3.9 μmol/liter. Its surface ECG showed slow (80 bpm) broad QRS complexes. Results from this rabbit were not included in the analysis.

**Effect of chronic amiodarone treatment on Ip**. Myocytes from 5 control rabbits and 5 rabbits treated with amiodarone were voltage-clamped at −40 mV, and Ip was measured using pipettes with Na<sup>+</sup> concentrations ([Na<sub>pip</sub>]) of 10 or 80 mM. Results from these experiments are summarized in figure 1A. When [Na<sub>pip</sub>] was 10 mM, mean Ip of 14 myocytes from rabbits treated with amiodarone was 0.24 ± 0.03 pA/pF, whereas mean Ip of 10 myocytes from rabbits given placebo was 0.36 ± 0.03 pA/pF. This 33% difference was statistically significant. Mean Ip in myocytes from the rabbits given placebo was similar to mean Ip in myocytes from untreated rabbits reported previously (Gray et al., 1997). When [Na<sub>pip</sub>] was 80 mM, amiodarone treatment resulted in a significant, 25% reduction in mean Ip (1.46 ± 0.07 pA/pF, n = 7, vs. 1.94 ± 0.05 pA/pF, n = 6). We conclude that chronic amiodarone treatment causes a reduction in overall Na<sup>+</sup>-K<sup>+</sup> pump capacity.

**Effect of chronic amiodarone on the affinity of the Na<sup>+</sup>-K<sup>+</sup> pump for extracellular K<sup>+</sup>**. Because it has been suggested that the effect of amiodarone on Na<sup>+</sup>-K<sup>+</sup>/ATPase is K<sup>+</sup>-dependent (Almotrefi and Dzimiri, 1991), we examined the effect of chronic amiodarone treatment on the apparent affinity of the pump for extracellular K<sup>+</sup> concentrations ([K<sub>e</sub>]). We measured changes in Ip in response to changes in [K<sub>e</sub>]. To facilitate the detection of small pump currents at low [K<sub>e</sub>], we used [Na<sub>pip</sub>] of 80 mM to induce near-maximal activation of the pump at intracellular sites.

After establishing the whole-cell configuration, we inactivated the pump by switching to a K<sup>+</sup>-free superfusate. This superfusate was nominally Ca<sup>2+</sup>-free and contained 2 mM CdCl<sub>2</sub> to eliminate Ca<sup>2+</sup> channel currents and Na<sup>+</sup>-Ca<sup>2+</sup> exchange current. Myocytes were voltage clamped at −40 mV, and Ip was identified as the membrane current resulting from reactivation of the pump on exposure to different [K<sub>e</sub>]. Each myocyte was exposed to a random sequence of at least three of the following [K<sub>e</sub>] concentrations (in mM): 0.5, 1, 2, 3, 5.6 and 15. The resulting currents were normalized relative to the current at 5.6 mM [K<sub>e</sub>], Ip (%). This concentration was therefore used in every series of exposures. Each exposure to a K<sup>+</sup> concentration was bracketed by reexposure to the K<sup>+</sup>-free superfusate until Ip had returned to its base-line level.

We previously published an illustration of the experimental protocol and representative traces of membrane currents (Gray et al., 1997). To examine whether pump run-down occurred, we concluded the protocol by reexposing a number of myocytes to the first [K<sub>e</sub>] concentration used. There was no...
Evidence of pump rundown in the time taken to conclude the experimental protocol (~18–20 min). We previously determined that K\(^+-\)induced shifts in holding currents attributed to the activation of the Na\(^+-\)K\(^+\) pump were not contaminated by other K\(^+\)-sensitive currents (Gray et al., 1997).

Experiments were performed on 19 myocytes from 5 amiodarone-treated rabbits and 17 myocytes from 4 control rabbits. The [K]o/I\(p\)\% relationships are summarized in figure 1B. When the Hill equation was fitted to the data, we obtained a K\(^+\) concentration for half-maximal pump activation (K\(0.5\)) of 2.3 mM for myocytes from amiodarone-treated rabbits and 2.7 mM for control rabbits. The Hill coefficients were 1.58 and 1.59, respectively. To allow statistical comparisons, we repeatedly fitted the Hill equation to randomly selected series of values for I\(p\) at all six [K]o concentrations tested. This allowed us to derive mean K\(0.5\) values and mean Hill coefficients for 10 derived [K]o activation curves for myocytes from the amiodarone-treated rabbits and 11 derived [K]o activation curves for myocytes from control rabbits. There was no significant difference between the mean values. We conclude that chronic amiodarone treatment does not affect the apparent K\(^+\) affinity of the Na\(^+-\)K\(^+\) pump.

**Effect of chronic amiodarone on the I\(p\)-V\(m\) relationship.** Because amiodarone is an amphiphile and amphiphiles can alter the voltage dependence of the Na\(^+-\)K\(^+\) pump (Läuger, 1991), we examined whether chronic amiodarone treatment affects the I\(p\)-V\(m\) relationship. After establishing the whole-cell configuration, we superfused myocytes with Ca\(^++\)-free modified Tyrode's solution, and we voltage-clamped the myocytes at −40 mV. We then applied 320-msec voltage steps in 20-mV increments to test potentials ranging from −100 to +60 mV. Averaged membrane currents at each test potential after superfusion of ouabain were subtracted from the respective averaged membrane currents before ouabain exposure to derive I\(p\) at each test potential. Experiments were performed at [Na]\(\text{pip}\) of both 10 and 80 mM. Figure 2A illustrates the voltage step protocol and an example of the resulting membrane currents for a myocyte studied using [Na]\(\text{pip}\) of 10 mM. Details of the experimental protocol and data analysis have been published previously (Gray et al., 1997).

The mean I\(p\)-V\(m\) relationships for 13 myocytes from 5 amiodarone-treated rabbits and 17 myocytes from 8 control rabbits at [Na]\(\text{pip}\) of 10 mM are illustrated in figure 2B. In this and all other I\(p\)-V\(m\) relationships, we normalized I\(p\) to the current measured at 0 mV to facilitate comparisons between experiments. The I\(p\)-V\(m\) relationships in myocytes from both groups of rabbits are near-linear and have a positive slope over the range of voltages examined. There were no major differences in the relationships for myocytes from the two groups. The mean I\(p\)-V\(m\) relationships for 7 myocytes from 3 amiodarone-treated rabbits and 8 myocytes from 3 control rabbits at [Na]\(\text{pip}\) of 80 mM are shown in figure 2C. The relationships in myocytes from both groups of rabbits are virtually superimposable, exhibiting a positive slope at negative potentials and a negative slope at positive potentials. We conclude that chronic amiodarone treatment does not affect the I\(p\)-V\(m\) relationship, at either physiological levels of intracellular Na\(^+\) or levels of intracellular Na\(^+\) expected to cause near-saturation of Na\(^+\) binding sites.

**Effect of chronic amiodarone on intracellular Na\(^+\).** If chronic amiodarone treatment inhibits Na\(^+-\)K\(^+\) pump activity, it may be expected to alter steady-state intracellular Na\(^+\) activity (a'\(\text{Na}\)). However, the direction and magnitude of any such change in a'\(\text{Na}\) cannot be assumed because amiodarone may also influence Na\(^+\) influx. We therefore examined the
effect of chronic amiodarone treatment on \( a^{1}_{Na} \). We determined \( a^{1}_{Na} \) in papillary muscles from 11 control rabbits and 11 rabbits treated with amiodarone for 4 weeks. We used ion-sensitive microelectrodes to measure \( a^{1}_{Na} \) as described previously (Hool et al., 1995). One or two determinations of \( a^{1}_{Na} \) were made with separate microelectrode impalements in tissue from each rabbit. Where two measurements were made, the mean value was used for statistical comparisons. Determinations of \( a^{1}_{Na} \) were made only after microelectrode recordings had been stable for \( \geq 20 \) minutes. The \( a^{1}_{Na} \) in control rabbits was \( 8.1 \pm 0.4 \) mM. In amiodarone-treated rabbits, \( a^{1}_{Na} \) was \( 9.8 \pm 0.8 \) mM. This difference was not statistically significant (\( P = .07 \)). Amiodarone is expected to reduce \( \text{Na}^{+} \) influx via \( \text{Na}^{+} \) channels (Mason et al., 1984); because amiodarone reduces cardiac metabolic rate (Charlier et al., 1988), treatment may have reduced \( \text{Na}^{+} \) influx via the \( \text{Na}^{+}\text{-H}^{+} \) exchanger. One may speculate that such decreases in \( \text{Na}^{+} \) influx partially offset the rise in \( a^{1}_{Na} \) expected from pump inhibition.

**Acute effect of amiodarone on \( I_p \).** We next examined whether acute exposure of myocytes to amiodarone affected the \( \text{Na}^{+}\text{-K}^{+} \) pump. For these experiments, myocytes were isolated from untreated rabbits and exposed to amiodarone in the tissue bath. After establishing the whole-cell configuration, we superfused myocytes with \( \text{Ca}^{2+} \)-free Tyrode’s solution that contained 2 mM BaCl\(_2\) and either amiodarone in 1% ethanol or 1% ethanol alone. The cells were exposed to this superfusate for a minimum of 6 minutes before changing to a superfusate that was similar except that it contained 100 \( \mu \)M ouabain. The duration of exposure was adopted from previous studies on \( \text{Na}^{+} \) channels (Follmer et al., 1989) in cardiac myocytes. These studies indicated that steady state effects were achieved after \( \approx 6 \)-min exposure to amiodarone \textit{in vitro}.

When \( \text{[Na]}_{\text{pip}} \) was 10 mM, mean \( I_p \) of 10 myocytes exposed to (nominally) 10 \( \mu \)M amiodarone in 1% ethanol was \( 0.19 \pm 0.02 \) pA/pF, whereas mean \( I_p \) of 10 myocytes exposed to ethanol alone was \( 0.31 \pm 0.03 \) pA/pF. This 39% reduction was statistically significant. Mean \( I_p \) of myocytes exposed to 1% ethanol was similar to mean \( I_p \) of myocytes from untreated rabbits not exposed to ethanol (Gray et al., 1997; Whalley et al., 1993), indicating that 1% ethanol does not affect \( I_p \) when membrane potential is held constant at -40 mV. We performed an additional four experiments to determine that the duration of exposure was adequate to achieve steady state effects. In these experiments, myocytes were exposed to amiodarone for \( \geq 20 \) min (range, 20 to 28 min) before \( I_p \) was measured. Mean \( I_p \) (0.21 \pm 0.01 pA/pF) was similar to the mean \( I_p \) of myocytes exposed to amiodarone for \( \approx 6 \) min only (0.19 \pm 0.02 pA/pF). This indicates that steady state was achieved using 6-min exposure.

To examine whether acute exposure of myocytes to amiodarone \textit{in vitro} also causes pump inhibition when intracellular \( \text{Na}^{+} \) is at high levels, we measured \( I_p \) using a \( \text{[Na]}_{\text{pip}} \) of 80 mM. Mean \( I_p \) of 6 myocytes was 1.90 \pm 0.19 pA/pF, which is not significantly different from mean \( I_p \) in 6 control myocytes (1.94 \pm 0.05 pA/pF). The mean \( I_p \) values measured using a \( \text{[Na]}_{\text{pip}} \) of either 10 and 80 mM in myocytes exposed to amiodarone and control myocytes are summarized in figure 3A. We conclude from these experiments that acute exposure to amiodarone inhibits \( \text{Na}^{+}\text{-K}^{+} \) pump function when intracellular \( \text{Na}^{+} \) is near physiological levels, whereas it has no
Effect of acute amiodarone on the affinity of the Na⁺-K⁺ pump for extracellular K⁺. To examine the effect of acute amiodarone exposure on K⁺ affinity, we measured Iₚ at different levels of [K]ₒ. We used a [Na]ₚ of 80 mM. After establishing the whole-cell configuration, we inactivated the Na⁺-K⁺ pump by superfusing myocytes with K⁺-free Tyrode’s solution. This superfusate also contained amiodarone. Myocytes were then voltage-clamped at −40 mV, and after a minimum of 6-min exposure to the amiodarone-containing superfusate, the pump was reactivated by exposing myocytes to a superfusate that was similar except that it contained different [K]ₒ values. In contrast to experiments examining the effects of chronic amiodarone treatment, each myocyte was exposed in a random sequence to all of the following [K]ₒ values (in mM): 1, 2, 3, 5.6 and 15. Results were normalized relative to 5.6 mM [K]ₒ. Each exposure to a [K]ₒ concentration was bracketed by return to the K⁺-free superfusate until Iₚ had returned to its baseline level. Control myocytes were maintained in the whole-cell configuration for the same duration as amiodarone-exposed myocytes before Iₚ was measured.

Nine myocytes were acutely exposed to amiodarone in vitro. When the Hill equation was fitted to the data, we obtained a Kₚ,5 value of 2.50 mM and a Hill coefficient of 1.84. To allow statistical comparisons, we fitted data from each experiment to the Hill equation to derive the respective mean values for Kₚ,5 and Hill coefficient. There was no significant difference between the mean values of either for myocytes exposed to amiodarone or control myocytes. The [K]ₒ/Iₚ% relationship for myocytes exposed to amiodarone has been plotted in figure 3B along with the relationship for control myocytes. We conclude from these experiments that acute exposure of myocytes to amiodarone has no effect on the apparent [K]ₒ affinity of the Na⁺-K⁺ pump.

Effect of acute amiodarone on the Iₚ-Vₚm relationship. We next examined whether acute exposure of myocytes to amiodarone affects the Iₚ-Vₚm relationship. After establishing the whole-cell configuration, the superfusate was changed to Ca⁺⁺-free Tyrode’s solution that contained either amiodarone in 1% ethanol or 1% ethanol alone. The myocytes were voltage-clamped at −40 mV; after a minimum exposure time of 6 min to this superfusate, we applied the voltage step protocol illustrated in figure 2A before and after exposure to 100 μM ouabain as outlined previously. Because in vitro exposure had no effect on Iₚ, when [Na]ₚ was 80 mM, these experiments were only performed at [Na]ₚ of 10 mM.

The mean Iₚ-Vₚm relationships for 9 myocytes exposed to amiodarone and 8 myocytes exposed to ethanol have been plotted in figure 3C. The Iₚ-Vₚm relationships in myocytes exposed to amiodarone and to ethanol demonstrated a positive slope at negative potentials and a negative slope at positive potentials. The negative slope at positive potentials is not seen in control myocytes not exposed to amiodarone or ethanol (Gray et al., 1997). It would thus appear that the negative slope at positive potentials is due to an effect of the vehicle, 1% ethanol, rather than an effect of amiodarone. We conclude that acute exposure to amiodarone has no effect on the Iₚ-Vₚm relationship.

Discussion

The major finding of our study is that both chronic and acute exposure to amiodarone decreased Na⁺-K⁺ pump activity in intact cardiac myocytes. After chronic treatment, pump activity was reduced at both low and high [Na]ₚ. This pattern suggests a decrease in overall pump capacity. Acute exposure to amiodarone in vitro decreased pump activity only when [Na]ₚ was near the physiological level for intracellular Na⁺ and had no significant effect at levels expected to saturate intracellular pump sites. This pattern is consistent with a decrease in the apparent affinity of the pump for intracellular Na⁺. A difference in effects between acute and chronic exposure is also well recognized for the clinical use of the drug (Ikeda et al., 1984) and for its effects on ion channel function (Varró et al., 1996). The [Na]ₚ-dependent difference in effects between chronic and acute exposure suggests that amiodarone affects the pump via two different mechanisms. With chronic exposure, amiodarone might affect the pump via both mechanisms: one related to a decrease in the abundance of Na⁺-K⁺ pumps and one due to a direct effect of the drug in the membrane. However, amiodarone induced a similar degree of inhibition with chronic and acute exposure when [Na]ₚ was 10 mM. This similarity might be explained by wash-out of amiodarone while cells from treated animals were maintained in amiodarone-free solutions for 2 to 10 hr before Iₚ was measured, with a loss of a direct effect of the drug bound to the membrane.

Two previous studies have examined the effect of chronic amiodarone treatment on the Na⁺-K⁺ pump. Prasada Rao et al. (1986) administered amiodarone parenterally to rats for 3 weeks. They found that amiodarone had no effect on activity of brain synaptosome Na⁺-K⁺/ATPase activity. To our knowledge, a study by Hensley et al. (1994) provides the only previous data on cardiac tissue. They examined the effects of 3- to 6-week parenteral amiodarone therapy on protein expression and mRNA levels for the alpha and beta subunits of the Na⁺-K⁺ pump in rat ventricular muscle. At 3 weeks, the abundance of the alpha-1, alpha-2 and beta-1 subunits was significantly decreased. This was accomplished by a 30% to 35% decrease in serum T₃ and T₄ levels consistent with systemic amiodarone-induced hypothyroid state. Because hypothyroidism reduces synthesis of Na⁺-K⁺ pump subunits in the heart (Horowitz et al., 1990, Kamitani et al., 1992), the decrease in subunit abundance at 3 weeks might be due to the effect of amiodarone on thyroid status. After 6-week therapy, T₃ and T₄ levels and alpha-1 and beta-1 subunit abundance had returned to control values, whereas the abundance of the alpha-2 subunit remained significantly depressed. Alpha-2 subunits do not appear to be quantitatively important in the rat, and Hensley et al. (1994) emphasized that the functional significance of altered alpha-2 expression was uncertain. The present study does not identify effects of amiodarone on specific subunits; however, it does indicate that chronic treatment has a functionally significant inhibitory effect on the sarcolemmal Na⁺-K⁺ pump.

Our study indicates that chronic treatment with amiodarone induces a decrease in Iₚ, when the pump is maximally stimulated. Maximal Iₚ is highly correlated with the number of Na⁺-K⁺ pump units expressed in the plasmalemma of voltage-clamped oocytes (Jaunin et al., 1992). If the same applies to pump units in the sarcolemmal membrane, our
results suggest that amiodarone induces a decrease in the number of functional Na\(^{+}\)-K\(^{+}\) pump units. The amiodarone-induced decrease in pump function occurred in the absence of systemic hypothyroidism. However, this does not rule out the possibility that amiodarone exerted its effect on the pump via interference with thyroid hormone action at the intracellular level because amiodarone inhibits the binding of T\(_3\) to its nuclear receptors (Drovota et al., 1994; Latham et al., 1987). The pattern of pump inhibition is similar to that reported by Doohan et al. (1995) in rabbits with experimentally induced hypothyroidism. It is also of interest to note that some of the electrophysiological effects of amiodarone, including prolongation of action potential duration, QT interval and sinus cycle length, are at least partly dependent on thyroid hormone activity and are greatly diminished or abolished by concurrent hypothyroidism (Polikar et al., 1986; Talajic et al., 1989).

Several previous studies have reported inhibition of purified cardiac Na\(^{+}\)-K\(^{+}\)/ATPase, the enzymatic equivalent of the Na\(^{+}\)-K\(^{+}\) pump, during acute exposure to amiodarone in vitro (Broekhuyzen et al., 1972; Dzimir and Almotrefi, 1991). Although these studies suggest that acute amiodarone exposure may inhibit the pump, their significance is unclear for several reasons. Isolation of Na\(^{+}\)-K\(^{+}\)/ATPase from the sarcoplasmic membrane necessarily involves removing the pump molecule from its native lipid environment. Because the lipid environment is an important regulator of pump activity (Cornelius, 1991) and may itself be influenced by amiodarone (Chatelain et al., 1985, 1986), the response of isolated Na\(^{+}\)-K\(^{+}\)/ATPase to amiodarone may not be representative of the behavior of the pump in the intact myocyte. In the studies by Broekhuyzen et al. (1972) and Dzimir and Almotrefi (1991), Na\(^{+}\)-K\(^{+}\)/ATPase was examined using saturating concentrations of Na\(^{+}\) and K\(^{+}\) to produce maximal pump activity. They therefore provide no information about the effect of amiodarone on the activity of the pump at physiological ligand concentrations. Finally, the concentrations of amiodarone necessary to induce significant ATPase inhibition were substantially higher than those encountered clinically.

In the only published study on intact cardiac muscle, Aomine (1989) indirectly determined the acute effects of amiodarone on pump activity in guinea pig papillary muscles using conventional microelectrode techniques. He found that exposure to 44 \(\mu\)M amiodarone decreased the postoverdrive hyperpolarization attributed to pump activity. Somewhat paradoxically, no effect was observed at the higher concentration of 440 \(\mu\)M.

Our in vitro study differs from previous studies in several aspects. We examined the effect of 0.3 to 1.2 \(\mu\)M amiodarone, a concentration range similar to that encountered clinically, and we examined the pump in its intact membrane while varying extracellular and intracellular ligand concentrations. In contrast to the studies on isolated ATPase, we found no effect on \(I_p\) measured under conditions expected to maximally stimulate the pump. However, pump inhibition could be demonstrated when we used a [Na\(_{p}\)] near physiological levels for intracellular Na\(^{+}\). This is consistent with a decrease in the apparent affinity of the pump for intracellular Na\(^{+}\) without an influence on overall pump capacity.

Although the mechanism for modulation of the Na\(^{+}\) affinity of the pump cannot be determined from our study, one may speculate that the effect of amiodarone could be due to modification of the physicochemical properties of the lipid membrane. Amiodarone is known to insert into the hydrocarbon core of the lipid bilayer and decrease membrane lipid mobility and membrane fluidity (Chatelain et al., 1986). Changes in membrane fluidity, in turn, may affect pump activity by impairing lateral movement of the pump molecule and hence conformational changes during the pump cycle (see Cornelius, 1991, for review). An alternative explanation may be provided by the cationic amphiphile nature of amiodarone. Cationic amphiphiles have been shown to decrease the apparent Na\(^{+}\) affinity of Na\(^{+}\)-K\(^{+}\)/ATPase reconstituted into liposomes (Cornelius, 1995).

Chronic treatment with amiodarone reduced \(I_p\) by 33% when [Na\(_{p}\)] was 10 mM. This inhibitory effect is similar to that estimated for clinical use of digoxin (Rasmussen et al., 1990; Schmidt et al., 1991). This finding may have implications for our understanding of the effect of the drug when used clinically. It prolongs action potential duration and myocardial refractoriness, effects that are believed to be largely mediated via block of voltage-gated K\(^{+}\) channels (Balser et al., 1991). The Na\(^{+}\)-K\(^{+}\) pump generates a hyperpolarizing membrane current that contributes to repolarization. This effect becomes more significant as intracellular Na\(^{+}\) increases, for example, during tachyarrhythmias (Gadsby, 1982). The inhibitory effect of amiodarone on the pump is therefore expected to contribute, at least in part, to the class III effects of the drug.

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