Role of Small Conductance Calcium-Activated Potassium Channels in Atrial Electrophysiology and Fibrillation in the Dog

Running title: Qi et al.; Ca\(^{+}\)-dependent K\(^{-}\)-current and AF

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Abstract

**Background**—Recent evidence points to functional Ca\(^{2+}\)‐dependent K\(^+\) (SK)‐channels in the heart that may govern atrial fibrillation (AF) risk, but the underlying mechanisms are unclear. This study addressed the role of SK‐channels in atrial repolarization and AF‐persistence in a canine AF‐model.

**Methods and Results**—Electrophysiological variables were assessed in dogs subjected to atrial remodeling by 7‐day atrial tachypacing (AT‐P), as well as controls. Ionic currents and single‐channel properties were measured in isolated canine atrial cardiomyocytes by patch‐clamp. NS8593, a putative highly‐selective SK‐blocker, suppressed SK‐current with an IC\(_{50}\) of \(~5\mu mol/L\), without affecting Na\(^+\), Ca\(^{2+}\), or other K\(^+\)‐currents. Whole‐cell SK‐current sensitive to NS8593 was significantly larger in pulmonary‐vein (PV) versus left‐atrial (LA) cells, without a difference in SK single‐channel open‐probability (Po), whereas AT‐P enhanced both whole‐cell SK‐currents and single‐channel Po. SK‐current block significantly increased APD in both PV and LA cells. SK2‐expression was more abundant at both mRNA and protein levels for PV versus LA in control dogs, in both control and AT‐P; AT‐P upregulated only SK1 at the protein level. Intravenous administration of NS8593 (5 mg/kg) significantly prolonged atrial refractoriness and reduced AF‐duration without affecting the Wenckebach cycle length (WCL), left ventricular refractoriness or blood pressure.

**Conclusions**  SK‐currents play a role in canine atrial repolarization, are larger in PVs than LA, are enhanced by atrial‐tachycardia remodeling, and appear to participate in promoting AF‐maintenance. These results are relevant to the potential mechanisms underlying the association between SK single nucleotide polymorphisms and AF, and suggest SK‐blockers as potentially interesting anti‐AF drugs.

**Key words:** action potentials, ion channels, antiarrhythmia agents, remodeling, electrophysiology
Introduction

Small-conductance Ca$^{2+}$-activated K$^+$ (SK) channels are widely expressed in different tissues, including nervous system, vascular endothelium, skeletal muscle, smooth muscle and cardiac myocytes.$^{1-3}$ SK-channels are encoded by at least three genes: SK1, SK2, and SK3 (KCNN1, KCNN2, and KCNN3).$^4$ SK-channels are characterized by their small unitary conductance (4-20 pS), sensitivity to submicromolar Ca$^{2+}$, very weak voltage sensitivity, and selective blockade by apamin.$^5-7$ A recently-described SK-blocker, (R)-N-(benzimidazol-2-yl)-1,2,3,4-tetrahydro-1-naphtylamine (NS8593), acts by decreasing the Ca$^{2+}$-sensitivity of SK-channels rather than blocking their pore.$^8$

After early interest in the function of Ca$^{2+}$-dependent K$^+$-channels in the heart, skepticism about the evidence led to a consensus that they do not play a significant role.$^9$ Interest was rekindled by the work of the Chiamvimonvat laboratory, which provided data suggesting functionally-significant atrial expression in mice.$^{10,11}$ Evidence was also provided that short-term atrial tachypacing of rabbit pulmonary veins induces action potential abbreviation by enhancing SK2-channel trafficking to the membrane.$^{12}$ SK2-current density appears to be enhanced in AF.$^{13}$ Recently, single nucleotide polymorphisms (SNPs) in the KCNN3-gene have been implicated in AF-risk.$^{14}$ NS8593 suppresses acetylcholine-induced AF in isolated rat and rabbit hearts, as well as with short-term rat-atrial tachypacing in vivo.$^{15}$

The present study was designed to assess the role of SK-channels in atrial electrophysiology and AF with the use of a dog model. Specifically, we aimed to evaluate: 1) SK-channel expression and role in repolarization of canine left-atrial (LA) and pulmonary-vein (PV) cardiomyocytes; 2) SK-channel changes in the AF-maintaining substrate induced by atrial tachypacing (AT-P); and 3) the potential role of SK-channels in maintaining AF in the
clinically relevant canine AT-P model.

Methods

A summary of principal methods is provided here- for details, see Data Supplement.

Animal Model

Animal care procedures were approved by the Animal Research Ethics Committee of the Montreal Heart Institute. A total of 71 adult mongrel dogs (22-30 kg) were studied, divided into control (n=43) and AT-P (n=32) groups.

To create AT-P dogs, atrioventricular block was created by radiofrequency ablation under ketamine (5.3 mg/kg IV)/diazepam (0.25 mg/kg IV)/1.5 %-isoflurane anesthesia. Unipolar pacing-electrode leads were inserted into the right-ventricular apex and right-atrial (RA) appendage, to pace the right ventricle and RA at 80 and 400 bpm respectively for 1 week. Atrial effective refractory period (aERP) and mean AF duration were measured as described previously. Hearts were then excised and immersed in oxygenated Tyrode’s solution.

Open-chest electrophysiological studies were performed in 14 dogs (7 controls, 7 AT-P), premedicated with ketamine (5.3 mg/kg IV)/diazepam (0.25 mg/kg IV) and anesthetized with 1.5%-isoflurane under mechanical ventilation. Teflon-coated stainless-steel electrodes were inserted into the left ventricle (LV) and left atrial (LA) appendage (LAA) for recording and stimulation. Following baseline recordings, 5 mg/kg NS8593 was injected, and 5 mg/kg/hr NS8593 was infused to produce stable electrophysiological effects. Recordings were then repeated. aERP was measured at basic cycle lengths (BCLs) of 200, 250, and 300 ms. LV ERP (vERP) was measured at a BCL of 300 ms. ERP was the longest S1-S2 failing to capture, with 5-ms S1-S2 decrements. AF was induced by burst-pacing at 50 Hz and 10 V. Mean AF duration
was based on 10 AF-inductions in each dog, or 5 if the mean duration of the first five episodes of AF was longer than 2 minutes. The Wenckebach cycle length (WCL) was measured as longest RA-BCL failing to conduct 1:1.

**Cardiomyocyte Isolation**

Single canine LA and pulmonary-vein (PV)-cardiomyocytes were isolated with previously-described methods.\(^\text{17}\) Isolated cardiomyocytes were stored in 200-μmol/L Ca\(^{2+}\)-containing Tyrode’s solution for action-potential (AP)-recording and in Kraftbruhe (KB) storage-solution for current recording.

**Cellular Electrophysiology**

All in-vitro recordings except for \(I_{Na}\) voltage-clamp and single-channel studies were obtained at 37°C. The whole-cell perforated-patch technique was used to record APs in current-clamp mode and tight-seal patch-clamp to record currents in voltage-clamp mode. Borosilicate glass electrodes (Sutter Instruments, Novato, CA) filled with pipette solution had tip resistances of 2-4 M\(\Omega\). For perforated-patch recording, nystatin-free intracellular solution was placed in the tip of the pipette, then pipettes were back-filled with nystatin-containing (600 μg/mL) solution. Data were sampled at 5 kHz and filtered at 1 kHz. Whole-cell currents are expressed as densities (pA/pF). Junction potentials averaged 10.5 mV and were corrected for APs only. For solution details, see Supplemental Methods. Cell capacitances averaged 90±3.5 and 89±2.9 pF (n=62/group; \(P=\text{NS}\) in control LA and PV cells.

SK-current was recorded using a voltage-ramp from -110 to +70 mV from a holding potential of -55 mV (**Figure 1**), as described previously.\(^\text{18}\) The extracellular solution for whole-cell SK-current recording contained (mmol/L): \(N\)-methylglucamine (NMG) 140; KCl 5.4; MgCl\(_2\) 1; glucose 5; HEPES 10 (pH 7.4, HCl). The internal solution consisted of (mmol/L): potassium-
gluconate 120; KCl 20; MgCl₂ 1.15; EGTA 5; HEPES 10 (pH 7.2, KOH) and CaCl₂ at selected concentrations.³⁰ Ca²⁺ was adjusted to yield a free [Ca²⁺], of 0.01, 100, 200, 500, or 1000 nmol/L with MaxChelator software. Paxillin (1-μmol/L) and TRAM-34 (1-μmol/L) were added to inhibit large-conductance and intermediate-conductance Ca²⁺-activated K⁺-current and niflumic acid (50-μmol/L) to block Ca²⁺-dependent Cl⁻-current.

For single-channel recordings, excised-patch inside-out mode was used to allow application of Ca²⁺ or Ca²⁺-chelator to the intracellular side of the channels. The bath solution was at room temperature and contained (mmol/L): KCl 130, MgCl₂ 1, EGTA 2, Glucose10, HEPES10 (pH 7.4 with 10 M KOH). The pipette solution contained (mmol/L): KCl 140, HEPES 10 (pH 7.4, KOH). Free Ca²⁺ on the intracellular side of the patch was adjusted to various concentrations based on MaxChelator software. Paxillin (1-μmol/L), niflumic acid (50-μmol/L) and TRAM-34 (1-μmol/L) were added in the bath solution. After establishment of the inside-out configuration, Ca²⁺-sensitivity of the channels in the patch was verified by superfusing the patch with Ca²⁺-free solutions until channel openings disappeared. If the channel openings did not disappear after 5 minutes (indicating non-SK channels), the recording was terminated. Open and closed levels were determined from all-point amplitude histograms (Supplemental Figure 1).

The open-state threshold was defined as 50% of single-channel current level. Leak-subtracted current records were idealized and used to construct the channel open probability (nPₒ). NPo was calculated by the equation:

\[ NPo = \left( \sum_{j=1}^{N} \frac{t_j}{T} \right) / T, \]

where Po is the single-channel open-state probability, T is the duration of the measurement, tj is the time spent with j = 1,2…N channels open, and N is the maximal number of simultaneous channel openings seen in the patch. NPo calculations were based on 5-second segments of single-channel recordings. The NPo averages of 24 to 36 segments.
(2-3 min) were used for statistical analysis. Values of $P_o$ were determined by dividing the $nP_o$ by the maximum number of simultaneous openings for each patch. Mean unitary single channel currents were measured at single holding membrane potential (-60 or +60 mV), from which mean single channel conductance was calculated by the equation: $\bar{g} = i / V$, where $\bar{g}$ is the single channel conductance, $i$ is the unitary single channel current and $V$ is the holding potential. Because all patches had more than one channel, open- and closed-times were not analyzed.

**Quantitative Polymerase Chain Reaction (qPCR)**

LA and PV cardiomyocytes were isolated by enzymatic digestion. Tissues were minced with scissors and then filtered on 100 μm mesh. To separate cardiomyocytes from fibroblasts, the cells were centrifuged and the pellet resuspended 3 times in KB (2 minutes, 500 rpm), before a final centrifugation (2 minutes, 1000 rpm) with the cardiomyocyte-containing pellet flash-frozen in liquid-N\textsubscript{2} for subsequent RNA-isolation. RNAs was isolated with Nucleospin RNA-II (Macherey Nagel), including DNase-treatment to prevent genomic contamination. Messenger RNAs were reverse-transcribed with the High-capacity Reverse Transcription kit (Applied Biosystems). Quantitative PCR was performed with TaqMan probes and primers from Applied Biosystems for SK1 (Assay ID: Cf02735889_mH), SK2 and SK3 (custom-designed). G6PD (Assay ID: Cf02646196_m1) was used for normalization. Quantitative PCR reactions were performed with Taqman Gene Expression Master Mix kit (Applied Biosystems) on a Stratagene MX3000. Standard curves were generated for each set of primers over a 2-log range. Relative gene-expression values were calculated by the $2^{-\Delta\Delta CT}$ method.

**Western Blot**

LA and PV cells were homogenized in RadioImmununo Precipitation Assay (RIPA) buffer as previously described.\textsuperscript{19} The homogenate was centrifuged (15,000 rpm, 20 minutes, 4°C). The
supernatant was used for protein concentration measurement by Bradford assay (Bio-Rad, Mississauga, Ontario) with BSA as a standard. For SK1-3, 40-μg protein samples were separated by 10% -Na-dodecylsulfate polyacrylamide-gel electrophoresis. After transfer to nitrocellulose membranes (Bio-Rad), membranes were incubated with anti-KCa2.1 (SK1) 1:200 (Alomone Labs, Jerusalem, Israel), anti-KCa2.3 (SK2) 1:200 (Alomone Labs), anti-KCa2.3 (SK3) 1:200 (Alomone Labs), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Research Diagnostics, Flanders, NJ), followed by goat anti-mouse or anti-rabbit (1/20,000, Santa Cruz Biotechnology, Dallas, TX) HRP-conjugated secondary antibodies. Signals were detected with Western Lightning Chemiluminescence Reagent-Plus (Perkin-Elmer Life Sciences, Waltham, MA) and quantified by videodensitometry.

Data Analysis

Clampfit 9.2 (Axon), GraphPad Prism 5.0, and Origin 5.0 were used for electrophysiological data handling and curve fitting. All data are expressed as mean±SEM. Data were analyzed statistically with repeated-measures mixed effects model when the same set of units of analysis (dogs or cells) was exposed to multiple interventions. Depending on experimental design, multi-level models were chosen to take into account correlation between multiple levels of within-dog and/or within-cell measurements. When analyses were performed for multiple cells per dog, the unit used for analysis was the independent variable dog. When applicable, heterogeneity of variance was accounted for in the models. Multiple-group comparisons and individual group-mean differences were studied using respectively main F-tests of the models and specific contrasts with Bonferroni correction. Adjusted P-values were calculated by multiplying original P-values by the number of comparisons (N) performed; values shown are adjusted values (NxP). The Ns used for correction are provided in the figure legends for all analyses. The statistical
approach used for each data set is now provided in the figure legend. All study data satisfied a Kolmogorov-Smirnov test for normal distribution, except for AF durations, which were normally distributed after log-transformation and were thus analyzed. All analyses were performed with SAS 9.3 (SAS Institute, Cary, North Carolina). \( P<0.05 \) was considered to represent statistical significance.

Results

Effects of NS8593 on Canine Atrial Ionic Currents

Figure 1A shows putative SK-currents recorded with a ramp protocol at baseline (CTL) and after the addition of 10-\( \mu \)mol/L NS8593. The current reversed close to -75 mV (corrected for junction potential) and was strongly inhibited by NS8593. Drug-sensitive current obtained by digital subtraction is shown in Figure 1B. The amplitude of 10-\( \mu \)mol/L NS8593-sensitive current was a function of \( \text{Ca}^{2+} \)-concentration, with a \( K_d \) of the order of 250 nmol/L and no difference between LA and PV cells (Supplemental Figure 2). With 500-nmol/L intracellular [\( \text{Ca}^{2+} \)], NS8593-sensitive current increased with increasing drug concentration (Figure 1C), with an estimated NS8593 IC\textsubscript{50} for current-inhibition of about 5 \( \mu \)mol/L, similar in LA and PV.

To confirm the selectivity of NS8593-effects in canine atrial myocytes, we recorded a variety of currents before and after exposure to 10-\( \mu \)mol/L NS8593. Figure 2A shows original Na\textsuperscript{+}-current (I\textsubscript{Na}) recordings before and after exposure to NS8593, along with mean current-density/voltage data. I\textsubscript{Na} density was similar before versus after the drug. Figure 2B shows original Ca\textsuperscript{2+}-current (I\textsubscript{Ca}) recordings before and after NS8593. As indicated in the mean data at the right, the current was unaffected by the compound. Figures 2C-F show respectively current-density/voltage relations for transient outward (I\textsubscript{o}), inward-rectifier (I\textsubscript{K1}), slow delayed-rectifier
(I_{Ks}) and rapid delayed-rectifier (I_{Kr}) K⁺-currents during voltage-steps according to the voltage-protocols shown in the insets (delivered at 0.1 Hz). Examples of original recordings for each type of current are provided in **Supplemental Figure 3**. We also sought to assess potential effects on the dog ultrarapid delayed rectifier current (I_{Kur.d}) that we previously reported in canine atrial myocytes.\textsuperscript{20} Despite significant effort, in the present experiments we were unable to record any significant current of the type we previously reported. I_{Kur.d} and any analogous currents would contribute to the end-pulse steady-state current (I_{ss}) after I_{to} inactivation.\textsuperscript{20,21} **Supplemental Figures 4A and B** show that NS8593 had no effect on I_{to} and I_{ss} current-voltage relations. We also verified whether NS8593 might have differential effects on LA versus PV I_{K1}, and no such differences were seen (**Supplemental Figures 4C and D**). In summary, NS8593 had no effect on any of the non-SK currents studied.

**Single-channel Properties and Effects of AT-P**

Single-channel recordings of SK-channels are shown in Figure 3. SK_{Ca} channels were characterized by their small conductance, sensitivity to Ca\textsuperscript{2+} and NS8593-inhibition. Figure 3A shows recordings from single patches under 0.01 nmol/L Ca\textsuperscript{2+}, 1 \mu mol/L Ca\textsuperscript{2+} and 1 \mu mol/L Ca\textsuperscript{2+}+10-\mu mol/L NS8593, with one set of recordings from a control LA-cell and the other from a PV-cell. Channel-activity is almost absent in the absence of Ca\textsuperscript{2+}, is greatly enhanced by adding Ca\textsuperscript{2+}, and is suppressed by adding NS8593. Overall mean open-probability data under various conditions are shown in **Supplemental Figure 1** (for control-cells) and **Supplemental Figure 5** (for AT-P). The strong dependence of channel open-probability on [Ca\textsuperscript{2+}], and the clear inhibitory effect of NS8593 are evident. Figure 3B shows corresponding recordings from AT-P dog-cardiomyocytes. Openings are almost absent with 0.01 nmol/L Ca\textsuperscript{2+}, are greatly enhanced by 1-\mu mol/L Ca\textsuperscript{2+} and are suppressed by 10-\mu mol/L NS8593, as in the control dogs, but
compared to control there are more openings in AT-P. **Figures 3C-E** show mean single-channel current-amplitude, conductance at +60 mV and open probability (Po) at 1-μmol/L Ca2+ in the groups studied. Single-channel conductance was of the range expected for SK-channels (about 6-8 pS) and was not different for LA versus PV, nor for AT-P versus control (**Figure 3D**). The same applies to single-channel current amplitude (**Figure 3C**). There were, however, significant differences in single-channel open probability, Po (**Figure 3E**), which was greater in AT-P than control.

**Figure 3** shows that single SK-channel opening is enhanced by AT-P. We also examined the effect of AT-P on whole-cell NS8593-sensitive currents. **Figure 4A** shows whole-cell currents elicited with a ramp protocol before and after NS8593 in LA and PV cells from one control dog. **Figure 4B** shows corresponding currents from an AT-P dog, which were larger (note the difference in current scale compared to **Figure 4A**). **Figures 4C and 4D** show mean data for whole cell NS8593 sensitive currents as a function of voltage in LA and PV cells respectively, for both control and AT-P dogs. Note that these currents were recorded with physiological transmembrane K+ concentration gradients, as for other K+ currents, so the reversal potential is close (around -70 mV after adjusting for junction-potential) to the physiological K+-equilibrum potential. AT-P significantly increased current-densities in both LA and PV cells. NS8593-sensitive current was significantly larger in PV vs LA cells: e.g. at -90 mV: -1.47±0.24 vs -0.58±0.10 pA/pF in CTL (P<0.01), and -2.64±0.31 vs. -1.20±0.13 pA/pF in AT-P (P<0.001).

**Molecular Correlates**

Our functional data indicate that SK-current is larger in canine PV than LA, and is upregulated by AT-P. The increased open-probability of SK-channels in AT-P versus control (**Figure 3E**)
could, at least partially, explain the increased SK-current with AT-P. However, this possibility does not exclude the participation of additional factors like differential subunit-expression, and the similar open-probability for PV versus LA indicates that kinetic factors cannot explain the PV-LA SK-current differences. To address a potential molecular basis at the level of channel-expression differences, we quantified the 3 known SK-subunits at mRNA and protein levels. **Figure 5A** shows mRNA-expression results. Of the 3 subunits tested, *KCNN2* (SK2) was the only differentially-expressed subunit at the mRNA-level, with stronger expression in PV than LA. Mean protein-expression data are shown in **Figure 5B**, with corresponding original Western blots in **Supplemental Figure 6**. Unlike mRNA-expression, SK1 protein-expression was upregulated by AT-P in both PV and LA, without LA-PV differences. SK2 protein-expression paralleled mRNA in being significantly stronger in PV versus LA. SK3 showed no significant expression differences between LA and PV or AT-P versus control. Overall, the expression data are in keeping with our functional findings but suggest a complex picture, with both SK1 and SK2 potentially contributing to AT-P versus control differences and only SK2 accounting for LA versus PV differences, but neither alone perfectly consistent with all findings.

**Role in Repolarization and AF-substrate**

In order to assess the potential functional importance of SK-current in repolarization, we recorded APs from canine LA and PV cardiomyocytes, and examined the effect of NS8593. **Figures 6A-D** show AP recordings from each region/condition studied, before and after 10-μmol/L NS8593. The drug clearly prolonged AP duration (APD) under all conditions, indicating a significant role in repolarization. No statistically-significant changes were seen in resting membrane potential or AP amplitude (**Supplemental Figure 7**) with SK-current block by NS8593.
In Vivo Effects of SK-current Inhibition

To test the potential role of SK-current in AF-maintenance, we administered NS8593 in vivo to CTL and AT-P dogs and studied resulting electrophysiological changes. NS8593 increased atrial ERP, consistent with the AP-changes that we noted. AT-P produced typical changes in atrial ERP, with substantial aERP-abbreviation and reversal of aERP-abbreviation with increased rate (reduced BCL). NS8593 produced substantial aERP increases, which were greater in AT-P dogs (e.g. 89.6±13.6% at BCL 300 ms) than in CTL dogs (15.2±6.3%). Ventricular ERP (Figure 7B) was unaffected by AT-P or NS8593. Wenckebach cycle length (WCL, measurable only in CTL dogs, since AT-P dogs had AV-block) was not altered by NS8593 (Figure 7C). Blood pressure was similarly not significantly altered by the drug (Figure 7D).

AF-responses are shown in Figure 8. CTL dogs had no AF-induction during ERP-testing, with similar results in the presence of NS8593. AT-P significantly increased the percentage of S2 stimuli that produced AF-episodes, and NS8593 significantly reduced AF-inducibility in AT-P dogs (Figure 8A). Figures 8B and C show examples of AF-induction by burst-pacing in control and AT-P dogs respectively, before and after NS8593. Mean AF duration was significantly reduced by NS8593, both under control and AT-P conditions (Figure 8D).

Discussion

In this study, we assessed the role of SK-channels in atrial repolarization and AF-vulnerability. Complementary pharmacological, biophysical and molecular methods were used, and demonstrated that SK-current is present in canine atrium, has greater macroscopic density in PV than LA, and is upregulated at the level of both whole-cell current and single-channel open-probability by AF-related AT-P remodeling. Complex differences in SK-subunit mRNA
and protein expression were noted, and in vivo studies pointed to roles for SK-current in atrial repolarization, AF-related remodeling and AF-susceptibility.

**SK-channels and Arrhythmias**

SK-channels are expressed in a wide variety of tissues, including nervous system, skeletal muscle and smooth muscle and play important roles in many physiological processes. For quite a while, the evidence for a functional role of SK-channels in the heart was considered questionable. An elegant series of papers from the Chiamvimonvat laboratory restored interest in cardiac SK-channels. They first demonstrated the presence of SK-currents in human and mouse hearts, and showed regional differences with atrial dominance, raising the possibility of atrial-selective therapeutic targeting. They then cloned the 3 principal forms of SK-subunits, SK1-3, from mouse hearts, confirming sequence homologies and atrial-predominance of isoforms 1 and 2 and equal atrial/ventricular expression of SK3. Ablation of SK-2 channels in mice led to APD-prolongation and atrial early-afterdepolarization (EAD)-related arrhythmia inducibility. Ozgen et al. showed that short-term burst-pacing of rabbit PVs reduced APD in a way that correlated with increases in apamin sensitive current and increases in SK2 mRNA and protein-expression. Li et al. subsequently showed increased SK-current and SK2-protein expression in patients with persistent AF. The potentially-important role of SK-channels in AF has been highlighted by the finding that variants in the gene (KCNN3) encoding SK3 are significantly related to AF-risk in man. Recent studies have also pointed to important contributions of SK-channels to ventricular repolarization and arrhythmogenesis in cardiac failure and infarction.

Our studies advance the understanding of the role and mechanisms of SK-channel involvement in atrial electrophysiology and fibrillation by providing a number of novel
observations. We demonstrate PV-selective localization of SK-currents and KCa2.2 subunits, which may contribute to the well-recognized but poorly-understood role of the PVs in AF. On the other hand, we also noted upregulation of KCa2.1 subunit protein expression with AT-P. We also performed single-channel analyses of SK-currents in cardiac tissue, which confirmed the biophysical properties of the channels. The increased Po that we observed with AT-P indicates that remodeling-induced increases in macroscopic currents do not arise solely from channel-subunit expression-changes, but that kinetic factors determining open-probability are also involved. Our work with blockers points to a contribution of SK-channels to atrial repolarization, remodeling and AF-susceptibility in a clinically-relevant paradigm of persistent AF-related atrial remodeling.

**SK-current as a Pharmacological Target**

Demonstration of atrial-selective expression of SK-channels pointed to the potential interest of targeting them as an antiarrhythmic strategy with reduced risk of ventricular proarrhythmia. NS8593 has been developed as a prototype membrane-permeable SK-channel blocker that can be given in vivo as an AF suppressing agent. The compound prolongs refractoriness and suppresses cholinergic AF in guinea pig and rabbit hearts. It is also effective in acute burst-pacing induced AF in the rat, an action shared with 2 other SK-blocking compounds. Additionally, NS8593 is effective in hypertensive rats with enhanced AF-vulnerability.

The present study confirms the selectivity of NS8593 for SK-channels, finding no effect on a broad range of other native K⁺-channels as well as native Na⁺ and Ca²⁺ currents. We also tested the drug for the first time in a large-animal model that presents a clinically-relevant paradigm of AF-associated remodeling, finding significant efficacy in both refractoriness-prolongation and suppression of AF-vulnerability. The lack of effect on ventricular refractoriness and blood
Several caveats must be recognized. The role of SK-channels in ventricular rhythm control appears complex. SK-channel activation has been linked to ventricular arrhythmogenesis in acute MI\textsuperscript{31} and experimental heart failure,\textsuperscript{29} suggesting that blocking SK-channels could have useful ventricular antiarrhythmic actions. On the other hand, SK-channel block causes frequency-dependent effects on the APD of failing rabbit ventricles, and blocking them can either promote arrhythmogenesis by impinging on repolarization reserve (predominant at long cycle-lengths) or prevent arrhythmogenesis by flattening the slope of the APD-restitution curve.\textsuperscript{30} Similarly, although in this study we demonstrate clear anti-AF effects in a clinically-relevant dog model, consistent with a number of previous analyses,\textsuperscript{15,33,34} loss of SK-channel function has previously been associated with AF-promoting EADs\textsuperscript{11} and more recently SK-block has been shown to favor the production of alternans, wave-break and atrial arrhythmias in isolated canine atrium.\textsuperscript{35} Finally, it remains to be seen whether targeting of cardiac SK-channels can be achieved without significant detrimental effects on other organ-systems in which SK-channels play important roles.\textsuperscript{1,22-27}

**Potential Limitations**

We used a specific model of AF in which to test the antiarrhythmic efficacy and atrial selectivity of NS8593. However, this is only one of several animal AF-models, each of which reproduces some but far from all aspects of clinical AF pathophysiology.\textsuperscript{32,36} Because of the large density of SK single-channels in canine atrium, we were unable to obtain patches containing only 1 channel, and were therefore unable to analyze precisely regional or remodeling dependent differences in channel kinetics (mean closed or open time values).

We attempted to record $I_{Kur,d}$ in the present study, and indeed in several other
experimental series over the past 10 years, and were unsuccessful. This differs from our experience in the 1990s,\textsuperscript{20} when we first reported this current in dog atrium and routinely saw robust currents. We are not sure whether the difference relates to differences in collagenase lot availability, to subtle changes in isolation technique or to the animals available. Other investigators have reported Kv1.5-type currents in canine atrium,\textsuperscript{21} which we are similarly unable to find. We can only assume that such currents are variously expressed and/or sensitive to unidentified technical details.

Our results regarding the effects of atrial-tachycardia remodeling on SK-currents differ from those of a previous study, which found downregulation of SK-current and proteins in atrial samples from AF-patients.\textsuperscript{37} These discrepancies may reflect species-dependent differences, effects of underlying heart disease in AF-patients, or other presently-unidentified factors.

The principal probe that we used to study $I_{SK}$ was NS8593, and no pharmacological compound is perfectly specific for the target of interest. NS8593 is part of a series of compounds that inhibit SK-currents by negative gating modulation, which results in a shift of the $Ca^{2+}$ concentration response curve towards higher $Ca^{2+}$ concentrations (i.e. reducing affinity of the channel for $Ca^{2+}$).\textsuperscript{38} While the mechanism is presumed to be quite selective and we showed experimentally high selectivity of NS8593 for SK-channels versus a variety of important currents in native cardiomyocytes, we cannot exclude the possibility of effects mediated by other physiological functions that we did not test.

In these experiments, we did not see a statistically-significant difference in resting membrane potentials among the different cell populations and conditions (\textit{Supplemental Figure 7}), although based on previous results in the literature we might have expected PV cells to be less polarized than LA\textsuperscript{39} and AT-P cells to be hyperpolarized relative to control\textsuperscript{40} because of...
differences in $I_{K1}$. Resting potential differences under these conditions are relatively small and may have been masked by inter-cell variability, particularly since $I_{K1}$ is very sensitive to cell isolation. In addition, in the present study we isolated PV-cells from the main body of the PV cardiomyocyte-sleeve, rather than as distally as possible in previous work. Changes in resting potential can have important effects on AF stability, and could be significantly modulated by a nonvoltage-gated $K^+$-conductance like that of SK-channels. In view of the acute rate-dependent atrial-cell $Ca^{2+}$-load resulting from AF, SK-current would be expected to increase and might cause substantial hyperpolarization. The issue of resting-potential changes, and how they are influenced by SK-channel function and remodeling, is therefore an important area to consider in future studies.

Conclusions

In the present study, we have shown: 1. that SK2 subunit-expression and SK-current are greater in canine PV versus LA; 2. that AT-P increases SK1 protein expression, SK-channel open-probability and SK current; and 3. that an SK current blocker increases atrial APD and ERP, while showing clear in vivo anti-AF effects. These results are relevant to the potential mechanisms underlying the association between $SK_{Ca}$ SNPs and AF and support the possibility that SK-blockers may be potentially interesting anti-AF drugs.

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**Conflict of Interest Disclosures:** JGD and MG are employees of Acesion Pharma, which owns the commercial rights to NS8593.

**References:**


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Figure Legends:

Figure 1. A, Examples of whole-cell SK-currents recorded from a canine PV-cardiomyocyte with the ramp-protocol shown, before and after 10-μmol/L NS8593 ([Ca^{2+}]i=500 nmol/L). B, NS8593-sensitive current obtained with digital subtraction; C, NS8593-sensitive currents recorded at -110 mV and 0.1 Hz in canine LA (n/N=5-11/3-7 cells/dogs per point) and PV (n/N=5-13/3-7 cells/dogs per point). Insets: Concentration-response curves.

Figure 2. A, Recordings and current-density versus voltage relations for I_{Na} before and after 10-μmol/L NS8593 in canine left-atrial cells (n/N=11/4 cells/dogs per group); B, Recordings of I_{Ca} and current-density at +10 mV before and after 10-μmol/L NS8593 in canine left-atrial cells (n/N=6/3 cells/dogs per group); C-F, Step-current versus voltage relations for I_{to}, I_{K1}, I_{Ks} and I_{Kr} before and after 10-μmol/L NS8593 (n/N=9-13/3-6 cells/dogs or /guinea pigs per group). I_{to}, I_{K1}, I_{Ks}, and I_{Kr} were recorded in canine left-atrial cells and I_{Kr} in guinea-pig ventricular cardiomyocytes. Statistical comparisons in panels C-F by multi-level repeated measures mixed effect models.

Figure 3. A, B. Examples of single-channel recordings at +60 mV in inside-out patches under conditions indicated for control (CTL, A) and AT-P (B) cells from canine PV or LA, as indicated in figure, at calculated free Ca^{2+}-levels shown. Channel openings are upward deflections (C=closed level, O=open-levels for various numbers of open channels). C, D, E, Mean±SEM data for single-channel current amplitude, unitary conductance, and open probability (Po) of SKCa (n/N=7-9/5 cells/dogs per group) *P<0.05, **P<0.01, CTL versus ATP. Statistical comparisons in panels C-E by two-way repeated measures ANOVA. CTL vs ATP comparison in
E by multi-level repeated-measures mixed-effect models (N=2 for Bonferroni-correction for individual-mean contrasts).

**Figure 4.** A, B, Whole-cell $I_{SK}$ recorded at 0.1 Hz ([Ca$^{2+}$]$_i$=500-nmol/L) with ramp-protocol shown from canine LA-cells (left) and PV-cells (right); C, Current-density voltage relation of NS8593-sensitive current from control (CTL) and AT-P canine LA cells; D, Current-density voltage relation of NS8593-sensitive current from CTL and AT-P canine PV cells (n/N=9-13/5 cells/dogs/group, as specified in figure). $P<0.0001$ for main effect, AT-P versus CTL in C, D (Multi-level repeated-measures mixed-effect model).

**Figure 5.** A, KCNN1-3 mRNA-expression. B, $K_{Ca}$ 2.1-2.3 protein-band intensities normalized to GAPDH. For examples of original Western blots, see Supplemental Figure 6. Statistical comparisons by two-way repeated measures ANOVA. Individual-mean comparison with Bonferroni-correction of individual-mean contrasts (N=4 for Bonferroni-correction).

**Figure 6.** A-D, AP-recordings at 1 Hz from canine LA and PV cardiomyocytes before and after 10-μmol/L NS8593. A, C, APD$_{90}$ in control (CTL) LA (n/N=14/6 cells/dogs per group), AT-P LA (n/N=24/6 cells/dogs per group), B, D CTL PV (n=15/6 cells/dogs per group), AT-P PV (n=26/6 cells/dogs per group) before and after 10-μmol/L NS8593. Comparisons by multi-level repeated-measures mixed effects model to compare before vs after drug (each comparison involves a separate set of cells studied before and after the drug shown).

**Figure 7.** A, Atrial effective refractory period (aERP) before and after NS8593. **$P<0.01$, 

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***P<0.001 control (CTL) versus AT-P; #P<0.05, ##P<0.01, NS8593 versus pre-NS8593 (N=4 for Bonferroni-correction). B, Ventricular effective refractory period (vERP) before and after NS8593; C, Wenckebach cycle length (WCL); D, Systolic (SBP) and diastolic (DBP) blood pressure. Statistical comparisons in A, B, D by multi-way repeated measures ANOVA. Individual-mean comparison with Bonferroni-correction (N=4 for Bonferroni-correction). Comparison in C by paired t-test.

**Figure 8.** A, AF inducibility (as % of dogs in which AF was induced during aERP-determination) before and after NS8593. B, C, ECG-recordings of AF-episodes induced by burst-pacing in control (CTL) and AT-P dogs before and after NS8593. D, AF-duration before and after NS8593. **P<0.01, ***P<0.001 NS8593 versus pre-NS8593. Statistical comparison by two-way repeated measures ANOVA (N=4 for Bonferroni-correction of individual-mean contrasts) in A and D.
Figure 1

A) Graph showing current amplitude over time with voltage changes from -55 mV to +70 mV. Cm = 108 pF. NS8593 sensitive current.

B) Graph showing NS8593 sensitive current over time.

C) Graph showing NS8593 sensitive current at -110 mV. IC50 = 5.6 µM for LA and IC50 = 4.7 µM for PV.
Figure 2
Figure 3
Figure 4

A

CTL

LA

PV

Cm=95 pF

Cm=110 pF

B

AT-P

Cm=112 pF

Cm=105 pF

NS8593-sensitive current

C

LA

PV

Current Density (pA/pF)

TP (mV)

P<0.001

D

Current Density (pA/pF)

TP (mV)

P<0.0001

Pre-NS8593

NS8593
Figure 5

*mRNA* and *Protein* expression levels of SK channels (SK1, SK2, SK3) and KCNN channels (KCN1, KCNN2, KCNN3, KCNN3) under LA and PV conditions.

*SK1*

- mRNA expression: LA CTL > PV CTL, LA AT-P > PV AT-P
- Protein expression: SK1/GAPDH ratio: LA > PV

*SK2*

- mRNA expression: KCNN2 expression in LA AT-P is significantly higher than in PV AT-P
- Protein expression: KCNN2/GAPDH ratio: LA > PV

*SK3*

- mRNA expression: LA (N=9) > PV (N=9)
- Protein expression: KCNN3/GAPDH ratio: LA (N=5) > PV (N=5)

*P* < 0.05, **P** < 0.01 for LA vs PV under corresponding condition.
Figure 6

Panel A: LA
- CTL
- P = 0.497
- BL: 14/6
- NS8593: 14/6

Panel B: PV
- P = 0.043
- BL: 15/6
- NS8593: 15/6

Panel C: AT-P
- P = 0.024
- BL: 24/6
- NS8593: 24/6

Panel D: AT-P
- P = 0.002
- BL: 26/6
- NS8593: 26/6
**P<0.001, pre-drug vs post-NS8593**

![Graph A](image1)

**P<0.01, ***P<0.001** pre-drug vs post-NS8593

![Graph D](image2)

**CTL**

Burst pacing

**CTL+NS8593**

Burst pacing

![Graph B](image3)

**20 s**

AT-P

AF episode

![Graph C](image4)

**AT-P+NS8593**

AF episode

![Graph D](image5)

**40 s**

Figure 8
Role of Small Conductance Calcium-Activated Potassium Channels in Atrial Electrophysiology and Fibrillation in the Dog

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SUPPLEMENTAL MATERIAL
Detailed Methods

Animal Model

All animal care procedures followed Canadian Council on Animal Care and National Institutes of Health guidelines and were approved by the Animal Research Ethics Committee of the Montreal Heart Institute. A total of 71 adult mongrel dogs (22-30 kg) were studied, divided into control (n=43) and AT-P (n=32) groups.

To create AT-P dogs, atrioventricular block was created by radiofrequency ablation following premedication with ketamine (5.3 mg/kg IV)/diazepam (0.25 mg/kg IV) and anesthesia with 1.5 %-isoflurane. Under aseptic conditions and fluoroscopic guidance, unipolar pacing-electrode leads were inserted via jugular veins into the right-ventricular apex and the right atrial (RA) appendage, and connected to pacemakers implanted in subcutaneous tissues of the neck. Following 24-hour recovery, the right ventricle was paced at 80 bpm and the RA was paced at 400 bpm for 1 week. On study days, dogs prepared for cell-isolation and ionic-current analysis were anesthetized with morphine (2 mg/kg SC) and α-chloralose (120 mg/kg IV load at 29.25 mg/kg per hour) and ventilated mechanically. After performance of a median sternotomy, the atrial effective refractory period and mean AF duration induced by burst pacing were measured as described previously. After open-chest study, hearts were excised through a median thoracotomy and immersed in oxygenated Tyrode’s solution. Atrial tissue was subjected to enzymatic digestion as described below for cell isolation.

Open-chest electrophysiological studies to study the in vivo effects of NS8593 were performed in 14 dogs (7 controls and 7 AT-P). Dogs were premedicated with ketamine (5.3 mg/kg IV)/diazepam (0.25 mg/kg IV) and anesthetized with 1.5%-isoflurane under
mechanical ventilation. In AT-P dogs, the tachypacemaker was deactivated. A median thoracotomy was performed. Through a small incision in the pericardium, teflon-coated stainless-steel electrodes were inserted into the left ventricle (LV) and left atrial (LA) appendage (LAA) for recording and stimulation. A programmable stimulator was used to deliver 2-ms twice-threshold current pulses. Following baseline recordings, 5 mg/kg NS8593 was injected as a bolus over 2 minutes, and an additional 5 mg/kg/hr NS8593 was infused for the remainder of the experiment (generally 20-30 min) to produce stable electrophysiological effects. Recordings were then repeated. Effective refractory period (ERP) in the LAA (aERP) was measured at basic cycle lengths (BCLs) of 200, 250, and 300 ms. LV ERP (vERP) was measured at a BCL of 300 ms. ERP was determined with 10 basic stimuli (S1) followed by a premature extrastimulus (S2) with 5-ms decrements. The longest S1-S2 failing to capture defined the ERP. The mean of 3 aERP values at each BCL was used for analysis. AF was induced with atrial burst-pacing at 50 Hz and 10 V. Mean AF duration was based on 10 AF inductions in each dog. If the mean duration of the first five episodes of AF was longer than 2 minutes, AF was only induced five times. The Wenckebach cycle length (WCL: longest RA-BCL failing to conduct 1:1 to the ventricles) was measured to evaluate effects on AV-nodal properties. Blood pressure and heart rate were monitored.

**Cardiomyocyte Isolation**

For in vitro study, dogs were anesthetized as described above, euthanized by cardiac excision, and atrial tissue taken for cell-isolation. Single canine LA and pulmonary-vein (PV)-cells were isolated with previously-described methods. The heart was removed.
after intra-atrial injection of heparin (10,000 U), immersed in 2-mmol/L Ca\(^{2+}\)-containing Tyrode’s solution, the left circumflex coronary artery was cannulated and perfused (along with the LA and PV tissue it supplies) with 2-mmol/L Ca\(^{2+}\)-containing Tyrode’s solution (37°C, 100% O\(_2\)), then with Ca\(^{2+}\)-free Tyrode’s solution (~10 minutes), followed by ~60-minute perfusion with the same solution containing collagenase (~0.48 mg/mL, CLSII, Worthington) and 0.1% bovine serum albumin (BSA, Sigma). Any leaking coronary-artery branches were ligated immediately after cannulation to ensure adequate tissue-perfusion. Tissues were minced and cardiomyocytes harvested. Isolated cardiomyocytes were stored in 200-μmol/L Ca\(^{2+}\)-containing Tyrode’s solution for action-potential (AP)-recording and in Kraftbruhe (KB) storage-solution for current recording.

**Cellular Electrophysiology**

All in-vitro recordings except for I\(_{\text{Na}}\) voltage-clamp and single-channel studies were obtained at 37°C. The whole-cell perforated-patch technique was used to record APs in current-clamp mode and tight-seal patch-clamp to record currents in voltage-clamp mode. Borosilicate glass electrodes (Sutter Instruments, Novato, CA) filled with pipette solution were connected to a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA). Electrodes had tip resistances of 2-4 MΩ. For perforated-patch recording, nystatin-free intracellular solution was placed in the tip of the pipette by capillary action (~30 s), then pipettes were back-filled with nystatin-containing (600-μg/mL) pipette solution. Data were sampled at 5 kHz and filtered at 1 kHz. Whole-cell currents are expressed as densities (pA/pF). Junction potentials between bath and pipette solutions averaged -10.5 mV and corrected values are shown for APs only. KB-solution contained
(mmol/L): KCl 20, KH$_2$PO$_4$ 10, dextrose 10, mannitol 40, L-glutamic acid 70, β-OH-butyric acid 10, taurine 20, and EGTA 10 and 0.1% BSA (pH 7.3, KOH). Tyrode’s solution contained (mmol/L): NaCl 136, CaCl$_2$ 1.8, KCl 5.4, MgCl$_2$ 1, NaH$_2$PO$_4$ 0.33, dextrose 10, and HEPES 5, titrated to pH 7.3 with NaOH. The pipette solution for AP-recording contained (mmol/L) GTP 0.1, potassium-aspartate 110, KCl 20, MgCl$_2$ 1, MgATP 5, HEPES 10, sodium-phosphocreatine 5, and EGTA 0.005 (pH 7.4, KOH). Cell capacitances averaged 90±3.5 and 89±2.9 pF (n=62 per group; $P$=NS) in control LA and PV cells.

SK-current was recorded using a ramp voltage-clamp protocol from -110 to +70 mV, with a slope of 360 mV/s from a holding potential of -55 mV (Figure 1), as described previously. SK-current was recorded with an extracellular solution containing (mmol/L): N-methylglucamine (NMG) 140; KCl 5.4; MgCl$_2$ 1; glucose 5; HEPES 10 (pH 7.4, HCl). The internal solution consisted of (mmol/L): potassium-gluconate 120; KCl 20; MgCl$_2$ 1.15; EGTA 5; HEPES 10 (pH 7.2, KOH) and CaCl$_2$ at selected concentrations. Ca$^{2+}$ was adjusted to yield a free [Ca$^{2+}$]$_i$ of 0.01, 100, 200, 500, or 1000 nmol/L based on calculations with MaxChelator software. Paxillin (1-µmol/L) and TRAM-34 (1-µmol/L) were added to inhibit large-conductance and intermediate-conductance Ca$^{2+}$-activated K$^+$-current respectively. Niflumic acid (50-µmol/L) was added to block Ca$^{2+}$-dependent Cl$^-$-current. NS8593-sensitive current was obtained using digital subtraction. Other K$^+$-currents were studied with Tyrode’s extracellular solution (a holding potential of -60 mV or more positive was used to inactivate Na$^+$-current) and internal solution containing (mmol/L): potassium-aspartate 110, KCl 20, MgCl$_2$ 1, MgATP 5, LiGTP 0.1, HEPES 10, sodium-phosphocreatine 5, and EGTA 5.0 (pH 7.3,
KOH). E-4031 (5-μmol/L) was added to block rapid delayed-rectifier ($I_{Kr}$) for slow delayed-rectifier ($I_{Ks}$)-recording. HMR1566 (500-nmol/L) was added to block $I_{Ks}$ for $I_{Kr}$-recording. For transient-outward ($I_{to}$) and inward-rectifier ($I_{K1}$) $K^+$-current recording, nifedipine was replaced by CdCl$_2$ (200-μmol/L). $I_{to}$ was studied in the presence of 10-mmol/L tetraethylammonium to inhibit the ultrarapid delayed-rectifier current, except in experiments attempting to record ultrarapid delayed-rectifier current or its counterpart, steady-state end-pulse current, $I_{ss}$. $I_{K1}$ was recorded as the 1-mmol/L Ba$^{2+}$-sensitive current.

The extracellular solution for Ca$^{2+}$-current ($I_{Ca}$) measurement contained (mmol/L): tetraethylammonium-chloride 136, CsCl 5.4, MgCl$_2$ 1, CaCl$_2$ 2, NaH$_2$PO$_4$ 0.33, dextrose 10, and HEPES 5 (pH 7.4, CsOH). Niflumic acid (50 μmol/L) was added to inhibit Ca$^{2+}$-dependent Cl$^-$-current, and 4-aminopyridine (2 mmol/L) to suppress $I_{to}$. The pipette solution for $I_{Ca}$-recording contained (mmol/L) CsCl 120, tetraethylammonium-chloride 20, MgCl$_2$ 1, EGTA 10, MgATP 5, HEPES 10, and Li-GTP 0.1 (pH 7.4, CsOH). For $I_{Na}$-recording, the external solution contained (mmol/L): NaCl 10, tetraethylammonium-Cl 126, MgCl$_2$ 3.0, CsCl 5.4, HEPES 10, 4-AP 2, glucose 5.5 (pH 7.35, CsOH). The internal solution contained (mmol/L): CsCl 120, Tetraethylammonium-Cl 20, MgCl$_2$ 1.0, HEPES 10, EGTA 10, MgATP 5, Li-GTP 0.1 (pH 7.2, CsOH). $I_{Na}$ was recorded at room temperature.

For single-channel recordings, excised-patch inside-out mode was used to allow application of Ca$^{2+}$ or Ca$^{2+}$-chelator to the intracellular side of the channels. The bath solution was at room temperature and contained (mmol/L): KCl 130, MgCl$_2$ 1, EGTA 2, Glucose10, HEPES10 (pH 7.4 with 10 M KOH). The pipette solution contained
(mmol/L): KCl 140, HEPES 10 (pH 7.4, KOH). Free Ca\textsuperscript{2+} on the intracellular side of the patch was adjusted to various concentrations based on MaxChelator software. Paxillin (1-\textmu mol/L), niflumic acid (50-\textmu mol/L) and TRAM-34 (1-\textmu mol/L) were added in the bath solution. After establishment of the inside-out configuration, Ca\textsuperscript{2+}-sensitivity of the channels in the patch was verified by superfusing the patch with Ca\textsuperscript{2+}-free solutions until channel openings disappeared. If the channel openings did not disappear after 5 minutes (indicating non-SK channels), the recording was terminated. Open and closed levels were determined from all-point amplitude histograms (Supplemental Figure 1). The open-state threshold was defined as 50% of single-channel current level. Leak-subtracted current records were idealized and used to construct the channel open probability (nPo). \( NP_o \) was calculated by the equation:

\[
NP_o = \left( \sum_{j=1}^{N} t_j \right) / T ,
\]

where \( Po \) is the single-channel open-state probability, \( T \) is the duration of the measurement, \( t_j \) is the time spent with \( j = 1,2...N \) channels open, and \( N \) is the maximal number of simultaneous channel openings seen in the patch. \( NP_o \) calculations were based on 5-second segments of single-channel recordings. The \( nP_o \) averages of 24 to 36 segments (2-3 min) were used for statistical analysis. Values of \( Po \) were determined by dividing the \( nP_o \) by the maximum number of simultaneous openings for each patch. Mean unitary single channel currents were measured at single holding membrane potential (-60 or +60 mV), from which mean single channel conductance was calculated by the equation: \( \gamma = i/V \), where \( \gamma \) is the single channel conductance, \( i \) is the unitary single channel current and \( V \) is the holding potential. Because all patches had more than one channel, open- and closed-times were not analyzed.
Quantitative Polymerase Chain Reaction (qPCR)

LA and PV cardiomyocytes were isolated by enzymatic digestion. Tissues were minced with scissors and then filtered on 100 µm mesh. To separate cardiomyocytes from fibroblasts, the cells were centrifuged and the pellet resuspended 3 times in KB (2 minutes, 500 rpm), before a final centrifugation (2 minutes, 1000 rpm) with the cardiomyocyte-containing pellet flash-frozen in liquid-N₂ for subsequent RNA-isolation. RNAs was isolated with Nucleospin RNA-II (Macherey Nagel), including DNase-treatment to prevent genomic contamination. Messenger RNAs were reverse-transcribed with the High-capacity Reverse Transcription kit (Applied Biosystems). Quantitative PCR was performed with TaqMan probes and primers from Applied Biosystems for SK1 (Assay ID: Cf02735889_mH), SK2 and SK3 (custom-designed). G6PD (Assay ID: Cf02646196_m1) was used for normalization. Quantitative PCR reactions were performed with Taqman Gene Expression Master Mix kit (Applied Biosystems) on a Stratagene MX3000. Standard curves were generated for each set of primers over a 2-log range. Relative gene-expression values were calculated by the $2^{-\Delta\Delta Ct}$ method.

Western Blot

LA and PV cells were homogenized in RadioImmuno Precipitation Assay (RIPA) buffer as previously described. The homogenate was centrifuged (15,000 rpm, 20 minutes, 4°C). The supernatant was used for protein concentration measurement by Bradford assay (Bio-Rad, Mississauga, Ontario) with BSA as a standard. For SK1-3, 40-µg protein samples were separated by 10% -Na-dodecylsulfate polyacrylamide-gel electrophoresis. After transfer to nitrocellulose membranes (Bio-Rad), membranes were
incubated with anti-KCa2.1 (SK1) 1:200 (Alomone Labs, Jerusalem, Israel), anti-KCa2.3 (SK2) 1:200 (Alomone Labs), anti-KCa2.3 (SK3) 1:200 (Alomone Labs), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Research Diagnostics, Flanders, NJ), followed by goat anti-mouse or anti-rabbit (1/20,000, Santa Cruz Biotechnology, Dallas, TX) HRP-conjugated secondary antibodies. Signals were detected with Western Lightning Chemiluminescence Reagent-Plus (Perkin-Elmer Life Sciences, Waltham, MA) and quantified by videodensitometry.

Data Analysis

Clampfit 9.2 (Axon), GraphPad Prism 5.0, and Origin 5.0 were used for electrophysiology data handling and curve-fitting. All data are expressed as mean±SEM. Data were analyzed statistically with repeated-measures mixed effects model when the same set of units of analysis (dogs or cells) was exposed to multiple interventions. Depending on experimental design, multi-level models were chosen to take into account correlation between multiple levels of within-dog and/or within-cell measurements. When analyses were performed for multiple cells per dog, the unit used for analysis was the independent variable dog. When applicable, heterogeneity of variance was accounted for in the models. Multiple-group comparisons and individual group-mean differences were studied using respectively main F-tests of the models and specific contrasts with Bonferroni correction. Adjusted P-values were calculated by multiplying original P-values by the number of comparisons (N) performed; values shown are adjusted values (N×P). The Ns used for correction are provided in the figure legends for all analyses. The statistical approach used for each data set is now provided
in the figure legend. All study data satisfied a Kolmogorov-Smirnov test for normal
distribution, except for AF durations, which were normally distributed after log-
transformation and were thus analyzed. All analyses were performed with SAS 9.3 (SAS
Institute, Cary, North Carolina).
References:


Supplemental Figure 1. A, SK single-channel recordings from canine LA and PV under conditions indicated. B, All-points histogram generated from current-recordings of the type shown in A. C, Channel open-probability (Po) of SK$_{Ca}$ channel openings under 0.01-nmol/L Ca$^{2+}$, 1-µmol/L Ca$^{2+}$ and 1-µmol/L Ca$^{2+}$+10-µmol/L NS8593 (n/N=7-8/6 cells/dogs per group). Statistical comparison by Statistical comparison by multi-level repeated-measures mixed-effect models (N=2 for Bonferroni-correction).
Supplemental Figure 2. Ca\(^{2+}\)-dependence of NS8595-sensitive I\(_{SK}\) dose-response curves at a test potential of +70 mV using [Ca\(^{2+}\)]\(_i\) from 0.01 to 1000 nmol/L from CTL canine LA (n/N=9-13/3-6 cells/dogs per point) and PV cells (n/N=7-13/3-6 cells/dogs per point), AT-P LA (n/N=7-14/3-5 cells/dogs per point) and PV cells (n/N=6-13/3-5 cells/dogs per point). Since a saturating effect of Ca\(^{2+}\) could not be achieved because of cell lethality at higher concentrations, the EC\(_{50}\) was based on the calculated concentration to achieve 50% of the maximum effect observed.
Supplemental Figure 3. A-C, Recordings of $I_{\text{to}}$, $I_{\text{K1}}$, $I_{\text{Ks}}$ from canine left-atrial cardiomyocytes before and after 10-µmol/L NS8593; D, $I_{\text{Kr}}$-recordings from guinea pig ventricular cardiomyocytes before and after 10-µmol/L NS8593.
Supplemental Figure 4. A, B. Current-density voltage relation for $I_{to}$ and end-pulse steady-state current ($I_{ss}$) from canine left-atrial cardiomyocytes before and after 10-μmol/L NS8593 (n/N=10/4 cells/dogs per group). C, D. Mean±SEM $I_{K1}$ density in control canine LA and PV cells and before and after 10-μmol/L NS8593 (n/N=10-17/4 cells/dogs per group). Statistical comparison by multi-level repeated-measures mixed-effect models.
Supplemental Figure 5. A, B. All-points histograms from canine AT-P LA and AT-P PV cells. C, Open probability (Po) of SKCa channel openings under 0.01-nmol/L Ca\(^{2+}\), 1-μmol/L Ca\(^{2+}\) and 1-μmol/L Ca\(^{2+}\)+10-μmol/L NS8593 from AT-P LA, AT-P PV cells. (n/N=9/6 cells/dogs per group). Statistical comparison Statistical comparison by multi-level repeated-measures mixed-effect models (N=2 for Bonferroni-correction).
**Supplemental Figure 6.** Examples of SK1 (KCa$_{2.1}$), SK2 (KCa$_{2.2}$), SK3 (KCa$_{2.3}$) and GAPDH immunoblots from dog tissue.
Supplemental Figure 7. Mean data for resting membrane potential (RP) and action potential amplitude (APA) from CTL LA (n/N=14/6 cells/dogs per group), CTL PV (n/N=15/6 cells/dogs per group), ATP LA (n/N=24/6 cells/dogs per group) and AT-P PV (n/N=26/6 cells/dogs per group) canine cells before and after 10-µmol/L NS8593. Statistical comparison by multi-level repeated-measures mixed-effect models.