Impaired Inactivation of L-Type Ca\(^{2+}\) Current as a Potential Mechanism for Variable Arrhythmogenic Liability of HERG K\(^{+}\) Channel Blocking Drugs

Jae Gon Kim\(^{1,2} \)†, Dong Jun Sung\(^{4} \)†, Hyun-ji Kim\(^{1} \)†, Sang Woong Park\(^{5} \), Kyung Jong Won\(^{5} \), Bokyung Kim\(^{6} \), Ho Chul Shin\(^{6} \), Ki-Suk Kim\(^{2,3} \), Chae Hun Leem\(^{7} \), Yin Hua Zhang\(^{8} \), Hana Cho\(^{1} \)*, Young Min Bae\(^{5} \)*

1 Department of Physiology and the Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon, South Korea, 2 Next-Generation Pharmaceutical Research Center, Korea Institute of Toxicology, Korea Research Institute of Chemical Technology, Daejeon, South Korea, 3 Human and Environmental Toxicology Program, University of Science and Technology, Daejeon, South Korea, 4 Division of Sport Science, College of Science and Technology, Konkuk University, Choongju, South Korea, 5 Department of Physiology, KU Open Innovation Center, Research Institute of Medical Science, Konkuk University School of Medicine, Chungju, South Korea, 6 Department of Veterinary Pharmacology and Toxicology, College of Veterinary Medicine, Konkuk University, Seoul, South Korea, 7 Department of Physiology, University of Ulsan College of Medicine, Seoul, South Korea, 8 Department of Physiology, Seoul National University College of Medicine, Seoul, South Korea

† These authors are joint co-first authors on this work.
* hanacho@skku.edu; ymbae30@kku.ac.kr

Abstract

The proarrhythmic effects of new drugs have been assessed by measuring rapidly activating delayed-rectifier K\(^{+}\) current (I\(_{Kr}\)) antagonist potency. However, recent data suggest that even drugs thought to be highly specific I\(_{Kr}\) blockers can be arrhythmogenic via a separate, time-dependent pathway such as late Na\(^{+}\) current augmentation. Here, we report a mechanism for a quinolone antibiotic, sparfloxacin-induced action potential duration (APD) prolongation that involves increase in late L-type Ca\(^{2+}\) current (I\(_{CaL}\)) caused by a decrease in Ca\(^{2+}\)-dependent inactivation (CDI). Acute exposure to sparfloxacin, an I\(_{Kr}\) blocker with prolongation of QT interval and torsades de pointes (TdP) produced a significant APD prolongation in rat ventricular myocytes, which lack I\(_{Kr}\) due to E4031 pretreatment. Sparfloxacin reduced peak I\(_{CaL}\) but increased late I\(_{CaL}\) by slowing its inactivation. In contrast, ketoconazole, an I\(_{Kr}\) blocker without prolongation of QT interval and TdP produced reduction of both peak and late I\(_{CaL}\), suggesting the role of increased late I\(_{CaL}\) in arrhythmogenic effect. Further analysis showed that sparfloxacin reduced CDI. Consistently, replacement of extracellular Ca\(^{2+}\) with Ba\(^{2+}\) abolished the sparfloxacin effects on I\(_{CaL}\). Acute exposure to sparfloxacin, an I\(_{Kr}\) blocker with prolongation of QT interval and torsades de pointes (TdP) produced a significant APD prolongation in rat ventricular myocytes, which lack I\(_{Kr}\) due to E4031 pretreatment. Sparfloxacin reduced peak I\(_{CaL}\), but increased late I\(_{CaL}\) by slowing its inactivation. In contrast, ketoconazole, an I\(_{Kr}\) blocker without prolongation of QT interval and TdP produced reduction of both peak and late I\(_{CaL}\), suggesting the role of increased late I\(_{CaL}\) in arrhythmogenic effect. Further analysis showed that sparfloxacin reduced CDI. Consistently, replacement of extracellular Ca\(^{2+}\) with Ba\(^{2+}\) abolished the sparfloxacin effects on I\(_{CaL}\). In addition, sparfloxacin modulated I\(_{CaL}\) in a use-dependent manner. Cardiomyocytes from adult mouse, which is lack of native I\(_{Kr}\), demonstrated similar increase in late I\(_{CaL}\) and afterdepolarizations. The present findings show that sparfloxacin can prolong APD by augmenting late I\(_{CaL}\). Thus, drugs that cause delayed I\(_{CaL}\) inactivation and I\(_{Kr}\) blockage may have more adverse effects than those that selectively block I\(_{Kr}\). This mechanism may explain the
reason for discrepancies between clinically reported proarhythmic effects and \(I_{Kr}\) antagonist potencies.

**Introduction**

Drug-induced QT interval prolongation and the appearance of torsade de pointes (TdPs) are recognized as potential risks associated with the use of a wide range of noncardiovascular drugs including antibiotics [1–4]. Quinolone antibiotics have been suggested to have a class effect of blocking the human \(\text{Ether-a-go-go-related gene (hERG)}\) \(K^+\) channel expressing the rapid component of the delayed rectifier current \(I_{Kr}\) in the human heart, and thus prolong action potential duration (APD), which is associated with QT interval prolongation. The quinolone antibiotic sparfloxacin (SPX) has been withdrawn from U.S. drug market, because it was shown to induce QT interval prolongation and ventricular arrhythmia [5, 6]. Another quinolone, grepafloxacin, was withdrawn because it induced TdP, a polymorphic ventricular tachycardia (VT) linked to excessive QT interval prolongation [7]. Concern over the proarrhythmic effects of many other quinolone antibiotics continues to grow. In nonclinical studies, the proarrhythmic effects of clinically used quinolone antibiotics have been assessed by measuring their associated \(I_{Kr}\) antagonist potency. However, discrepancies between clinically reported proarrhythmic effects and in vitro observations exist. For example, the antibiotic moxifloxacin blocks \(I_{Kr}\) but has been associated with drug-induced long QT syndrome (LQT) only very rarely [8, 9].

Cardiac rhythm and contractility are regulated by the composite functions of cardiac myocyte ion channels. Specifically, the lengthening and flattening of action potential (AP) plateaus are determined by the sum of inward and outward currents. \(I_{CaL}\) contribute to inward currents, maintaining the plateau phase of ventricular AP (phase 2). Inhibition of \(I_{CaL}\) shortens the AP, whereas inhibition of outward \(I_{Kr}\) results in AP prolongation. Therefore, if both \(K^+\) and \(Ca^{2+}\) channels are inhibited, \(I_{CaL}\) inhibition may counteract the \(I_{Kr}\)-blocking effects of quinolone antibiotics. Indeed, Xu *et al.* (2003) reported that drugs with dual blocking action against hERG \(K^+\) and \(Ca^{2+}\) channels are less likely to cause arrhythmias than drugs with selective blocking activity against hERG \(K^+\) current [10]. On the other hand, enhancing \(I_{CaL}\) while blocking \(I_{Kr}\) may aggravate APD prolongation and/or generate early afterdepolarization (EAD) upstrokes.

During a normal AP, \(I_{CaL}\) peaks early, triggering robust sarcoplasmic reticulum \(Ca^{2+}\) release before partially inactivating due to two processes: \(Ca^{2+}\)-dependent inactivation (CDI), mediated by \(Ca^{2+}\) binding to calmodulin (CaM) tethered to the C-terminus of the channel, and voltage-dependent inactivation (VDI). The rate and degree of \(I_{CaL}\) inactivation due to these processes during the late phase of the AP has a major effect on repolarization. This raises the possibility that instead of potentiating \(I_{CaL}\), modifying its shape by altering its inactivation kinetics might lead to APD prolongation and EAD. In a recent study, elimination of CDI in guinea pig ventricular myocytes via expression of \(Ca^{2+}\)-insensitive CaM (CaM1234) was shown to produce “ultralong” APs [11].

In the present study, we sought to determine the relevance of \(I_{CaL}\) in APD prolongation effects of SPX. We analyzed the biophysical properties of L-type \(Ca^{2+}\) channels affecting APD. SPX reduced peak \(I_{CaL}\). However this quinolone antibiotic augmented late \(I_{CaL}\) by attenuating CDI, which promoted APD prolongation in cardiac myocytes. In contrast, \(I_{Kr}\) blockers not associated with serious arrhythmias—ketoconazole, ciprofloxacin, enoxacin, ofloxacin, and levofloxacin produced no change in \(I_{CaL}\) or decreased both peak and late \(I_{CaL}\), suggesting the role of increased late \(I_{CaL}\) in arrhythmogenic effect. Our results suggest an importance of calcium channel inactivation in producing the arrhythmogenic effects of SPX and, as such, it is
necessary to consider $I_{\text{CaL}}$ property changes when assessing drugs for QT prolonging and arrhythmogenic liability.

**Materials and Methods**

**Cardiomyocyte isolation and culture**

All animal care and experimental procedures complied with the National Institutes of Health guidelines, and the Institutional Animal Care and Use Committee of Konkuk University and Sungkyunkwan University approved this study. Neonatal ventricular myocytes were isolated from 1 to 2-day-old Sprague-Dawley (SD) rats (Nara Biotech, Seoul, Korea) by using a previously reported method [12]. Ventricular regions of neonatal rat hearts were excised (approximately, the lower third) and the tissues (approximately 1–2 mm) were minced on ice. The minced tissues were treated with a solution containing 0.1% collagenase (Wako, Japan), 0.1% trypsin, and 1% glucose in phosphate-buffered saline (Ca$^{2+}$/Mg$^{2+}$-free) at 37°C for 10 min. After the supernatant from the first digestion was removed, three 10-min digestions were performed using the same enzyme solution. The supernatants were stored in DMEM/F-12 culture medium containing 10% fetal bovine serum, 5% horse serum, penicillin-streptomycin (100 U/ml and 100 μg/ml, respectively) in a 4°C ice chamber and the centrifuged for 7 min at 700 × g. The cell pellets were incubated at 37°C in a 95% O$_2$ incubator for 1.5 h to attach non-cardiac myocytes to microscope cover glass. The cells were subsequently cultured on the cover glass for 3 days at 37°C and 95% O$_2$. Cells cultured for 3–5 days were used for $I_{\text{CaL}}$ current recordings.

Ventricular myocytes from adult mice (> 5 months) or 2-week-old SD rats were isolated as previously described with minor modifications [13]. Briefly, ventricular myocytes were isolated by perfusing Ca$^{2+}$-free NT solution containing collagenase (1 mg/ml, 4176, Worthington) and DL-dithiothreitol (1 mg/ml, D-0632, Sigma, St. Louis, MO, USA) through Langendorff columns at 37°C.

**Electrophysiological recording**

Cardiac myocytes were subjected to patch-clamp experiments. Whole-cell Ca$^{2+}$ and Ba$^{2+}$ currents were recorded using a conventional whole-cell patch-clamp configuration, outfitted with an EPC 8 patch-clamp amplifier (Heka, Germany). Voltage pulse generation was controlled using R-clamp software (R-clamp; provided by Dr. S.Y. Ryu). The data were digitized using the R-clamp software at a sampling rate of 5 kHz, after being low-pass filtered at 1 kHz. The patch pipettes were created from borosilicate glass capillaries (Clark Electromedical Instruments, Pangbourne, UK) by using a puller (PP-83; Narishige, Japan). Patch pipettes producing resistances of 1.5–2.5 MΩ in bathing solution were used. All experiments were performed at room temperature (20–25°C).

APs were recorded using a nystatin-perforated patch-clamp configuration, with an EPC10 patch-clamp amplifier (Heka, Germany). Data were digitized and current injection (125–175 pA, 9 ms) for AP generation were both controlled using Patch-Master software.

**Solutions and Drugs**

To record $I_{\text{CaL}}$ in cardiac myocytes, NMDG-Tyrode’s [143 mM N-methyl-D-glucamine (NMDG)-Cl, 5.4 mM CsCl, 0.33 mM NaH$_2$PO$_4$, 5 mM HEPES, 0.5 mM MgCl$_2$, 1.8 mM CaCl$_2$, 11 mM D-glucose, pH adjusted to 7.4 with HCl] was used as the bathing solution. The pipette solution contained 115 mM CsCl, 5 mM Mg-ATP, 10 mM HEPES, 5 mM ethylene glycol-bis (2-aminoethyl ether)-N,N,N,N’-tetraacetic acid (EGTA), and 5 mM creatine phosphate (disodium salt). The pH was adjusted to 7.3 by using CsOH. APs were recorded from single isolated
myocytes in a perforated patch configuration by using nystatin (200 μg/ml). Normal Tyrode’s solution (143 mM NaCl, 5.4 mM KCl, 0.33 mM NaH2PO4, 5 mM HEPES, 0.5 mM MgCl2, 1.8 mM CaCl2, 11 mM d-glucose, pH adjusted to 7.4 with NaOH) was used as the bathing solution. The pipette solution for recording APs contained 140 mM KCl, 10 mM HEPES, 5 mM EGTA, and 1 mM MgCl2, and the pH was adjusted to 7.2 with KOH.

To record INa in cardiac myocytes, 140 mM NaCl, 5 mM CsCl, 1.8 mM CaCl2, 11 mM MgCl2, 11 mM glucose, 10 mM HEPES adjusted with NaOH (pH 7.4) was used as the bath solution. Nifedipine (1 μM), SN-6 (10 μM), and CdCl2 (100 μM) were used to block L-type Ca2+ currents, NCX currents, and T-type Ca2+ currents, respectively. The pipette solution contained 20 mM CsCl, 100 mM Cs-Asp, 10 mM EGTA, 10 mM HEPES, 20 mM TEA-Cl, 5 mM Mg-ATP adjusted to 7.25 with CsOH.

Unless otherwise stated, all chemicals and drugs were purchased from Sigma-Aldrich; SPX (Fluka, S6968) and E4031 (Sigma, M5060) were prepared as stock solutions in dimethyl sulfoxide. The drugs were diluted in the bathing solution on the day of the experiment.

Statistical analysis
The results are shown as mean ± standard error of the mean. Student’s t-tests or Fisher’s exact test were performed to test for significance as appropriate using SigmaPlot. P values < 0.05 were deemed to be statistically significant.

Results
SPX induces APD prolongation in the presence of IKr blocker
To test the hypothesis that factors other than IKr modulation play important roles in SPX-induced APD prolongation, the effects of SPX applied at concentrations between 10 and 300 μM were investigated in 2-week-old rat ventricular myocytes in the presence of E4031, a selective IKr blocker. APs were elicited by electrical stimulations delivered using a patch pipette in current clamp mode, at a stimulation frequency of 2 Hz. As shown in Fig 1A, 30 μM SPX significantly prolonged APD in the presence of 1 μM E4031. The time required for 90% repolarization (APD90), increased from 54.5 ± 3.3 to 95.0 ± 13.6 ms after 10 min of 30-μM SPX treatment (paired t-test; n = 7, P < 0.05).

No changes in resting membrane potential (RMP) or in AP overshoot potentials were observed during SPX treatments. The mean RMP and overshoot potential values after SPX treatment were −68.5 mV (n = 7; P > 0.05 vs. −70.8 mV in the absence of SPX) and 53.5 mV (n = 7; P > 0.05 vs. 52.7 mV in the absence of SPX), respectively. The steady-state APD90 values obtained at various SPX concentrations are summarized in Fig 1B. These data indicate that SPX-induced APD prolongation is not only attributable to blocking IKr, but is also influenced by additional channel modulation.

Effects of SPX on ICaL
We next examined whether SPX enhances ICaL in neonatal cardiomyocytes. By holding Em at −50 mV, we could successfully isolate ICaL from ICaT measurements (S1 Fig). Therefore, we used holding potential of −50 mV for all voltage-clamp experiments, excluding those reported in Fig 2, in which we applied a double pulse at −40 mV followed by 0 mV from a holding potential of −80 mV [14]. To eliminate voltage-gated K+ currents and Na+ currents, a Cs+ -rich pipette solution and Na+ -free (substituted with NMDG+) bath solution was used. Fig 2A shows representative ICaL measurements under control conditions and in the presence of 300 μM SPX, recorded from the same ventricular myocytes. The amplitude of the peak ICaL was
reduced after SPX treatment. The I–V curves indicated that the peak $I_{Ca,L}$ was decreased after 300 μM SPX treatment at all potentials ranging from −40 to +50 mV, without altering the I–V relationship ($n = 10$; Fig 2B). Inactivation, however, was slowed by SPX treatment. Slower $I_{Ca,L}$ decay values resulted in larger current amplitudes in the presence of SPX at the end of the 200 ms pulse (Fig 2C). The amplitude of this current was larger over the voltage range 0 to +20 mV—i.e. the range in which the AP plateau typically occurs.

We also examined the effects on $I_{Ca,L}$ of multiple $I_{Kr}$ blockers that have not been associated with severe arrhythmias. Ciprofloxacin, enoxacin, ofloxacin, and levofloxacin are quinolone antibiotics with variable $I_{Kr}$ potencies that are rarely associated with LQTS risk [15, 16]. They had no effect on $I_{Ca,L}$ (S2 Fig). We also examined the effect on $I_{Ca,L}$ of ketoconazole an antifungal agent which is known to block $I_{Kr}$ but is not associated with TdP risk [17, 18]. Ketoconazole did decrease $I_{Ca,L}$ (Fig 2D). In contrast to SPX, however, it reduced late $I_{Ca,L}$ as well as peak $I_{Ca,L}$ (Fig 2E). Taken together, these data suggest that SPX increased late $I_{Ca,L}$ that might be related with its ability to induce arrhythmias.

**Effect of SPX on the Inactivation Kinetics of $I_{Ca,L}$**

To further investigate the effect of SPX on inactivation kinetics, the decay phase of $I_{Ca,L}$ was fitted using an exponential function. When the decay phase was fitted using a monoeponential function, the time constant (τ) was significantly increased by SPX treatment (Fig 3A). The voltage dependence of τ remained unchanged in the presence of SPX. As shown in Fig 3B, the effects of SPX on inactivation kinetics were dose-dependent. Taken together, these results suggest that SPX induces APD prolongation by slowing $I_{Ca,L}$ inactivation.
SPX reduces Ca\textsuperscript{2+}-dependent \(I_{\text{CaL}}\) inactivation

The effects of SPX on late \(I_{\text{CaL}}\) inactivation were investigated using conventional double-pulse protocol. Prepulses 1000 ms in duration and at various potentials ranging from −50 to +50 mV preceded a 100-ms test pulse at 0 mV. The superimposed current responses to the test pulse (0 mV) are shown in Fig 4A (left, control; right, 300 \(\mu\)M SPX). The slow inactivating \(I_{\text{CaL}}\) in the presence of SPX indicates that SPX exerts a pharmacological effect (Fig 4A, right).

Under control conditions, inactivation increased sharply as the prepotential increased from −40 to −20 mV, reaching a maximum of ~95.8% at +10 mV (Fig 4B). At prepulse potentials greater than +20 mV, the extent of inactivation decreased, resulting in a U-shaped \(I_{\text{CaL}}\) inactivation curve. The data from −100 to +10 mV were fitted using the following Boltzmann equation:

\[
y = \frac{A_1 - A_2}{1 + \exp\left[\frac{(V - V_{1/2})}{\kappa}\right]} + A_2
\]

\[\text{Fig 2. Effect of SPX on } I_{\text{CaL}}.\] A, Representative traces of \(I_{\text{CaL}}\) during a 200-ms voltage-clamp pulse from −40 to 0 mV before (black) and after (red) exposure to 300 \(\mu\)M SPX. B–C, current–voltage (\(I\)-\(V\)) relationships of \(I_{\text{peak}}\) (●, B) and \(I_{\text{end of pulse}}\) (●, C) under control conditions (black) and after application of 300 \(\mu\)M SPX (red) (\(n = 10\)). D, Representative traces of \(I_{\text{CaL}}\) to 1 \(\mu\)M and 10 \(\mu\)M of ketoconazole (blue) (\(n = 4\)). E, The comparison of \(I_{\text{peak}}\) (●, left) and \(I_{\text{s-s}}\) (●, right) from \(I_{\text{CaL}}\) before and after 300 \(\mu\)M SPX (\(n = 10\)) or 10 \(\mu\)M ketoconazole (\(n = 4\)). *\(P < 0.05\) **\(P < 0.01\).

doi:10.1371/journal.pone.0149198.g002
where $V$ is the membrane potential, $V_{1/2}$ is the membrane potential of half-maximum inactivation, and $k$ is the slope of the inactivation curve. $A_1$ represents the maximal amplitude and $A_2$ is the amplitude of the non-inactivating component of $I_{Ca,L}$. $V_{1/2}$ was $-26.2 \pm 1.4$ mV and $k$ was $+4.9 \pm 0.7$ mV under control condition.

The current availability curves produced in the presence of SPX (Fig 4B) indicate that SPX significantly reduced steady-state inactivation. When the data from $-100$ to $+10$ mV were fitted using the Boltzmann equation, the $A_2$ value, the amplitude of the non-inactivating component of $I_{Ca,L}$, was increased by SPX ($0.087 \pm 0.008$ vs. $0.042 \pm 0.012$ in control, $n = 5$, $P < 0.01$). Neither $V_{1/2}$ nor $k$ were affected ($-26.6 \pm 1.5$ mV and $+4.8 \pm 0.8$ mV, respectively; $P > 0.05$). In addition, at prepulse potentials greater than 0 mV, $I_{Ca,L}$ amplitudes in the presence of SPX were greater than those under control conditions ($P < 0.01$; paired $t$-test; $n = 5$).

We confirmed that no differences in the steady-state activation curves (Fig 4C) were present before and after SPX treatment. The voltages for half-activation were $-22.2 \pm 0.1$ mV ($n = 10$) in the control and $-23.2 \pm 1.2$ mV ($n = 10$) in the presence of SPX (paired $t$-test, $P > 0.05$). These data suggest that SPX attenuates inactivation, leading to slower $I_{Ca,L}$ decay.

L-type Ca$^{2+}$ channels can be inactivated by two different mechanisms: CDI and VDI. The Ca$^{2+}$-dependent aspect of L-type Ca$^{2+}$ channel inactivation is dependent on Ca$^{2+}$ entry. Therefore, essentially all inactivation of Ba$^{2+}$ current through the L-type Ca$^{2+}$ channels is voltage dependent. The substitution of Ba$^{2+}$ ions for Ca$^{2+}$ has been used widely to separate the contribution VDI from CDI to the macroscopic $I_{Ca,L}$. We confirmed the results reported previously, showing the Ca$^{2+}$ dependence of CDI in our cells and that the effects of SPX are changed by replacing Ca$^{2+}$ with Ba$^{2+}$ (Fig 5). As shown in Fig 5A, SPX did not slow Ba$^{2+}$ current inactivation. SPX did not increase the inactivation time constant, but instead reduced it, indicating that the attenuation of $I_{Ca,L}$ inactivation by SPX was abolished (Fig 5B). In addition, SPX had little effect on the steady-state inactivation of Ba$^{2+}$ currents (Fig 5C). These results confirm that SPX specifically modulates CDI.
Since SPX slowed the inactivation of $I_{CaL}$ by inhibiting CDI, it is expected that repetitive application of depolarizing voltage steps may cause less accumulation of $I_{CaL}$ inactivation in the presence of SPX. In order to prove this hypothesis, a series of depolarizing step pulses at frequencies of 2Hz were applied (Fig 6A, inset). Fig 6A and 6B shows superimposed current traces of cells in the absence and presence of SPX, respectively. When the depolarizing step pulses were applied repetitively under control conditions, the $I_{CaL}$ peak amplitudes were gradually decreased (50.6 ± 7.3% of the initial level at pulse 13; Fig 6C). In the presence of SPX (300 μM), however, this gradual decrease was significantly attenuated, with the $I_{CaL}$ peak amplitude being 63.3 ± 5.7% of the initial level by pulse 13 (Fig 6C). Fig 6C illustrates the gradual decrease of $I_{CaL}$ peak amplitudes during repetitive pulses in the absence and presence of SPX.
Recovery from inactivation

Inactivation and recovery from inactivation are closely related processes and are critical factors that determine channel function. Recovery from inactivation was investigated by eliciting sustained depolarization (200 ms), followed by recovery intervals of increasing durations, and then applying a subsequent test pulse (Fig 7A, inset). In comparison to the control, recovery from inactivation was not changed by SPX treatment (Fig 7A and 7B). Data were fitted to a single exponential function. The time constants for recovery from inactivation were 365.4 ± 20.0 ms (n = 7) in the control and 382.6 ± 22.9 ms (n = 7) in the presence of SPX (paired t-test, P > 0.05).

Effect of SPX on \( I_{CaL} \) and AP in adult mouse cardiac myocytes

We then examined whether SPX still increases late \( I_{CaL} \) and APDs in cardiac myocytes from adult mouse (6 month old), which is lack of native \( I_{Kr} \). We performed experiments similar to that shown in Fig 2. Fig 8A and 8C show that late \( I_{CaL} \) in adult mouse ventricular myocytes was significantly increased by exposure to 300 μM SPX for 10 min. Interestingly, SPX-induced
reduction of peak $I_{CaL}$ in adult mouse ventricular myocytes was not so pronounced as that observed in neonatal cardiac myocytes (Fig 8B). Consistent with increase in late $I_{CaL}$, AP prolongation was observed when adult mouse cardiac myocytes were treated with SPX (300 μM) (Fig 8D & 8E). Fig 8E summarizes these results and shows that SPX prolonged APD$_{90}$ over a range of stimulation rate in adult mouse cardiomyocytes. In addition, with exposure to SPX, triggered beats arising from early and delayed afterdepolarizations were observed in all cardiac myocytes examined at slow stimulation rate ($n = 4$); an example is shown in Fig 8F. In the absence of drug exposure, no afterdepolarizations were observed in cells ($n = 4$, Fig 8G). These findings exclude the potential E4031 effect and support the idea that SPX can increase late $I_{CaL}$.

Fig 6. Use-dependency of SPX on $I_{CaL}$. A, Repetitive application of depolarizing pulses (shape is shown in the figure inset) to 0 mV from a holding potential of −50 mV gradually decreased the $I_{CaL}$ amplitude. The frequency of depolarizing pulses was 2 Hz. In the presence of SPX, the gradual decrease of $I_{CaL}$ was significantly attenuated. B, Summary of the use-dependent effect of SPX. Normalized current levels are plotted against pulse number (A and B, $n = 6$; black, control; red, SPX). The amplitudes of steady-state currents of both control and SPX group were normalized by the first current of the control. *$P < 0.05$ **$P < 0.01$ ($n = 6$).
and APDs in cardiac myocytes. Recently it was demonstrated that chronic exposure to some $I_{Kr}$ blockers also increases cardiac late $Na^+$ current, which is probably regarded as another mechanism for the drug-induced Q-T prolongation and TdP in patients chronically exposed to (non)-cardiac drugs in clinics [19]. We examined whether chronic exposure (5 hrs) to SPX enhances late $Na^+$ currents in adult mouse ventricular myocytes. However no differences in late $Na^+$ currents were observed between control and SPX-treated cells (S3 Fig), suggesting that the SPX effect on APs could not be attributed to a change in late $Na^+$ currents.

Fig 7. Effect of SPX on time course of the recovery from inactivation. A. Raw current tracings in the absence and presence of SPX. The voltage pulse protocol is shown as a figure inset. B. Summary of time courses of the recovery from inactivation in the absence and presence of SPX (n = 6; black, vehicle; red, SPX). *P < 0.05 **P < 0.01.
Discussion

It has been argued that the extent of $I_{Kr}$ block is imperfect at best as a predictor of effects of a drug in a human subject [20, 21]. Proposed reasons for this discrepancy include a time-dependent effect on biosynthesis of hERG channel or on cell surface trafficking [22, 23] or failure of in vitro testing to consider other ion channel actions such as $I_{CaL}$ or late Na$^+$ currents [19, 24]. Our data showed that SPX markedly prolonged APD in a concentration-dependent manner in cardiac myocytes that lack $I_{Kr}$, suggesting that SPX can be arrhythmogenic via an $I_{Kr}$-independent pathway. SPX reduced peak $I_{CaL}$ but augmented late $I_{CaL}$ recorded several hundred milliseconds after a step depolarization and thus associated with APD prolongation. This effect is not seen with $I_{Kr}$ blockers not associated with severe arrhythmias (ciprofloxacin, enoxacin, ofloxacin, and levofloxacin). We further showed that an antifungal agent ketoconazole, a potent $I_{Kr}$ blocker without severe arrhythmias reduced both peak and late $I_{CaL}$, suggesting a...
close relationship between late $I_{CaL}$ and arrhythmogenesity. Detailed analysis showed that SPX treatment reduced the Ca$^{2+}$-dependent component of steady-state inactivation, indicating that SPX attenuated CDI. Consequently, the steady-state levels of $I_{CaL}$ were increased in the presence of SPX compared to that of the control. Consistent with the observed SPX-induced CDI attenuation, SPX had little effect on the inactivation time constant and steady-state inactivation once extracellular Ca$^{2+}$ was replaced with Ba$^{2+}$, a scenario in which essentially all inactivation is voltage dependent. The progressive use-dependent decrease of $I_{CaL}$, which was assessed by applying repetitive voltage pulses at 2 Hz, was less pronounced in the presence of SPX, indicating the positive effect of SPX on $I_{CaL}$ occurred in a use-dependent manner. The recovery from inactivation of $I_{CaL}$ was not altered by SPX. Taken together, our data suggest that SPX attenuates CDI, and the resulting slower $I_{CaL}$ decay might contribute to SPX-associated EAD and TdP.

The positive effects of SPX on $I_{CaL}$ were concentration dependent, and SPX started to slow Ca$^{2+}$ channel inactivation at a treatment concentration of 10 μM (Fig 3). Moreover, SPX-induced APD prolongation in the presence of E4031 was evident at 30 μM (Fig 1). Because the steady-state plasma concentration of SPX in healthy volunteers and patients was 1.8 μM and the hERG IC$_{50}$ value is 18 μM[16], these results suggest that the slowing of $I_{CaL}$ inactivation may attributable to SPX-induced LQT or arrhythmia under clinical conditions.

We demonstrated that SPX attenuated late $I_{CaL}$ inactivation, especially at depolarized potentials ($\geq$ 0 mV) without voltage shift of steady-state curve. Therefore, inactivation curve was more U-shaped in the presence of SPX (Fig 4). These results suggest that SPX specifically interrupts the Ca$^{2+}$-dependent component of $I_{CaL}$ inactivation, having little effect on the voltage-dependent component. In support of this hypothesis, when Ba$^{2+}$ was used as the $I_{CaL}$ charge carrier (Fig 5), SPX-induced inactivation slowing and the consequent increase in the late $I_{CaL}$ were abolished (Fig 5).

Our data showed that SPX treatment reduced peak $I_{CaL}$ amplitude as well as slowed its inactivation. These two changes have opposing effects on APD. However, the results of previous studies suggest that the kinetics of $I_{CaL}$ inactivation, rather than the amplitude modulates its effects on the APD restitution slope and reentry [25]. Consistent with this concept, SPX induced APD prolongation, because of the dominance of suppressed $I_{CaL}$ inactivation in controlling APD compared to the ongoing reduction of $I_{CaL}$ amplitude. In a context in which there is a concomitant reduction of repolarizing current, which should shorten APs, the slowed $I_{CaL}$ decay is an important factor in tipping the balance towards EAD formation.

Although the precise molecular sites that are responsible for the CDI is not entirely clear yet, it has been demonstrated that two kinds of Ca$^{2+}$-binding sites model (i.e., high-affinity slow and low-affinity fast kinetic binding sites) successfully simulated the CDI obtained by experiments [26,27]. The high affinity binding site was expected to be present very near at inner channel mouth and not to be accessible by intracellular Ca$^{2+}$ buffers such as EGTA or BPATA. Therefore, this CDI is attributable by the influx of Ca$^{2+}$ itself and can’t be excluded by pipette EGTA or BAPTA. It can only be excluded by the substitution of Ca$^{2+}$ with other ions such as Ba$^{2+}$ for the charge carrier. Classically, the 'domain' model of CDI can explain well this high affinity Ca$^{2+}$ binding site model [26–28]. The low-affinity Ca$^{2+}$ binding site can explain well the 'shell' model of CDI, in which global increase in cytosolic [Ca$^{2+}$]i mediates the CDI [27,28]. The Ca$^{2+}$ that are released from intracellular store such as sarcoplasmic reticulum (SR) took the biggest part in the 'shell' or 'low-affinity Ca$^{2+}$ binding site' models [27]. Therefore, the release-dependent inactivation (RDI) was primarily responsible for CDI in the 'low-affinity Ca$^{2+}$ binding site' model [27]. High concentrations of pipette Ca$^{2+}$ buffer such as BAPTA can effectively exclude this CDI that is mediated by the low-affinity Ca$^{2+}$ binding site. Since we used pipette solution with 10 mM EGTA in the present study, it is expected that SR
Ca\textsuperscript{2+} is depleted and the CICR is largely prevented. Therefore, the CDI of this study is thought to be primarily mediated by the high affinity Ca\textsuperscript{2+} binding site probably very near at the channel mouth. Taken together, the slowing of inactivation time course of \( I_{\text{CaL}} \) by SPX was not secondary phenomenon due to the decreases in peak \( I_{\text{CaL}} \) and intracellular [Ca\textsuperscript{2+}], but due to SPX-induced specific inhibition of CDI that is mediated by high affinity Ca\textsuperscript{2+}-binding site (that is, an EGTA-insensitive site). Moreover, lack of effects on the inactivation time courses by ketoconazole (Fig 2), at concentrations that inhibit the peak \( I_{\text{CaL}} \) similarly to those of SPX, also indicates that the CDI of the present study is not mediated by the global intracellular [Ca\textsuperscript{2+}] increase.

In conclusion, the present findings demonstrate the role of \( I_{\text{CaL}} \) in SPX-induced APD prolongation. Our results suggest that modification of \( I_{\text{CaL}} \) properties, in addition to \( I_{\text{Kr}} \) antagonistic activities, should be considered when assessing the proarrhythmic potential of drugs. Especially new drug evaluation will need to look beyond effect on peak \( I_{\text{CaL}} \) and examine drug effects on late \( I_{\text{CaL}} \) of which perturbation induces abnormal repolarization.

Supporting Information

**S1 Fig. L-type and T-type Ca\textsuperscript{2+} channels in neonatal rat cardiomyocytes** A, Ca\textsuperscript{2+} currents were elicited by depolarizing voltage steps from a holding potential of −80 mV in the absence and presence of nifedipine (1 \( \mu \)M) or nifedipine (1 \( \mu \)M) plus NiCl\textsubscript{2} (100 \( \mu \)M). B, Current–voltage (\( I–V \)) relationships of the peak Ca\textsuperscript{2+} current (holding potential −80 mV) in the absence and presence of Ca\textsuperscript{2+} channel inhibitors (black, control; red, Nifedipine; green, Nifedipine + NiCl\textsubscript{2}). C, Ca\textsuperscript{2+} currents were elicited by depolarizing voltage steps from a holding potential of −50 mV in the absence and presence of nifedipine (1 \( \mu \)M). D, Current–voltage (\( I–V \)) relationships of the peak Ca\textsuperscript{2+} currents in the absence and presence of nifedipine (holding potential −50 mV; black, control; red, nifedipine).

(TIF)

**S2 Fig. Effects of quinolones on \( I_{\text{CaL}} \)** Representative traces showing the effect of 1 mM ciprofloxacin (A), 1 mM enoxacin (B), 1 mM ofloxacin (C), and 1 mM levofloxacin (D) on \( I_{\text{CaL}} \). Representative traces of \( I_{\text{CaL}} \) during a 200-ms voltage-clamp pulse from −40 to 0 mV before (black) and after (red) exposure to 300 \( \mu \)M SPX. Lower panels (E–H) summarize the concentration-response of the quinolones.

(TIF)

**S3 Fig. Chronic exposure to SPX does not increase late Na\textsuperscript{+} current** A, Examples of Na\textsuperscript{+} current recorded 5 hours after isolation in the absence (vehicle; left), or in the presence of SPX (right). The selective late current blocker ranolazine did not affect Na\textsuperscript{+} current in SPX-treated cells as well as cells under control condition. B, Summary data show that there was no effect on late Na\textsuperscript{+} current of 5-hour exposure to SPX in adult mouse ventricular myocytes.

(TIF)

Acknowledgments

This work was supported by Grant [08172KFDA508] from the Korea Food and Drug Administration in 2008, Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education [NRF-2009-0071242], and Basic Science Research Program through the NRF funded by the Ministry of Science, ICT & Future Planning [NRF-2012R1A2A2A01046878].
Author Contributions
Conceived and designed the experiments: BK HCS HC YMB. Performed the experiments: JGK DJS SWP HK KJW. Analyzed the data: JGK CHL YHZ KSK. Wrote the paper: HC YMB.

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