Amiodarone Inhibits Apamin-Sensitive Potassium Currents

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Abstract

Background: Apamin sensitive potassium current (IKAS), carried by the type 2 small conductance Ca²⁺-activated potassium (SK2) channels, plays an important role in post-shock action potential duration (APD) shortening and recurrent spontaneous ventricular fibrillation (VF) in failing ventricles.

Objective: To test the hypothesis that amiodarone inhibits IKAS in human embryonic kidney 293 (HEK-293) cells.

Methods: We used the patch-clamp technique to study IKAS in HEK-293 cells transiently expressing human SK2 before and after amiodarone administration.

Results: Amiodarone inhibited IKAS in a dose-dependent manner (IC₅₀ 2.67±0.25 μM with 1 μM intrapipette Ca²⁺). Maximal inhibition was observed with 50 μM amiodarone which inhibited 85.6±3.1% of IKAS induced with 1 μM intrapipette Ca²⁺ (n = 3). IKAS inhibition by amiodarone was not voltage-dependent, but was Ca²⁺-dependent: 30 μM amiodarone inhibited 81.5±1.9% of IKAS induced with 1 μM Ca²⁺ (n = 4), and 16.4±4.9% with 250 nM Ca²⁺ (n = 5). Desethylamiodarone, a major metabolite of amiodarone, also exerts voltage-independent but Ca²⁺ dependent inhibition of IKAS.

Conclusion: Both amiodarone and desethylamiodarone inhibit IKAS at therapeutic concentrations. The inhibition is independent of time and voltage, but is dependent on the intracellular Ca²⁺ concentration. SK2 current inhibition may in part underlie amiodarone’s effects in preventing electrical storm in failing ventricles.

Introduction

Heart failure is a major public health problem with 300,000 directly attributable deaths annually, in the United States alone. It has a prevalence of 5.8 million in the U.S. and over 23 million worldwide [1]. Ventricular arrhythmias are a major cause of morbidity and mortality in heart failure [2]. Today, many patients with heart failure receive an implantable cardioverter defibrillator (ICD) for primary or secondary prevention of arrhythmic death. However, ICD itself does not reduce the incidence of arrhythmias. Electrical storm (ES) defined as recurrent ventricular arrhythmias in a short period of time, remains a frequent complication and a strong independent predictor of poor outcome even in patients with ICDs [3,4]. Amiodarone is effective in the treatment of recurrent ventricular tachycardia or fibrillation [5] and is commonly used as the first line therapy for ES [6,7]. However, the mechanism behind amiodarone’s effectiveness in treating ES remains poorly understood.

Ca²⁺ activated K⁺ channels integrate intracellular calcium handling with membrane repolarization in various tissues including brain, peripheral nerve, endothelium, leukocytes, erythrocytes, heart, skeletal and smooth muscle [8]. They are classified into three types based on their conductance pattern: large (BK), intermediate (IK) and small (SK) conductance Ca²⁺ activated K⁺ channels. SK channels show weak voltage dependence, susceptibility to the bee venom toxin apamin, and they are highly Ca²⁺ sensitive [9]. However, the role of these channels in the heart is poorly understood.

Xu et al. identified three isoforms of SK channels (SK1, SK2 and SK3) in the mouse and human heart, and found that they play important roles in the maintenance of action potential duration (APD) in atrial myocytes and pacemaking tissues [10]. Subsequently, the same group demonstrated that mice engineered to lack SK2 have prolonged atrial APD and higher susceptibility to atrial fibrillation [11]. Interestingly, SK2 expression is strikingly higher in normal mouse, cat and human atria than their respective ventricles [10]. This preferential expression led researchers to
propose SK2 as a target for treating atrial arrhythmias without ventricular proarrhythmic risk. However, in our recent study, we demonstrated that SK2 expression is significantly upregulated in failing ventricles compared to normal ones [12]. Moreover, upregulation of SK2 channels contributes to the development of ES in heart failure [13,14] and apamin, a specific inhibitor of SK2 channels [15,16], can prevent the post-shock APD shortening and ES in failing rabbit ventricles [13].

These findings led us to hypothesize that amiodarone, the most effective and commonly used antiarrhythmic agent for the treatment of ES, can inhibit SK2 channels. However, amiodarone also inhibits various ion currents (e.g., I\textsubscript{Na}, I\textsubscript{Ca}, I\textsubscript{K}, I\textsubscript{Ks}, and I\textsubscript{Kr}) as well as β-adrenergic receptors. One possible approach to test the effects of amiodarone on SK2 currents is to first inhibit other major repolarization currents (such as I\textsubscript{Kr} and I\textsubscript{Ks}) before administering amiodarone in cardiac cells. However, we found that chromanol 293B and E-4031 (known inhibitors of I\textsubscript{Kr}, I\textsubscript{Ks}, and I\textsubscript{Ks}) are also inhibitors of SK2 channels. Using these two drugs in cardiac cells may prevent us to accurately study the effects of amiodarone on SK2 currents. Therefore, to test amiodarone’s effects by itself on SK2 channels, we expressed the human SK2 in human embryonic kidney 293 (HEK-293) cells by transiently constructing its coding gene in pCMV6-XL plasmids. Naïve HEK-293 cells in Medium supplemented with 10% FBS.

Our findings suggest that the antiarrhythmic action of amiodarone prevents postshock APD shortening in failing rabbit ventricles. The change in I\textsubscript{K} amplitude was monitored using repetitive ramp pulses that were applied every 10 seconds (holding membrane potential –80 mV; test potentials from +20 mV to –120 mV for 400 ms). Once I\textsubscript{K} became steady, a step-pulse protocol was also used to record baseline I\textsubscript{K} (holding membrane potential –80 mV; test potentials in 10 mV steps from –140 mV to +40 mV for 300 ms). While I\textsubscript{K} were monitored with ramp-pulses, amiodarone in various concentrations was applied to the bath solution. After maximum inhibition of I\textsubscript{K} was achieved, above step pulse protocol was applied again to record the I\textsubscript{K} under drug effect. After recordings with amiodarone were obtained, the drug was washed out. Finally, apamin (100 nM) was applied to ensure the measured I\textsubscript{K} were apamin-sensitive. I\textsubscript{K} after 100 nM apamin application were subtracted from the I\textsubscript{K} under no drug application and the current difference was defined as I\textsubscript{KAS} that is carried by SK2 channels. Inhibition of I\textsubscript{K} under various concentrations of amiodarone was compared and normalized to the inhibition of I\textsubscript{K} with 100 nM apamin (i.e., I\textsubscript{KAS}).

**Materials and Methods**

All experimental protocols were approved by the Institutional Committee of Animal Use and Care, and the Institutional Committee of Human and DNA Research at Indiana University.

HEK-293 cell preparation and transfection

HEK-293 cells obtained from American Type Cell Culture were grown at 37°C and 5% CO\textsubscript{2} in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS. KC\textsubscript{N2}, the gene encoding human small conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel, subfamily N, member 2 (SK2), transcript variant 1 (Gene Bank Accession # NM_021614.2), was obtained from OriGene (Rockville, MD) and constructed in pCMV6-XL plasmids. Naïve HEK-293 cells in 35 mm dishes were co-transfected with 4 μg of the expression construct and 0.5 μg of pEGFP-C3 plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfected cells were incubated at 37°C and 5% CO\textsubscript{2} for 48 hours prior to patch-clamp experiments. Cells showing green fluorescence were chosen to study the effects of amiodarone on SK2.

Expression of SK2 and I\textsubscript{KAS}

The HEK-293 cells transfected only with pEGFP-C3 were used as negative control for the patch clamp experiments. These did not generate significant I\textsubscript{K} with 1 μM intrapipette Ca\textsuperscript{2+} (Figure S1 in Data S1). Since apamin effectively suppressed almost all K\textsuperscript{+} currents in the transfected cells, nearly all currents in this study were apamin sensitive (I\textsubscript{KAS}). Apamin concentration used in our study (100 nM) was higher than the concentration shown to exert maximal inhibition on SK2 channels in other studies [15,17]. DMSO as a vehicle at 0.1% had no effects on I\textsubscript{KAS} (data not shown).

**Patch-clamp Experiments**

Effects of amiodarone on I\textsubscript{KAS} were studied using the whole-cell patch-clamp technique. Pipette resistances were 2–4 MΩ when filled with pipette solution. After whole-cell patch was obtained in Tyrode’s solution, chamber bath was changed to a solution containing N-methylglucamine. Capacitance currents were monitored with repetitive 5 mV pulses for at least five minutes to measure the cell capacitance. Whole-cell compensation was not used for I\textsubscript{KAS} measurements. All experiments were performed at 36°C. Voltage pulse protocols were generated with an Axopatch 200B amplifier using pCLAMP-10 software (Molecular Devices/Axon, Sunnyvale, CA). The data were filtered with a built-in four-pole low-pass Bessel filter at 2 kHz and digitized at 5 kHz. Extracellular solution contained (in mM): NMG, 140; KCl, 4; MgCl\textsubscript{2}, 1; glucose, 5; and HEPES, 10 (pH 7.4 with HCl). Pipette solution contained (in mM): potassium gluconate, 144; MgCl\textsubscript{2}, 1.15; EGTA, 1; HEPES, 10; MgATP, 2; and CaCl\textsubscript{2}, 0.85 (pH 7.25 with KOH). This composition yields 1 μM free Ca\textsuperscript{2+} at 36°C based on the calculation method by Bers et al. [18]. For study of Ca\textsuperscript{2+}-dependency, various combinations of EGTA and CaCl\textsubscript{2} were used in the pipette solution to achieve different intracellular [Ca\textsuperscript{2+}].

Since SK2 channels are activated by intracellular Ca\textsuperscript{2+}, it usually took several minutes for the intracellular environment to reach equilibrium after whole-cell configuration was established. The change in I\textsubscript{K} amplitude was monitored using repetitive ramp pulses that were applied every 10 seconds (holding membrane potential –80 mV; test potentials from +20 mV to –120 mV for 400 ms). Once I\textsubscript{K} became steady, a step-pulse protocol was also used to record baseline I\textsubscript{K} (holding membrane potential –80 mV; test potentials in 10 mV steps from –140 mV to +40 mV for 300 ms). While I\textsubscript{K} were monitored with ramp-pulses, amiodarone in various concentrations was applied to the bath solution. After maximum inhibition of I\textsubscript{K} was achieved, above step pulse protocol was applied again to record the I\textsubscript{K} under drug effect. After recordings with amiodarone were obtained, the drug was washed out. Finally, apamin (100 nM) was applied to ensure the measured I\textsubscript{K} were apamin-sensitive. I\textsubscript{K} after 100 nM apamin application were subtracted from the I\textsubscript{K} under no drug application and the current difference was defined as I\textsubscript{KAS} that is carried by SK2 channels. Inhibition of I\textsubscript{K} under various concentrations of amiodarone was compared and normalized to the inhibition of I\textsubscript{K} with 100 nM apamin (i.e., I\textsubscript{KAS}).

**Drugs and Reagents**

Apamin was purchased from Tocris (catalog#1652), and was dissolved in water for a 500 μM stock solution. Amiodarone-HCl was also purchased from Tocris (catalog# 4095), and was dissolved in DMSO or ethanol for a 25 mM stock solution. All other chemicals were purchased from Sigma (St. Louis, MO).

**Ethics Statement**

The protocol for animal experiments was approved by the Indiana University Institutional Animal Care and Use Committee.

**Data Analysis**

Patch-clamp data were analyzed using Clampfit (Molecular Devices/Axon, Sunnyvale, CA). Inhibition of I\textsubscript{K} with amiodarone was compared and normalized to I\textsubscript{KAS} (I\textsubscript{K} with 100 nM apamin) and was plotted as a function of amiodarone concentration. The data were fitted with the Hill equation: \(y = 1 / (1 + (IC_{50}/X)^n)\), where \(y\) indicates normalized I\textsubscript{KAS}, \(X\) is concen-
Amiodarone inhibits $I_K_{AS}$

We first tested whether the extracellularly applied amiodarone can inhibit $I_K_{AS}$. $I_K$ was activated with various intrapipette Ca$^{2+}$ concentrations. Figure 1 shows representative traces of 1 mM Ca$^{2+}$-activated $I_K$ obtained with a step-pulse protocol in the absence (Figure 1A) and in the presence of 10 mM amiodarone (Figure 1B) and 100 nM apamin (Figure 1C). Amiodarone significantly decreased $I_K$ in a time-independent manner. Figure 1D illustrates the current-voltage ($I-V$) relationship in the absence and presence of these drugs. Inhibition by amiodarone (10 mM) was observed at all membrane potentials.

Inhibition of $I_K_{AS}$ with amiodarone is reversible

We next tested whether the inhibitory effect of amiodarone was reversible. $I_K$ was recorded with a repetitive ramp pulse protocol. Figure 2A shows representative $I_K$ traces obtained in the absence (baseline) and in the presence of amiodarone (0.1 mM) and apamin (100 nM). While the inhibitory effect of amiodarone was completely reversed after washout, the inhibitory effect of apamin was only partially reversible. Figure 2B demonstrates the time course of $I_K$ measured at a membrane potential of 20 mV. We also performed experiments in which apamin (100 nM) was added first, followed by amiodarone administration. In these experiments, amiodarone did not further reduce the total $I_K$.

Inhibition of $I_K_{AS}$ with amiodarone is dose-dependent

To study whether amiodarone’s effects on $I_K_{AS}$ were dose-dependent, various consecutive concentrations of amiodarone were applied to the chamber after achieving steady-state $I_K_{AS}$ with repetitive ramp-pulse protocols. $I_K_{AS}$ was induced with an intrapipette Ca$^{2+}$ concentration of 1 mM. As indicated in Figure 3A, $I_K_{AS}$ was inhibited by amiodarone in a dose-dependent manner. In Figure 3B, $I_K_{AS}$ is shown in the presence of various concentrations of amiodarone obtained at a membrane potential of 20 mV and is plotted as a function of time. Figure 3C shows the dose-dependency of the inhibition of $I_K_{AS}$ by amiodarone. The inhibition of $I_K_{AS}$ with various amiodarone concentrations was normalized to the inhibition with 100 nM apamin (i.e. total $I_K_{AS}$), and plotted as a function of amiodarone concentration. Data were fit with the Hill equation, yielding an IC$_{50}$ of 2.67$\pm$0.25 mM, and a Hill coefficient of 0.51$\pm$0.02.

Figure 1. Inhibitory effect of amiodarone on $I_K_{AS}$

(A) Representative superimposed whole-cell $I_K$ traces obtained by the pulse protocol shown in the inset. The pipette solution contained 1 mM free Ca$^{2+}$ to activate $I_K_{AS}$. (B) Superimposed $I_K$ traces in the presence of 10 mM amiodarone. (C) Superimposed $I_K$ traces after adding 100 nM apamin to the same preparation. (D) Current-voltage ($I-V$) relationships obtained in the absence and presence of 10 mM amiodarone, then 100 nM apamin. $I_K$ was measured between 280 ms and 290 ms of the test pulse, and plotted against membrane potentials. Similar results with 10 mM amiodarone were observed in 5 cells.

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Inhibition of $I_{KAS}$ with amiodarone is affected by intracellular Ca$^{2+}$ concentration

The open-probability of SK2 channels is dependent on intracellular concentration of Ca$^{2+}$ which is suggestive of different conformational states for SK2 channels at different Ca$^{2+}$ concentrations [19]. Therefore, we questioned whether the extent of inhibition might also be Ca$^{2+}$-dependent. To test this hypothesis, we induced $I_{KAS}$ with various intrapipette Ca$^{2+}$ concentrations and studied its inhibition by amiodarone. **Figure 4A** shows representative $I_K$ traces induced with an intrapipette Ca$^{2+}$ concentration of 500 nM at baseline and in the presence of various concentrations of amiodarone. Amiodarone exerts less $I_{KAS}$ inhibition, when $I_{KAS}$ is induced with 500 nM intrapipette Ca$^{2+}$ compared to its inhibition of the $I_{KAS}$ induced with 1 μM intrapipette Ca$^{2+}$ (Figure 3A). Furthermore, the inhibitory effects of amiodarone (30 μM) on $I_{KAS}$ were even smaller when the currents were induced with 250 nM rather than with 500 nM intrapipette Ca$^{2+}$ (Figure 4B).

**Figure 5A** shows $I_{KAS}$ densities induced by various intrapipette Ca$^{2+}$ concentrations. $I_{KAS}$ density was significantly larger when induced with 1 μM Ca$^{2+}$ compared to 500 nM and 250 nM. **Figure 5B** shows that the extent of amiodarone’s inhibitory effect on $I_{KAS}$ is dependent on whether the $I_{KAS}$ is induced with 1 μM, 500 nM or 250 nM intrapipette Ca$^{2+}$ (i.e. different channel conformations respond differently to amiodarone). We used three different therapeutic amiodarone concentrations (1, 10 and 30 μM) to inhibit $I_{KAS}$. The extent of inhibition in each condition was normalized to the extent of inhibition achieved by 100 nM apamin of the 1 μM intracellular Ca$^{2+}$ induced $I_{KAS}$. With both 1 μM and 10 μM amiodarone, the extent of inhibition we observed was significantly smaller when the currents were activated with 500 nM intrapipette Ca$^{2+}$ (light grey boxes) compared to 1 μM intrapipette Ca$^{2+}$ (white boxes). The inhibition of $I_{KAS}$ with 50 μM amiodarone was also highest when $I_{KAS}$ was induced with 1 μM intrapipette Ca$^{2+}$. Amiodarone, at 30 μM, inhibited the $I_{KAS}$ induced by 500 nM Ca$^{2+}$ less than by 1 μM Ca$^{2+}$, and barely inhibited the $I_{KAS}$ induced by 250 nM intrapipette Ca$^{2+}$ (dark grey box on the far right). These results indicate that the inhibition of $I_{KAS}$ by amiodarone is Ca$^{2+}$ dependent.

Desethylamiodarone, an amiodarone metabolite, also inhibits $I_{KAS}$

Once amiodarone is absorbed in the human body, it is extensively metabolized in the liver by cytochrome P450 3A4. The main metabolite is desethylamiodarone (DEA), which also has antiarrhythmic properties as the parent compound [20]. Therefore, we also studied the effects of DEA on $I_{KAS}$. **Figure 6A-C** shows representative $I_K$ traces obtained with a step-pulse protocol in the absence of DEA (**Panel A**), in the presence of 20 μM DEA (**Panel B**) and after addition of 100 nM apamin (**Panel C**). **Figure 6D** illustrates the current-voltage (I-V) relationships of these currents. Similar to amiodarone, DEA inhibited $I_{KAS}$ in a time and voltage independent manner. The extent of inhibition with DEA (20 μM) was 79.0% [74.3; 86.8] of 1 μM intrapipette Ca$^{2+}$ induced $I_{KAS}$ (n = 6).

**Discussion**

In this study, we examined the modulatory effects of amiodarone on human SK2 channels. Our novel findings are: (1) amiodarone reversibly inhibits the SK2 channels in a dose-dependent manner; (2) the inhibitory effect of amiodarone is time-independent and voltage independent; (3) the degree of $I_{KAS}$ inhibition by amiodarone is dependent on the intracellular calcium concentration; and (4) desethylamiodarone, the main metabolite of amiodarone, also inhibits SK2 channels.
Comparison with previous studies

Since the role of SK2 channels in ventricular arrhythmias of the failing heart had not been fully elucidated, effects of antiarrhythmic agents on SK2 channels were not studied systematically. Most studies about SK2 channels come from the neuroscience literature. Dreixler et al. [21] studied the effects of anesthetic agents on these channels and found that lidocaine inhibits SK2 channels expressed in HEK-293 cells. However, the IC50 they reported was 4.44 mM, which is much higher than the antiarrhythmic therapeutic concentration range (in μM) [22]. Although the underlying molecular mechanisms of lidocaine’s effect on SK2 channels were not elucidated in the study, it was speculated that lidocaine interfered with the regulation of intracellular Ca2+, resulting in a change in SK2 activation. In our study, the intracellular Ca2+ concentration was clamped through the pipette solution and the extracellular buffer did not contain Ca2+. Therefore, it is unlikely that amiodarone inhibited SK2 channels by affecting intracellular Ca2+ in HEK-293 cells.

Very recently, Diness et al. [23] showed that three SK channel inhibitors (UCL1684, N-(pyridin-2-yl)-4-(pyridin-2-yl) thiazol-2-amine (ICA), and NS8593) successfully prevented the initiation of AF in various animal models. Since amiodarone (10 μM) also effectively prevented AF initiation in their models, they tested effects of 10 μM amiodarone on whole-cell SK2 currents expressed in HEK-293 cells. Surprisingly in their study, the intracellular Ca2+ concentration was clamped through the pipette solution and the extracellular buffer did not contain Ca2+. Therefore, it is unlikely that amiodarone inhibited SK2 channels by affecting intracellular Ca2+ in HEK-293 cells.

Antiarrhythmic effects of amiodarone

Since amiodarone affects various ion currents (e.g., IKs, ICaL, IKr, and If) as well as β-adrenergic receptors, it is not straightforward to determine its most clinically important target for its anti-arrhythmic activity. Our study adds a new target ion channel to this list.

One mechanism for amiodarone’s effectiveness in the treatment of electrical storm can be through prevention of excessive APD shortening after successful defibrillation. Persistence of Ca2+ elevation after the conclusion of repolarization results in depolarizing Na+ currents through the Na+/Ca2+ exchanger, which in turn results in late phase 3 EADs and triggered activity [13,26–28]. Targeting the mechanism behind post-shock APD shortening would act as a specific therapeutic maneuver to prevent ES. In a recent study, we were able to effectively prevent spontaneous VF after defibrillation by the selective SK channel inhibitor apamin.
Using the same rabbit heart failure model, we demonstrated that amiodarone can decrease post-shock APD shortening, which underlies its effectiveness in treatment of ES (Figure S2 in Data S2).

Another mechanism behind amiodarone’s effectiveness for ES could be through its effects on APD restitution. Previously, Omichi et al. reported that amiodarone flattened the APD restitution slope which in turn resulted in termination of VF in isolated swine ventricles [29]. Since VF induces Ca\(^{2+}\) accumulation due to high frequency depolarizations [30–33], it is reasonable to speculate that amiodarone’s inhibitory action on SK2 channels are responsible for flattening of the APD restitution slope. This

![Figure 4](image)

**Figure 4. Inhibition of \(I_{KAS}\) with amiodarone is state-dependent.** (A) Superimposed \(I_{K}\) traces in various conditions as labeled. The intrapipette Ca\(^{2+}\) was 500 nM. \(I_{K}\) was obtained with the same ramp pulse protocol used in Figure 2 (also shown in the inset). (B) Superimposed \(I_{K}\) traces induced with 250 nM intrapipette Ca\(^{2+}\). Similar results were observed in 5 cells. AMD indicates amiodarone.

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![Figure 5](image)

**Figure 5. Density of \(I_{KAS}\) and its inhibition by amiodarone is dependent on the intracellular Ca\(^{2+}\) concentration.** (A) \(I_{KAS}\) densities obtained with three different intrapipette Ca\(^{2+}\) concentrations. The box-plot indicates the median and 25 and 75 percentile values. The whiskers indicate the minimum and maximum values. (B) Inhibitory effects of various concentrations of amiodarone on \(I_{KAS}\) induced with various intrapipette Ca\(^{2+}\) concentrations as shown in the top labels. In the left panel, inhibitory effects of 1 \(\mu\)M amiodarone were tested on \(I_{KAS}\) induced with two different intrapipette Ca\(^{2+}\) concentrations (1 \(\mu\)M and 500 nM). In the mid panel, inhibitory effects of 10 \(\mu\)M amiodarone were tested on \(I_{KAS}\) induced with two different intrapipette Ca\(^{2+}\) concentrations (1 \(\mu\)M and 500 nM). In the right panel, inhibitory effects of 30 \(\mu\)M amiodarone were tested on \(I_{KAS}\) induced with three different Ca\(^{2+}\) concentrations (1 \(\mu\)M, 500 nM and 250 nM). Inhibition of 250 nM Ca\(^{2+}\) induced \(I_{KAS}\) by 1 \(\mu\)M and 10 \(\mu\)M amiodarone is not shown since this was almost undetectable. The numbers in parentheses indicate the number of patches.

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hypothesis is supported by a recent study that showed apamin flattens APD restitution curve at fast pacing rates [34].

We found that the inhibitory effects of amiodarone on SK2 channels were state-dependent (i.e., more inhibition for more current). Cytoplasmic domains of SK2 channels bind to calmodulin, and binding of Ca\(^{2+}\) to calmodulin may affect the conformation of SK2 channels and its gating kinetics [35]. This Ca\(^{2+}\)-dependent conformational change may contribute to the state-dependent inhibition of SK2 by amiodarone. The state-dependent inhibition may be clinically important, since amiodarone may affect the channel only when the intracellular Ca\(^{2+}\) is elevated. This makes it particularly important in patients with heart failure or electrical storm where the basal intracellular Ca\(^{2+}\) is elevated.

In our study, an IC\(_{50}\) of amiodarone on whole-cell \(I_{KAS}\) induced with 1\(\mu\)M Ca\(^{2+}\), was 2.67\(\mu\)M. Since acute and chronic amiodarone administration results in plasma levels of approximately 0.16 to 10\(\mu\)M [36], inhibitory effects of amiodarone on SK2 channels can be seen with doses used in the clinical setting. Le Bouter et al. [37] demonstrated that mice fed with clinically used doses of amiodarone for 6 weeks showed significant increase of SK2 mRNA levels in their total heart tissue. Although \(I_{KAS}\) were not determined in that study, it is reasonable to hypothesize that the transcripts could have increased as a compensatory mechanism to the inhibition of SK2 channels with chronic amiodarone therapy.

**Study Limitations**

Our study has several limitations: (1) we studied only one variant of SK2 channel (KCNN2 transcript variant 1). Several different SK2 variants are known, although cardiac expression of these variants remains unclear. In addition, other subtypes of SK channels (e.g., SK1 and SK3) exist in cardiomyocytes, and they may form heteromers with SK2 channel resulting in different sensitivity to amiodarone [38] in human hearts; (2) Clearly cytoplasmic environment of HEK-293 cells are very different from human cardiomyocytes. Since SK2 channels are regulated by various signal transduction pathways such as calmodulin, protein kinase CK2, and protein phosphatase 2A [35], effects of amiodarone on SK2 currents may differ between HEK-293 cells and human cardiomyocytes. To overcome this problem, testing amiodarone’s effects on SK2 currents in cardiomyocytes or whole heart would have been helpful. However, since amiodarone inhibits various ion channels including \(I_{Kr}\), \(I_{Ks}\), and \(I_{To}\) in cardiomyocytes, specific inhibitors of these channels need to be used to isolate amiodarone’s effects on SK2 currents. After attempting to study amiodarone effects in cardiomyocytes, we found that specific inhibitors of delayed rectifier K\(^{+}\) channels (\(I_{Kr}\) and \(I_{To}\)); chromanol 293B and E4031 [39,40] also inhibit SK2 currents complicating the interpretation of data we would obtain using cardiomyocytes (Figure S3 in Data S3, Figure S4 in Data S4). To avoid the non-specific effects of various “specific” ion channel inhibitors, genetically manipulated models are needed. However, it is beyond a scope of this study; (3) Currently we do not know whether apamin can prevent electrical storm in human since it is a neurotoxin and cannot be used in the clinical setting. However, our recent study using rabbit heart failure models demonstrated that \(I_{KAS}\) were upregulated and resulted in shortening of the APD after VF termination, predisposing the failing heart to spontaneous recurrence of VF. Apamin, through inhibition of SK2 channels, prevented the APD shortening, recurrence of VF and electrical storm. Similarly, we
have shown that SK2 currents were upregulated in failing human ventricular myocytes compared with the non-failing myocytes, and Apamin prolonged the APD in failing human myocytes but not in the non-failing ones [12]. These facts, taken together, allow us to speculate that the inhibition of SK2 currents with amiodarone may, at least in part, underlie amiodarone’s effects in termination and prevention of ES in human; (4) We assumed that the SK2 expression level is similar among different experiments, and thus $I_{KAS}$ density can reflect the open probability of the channel. Our assumption was based on our consistent cell culture and transfection protocols. However, we do not have single-channel recording data. Therefore, we do not know the detailed molecular mechanisms behind this inhibition. In spite of these limitations, the results of this study showed for the first time that amiodarone is a potent inhibitor of $I_{KAS}$. It is possible that $I_{KAS}$ inhibition may partially account for the antiarrhythmic efficacy of amiodarone.

### Supporting Information

**Data S1.** Control experiments. Figure S1. Superimposed $I_K$ traces obtained from an HEK-293 cell with mock transfection using the pEGFP-C3 vector. The $I_K$ were recorded with an intra-pipette Ca$^{2+}$ concentration of 1 μM. The voltage was clamped by the ramp-pulse protocol shown in the inset. Eighty repetitive ramp-pulses were applied every 10 seconds for approximately 5 minutes after the formation of whole-cell configuration.

**Data S2.** Effects of amiodarone on the post-shock action potential durations. We sought to investigate whether amiodarone can antagonize the post-shock action potential (APD) shortening. For this, we used pacing-induced rabbit heart failure models since induction of ventricular fibrillation (VF) was difficult in human ventricle wedge preparations. The protocol was approved by the Indiana University Institutional Animal Care and Use Committee. Pacing-induced rabbit heart failure model was created and optical mapping studies in the Langendorff-perfused hearts were performed as previously described [1]. Failing hearts were stained with RH237 for measurement of the membrane potential ($E_m$). Rapid ventricular pacing and 3 to 5 ventricular fibrillation-defibrillation episodes were mapped in each heart. Amiodarone (10 μM) was added to the perfusate for 30 minutes and then the same protocol was repeated. Figure S2A shows a representative action potential (AP) recording at a pacing cycle length (PCL) of 300 ms obtained from the anterobasal left ventricle without amiodarone. The average APD$_{90}$ obtained was 176 ms. VF was induced from the anterobasal left ventricle without amiodarone. The baseline (baseline: 116.3 ± 6.0, n = 12 beats) reached steady state (baseline) within a few minutes after the formation of whole-cell configuration. E4031 (0.5 μM) reversibly blocked the $I_K$. Percent inhibition of $I_K$ by E4031 was 14.9% (n = 5).

**Figure S3.** Effect of chromanol 293B on $I_{KAS}$ in failing human ventricular myocytes. (A) Representative $I_K$ traces obtained with two different 293B concentrations. The ramp-pulse protocol used is shown in the inset. Note that apamin (100 nM) almost completely inhibited the $I_K$ meaning that the $I_K$ inhibited by 293B is $I_{KAS}$. (B) The time course of the $I_K$ measured at +20 mV. The $I_K$ reached the steady state (baseline) within a few minutes of whole-cell configuration. Chromanol 293B inhibited the $I_K$ in a dose-dependent manner. Subsequent application of apamin (100 nM) inhibited almost all of the $I_K$.

**Data S4.** Inhibitory effects of E4031 on SK2 currents. Next, we tested the effect of E4031 (a specific $I_{KAS}$ blocker) on $I_{KAS}$. The IC$_{50}$ of E4031 on $I_K$ was 397 nM, and 5–30 μM of E4031 is necessary to block both $I_K$ [2,5]. Similar to 293B, E4031 also inhibited $I_{KAS}$ induced with 1 μM origanum Ca$^{2+}$ and repetitive ramp-pulse protocols (Figure S4A in Data S4). Figure S4B demonstrates that the inhibition of $I_{KAS}$ by E4031 is reversible. Percent-inhibition of $I_{KAS}$ with 500 nM E4031 was 37.6 ± 14.9% (n = 5). Figure S4. Effect of E4031 on SK2 currents. (A) Representative $I_K$ traces in various conditions. The ramp-pulse protocol used is shown in the inset. (B) Time course of the $I_K$ measured at +20 mV. The $I_K$ reached the steady state (dotted line) within a few minutes after the formation of whole-cell configuration. E4031 (0.5 μM) reversibly blocked the $I_K$. Subsequent application of apamin (100 nM) inhibited most of the $I_K$.

**References**

S1.
Conceived and designed the experiments: IT CCY YS P-SC TA.

Performed the experiments: IT CCY P-GC TA. Analyzed the data: IT CCY TA. Contributed reagents/materials/analysis tools: ZC S-FL. Wrote the paper: IT TA.

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