Short- and Long-Term Effects of Amiodarone on the Two Components of Cardiac Delayed Rectifier K⁺ Current

Kaichiro Kamiya, MD; Atsushi Nishiyama, MD; Kenji Yasui, MD; Mayumi Hojo, BS; Michael C. Sanguinetti, PhD; Itsuo Kodama, MD

Background—Amiodarone is the most promising drug for the treatment of life-threatening tachyarrhythmias in patients with structural heart disease. The pharmacological effects of amiodarone on cardiac ion channels are complex and may differ for short-term and long-term administration.

Methods and Results—The delayed rectifier K⁺ current (I_K) of ventricular myocytes isolated from rabbit hearts was recorded with the whole-cell voltage-clamp technique. I_K was separated into 2 components by use of specific blockers for either I_Kr (chromanol 293B, 30 μmol/L) or I_Ks (E-4031, 10 μmol/L). Short-term application of amiodarone caused a concentration-dependent decrease in I_K with an IC₅₀ of 2.8 μmol/L (n=8) but only a minimal reduction in I_Kr. The short-term effects of amiodarone were also determined in Xenopus oocytes expressing the cloned human channels that conduct I_Kr and I_Ks (HERG and KvLQT1/minK). HERG current in oocytes was reduced by amiodarone (IC₅₀=38 μmol/L), whereas KvLQT1/minK current was unaffected by 300 μmol/L amiodarone. To study the effects of long-term drug administration, rabbits were treated for 4 weeks with oral amiodarone (100 mg · kg⁻¹ · d⁻¹) before cell isolation. Long-term administration of amiodarone decreased I_K to 55% (n=10) in control rabbits and altered the relative density of I_Kr and I_Ks. The majority (92%) of current was I_Kr. mRNA levels of rabbit ERG, KvLQT1, and minK in left ventricular myocardium did not differ between control and long-term amiodarone.

Conclusions—Amiodarone has differential effects on the 2 components of I_K, depending on the application period; short-term treatment inhibits primarily I_Kr, whereas long-term treatment reduces I_Ks. (Circulation. 2001;103:1317-1324.)

Key Words: amiodarone • potassium • mRNA

Amiodarone is an effective drug for the treatment of life-threatening ventricular tachyarrhythmias. Amiodarone has been referred to as a class III antiarrhythmic agent, but its pharmacological action is in fact very complex. It prolongs both action potential duration and refractory period when administered long term but blocks Na⁺ and Ca²⁺ channels after short-term administration. Amiodarone also has noncompetitive antisympathetic effects and modulates thyroid function, phospholipid metabolism, and production of certain cytokines. What specific action or combination of actions is fundamental and salutary for potent antiarrhythmic activity is not known. As recently reviewed, the short-term and long-term effects of amiodarone on action potentials and ionic currents of cardiac cells are quite different.

Delayed rectifier K⁺ currents (I_K) are important determinants of cardiac repolarization. I_K is composed of 2 distinctive components: a rapidly activating component showing inward rectification (I_Kr) and a slowly activating component (I_Ks) with a linear current-voltage (I-V) relationship. K⁺ channel genes that encode I_Kr and I_Ks are HERG and KvLQT1 plus minK, respectively. The effects of amiodarone on I_K are still limited and controversial. Therefore, in the present study, we examined the short- and long-term effects of amiodarone on I_Kr and I_Ks in rabbit ventricular myocytes. In addition, we studied the short-term effects of amiodarone on human ERG and KvLQT1/minK channels expressed heterologously in Xenopus oocytes. The effects of long-term amiodarone on rabbit ERG, KvLQT1, and minK mRNA were also examined.

Methods

Rabbit Ventricular Myocytes

Japanese white rabbits of either sex weighing 1.5 to 2.2 kg were killed under anesthesia with thymalir sodium (30 mg/kg IV), and the hearts were removed. Single myocytes were isolated enzymatically from the apical region of the left ventricle (approximately one third in the apex/base axis) as described previously. To observe the long-term effects of amiodarone, the rabbits were administered oral amiodarone (100 mg · kg⁻¹ · d⁻¹) for 4 weeks before cell isolation.

Electrophysiological Recording

The single-pipette, whole-cell, voltage-clamp technique was used for recording membrane currents. Cell capacitance was measured by...
integrating the capacitive transient evoked by applying a hyperpolarizing pulse to \(-5\) mV from a holding potential of \(-50\) mV. The cell capacitance and series resistance were electrically compensated by 60% to 90%. Command potential generation and data acquisition were controlled by pCLAMP software (version 6.0.3, Axon Instruments) and an IBM-compatible computer. Current signals were filtered at 1 kHz and digitized at a sampling frequency of 2 kHz. Current measurement was started after 40-minute superfusion with normal Tyrode's solution to avoid possible retention of amiodarone and its active metabolite (desethylamiodarone) on the cell surface in rabbits with oral treatment.

**cRNA Injection and Voltage-Clamp of Oocytes**

Isolation and maintenance of *Xenopus* oocytes and injection with HERG cRNA were performed as described previously. Stage V and VI oocytes were injected with 10 ng HERG cRNA. *KVLQT1* cRNA (5 ng) and *minK* cRNA (1 ng) were co-injected to induce \(I_{Kc}\). Currents were recorded at room temperature (22°C to 24°C) by standard 2-microelectrode voltage-clamp techniques to 2 to 4 days after cRNA injection.

**Ribonuclease Protection Assay**

For the ribonuclease protection assay, rabbit *ERG, KVLQT1,* and *minK* subunit cDNA fragments were amplified by reverse-transcriptase polymerase chain reaction. The nucleotide sequences of the primers and the amplified regions are listed in Table 1. Gene bank accession numbers are U75212 (nucleotides 187 to 456) for *ERG*, U70068 (nucleotides 502 to 1133) for *KVLQT1*, and L41659 (nucleotides 239 to 477) for *minK*. A HindIII site (AAGCTT) was introduced into the 5' end of the sense primers of the rabbit *ERG, KVLQT1,* and *minK*. The amplified cDNA was cloned into pGEM-T vector with the TA cloning system (Promega). The ribonuclease protection assay was performed as described previously.

**Solution and Drugs**

The Tyrode’s solution used for cell isolation and the single myocyte experiments was composed of (mmol/L) NaCl 143, KCl 5.4, CaCl\(_2\) 1.8, MgCl\(_2\) 0.5, Na\(\text{H}_2\)PO\(_4\) 0.25, HEPES 5.0, and glucose 5.6; pH was adjusted to 7.4 with NaOH. The glass pipette had a resistance of 3 to 5 MΩ after filling with the internal pipette solution containing (mmol/L) KOH 60, KCl 80, aspartate 40, HEPES 5, MgATP 5, sodium creatinine phosphate 5, and CaCl\(_2\) 0.65 (P\(\text{Ca}\) 8.0) at pH 7.2. The external solution used to measure \(I_{K1}\) currents was maintained at 34°C and was composed of the following (mmol/L):
**TABLE 2. Activation and Deactivation Time Constants of I_{Kr} and I_{Ks} in Rabbit Ventricular Myocytes Isolated From Hearts After Long-Term Amiodarone Treatment**

<table>
<thead>
<tr>
<th></th>
<th>Activation Time Constants, τ, ms</th>
<th>Deactivation Time Constants, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>293B-resistant current (I_{Kr})</td>
<td>482±41</td>
<td>191±26</td>
</tr>
<tr>
<td>293B-sensitive current (I_{Ks})</td>
<td>585±34</td>
<td>205±41</td>
</tr>
<tr>
<td>Chronic amiodarone (n=8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>293B-resistant current (I_{Kr})</td>
<td>465±43</td>
<td>221±21</td>
</tr>
</tbody>
</table>

N-methyl-D-glucamine 149, MgCl₂ 5, HEPES 5, and nisoldipine 0.003. Iₖᵣ and Iₖₛ were separated by applying 2 specific blockers, chromanol 293B (30 μmol/L)¹⁷ and E-4031 (10 μmol/L), respectively.

Oocytes were bathed in a modified ND96 solution containing (mmol/L) NaCl 94, KCl 4, MgCl₂ 2, CaCl₂ 0.1, and HEPES 5 (pH 7.6). CaCl₂ was reduced to 0.1 mmol/L to suppress endogenous Ca²⁺-activated Cl⁻ current.

Amiodarone hydrochloride (Sigma Chemical Co) was dissolved in dimethyl sulfoxide to prepare a stock solution of 300 mmol/L. On the day of experiments, aliquots of the stock solution were diluted with the bath solution. Dimethyl sulfoxide at 0.1% had no significant effect on outward currents in rabbit ventricular myocytes or Xenopus oocytes. E-4031 was kindly provided by Eisai Pharmaceuticals (Tokyo, Japan); chromanol 293B, by Aventis Pharma (Frankfurt, Germany).

**Statistical Analysis**

Data are presented as mean±SEM unless otherwise specified. When relative densities of Iₖᵣ and Iₖₛ and effects of long-term amiodarone administration on the ratio were determined, current amplitudes from 2 to 3 myocytes per each rabbit heart were averaged and served as 1 datum point. Statistical comparisons between the different experimental groups were obtained by ANOVA. Comparisons between multiple group means were performed with a Bonferroni-corrected t test for all group comparisons. Differences were considered significant at P<0.05. Concentration-response relationships were fit to the Hill equation to determine the concentration of drug required for 50% inhibition (IC₅₀). A nonlinear least-squares curve-fitting program (CalmWfit 6.0) was used to analyze deactivation kinetics.

**Results**

Iₖᵣ and Iₖₛ of Rabbit Ventricular Myocytes

The relative contributions of chromanol 293B–resistant and sensitive components of Iₖ in rabbit isolated ventricular

Figure 2. Short-term effects of amiodarone on I_{Kᵣ}. I_{Kᵣ} was elicited by same voltage-clamp pulse protocol as in Figure 1. A, Representative current traces in ventricular myocyte pretreated with 30 μmol/L chromanol 293B. Bath application of 10 μmol/L amiodarone caused marked inhibition of 293B-resistant component of I_{Kᵣ} (I_{Kᵣ}). Additional application of 10 μmol/L E-4031 resulted in complete elimination of time-dependent currents. B, Iₖᵣ-V relationship for I_{Kᵣ} before and after application of 1 and 10 μmol/L amiodarone. Density of peak tail current (mean±SE, n=8) was plotted vs test potentials (P<0.05 vs control). C, Concentration-response relationship for block of tail current after pulse to 50 mV by amiodarone. IC₅₀ and Hill coefficient were 2.8 μmol/L and 0.91, respectively (n=8).
myocytes are illustrated in Figure 1. Depolarizing pulses for 2 seconds from −40 to 70 mV were applied from the holding potential at −75 mV at 0.03 Hz. The tail current (I_K, tail) was measured on repolarization back to −50 mV (Figure 1A). Application of 30 μmol/L chromanol 293B decreased the time-dependent outward current during depolarization and the tail current on repolarization. Additional application of 10 μmol/L E-4031 eliminated most time-dependent current. Figure 1B illustrates the chromanol 293B–sensitive component obtained by subtraction of the current traces. The chromanol 293B–resistant and –sensitive components of I_K were used in this study to define I_Kr and I_Ks, respectively.

Figure 1C shows a tail current-voltage (I-V) relationship for total I_K, I_Kr, and I_Ks in control rabbit ventricular myocytes (n=10, 22 cells). Based on sensitivity to chromanol 293B, 71% of the tail current amplitude after depolarization to 50 mV was attributable to I_Kr and 29% to I_Ks. Tail current amplitudes were normalized to peak values, and the resulting I-V relation was fitted to a Boltzmann equation to construct isochronal activation curves (Figure 1D).

The time courses of activation at 50 mV and deactivation at −50 mV were determined by exponential fitting. The time constants of I_Kr and I_Ks in control myocytes are summarized in Table 2. There were no significant differences between the 2 components of I_K at these potentials.

Short-Term Effects of Amiodarone on I_Kr and I_Ks

The short-term effects of amiodarone on I_Kr were examined in ventricular myocytes pretreated with 10 μmol/L E-4031 (Figure 2). Application of 10 μmol/L amiodarone caused minimal inhibition of E-4031–resistant component of I_Kr (a, b). Additional application of 30 μmol/L 293B resulted in complete elimination of the time-dependent currents (c). Changes in 293B-sensitive component of I_Kr after application of 10 μmol/L amiodarone are shown by digital subtraction (a-c, b-c). B, I-V relationship for I_Ks before and after 10 μmol/L amiodarone (n=10; *P<0.05 vs control).

Short-term effects of amiodarone on I_Ks were examined in myocytes pretreated with 10 μmol/L E-4031 (Figure 3). Application of 10 μmol/L amiodarone to these myocytes caused only a slight inhibition of currents. The residual time-dependent currents in the presence of 10 μmol/L amiodarone were abolished after additional application of 30 μmol/L 293B. To estimate the short-term effects of amiodarone on I_Ks more precisely, 293B-sensitive components were obtained by subtraction of current traces in the absence and presence of 30 μmol/L 293B (Figure 3B, a-c and b-c). A minimal reduction in both time-dependent outward currents and tail currents by 10 μmol/L amiodarone was recognized more clearly in such subtracted current traces. Figure 3B shows the I-V relationship of I_Ks, in which the tail current amplitude in the subtracted current was plotted against test potential (n=9). Amiodarone (10 μmol/L) caused a slight reduction in currents that was associated with a 15-mV shift in the I-V relationship.

Effects of Amiodarone on HERG and KvLQT1/minK Currents

HERG currents were measured with a 2-step pulse protocol at a frequency of 0.03 Hz. From a holding potential at −90 mV,
a 2-second depolarization (−80 to 50 mV) was applied to activate outward currents, followed by return of the membrane potential to −70 mV to evoke tail currents (Figure 4A). The amplitude of outward tail currents exceeded the amplitude of the activating currents, characteristic of HERG channel behavior.11,18 Bath application of amiodarone (1 to 100 μmol/L) for 30 minutes resulted in a concentration-dependent decrease in outward currents during depolarization and tail currents. Figure 4B and 4C shows the I-V relationship for currents at the end of the depolarization step and at the peak of the tail after repolarization, respectively (n=5). The IC₅₀ for amiodarone block of the HERG channel tail current was 37.9 μmol/L (Hill coefficient=0.61; n=5).

Injection of oocytes with cRNA encoding KVLQT1 and minK subunits induced Iₖₛ, characterized by a linear I-V relationship (Figure 4D).9,9 Currents were measured during a 7.5-second depolarizing pulse to potentials ranging from −80 to 40 mV, with tail currents measured at −80 mV. Pulses were applied at a frequency of 0.03 Hz. Bath application of amiodarone did not affect the time-dependent currents even at the highest concentration tested (300 μmol/L). Figure 4E and 4F shows the I-V relationships for currents during the test pulse (step) and for tail currents. Unlike what was observed for Iₖₛ in rabbit myocytes, the averaged I-V curve was not shifted by 300 μmol/L amiodarone.

Long-Term Effects of Amiodarone on Iₖₑ and Iₖₛ

Figure 5 shows Iₖₑ and Iₖₛ in ventricular myocytes isolated from rabbits treated with oral amiodarone (100 mg · kg⁻¹ · d⁻¹) for 4 weeks. Averaged current densities of total Iₖ in these myocytes were significantly less than total Iₖₑ measured in cells isolated from control rabbits. The tail current density after depolarization to 50 mV was 0.81±0.13 pA/pF in controls (n=10; 22 cells) and 0.45±0.04 pA/pF in cells isolated from rabbits treated long term with amiodarone (n=9; 26 cells). Application of 30 μmol/L 293B caused no substantial change in both time-dependent outward currents and tail currents (Figure 5A). Additional application of 10
μmol/L E-4031 resulted in complete elimination of the time-dependent currents. These findings indicate that I_{Ks} was very small in cells isolated from rabbits treated long term with amiodarone.

Figure 5B shows I_{V}–I_{R} relationships for total I_{K}, I_{Kr}, and I_{Ks} in myocytes treated with long-term amiodarone (n=9; 26 cells). After depolarization to 50 mV, 92% of the tail current was I_{Kr} resistant (I_{Kr}), and only 8% was chromanol 293B sensitive (I_{Ks}). The tail current density of I_{Kr} in the amiodarone-treated myocytes (0.41±0.04 pA/pF at 50 mV) was reduced by 29% (P<0.05) compared with control myocytes (0.58±0.11 pA/pF at 50 mV). In contrast, the tail current density of I_{Ks} in the amiodarone-treated myocytes (0.04±0.02 pA/pF at 50 mV) was reduced by 83% (P<0.05) compared with control (0.23±0.02 pA/pF at 50 mV). Figure 5C shows isochronal activation curves for total I_{K} and its 2 components. V_{1/2} of total I_{K} and I_{Kr} did not differ significantly from the corresponding values in control myocytes. The amplitude of I_{Ks} was too small to analyze precisely, but its activation curve (Figure 5C, dotted line) was apparently shifted toward more positive potentials compared with those of I_{K} and I_{Kr}. There were no significant differences between control and amiodarone-treated myocytes in the activation and deactivation time constants of I_{Kr} (Table 2).

Channel Subunit Expression
The effects of long-term treatment of amiodarone on mRNA encoding ERG, KVLQT1, and minK subunits were measured with the ribonuclease protection assay using hearts obtained from control and amiodarone-treated rabbits killed after 28 days. Cyclophilin mRNA expression levels were used for the internal control. As shown in Figure 6, the levels of rabbit ERG, KVLQT1, and minK mRNAs did not exhibit a significant difference between control and amiodarone-treated rabbits.

Discussion
Short-Term Effects of Amiodarone on I_{Kr} and I_{Ks}
We used myocytes isolated from the apical region of the left ventricle to minimize cell-to-cell variation of I_{Ks} and I_{Kr}.
The most prominent effect of long-term amiodarone on cardiac muscles is a moderate and frequency-independent prolongation of action potential duration. Information available to explain the mechanism underlying the action potential duration prolongation is limited. It was shown in our previous study in ventricular myocytes isolated from rabbits treated with oral amiodarone (100 mg·kg\(^{-1}\)·d\(^{-1}\) for 4 weeks) that the current densities of \(I_{Kr}\) and \(I_{Ks}\) were decreased significantly compared with control rabbits (by \(\approx 50\%\) and \(\approx 30\%\), respectively), without any appreciable changes in their voltage dependence. Qualitatively similar findings have been reported by Varró et al.\(^{12}\) In these reports, \(I_{Ks}\) was not separated into the 2 components \(I_{Kr}\) and \(I_{Ks}\). In the present study, the tail current density of total \(I_{K}\) in amiodarone-treated rabbits was decreased by 29%, whereas the tail current density of \(I_{Ks}\) was reduced by 83% from controls. This indicates that \(I_{K}\) inhibition by long-term amiodarone is predominantly due to a reduction in \(I_{Ks}\).

Recently, Bosch et al.\(^{13}\) reported that long-term treatment of guinea pigs with intraperitoneal amiodarone (80 mg·kg\(^{-1}\)·d\(^{-1}\) for 7 days) caused a substantial reduction in the current density of \(I_{Kr}\) and \(I_{Ks}\) of ventricular myocytes to a similar extent (\(\approx 60\%\) reduction) without affecting their voltage-dependence or kinetics. Different periods of amiodarone administration (4 versus 1 week), different experimental protocols, and different animal species (rabbits versus guinea pigs) may underlie the discrepancy between our data and their observations.

### Study Limitations

We focused our investigation on the short- and long-term effects of amiodarone on \(I_{Kr}\) and \(I_{Ks}\) because block of these currents is an obvious candidate mechanism for the class III properties of this drug. However, the antiarrhythmic action of amiodarone likely results from inhibition of multiple channels and receptors.\(^{3,5}\) In addition, long-term effects of amiodarone on the heart are modulated by plasma and tissue accumulation of the parent drug and its active metabolite desethylamiodarone, which would impose their direct effects.\(^{3,5}\)

mRNA levels of \(ERG, KVLQT1\), and \(minK\) potassium channel subunits were not affected by long-term amiodarone, indicating that the reduction in \(I_{Ks}\) density cannot be ascribed to reduced transcription of mRNA. The mechanism of reduced \(I_{Ks}\) is unknown but may be due to an effect on the posttranscriptional processes of channel protein synthesis.

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