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

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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 selective SK blocker, suppressed SK current with an IC_{50} of ≈5 μmol/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 (P_{o}), whereas AT-P enhanced both whole-cell SK currents and single-channel P_{o}. SK-current block increased action potential duration in both PV and LA cells after AT-P; but only in PV cells in absence of AT-P. 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, 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. (Circulation. 2014;129:430-440.)

Key Words: action potentials ■ anti-arrhythmia agents ■ electrophysiology ■ ion channels

Small-conductance Ca^{2+}-activated K^+ (SK) channels are expressed widely 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

Clinical Perspective on p 440

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 (AT-P) of rabbit pulmonary veins (PVs) induces action potential (AP) abbreviation by enhancing SK2-channel trafficking to the membrane.12 SK2-current density appears to be enhanced in atrial fibrillation (AF).13 Recently, single-nucleotide polymorphisms 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 AT-P in vivo.15

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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 the following: (1) SK-channel expression and role in repolarization of canine left atrial (LA) and PV cardiomyocytes; (2) SK-channel function and expression changes in the AF-maintaining substrate induced by AF-P; and (3) the potential role of SK channels in maintaining AF in the clinically relevant canine AF-P model.

Methods

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

Animal Model

Animal care procedures were approved by the Animal Research Ethics Committee of the Montreal Heart Institute. A total of 75 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. After baseline recordings, 5 mg/kg 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 and left-atrial (LA) appendage for recording and stimulation. After baseline recordings, 5 mg/kg NS8593 was injected, and 5 mg·kg⁻¹·h⁻¹ 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. Left ventricular ERP 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 5 episodes of AF was longer than 2 minutes. The Wenckebach cycle length was measured as the longest RA BCL failing to conduct 1:1.

Cardiomyocyte Isolation

Single canine LA and PV cardiomyocytes were isolated with previously described methods. Isolated cardiomyocytes were stored in 200 μmol/L Ca²⁺-containing Tyrode’s solution for AP recording and in Kraftbruhe storage solution for current recording.

Cellular Electrophysiology

All in vitro recordings except for Na⁺ current 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 to 4 mol/L. For perforated-patch recording, nystatin-free intracellular solution was placed in the tip of the pipette, and then pipettes were backfilled 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 (picoamperes per picofarads). Junction potentials averaged 10.5 mV and were corrected for APs only. For solution details, see online-only Data Supplement Methods. Cell capacitances averaged 90±3.5 and 89±2.9 pF (n=62 per group; P value is nonsignificant) 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. The extracellular solution for whole-cell SK-current recording contained the following (in mmol/L): 140 N-methylglucamine, 5.4 KCl, 1 MgCl₂, 5 glucose, and 10 HEPES (pH 7.4, HCl). The internal solution consisted of the following (in mmol/L): 120 potassium gluconate, 20 KCl, 1.15 MgCl₂, 5 EGTA, 10 HEPES (pH 7.2, KOH), and CaCl₂ at selected concentrations. 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-[[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole) (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 Ca\textsuperscript{2+} or Ca\textsuperscript{2+} chelator to the intracellular side of the channels. The bath solution was at room temperature and contained the following (in mmol/L): 130 KCl, 1 MgCl\textsubscript{2}, 2 EGTA, 10 glucose, and 10 HEPES (pH 7.4 with 10 mol/L KOH). The pipette solution contained the following (in mmol/L): 140 KCl and 10 HEPES (pH 7.4, KOH). Free Ca\textsuperscript{2+} on the intracellular side of the pipette solution contained the following (in mmol/L): 140 KCl and 10 glucose, and 10 HEPES (pH 7.4, KOH). Free Ca\textsuperscript{2+} on the intracellular side of the channels. The bath solution was at room temperature and contained the following (in mmol/L): 130 KCl, 1 MgCl\textsubscript{2}, 2 EGTA, 10 glucose, and 10 HEPES (pH 7.4 with 10 mol/L KOH). The pipette solution contained the following (in mmol/L): 140 KCl and 10 HEPES (pH 7.4, KOH). Free Ca\textsuperscript{2+} on the intracellular side of the pipette solution contained the following (in mmol/L): 140 KCl and 10 glucose, and 10 HEPES (pH 7.4, KOH). Free Ca\textsuperscript{2+} on the intracellular side of the pipette solution contained the following (in mmol/L): 140 KCl and 10 glucose, and 10 HEPES (pH 7.4, KOH).

\[
i_{\gamma} = \frac{\sum_{j=1}^{N} i_{j} T_{j}}{N}
\]

where \(P\) is the single-channel open-state probability, \(T\) is the duration of the measurement, \(i\) is the time spent with \(j=1,2,\ldots,N\) channels open, and \(N\) is the maximal number of simultaneous channel openings seen in the patch. \(N_{o}\) calculations were based on 5-second segments of single-channel recordings. The \(N_{o}\) averages of 24 to 36 segments (2–3 minutes) were used for statistical analysis. Values of \(P\) were determined by dividing the \(N_{o}\) by the maximum number of simultaneous openings for each patch. Mean unitary single-channel currents were calculated at single holding membrane potential (–60 or +60 mV), from which mean single-channel conductance was calculated by the following 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**

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 was resuspended 3 times in Kraftbruhe solution (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. RNA was isolated with Nucleospin RNA-II (Macherey-Nagel), including deoxyribonuclease treatment to prevent genomic contamination. mRNA were reverse transcribed with the High-Capacity Reverse Transcription kit (Applied Biosystems). Quantitative polymerase chain reaction was performed with TaqMan probes and primers from Applied Biosystems for SK1 (assay identification no. C\textsuperscript{0}2735889\textunderscore mH), SK2, and SK3 (custom designed). Glucose-6-phosphate dehydrogenase (assay identification no. C\textsuperscript{0}2646196\textunderscore m1) was used for normalization. Quantitative polymerase chain reaction 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\textsuperscript{–}\(\Delta\Delta C\text{t}\) method.

**Western Blot**

LA and PV cells were homogenized in radioimmunoprecipitation assay buffer as described previously. 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, Canada) with BSA as a standard. For

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SK1–SK3, 40-µg protein samples were separated by 10% SDS-PAGE. After transfer to nitrocellulose membranes (Bio-Rad), membranes were incubated with anti-KCa2.1 (SK1) at 1:200 (Alomone Labs, Jerusalem, Israel), anti-KCa2.3 (SK2) at 1:200 (Alomone Labs), anti-KCa2.3 (SK3) at 1:200 (Alomone Labs), and antiglyceraldehyde-3-phosphate dehydrogenase (Research Diagnostics, Flanders, NJ), followed by goat antimouse or antirabbit (1:20,000, Santa Cruz Biotechnology, Dallas, TX) horseradish peroxidase-conjugated secondary antibodies. Signals were detected with Western Lightning Chemiluminescence Reagent-Plus (PerkinElmer Life and Analytical Sciences, Waltham, MA) and quantified by video densitometry.

Data Analysis
Clampfit 9.2 (Molecular Devices), 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, multilevel models were chosen to take into account correlation between multiple levels of within-dog 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 main F tests of the models and specific contrasts with Bonferroni’s correction, respectively. 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 N values used for correction are provided in the figure legends for all analyses. The statistical approach used for each dataset is 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, NC). 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 (control) and after the addition of 10 µmol/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 µmol/L NS8593-sensitive current was a function of Ca2+ concentration, with a Kd of the order of 250 nmol/L and no difference between LA and PV cells (see Figure II in the online-only Data Supplement). With 500 nmol/L [Ca2+]i NS8593-sensitive current increased with increasing drug concentration (Figure 1C), with an estimated NS8593 IC50 for current inhibition of ~5 µmol/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 µmol/L NS8593. Figure 2A shows original Na+ current recordings before and after exposure to NS8593, along with mean current–density/voltage data. Na+ current density
was similar before versus after the drug. Figure 2B shows original Ca\(^{2+}\) current recordings before and after NS8593. As indicated in the mean data at the right, the current was unaffected by the compound. Figure 2C through 2F shows, respectively, current–density/voltage relations for transient outward, inward-rectifier, slow delayed-rectifier, and rapid delayed-rectifier 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 Figure III in the online-only Data Supplement. We also sought to assess potential effects on the dog ultrarapid delayed rectifier current that we reported previously in canine atrial myocytes.\(^{20}\) Despite significant effort, in the present experiments, we were unable to record any significant current of the type we reported previously. The ultrarapid delayed rectifier current and any analogous currents would contribute to the end-pulse steady-state current after transient outward current inactivation.\(^{20,21}\) Figure IVA and IVB in the online-only Data Supplement shows that NS8593 had no effect on transient outward and steady-state current–voltage relations. We also verified whether NS8593 might have differential effects on LA versus PV inward-rectifier current, and no such differences were seen (see Figure IVC and IVD in the online-only Data Supplement). In summary, NS8593 had no effect on any of the non-SK currents studied.

**Figure 4.** A and B, Whole-cell Ca\(^{2+}\)-dependent K\(^{+}\) current recorded at 0.1 Hz ([Ca\(^{2+}\)]\(_i\)) of 500 nmol/L with ramp protocol shown from canine left atrial (LA) cells (left) and pulmonary vein (PV) cells (right). C, Current–density voltage relation of NS8593-sensitive current from control (CTL) and atrial tachypacing (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). P<0.001 and P<0.0001 for main effect, AT-P vs CTL in C and D, respectively (multilevel repeated-measures mixed-effect model).

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

Single-channel recordings of SK channels are shown in Figure 3. SK\(_{\text{Ca}}\) channels were characterized by their small conductance, sensitivity to Ca\(^{2+}\), and NS8593 inhibition. Figure 3A shows recordings from single patches under 0.01 nmol/L Ca\(^{2+}\), 1 µmol/L Ca\(^{2+}\), and 1 µmol/L Ca\(^{2+}\) plus 10 µmol/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\(^{2+}\), is greatly enhanced by adding Ca\(^{2+}\), and is suppressed by adding NS8593. Overall mean \(P_o\) data under various conditions are shown in Figure I in the online-only Data Supplement (for control cells) and Figure V in the online-only Data Supplement (for AT-P). The strong dependence of channel \(P_o\) on [Ca\(^{2+}\)], 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\(^{2+}\), are greatly enhanced by 1 µmol/L Ca\(^{2+}\), and are suppressed by 10 µmol/L NS8593, as in the control dogs, but compared with controls, there are more openings in AT-P. Figure 3C through 3E shows mean single-channel current amplitude, conductance at +60 mV, and \(P_o\) at 1 µmol/L Ca\(^{2+}\) in the groups studied. Single-channel conductance was of the range expected for SK channels (~6–8 pS) and was not different for LA versus PV nor for AT-P versus control.
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Figure 3D. The same applies to single-channel current amplitude (Figure 3C). However, there were significant differences in single-channel $P_o$ (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 that were larger (note the difference in current scale compared with Figure 4A). Figure 4C and 4D shows 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 (approximately $-70 \text{ mV}$ after adjusting for junction potential) to the physiological K+ equilibrium potential. AT-P significantly increased current densities in both LA and PV cells. NS8593-sensitive current was significantly larger in PV versus LA cells: at $-90 \text{ mV}$, $-1.47\pm0.24 \text{ versus } -0.58\pm0.10 \text{ pA/pF in controls (P}<0.01) \text{ and } -2.64\pm0.31 \text{ versus } -1.20\pm0.13 \text{ pA/pF in AT-P (P}<0.001)$.

Molecular Correlates
Our functional data indicate that SK current is larger in canine PV than LA and is upregulated by AT-P. The increased $P_o$ 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, such as differential subunit expression, and the similar $P_o$ 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 Figure VI in the online-only Data Supplement. 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 SK1 potentially contributing to AT-P versus control differences and...
SK2 accounting for LA versus PV differences, but neither alone perfectly consistent with all findings.

Role in Repolarization and AF Substrate
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. Figure 6A through 6D shows AP recordings from each region/condition studied, before and after 10 μmol/L NS8593. The drug clearly prolonged AP duration (APD) for all but control LA conditions, indicating a significant role in repolarization. No statistically significant changes were seen in resting membrane potential or AP amplitude (see Figure VII in the online-only Data Supplement) 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 control and AT-P dogs and studied resulting electrophysiological changes. NS8593 increased aERP, consistent with the AP changes that we noted. AT-P produced typical changes in aERP, 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 (eg, 89.6±13.6% at BCL of 300 ms) than in control dogs (15.2±6.3%). Ventricular ERP (Figure 7B) was unaffected by AT-P or NS8593. Wenckebach cycle length (measurable only in control dogs because 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. Control 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). Figure 8B and 8C shows 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, under both 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 $P_o$ 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–SK3, from mouse hearts, confirming sequence homologies and atrial predominance of isoforms 1 and 2 and equal atrial/ventricular expression of SK3. Ablation of SK2 channels in mice led to APD prolongation and atrial early afterdepolarization-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 AF and atrial electrophysiology 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. Conversely, 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 \( P_o \) 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 \( P_o \) 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⁺ currents, 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 pressure is encouraging. Several caveats must be recognized. The role of SK channels in ventricular rhythm control appears complex. SK-channel activation has been linked to ventricular...

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**Figure 7.** A, Atrial effective refractory period (aERP) before and after NS8593. **\( P<0.01 \), ***\( P<0.001 \) control (CTL) vs atrial tachypacing (AT-P); **\( P<0.05 \), ***\( P<0.01 \), NS8593 vs pre-NS8593 \( n=4 \) for Bonferroni’s 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, and D by multway repeated-measures ANOVA. Individual-mean comparison with Bonferroni’s correction \( n=4 \) for Bonferroni’s correction. Comparison in C by paired \( t \) test. BCL, basic cycle length.
Arrhythmogenesis in acute myocardial infarction and experimental heart failure, suggesting that blocking SK channels could have useful ventricular antiarrhythmic actions. Conversely, 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. Similarly, although in this study we demonstrate clear anti-AF effects in a clinically relevant dog model, consistent with a number of previous analyses, loss of SK-channel function has been associated previously with AF-promoting early afterdepolarizations and, more recently, SK block has been shown to favor the production of alternans, wavebreak, and atrial arrhythmias in isolated canine atrium. 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.

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. 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 ultrarapid delayed rectifier current 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, 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, subtle changes in isolation technique, or the animals available. Other investigators have reported Kv1.5-type currents in canine atrium, which we are similarly unable to find. We can only assume that such currents are variously expressed 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 that found downregulation of SK current and proteins in atrial samples from AF patients. 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 Ca^{2+}-dependent K^+ current 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 toward higher Ca^{2+} concentrations (ie, reducing affinity of the channel for Ca^{2+}). Although the mechanism is presumed to be quite selective and we showed experimentally high selectivity of NS8593 for SK...

Figure 8. A, Atrial fibrillation (AF) inducibility (as percentage of dogs in which AF was induced during atrial effective refractory period [aERP] determination) before and after NS8593. B and C, Atrial electrogram recordings during AF episodes induced by burst pacing in control (CTL) and atrial tachypacing (AT-P) dogs before and after NS8593. D, AF duration before and after NS8593. **P<0.01, ***P<0.001, NS8593 vs pre-NS8593. Statistical comparison by two-way repeated-measures ANOVA (n=4 for Bonferroni’s correction of individual-mean contrasts) in A and D.
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 (see Figure VII in the online-only Data Supplement), although based on previous results in the literature, we might have expected PV cells to be less polarized than LA and AT-P cells to be hyperpolarized relative to control because of differences in inward-rectifier current. Resting potential differences under these conditions are relatively small and may have been masked by intercell variability, particularly because inward-rectifier current 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 because of differences in inward-rectifier current.

In the present study, we have shown the following: (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 $P_e$ 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 $K_{Ca}$ single-nucleotide polymorphisms and AF and support the possibility that SK blockers may be potentially interesting anti-AF drugs.

Conclusions

We thank France Thériault for excellent secretarial help, Annik Fortier, MSc, and Marie-Claude Guertin, PhD, for expert statistical advice and help, and Nathalie L’Heureux and Chantal St-Cyr for experimental assistance.

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Disclosures

Drs Dines and Grunnet are employees of Acesion Pharma, which owns the commercial rights to NS8593. The other authors report no conflicts.

References


**CLINICAL PERSPECTIVE**

There is increasing evidence for a role of calcium-dependent potassium (SK) channels in cardiac electrophysiology, including gene-wide association studies showing that variants of a calcium-dependent potassium-channel gene (*KCNN3*) are an important risk factor for atrial fibrillation (AF) in man. In this study, we examined the role of SK channels in the electrophysiological properties of canine atrial tissue. We used patch-clamp methods to record whole-cell and single-channel SK currents from dog left atrial (LA) and pulmonary vein (PV) cells, in both normal dogs and dogs subjected to 1 week of rapid atrial pacing to mimic AF. In addition, we exploited a selective SK channel channel blocker (NS8593) to study the functional role of the channel. Measurable whole-cell SK current was detected in dog atrial cells, larger in PV than LA. In addition, atrial tachypacing enhanced SK-current amplitude. Consistent with the ion-current data, blocking SK channels prolonged atrial repolarization, with larger changes in PV than LA and larger increases in cells from tachypaced dogs than normal dogs. Single-channel open probability was enhanced by atrial tachypacing but was not significantly different in PV versus LA. Measurements of SK-channel subunit protein expression showed differences consistent with the relative ion-current differences between PV and LA and between tachypacing and control. In vivo SK-channel block with NS8593 prolonged refractoriness and suppressed AF, implying that SK channels are involved in atrial repolarization and contribute to the control of AF sustainability. These findings suggest that SK-channel gene variants may modulate the risk of AF by altering atrial repolarization and that SK-channel blockers might be an interesting target for AF-suppressing therapy.
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.² 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-\(\mu\)mol/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 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-\(\mu\)g/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 CdCl₂ (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 Ba²⁺-sensitive current.

The extracellular solution for Ca²⁺-current (I_{Ca}) measurement contained (mmol/L): tetraethylammonium-chloride 136, CsCl 5.4, MgCl₂ 1, CaCl₂ 2, NaH₂PO₄ 0.33, dextrose 10, and HEPES 5 (pH 7.4, CsOH). Niflumic acid (50 μmol/L) was added to inhibit Ca²⁺-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, MgCl₂ 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, MgCl₂ 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, MgCl₂ 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 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 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 (nP\textsubscript{o}). N\textsubscript{Po} was calculated by the equation:

\[
N\text{Po} = \frac{\sum_{j=1}^{N} t_{j} \cdot j}{T},
\]

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. N\text{Po} calculations were based on 5-second segments of single-channel recordings. The nP\textsubscript{o} averages of 24 to 36 segments (2-3 min) were used for statistical analysis. Values of Po were determined by dividing the nP\textsubscript{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-N$_2$ 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 $2^{-ΔΔT}$ 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 SKCa channel openings under 0.01-nmol/L Ca²⁺, 1-µmol/L Ca²⁺ and 1-µmol/L Ca²⁺+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\textsuperscript{2+}-dependence of NS8595-sensitive I\textsubscript{SK} dose-response curves at a test potential of +70 mV using [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+} could not be achieved because of cell lethality at higher concentrations, the EC\textsubscript{50} was based on the calculated concentration to achieve 50\% of the maximum effect observed.
Supplemental Figure 3. A-C, Recordings of $I_{to}$, $I_{K1}$, $I_{Ks}$ from canine left-atrial cardiomyocytes before and after 10-$\mu$mol/L NS8593; D, $I_{Kr}$-recordings from guinea pig ventricular cardiomyocytes before and after 10-$\mu$mol/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 Ca²⁺, 1-µmol/L Ca²⁺ and 1-µmol/L Ca²⁺+10-µmol/L NS8593 from AT-P LA, AT-P PV cells. (n/N=9/6 cells/dogs per group). Statistical comparison by multilevel 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.