Small-conductance calcium-activated potassium (SK) channels contribute to action potential repolarization in human atria

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Received 16 September 2013; revised 2 April 2014; accepted 8 April 2014; online publish-ahead-of-print 9 May 2014

Time for primary review: 27 days

Aims
Small-conductance calcium-activated potassium (SK) channels are expressed in the heart of various species, including humans. The aim of the present study was to address whether SK channels play a functional role in human atria.

Methods and results
Quantitative real-time PCR analyses showed higher transcript levels of SK2 and SK3 than that of the SK1 subtype in human atrial tissue. SK2 and SK3 were reduced in chronic atrial fibrillation (AF) compared with sinus rhythm (SR) patients. Immunohistochemistry using confocal microscopy revealed widespread expression of SK2 in atrial myocytes. Two SK channel inhibitors (NS8593 and ICAGEN) were tested in heterologous expression systems revealing ICAGEN as being highly selective for SK channels, while NS8593 showed less selectivity for these channels. In isolated atrial myocytes from SR patients, both inhibitors decreased inwardly rectifying K+ currents by ≏15% and prolonged action potential duration (APD), but no effect was observed in myocytes from AF patients. In trabeculae muscle strips from right atrial appendages of SR patients, both compounds increased APD and effective refractory period, and depolarized the resting membrane potential, while only NS8593 induced these effects in tissue from AF patients. SK channel inhibition did not alter any electrophysiological parameter in human interventricular septum tissue.

Conclusions
SK channels are present in human atria where they participate in repolarization. SK2 and SK3 were down-regulated and had reduced functional importance in chronic AF. As SK current was not found to contribute substantially to the ventricular AP, pharmacological inhibition of SK channels may be a putative atrial-selective target for future antiarrhythmic drug therapy.

Keywords
Small-conductance calcium-activated potassium channel • Atrial-specific SK channel • Atrial fibrillation • Human heart • Antiarrhythmic drug therapy

1. Introduction
Current options for pharmacological therapy of atrial fibrillation (AF) are limited by low efficacy and side effects, including life-threatening ventricular arrhythmias and severe extracardiac toxicities.1 Thus, a need for more efficacious and safe treatment of AF exists. To this end, developing drugs that are predominantly active in atria when compared with ventricles appear promising.

Relative atrial selectivity can be achieved either by targeting ion channels primarily expressed in the atria or by blocking Na+ channels in a state-dependent manner favouring blockage of atrial rather than ventricular sodium conductance.2 Until recently, the acetylcholine-activated potassium current (I_{K,ACh}) and the ultra-rapid delayed rectifier potassium current (I_{Kur}) were the only currents known to be functionally more important in atria than in ventricles. However, within the last years, an increasing amount of evidence has revealed that cardiac small-conductance
Ca^{2+}-activated K^+ (SK) channels may represent a new atrial-selective target in both animals and humans.\textsuperscript{3–5} Cardiac SK channels are functionally linked to voltage-gated Ca^{2+} channels\textsuperscript{11} and are expected to be activated during systole thereby participating in the repolarization of the cardiac action potential (AP). As a consequence of their activation by increased intracellular Ca^{2+}, SK channels might be especially important during rapid cardiac electrical activity such as AF. Furthermore, SK channels seem to be involved in atrial remodelling in experimental AF models.\textsuperscript{5,6} Interestingly, Li et al.\textsuperscript{5} described AP prolongation and AF in a KCNN2 (SK2) knockout mouse model, whereas Mahida et al.\textsuperscript{12} recently reported that overexpression of the KCNN3 gene, encoding SK3, in mice, resulted in sudden cardiac death and atrial arrhythmias. Moreover, KCNN3 is one of the few genes directly linked to clinical AF,\textsuperscript{13,14} indicating that Ca^{2+}-activated SK channels are important in human atria.

The function of SK channels in the human myocardium has not been clearly established. Xu et al.\textsuperscript{3} observed a profound effect on AP morphology when blocking SK channels using apamin in isolated human atrial myocytes. In several animal models of experimental AF, we found SK inhibition to be antiarrhythmic.\textsuperscript{7,8,15,16} The toxin apamin, isolated from bee venom, is a pan-selective SK inhibitor\textsuperscript{17} and considered the key tool for addressing SK function in the brain as well as in peripheral tissue.\textsuperscript{18} However, for unclear reasons, apamin does not always provide a complete inhibition of SK channels and seems to have very low efficacy particularly in cardiac tissue.\textsuperscript{15,19,20} New reliable tool compounds with high potency and selectivity for the inhibition of cardiac SK channels are thus needed.

All three SK channel subunits have been detected in human cardiac tissue from atria and ventricles.\textsuperscript{10,21} They can form both homo- and heteromultimeric complexes, as shown by co-immunoprecipitation experiments.\textsuperscript{10,21} Reportedly, SK currents are reduced in atrial myocytes from chronic AF patients, which are supported by a reduction in SK1–3 mRNA and protein expression.\textsuperscript{10,22} However, increased SK current density has also been described in atrial myocytes from patients in persistent AF.\textsuperscript{23} To address the potential role of SK channels in human cardiac tissue, and more specifically to investigate whether SK channel inhibition would mediate any effect on the human cardiac AP morphology, we took advantage of two potent small molecule inhibitors of SK channels, NS8593\textsuperscript{24} and ICAGEN,\textsuperscript{25} which have previously been studied ex vivo and in vivo.\textsuperscript{7,8,15,16}

2. Methods

A more thorough description of some of the methods used is found in Supplementary material online.

2.1 Human tissue

All work with human samples conforms to the Declaration of Helsinki. The study was approved by the Ethics Committee of the Dresden University of Technology (No. EK790799). Each patient gave written, informed consent. Right atrial appendages (RAAs) were obtained from 65 patients in sinus rhythm (SR) and 22 patients with chronic AF (clinically diagnosed AF lasting >6 months) undergoing coronary artery bypass surgery and/or valve replacement. The exact duration of AF and latest attempts of cardioversion are unknown factors; thus, the degree of structural and electrical remodelling could vary in this group. A significant difference between the SR and AF groups was found for pulmonary hypertension (Table 1). AF patients more often received digitalis and diuretics. None of the patients were considered lone AF patients. Biopsies from interventricular septum were obtained from 15 patients undergoing aortic valve replacement. Table 1 gives a detailed overview of patient characteristics.

<table>
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<th>Table 1 Patients characteristics</th>
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<td>Patients, n</td>
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<td>Nitrates, n</td>
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<td>Lipid-lowering drugs, n</td>
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SR, sinus rhythm; AF, chronic atrial fibrillation; CAD, coronary artery disease; MVD, mitral valve disease requiring valve replacement; AVD, aortic valve disease requiring valve replacement; LVEF, left ventricular ejection fraction; ACE, angiotensin-converting enzyme; AT, angiotensin receptor.

2.2 Quantitative real-time PCR

mRNA expression in human RAAs from 10 patients in SR and 14 in AF was analysed by quantitative real-time PCR (qPCR), as previously described.\textsuperscript{26} Applied Biosystems custom designed assays were used (KCNN1: Hs00158457_m1, KCNN2: Hs00222059_m1, KCNN3: Hs01546821_m1, KCNN4: Hs99999994_m1, GATA4: Hs00171403_m1, MLC2V: Hs00166405_m1). Normalized gene expression levels were calculated by the 2\(^{-}\Delta\Delta Ct\) method,\textsuperscript{27} using the expression of GATA4 as reference. Delta cycle threshold (\(\Delta Ct\)) values were calculated by subtracting the C\(_{t}\) value of a target gene from the C\(_{t}\) value of GATA4 for each sample. Values for the relative expression were obtained by using the following formula: 1/(2 \(− \Delta C\_t\)).

2.3 Immunofluorescence

Cryosections from human atrial trabeculae and left ventricular endocardial biopsies were fixed with paraformaldehyde, and immunohistochemistry with confocal microscopy for SK2 protein was performed as specified (see Supplementary material online, Methods).

2.4 Heterologous expression and selectivity profiling

The ion channel selectivities of the two potent SK channel inhibitors NS8593 and ICAGEN were studied in CHO or HEK293 cells in either transiently or stably transfected cell lines (see Supplementary material online, Methods), using either Q-patch, described in Schroeder et al.\textsuperscript{28} or regular patch-clamp techniques.
2.5 Electrophysiology

Atrial myocytes were isolated and patch-clamp recordings performed as previously described. All experiments were done at 37°C. For measurements of inwardly rectifying K⁺ currents, the extracellular solution consists of (in mM): NaCl 150, KCl 20, MgCl₂ 1, glucose 10, and HEPES 10 (pH 7.4 with NaOH), and the pipette solution: potassium aspartate 80, KCl 40, NaCl 8, EGTA 5, GTP-Tris 0.1, Mg-ATP 5, and HEPES 10 (pH 7.4 with KOH). CaCl₂ was added to give a free Ca²⁺ concentration of 300 nM. Currents were measured at a frequency of 0.5 Hz using a ramp protocol from −120 to +40 mV over 1550 ms with a holding potential at −80 mV. In atrial myocytes from AF patients and ventricular myocytes, the effects of SK channels inhibitors were best described by fitting a sigmoidal function to the data: 

\[ y = C + \left(1 - C\right) \times \frac{\text{Fraction 1}}{1 + 10^{-\text{logIC50}_{1}} + \left(1 - C\right) \times \frac{\text{Fraction 2}}{1 + 10^{-\text{logIC50}_{2}}}} \]

where C is the fraction of non-blocked current. In contrast, in atrial myocytes from SR patients, a two component Hill equation was a more appropriate fit to the data: 

\[ y = C + \left(1 - C\right) \times \left(\frac{1}{1 + 10^{-\text{logIC50}_{1}}} + \frac{1}{1 + 10^{-\text{logIC50}_{2}}}ight) \]

GraphPad Software, San Diego, CA, USA). For AP measurements, the solutions were similar with 5.4 mM extracellular KCl and 35 mM free Ca²⁺ in the pipette. APs were triggered by 2 ms long depolarizing pulses at a frequency of 0.5 Hz.

2.6 Electrophysiological recordings in intact muscle tissue

APs were recorded with standard intracellular microelectrodes in right atrial trabeculae muscle and ventricular septal biopsies as in Wettwer et al. and thoroughly described in Supplementary material online. Methods.

2.7 Drugs and chemicals

Unless otherwise mentioned, all chemicals used were of analytical grade and obtained from Sigma-Aldrich (Steinheim, Germany). The two SK channel inhibitors used in this study; N-((1R)-1,2,3,4-tetrahydro-1-naphthalenyl)-1H-benzimidazol-2-amine hydrochloride (NS8593) and N-(pyridin-2-yl)-4-(pyridin-2-yl)thiazol-2-amine (ICAGEN) were synthesized at NeuroSearch A/S, Ballerup, Denmark.

2.8 Data analysis

For data analysis and drawings, Chart 7 software (ADInstruments, Dunedin, New Zealand) and GraphPad Prism software (GraphPad Software, San Diego, USA) were used. All AP parameters were analysed as an average of >10 APs. Data acquired in intact cardiac muscle preparations (see Supplementary material online, Table S2) were analysed using paired Student’s t-test. In Figures 1–3, data were analysed using unpaired Student’s t-test. Data-sets in Figure 4 were analysed using one-way ANOVA with Bonferroni’s post-test for multiple comparisons. Frequency data (Figure 7) were analysed using repeated-measures two-way ANOVA with Sidak’s post hoc test for multiple comparisons. Values of P < 0.05 were considered statistically significant (in figures, * denotes P-values below 0.05, 0.01, and 0.001, respectively).

3. Results

3.1 Quantification of SK channel mRNA in SR and AF

The expression of the three SK isoforms, SK1, SK2, and SK3, was quantified in right atrial tissue from 10 SR and 14 AF patients using qPCR. The expression of four reference genes, including three cardiac-specific genes, was similar in SR and AF samples, indicating comparable levels of cardiac transcripts in the two groups (Figure 1A). In both SR and AF, SK2 (KCNN2) and SK3 (KCNN3) expression levels were 10-fold higher than that of SK1 (KCNN1) (Figure 1B). KCNN2 and KCNN3 expression in AF tissue were ~50% of the expression levels in SR tissue, indicating that these genes undergo pronounced remodelling, but are still present in tissue from patients with long-lasting AF.

3.2 Immunohistochemistry and confocal microscopy of atrial and ventricular tissue

Protein distribution of SK2 channels, which showed the highest mRNA expression, was addressed by immunohistochemistry and confocal microscopy. SK2 protein has intensively been studied in animal heart expression, was addressed by immunohistochemistry and confocal microscopy. SK2 and SK3 mRNA were significantly down-regulated in chronic AF tissue compared with SR (reductions of 53.4 ± 14.7%, P < 0.01 and 40.4 ± 17.7%, P < 0.05 for SK2 and SK3, respectively).

Figure 1 mRNA expression was quantified by qPCR in SR (n = 10) and AF tissue (n = 14). (A) Expression levels of reference genes: cyclophilin A (CYP), ryanodine receptor 2 (RYR2), GATA-binding protein 4 (GATA4), and myosin-light chain 2V (MLC2V). (B) Expression levels of KCNN1 (SK1), KCNN2 (SK2), and KCNN3 (SK3) were normalized to the expression of the cardiac-specific transcription factor GATA4. SK2 and SK3 mRNA were significantly down-regulated in chronic AF tissue compared with SR (reductions of 53.4 ± 14.7%, P < 0.01 and 40.4 ± 17.7%, P < 0.05 for SK2 and SK3, respectively).
pattern seemed to be more disrupted and irregular, as shown before, and a clear membrane localization of SK2 protein was absent. In human ventricular tissue, SK2 staining showed a lower overall intensity compared with atrial tissue, but revealed intense bands located at the intercalated discs. The characteristic Z-line distribution was absent in the ventricles, whereas a longitudinal striated pattern was observed (see Supplementary material online, Figure S5). Co-staining for SK2 and the gap junction protein connexin 43 (Cx43), which is expressed in the intercalated discs, revealed co-localization of the two proteins (see Supplementary material online, Figures S4 and S5).

3.3 Selectivity profiling of NS8593 and ICAGEN

NS8593 and ICAGEN represent two distinct chemical families and have different modes of action. NS8593 acts as a negative allosteric modulator, whereas ICAGEN is a traditional pore blocker of SK channels. A thorough investigation of the selectivity profiles of the compounds using heterologous expressing systems (Table 2) revealed that NS8593 at high concentrations inhibits $I_{Ca,L}$, $I_{Ca,T}$, $I_{Na}$, $I_{Kr}$, $I_{Ks}$, and $I_{K,Ach}$, although at IC50 values 5–50 times higher than those reported for $I_{SK}$. In contrast, ICAGEN only showed minor inhibitory effect on $I_{K1}$ (IC50 = 21 μM) and $I_{K1}$ (IC50 > 100 μM) and had no effect on other tested ion channels even at the highest concentrations tested. Both NS8593 and ICAGEN have previously been reported to have no effect on either large- or intermediate-conductance calcium-activated potassium channels.

3.4 SK channel inhibitors reduce inwardly rectifying K+ currents and prolong action potential duration in isolated atrial myocytes

The effects of NS8593 and ICAGEN were tested on inwardly rectifying K+ currents using 300 nM free Ca2+ in the pipette solution. High
extracellular $K^+$ ($20$ mM) was used to shift the reversal potential to about $-48$ mV. Current amplitude was analysed at $-60$ mV. NS8593 clearly blocked inwardly rectifying currents in SR atrial myocytes at concentrations from $10$ μM (Figure 2). Between $0.3$ and $3$ μM, a small reduction that was difficult to distinguish from run-down of current was observed (see Supplementary material online, Figure S1), but showed significant difference at $1$ μM compared with time-matched control (TMC) run-down (Figure 2, left). Datapoints were best fitted with a two component Hill function, suggesting that NS8593 blocked inwardly rectifying current in two distinct concentration ranges. A fraction of $13\%$ of total current was highly sensitive to NS8593 with an estimated IC$_{50}$ value of $258$ nM, comparable with the IC$_{50}$ for $I_{SK}$ block in expression systems (Table 2). The fraction more resistant to NS8593 amounted to $82\%$ and had an estimated IC$_{50}$ of $21$ μM, a value similar to the one for $I_{K1}$ inhibition (Table 2). A small fraction of current ($5\%$) was not blocked by NS8593. In ventricular cells, current blocked by NS8593 was best fitted with a sigmoidal function with an IC$_{50}$ of $\approx 15$ μM (Figure 2, right), and an estimated $15\%$ of total current not blocked at all. Spontaneous current run-down in ventricular TMCs was larger than in atrial TMCs. In contrast to atrial cells, $1$ μM NS8593 did not affect current decline in ventricular TMCs. These data are in line with the presence of a small fraction of SK channels contributing to inwardly rectifying current in atrial, but not in ventricular, cells.

Similar to NS8593, increasing concentrations of ICAGEN reduced inwardly rectifying currents in SR atrial myocytes in a biphasic manner (Figure 3, left). By adapting a two component Hill equation to the data, we identified a sensitive fraction which represented $15\%$ of the total current with a calculated IC$_{50}$ value of $\approx 143$ nM; and a resistant fraction which amounted to $26\%$ with an IC$_{50}$ of $\approx 10$ μM. The IC$_{50}$ value of the sensitive fraction was comparable with the IC$_{50}$ reported for SK channel block by ICAGEN in expression systems (Table 2). In contrast to NS8593, a large fraction of current ($61\%$) was not affected by the drug. In AF atrial myocytes, only the more resistant fraction was observed with an IC$_{50}$ of $\approx 5$ μM ICAGEN. This fraction represented $23\%$ of the total current. Comparison with TMCs confirmed that $1$ μM ICAGEN significantly reduced current amplitude in SR myocytes, but did not affect currents measured in AF cells to any significant extent (Figure 3, middle). Finally in ventricular myocytes, no effect of ICAGEN was observed when compared with TMCs (Figure 3, right).

The statistically significant difference between current decrease in cells exposed to low drug concentrations and TMC could merely reflect different individual rates of spontaneous current run-down. To estimate the impact of such possibility, we calculated the individual run-down in each cell within the $200$-s period immediately preceding drug application (or TMCs) and during drug application (or TMCs). This comparison showed that rates of run-down during the initial

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**Figure 3** Effect of ICAGEN on inwardly rectifying $K^+$ currents in atrial myocytes from SR (left) and AF patients (middle), and ventricular myocytes (right). Top panel: representative current recordings under control conditions and in the presence of 1, 10, and 100 μM ICAGEN. Middle panel: concentration–response curves for ICAGEN (full lines) and TMC (dotted lines). Lower panel: comparison of the effect of ICAGEN to the corresponding TMCs.
phase of the experiment were indeed different between the various groups of cells (see Supplementary material online, Table S1). However, it slowed down during the following phase in TMCs, supporting an exponential time course of run-down in that group, but was increased in the drug-exposed cells, providing evidence for drug-induced block rather than mere run-down.

We further tested the effect of 1 μM NS8593 and 1 μM ICAGEN on APs measured in single atrial myocytes. According to our results on inwardly rectifying currents, only the more sensitive population of channels should be blocked by this concentration. As shown in Figure 4A, NS8593 and ICAGEN significantly prolonged action potential duration (APD)₉₀ in SR myocytes. In contrast, AP parameters in AF cells were not affected by 1 μM ICAGEN, confirming the absence of the more sensitive population of inwardly rectifying K⁺ channels in remodelled AF myocytes (Figure 4B). To ascertain that the prolongation of APD observed was not due to unspecific inhibition of I_{Kr}, we tested the human Ether-a-go-go-Related Gene (hERG1) channel blocker E-4031. As shown in Figure 4A, 5 μM E-4031 had no effect on AP parameters in SR myocytes.

### 3.5 Effect of NS8593 and ICAGEN on AP parameters in human atrial muscle preparations

To further understand the role of I_{Kr}, we performed AP recordings on excised human muscle strips, which have intact coupling and ion channel surface expression in contrast to isolated cardiomyocytes. AP recordings in atrial trabeculae from SR patients revealed a spike and dome morphology as described previously. Fast depolarization to positive potentials was immediately followed by repolarization to a potential around −20 mV, after which a plateau phase was followed by slow repolarization to the resting membrane potential (RMP). Superfusion of SR tissue with 30 μM NS8593 resulted in significant changes of AP morphology. While APD₉₀ was not changed, APD₉₀ was increased by 11% and atrial effective refractory period (ERP) by 26%. Furthermore, the plateau potential, defined as the mean potential in the time window between 20 and 30% of APD₉₀ (PLT₂₀), was significantly reduced, probably due to unselective actions of NS8593. About 10 μM ICAGEN induced an increase in APD₉₀ of 19% and in ERP of 38%, while no effect was observed on PLT₂₀ or APD₅₀. A detailed presentation of drug effects on APD₉₀ and ERP for each single experiment is depicted in Supplementary material online, Figure S2.

AP morphology in AF tissue was considerably changed when compared with SR tissue (Figure 6). RMP was more hyperpolarized and instead of the characteristic spike and dome morphology, a more triangular shape with shorter APD was observed. Application of NS8593 prolonged both APD₉₀ and ERP, and resulted in a more negative PLT₂₀. In contrast, ICAGEN did not change any of these parameters. In TMCs, PLT₂₀, APD₉₀, and ERP did not change over a period of 60 min (see Supplementary material online, Table S2).

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Figure 4 Representative recordings of single-cell atrial APs from patients in SR (A) or AF (B) using the current-clamp technique before and after application of either 1 μM NS8593, 1 μM ICAGEN, or 5 μM E-4031. A holding current was applied to maintain the resting membrane potential (RMP) around −80 mV. (C) Effects of the drugs on RMP, duration at 90% of repolarization (APD₉₀) and upstroke velocity (dV/dt max). Data were normalized to pre-drug controls and compared with TMCs.
3.6 Frequency dependence of SK channel inhibition

To investigate a possible frequency dependence of the effect on APD and ERP by the two SK channel inhibitors, experiments were performed at different pacing rates (0.5, 1, and 3 Hz) (Figure 7). In SR tissue, application of NS8593 resulted in increased APD90 and ERP at 0.5 and 1 Hz, whereas no significant effects were observed at 3 Hz. It should be noted that after drug application, the excitability of SR tissue was reduced to such an extent that the tissue could only follow 3 Hz stimulation in some experiments (n = 5 of 12), impairing the statistical interpretation. In AF tissue, application of NS8593 increased APD90 and ERP at 0.5 and 1 Hz, as in SR tissue, although APD90 was also significantly prolonged at 3 Hz. In contrast to SR tissue, all AF preparations remained excitable at 3 Hz pacing after application of NS8593, probably due to significantly shorter initial APD and ERP, and a more hyperpolarized RMP, as previously observed in AF tissue.32 Similar to what we observed with NS8593, application of ICAGEN in SR tissue significantly increased APD90 and ERP at 0.5 and 1 Hz, but only ERP was significantly prolonged at 3 Hz. After application of ICAGEN, 4 of 9 experiments could still be excited at 3 Hz pacing. In AF tissue, neither APD90 nor ERP were affected by ICAGEN at any of the tested frequencies. All AF trabeculae followed pacing frequencies of 3 Hz, both before and after application of ICAGEN. Neither of the two tested drugs affected APD50 at any given frequency. A presentation of all AP parameters is found in Supplementary material online, Table S2.

3.7 SK channel inhibition induces a depolarization of RMP and reduced excitability

NS8593 and ICAGEN induced a significant depolarization of the RMP at 0.5, 1, and 3 Hz in SR atrial tissue of 2–4 mV (see Supplementary material online, Table S2). In AF tissue, NS8593 depolarized the RMP to the same extent as in SR tissue, while ICAGEN only showed a small but significant depolarization at 1 Hz frequency. Both AP amplitude (APA) and maximum upstroke velocity (dV/dt_max) were significantly decreased at all pacing frequencies by NS8593 and at 1 Hz by ICAGEN. For NS8593, RMP depolarization may partly result from $I_{K1}$ and $I_{K,ACh}$ inhibition, while reduced upstroke velocity and APA can be explained by indirect or direct $I_{Na}$ inhibition.

3.8 Human ventricular muscle preparations

Human ventricular septal tissue was acquired from patients who were clinically characterized as non-heart failure patients. We investigated the potential effect of SK channel inhibition on ventricular electrophysiology by superfusing ventricular septal muscle strips with 30 μM NS8593 and found that neither RMP and AP parameters, nor ERP, at...
0.5 or 1 Hz were affected (Figure 6). Supporting these results, two experiments with 10 μM ICAGEN showed no change in any parameter. Taken together, these findings indicate that I_{SK} does not play a prominent role in the ventricles.

### Table 2: Selectivity profile of NS8593 and ICAGEN on different cardiac relevant ion currents

<table>
<thead>
<tr>
<th>Ion channel</th>
<th>Ion current</th>
<th>IC_{50} (μM)</th>
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<tr>
<td></td>
<td>NS8593</td>
<td>ICAGEN</td>
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<tr>
<td>hSK2/hSK3</td>
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<td>0.6/0.72^25</td>
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<td>hERG1 (Kv1.1)</td>
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### 4. Discussion

Recent evidence has linked SK channels to cardiac electrophysiology, suggesting that, as they are more abundant in the atria compared with the ventricles, these channels could be an interesting atrial-selective drug target candidate for the treatment of atrial arrhythmias.\(^{3,4,20,35}\)

The present study investigated SK channel expression and functional importance in human atrial and ventricular tissue.

### 4.1 SK channel expression in the human heart

Transcripts for SK2 and SK3 were found at significantly lower levels in tissue from patients with chronic AF compared with SR, suggesting a down-regulation of the expression of these proteins during AF. Similar findings have been reported previously,\(^{10,22}\) while others have described increased atrial SK2 current in patients with persistent AF.\(^{23}\) We hypothesize that the expression level of SK channels depends on the duration of AF, an initial up-regulation of SK expression might be followed by down-regulation during long-lasting AF, as the atria undergo extensive structural and electrical remodelling.

In atrial SR tissue, SK2 channels were distributed in the sarcolemmal membrane and intracellularly in a distinct striated Z-band pattern across the myocytes. This pattern is consistent with earlier reports...
documenting an overlap in the distribution of SK1–3 channel isoforms along the Z-lines in isolated mouse atrial myocytes. It has been suggested earlier that SK2 channels interact with α-actinin cytoskeletal proteins, which anchor the channels at the surface membrane and along the tubular structure in cardiomyocytes. The authors further hypothesized that, under pathophysiological conditions, compromised α-actinin expression might lead to an impaired trafficking of SK channels to the surface membrane, which could explain the differences we found in SK2 distribution between SR and AF cells. Further supporting our observations, previous studies describing cellular remodelling in fibrillating and dilated atria have reported a disrupted sarcomeric apparatus in right atrial myocytes, with fragmented cardiomyocyte structure and irregular tissue morphology. In ventricular tissue, SK2 channels were detected, although at lower intensity, but with bright bands at the intercalated discs. Co-localization with the gap junction-forming protein connexin 43 suggests that SK channels might be involved in intercellular communication between ventricular cardiomyocytes, although at present this is a very speculative assumption. Tuteja et al. elegantly showed that human and mouse cardiac SK channel subunits form heteromultimers through direct interaction of C-terminal domains, and that SK2 and SK3 subunits co-localize in clusters. This finding suggests that the SK2 channel localization found in our study may also be representative for SK3.

4.2 SK channel function in human atria
Under high [Ca^{2+}], conditions, both SK channel blockers reduced total inwardly rectifying K\textsuperscript{+} currents in atrial myocytes from SR patients in a biphasic concentration-dependent manner. The more sensitive fraction exhibited IC\textsubscript{50} values (258 nM for NS8593 and 143 nM for ICAGEN) comparable with values previously reported, providing evidence for contribution of SK channels in atrial cells. At −60 mV, this current contributed ~15% of total inward current.

Blocking putative I\textsubscript{SK} resulted in the prolongation of AP duration in SR myocytes, which is in agreement with what has been reported for other SK channel blockers, including apamin, and confirms that I\textsubscript{SK} could participate in the repolarization of APs in such cells. In contrast, in atrial myocytes from AF patients, the fraction of current sensitive to low concentrations of ICAGEN was not detected, which is consistent with the down-regulation of SK channel expression in AF tissue.

In SR myocytes, the second fraction of current blocked by NS8593 represented almost all the current left (~82%) and the IC\textsubscript{50} value (~21 μM) suggests the block of I\textsubscript{K1} or I\textsubscript{K,ACh}. As constitutively active I\textsubscript{K,ACh} is believed to be present only in patients suffering from chronic AF, the current blocked by NS8593 in SR patients most likely represents I\textsubscript{K1}.

High concentrations of ICAGEN blocked ~25% of total inwardly rectifying K\textsuperscript{+} current in both SR and AF atrial myocytes. The IC\textsubscript{50}, in the μM
SK channels in the human heart

range, is consistent with inhibition of $I_{K1}$ or $I_{K,ACh}$. Interestingly, ICAGEN did not, at any concentration, reduce inwardly rectifying currents in ventricular cells, suggesting that this ICAGEN-sensitive current is only present in atrial cells or that the channel subunits responsible for $I_{K1}$, which are expressed at different ratios between atrial and ventricular tissue, are less sensitive to the drug in ventricular cells. A differential expression of channel isoforms might also underlie the larger current run-down observed in ventricular myocytes.

The absence of effect of the hERG channel blocker, E-4031, on AP parameters, confirmed that hERG channels were destroyed during the enzymatic digestion of the cell isolation procedure, and that inhibition of $I_{Kr}$ could not account for APD prolongation upon application of NS8593 or ICAGEN. In intact atrial muscle preparations, equivalent concentrations of E-4031 considerably prolonged APD in both SR and AF tissue.

To further investigate the function of $I_{Sk}$, APs were measured in atrial trabeculae strips from patients in SR and AF. We observed a significant prolongation of APD$_{90}$ and ERP in SR preparations, in the presence of both SK channel inhibitors. In contrast, in AF preparations, significant effects on APD and ERP were only observed in the presence of 30 μM NS8593, whereas 10 μM ICAGEN did not affect APO and ERP. This is in line with our experiments on isolated cells and the down-regulation of SK channels in AF tissue. We therefore assume that the prolongation of APD and ERP following ICAGEN application in SR preparations resulted from the specific block of SK channels. These findings imply that, together with $I_{K1}$, $I_{K,ACh}$ and $I_{Ca,L}$, SK channel conductance plays an important role in late atrial repolarization. The effects observed in both SR and AF tissue upon application of NS8593 were probably not due to the sole block of SK channels, but might also result from the action of the drug on other currents contributing to the repolarization reserve.

In chronic AF patients, $I_{K1}$ and the constitutively active component of $I_{K,ACh}$ are up-regulated, while the activated $I_{K,ACh}$ component is down-regulated. In the present study, we suggest that SK channels are down-regulated in patients with chronic AF, but it is important to bear in mind that alterations of other inwardly rectifying $K^+$ channels might influence our findings. Furthermore, the loss of L-type Ca$_{2+}$ current reported in patients with persistent AF is also likely to have a significant impact on SK current amplitude.

### 4.3 Shift in atrial RMP causes an indirect Na$^+$ block

SK channel inhibition by both NS8593 and ICAGEN induced a significant depolarization of RMP in atrial multicellular muscle preparations. As mentioned above, the effects observed with NS8593 could not only result from the block of $I_{Sk}$, but also from the inhibition of $I_{K1}$ or $I_{K,ACh}$. However, since ICAGEN does not seem to directly affect other inwardly rectifying $K^+$ currents in muscle preparations, the results suggest that $I_{Sk}$ participates in setting the RMP. As we did not observe any direct effect by ICAGEN on Na$^+$ channels in expression systems and single-cell APs, we presume that the reduction of both APA and $dV/dt_{max}$ following SK channel inhibition was due to the depolarization of RMP, which consequently decreased availability of Na$^+$ channels as a higher fraction of these channels are inactivated at more depolarized potentials. This would also explain the decreased excitability we observed at high pacing frequencies. Through a further prolongation of ERP resulting in post-repolarization refractoriness and reduced excitability, this indirect block of Na$^+$ channels might contribute to the anti-arrhythmic effects of SK channel inhibition we and others have previously observed. NS8593, ICAGEN, and apamin have shown clear antiarrhythmic effects in animal models, with no signs of pro-arrhythmic side effects.

Mechanistically, the antiarrhythmic potential of SK channel inhibition could thus result from the synergy of direct $I_{Sk}$ block and indirect $I_{Na}$ reduction following RMP depolarization.

### 4.4 No effects of SK inhibition in ventricular tissue

In ventricular myocytes, only high concentrations of NS8593 affected inwardly rectifying K$^+$ currents, suggesting a block of $I_{K1}$. The lack of effect of NS8593 on electrophysiological parameters in ventricular tissue, including RMP, indicates that the applied concentration of NS8593 does not inhibit $I_{K1}$, $I_{K,ACh}$, $I_{Na}$, and $I_{Ca,L}$ to any functionally important extent in this tissue. The absence of any effect in single cells and muscle preparations following ICAGEN application further support the notion that SK channels do not play a functional role in ventricular tissue. Other studies have documented functional SK channels in human heart failure patients. In isolated cardiomyocytes from heart failure patients, apamin prolonged the ventricular AP to a substantial degree, but did not affect the AP in non-heart failure patients. The same group recently reported antiarrhythmic effects of apamin on ventricular fibrillation in failing rabbit Langendorff-perfused hearts. We and others have detected expression of mRNA and protein of SK channels in non-diseased human ventricles without any apparent functional role under physiological conditions. This finding could be due to a greater repolarization reserve combined with a lower relative SK channel expression in the ventricles compared with atria.

### 5. Study limitations

In this study, long incubation times of muscle strips were necessary before steady-state drug effects could be achieved. Compared with earlier studies, 3- to 10-fold higher concentrations of the lipophilic NS8593 and ICAGEN compounds were required for significant effects. Furthermore, drug concentrations 10- to 30-fold higher than in single-cell experiments were needed to reach significant levels of effect, reflecting the difficulties of lipophilic drug molecules to reach their targets when superfused in intact muscle preparations. We have previously reported this phenomenon using tertiaripin-Q, a very potent and selective blocker of $I_{K,ACh}$ which showed full inhibitory effect in isolated atrial myocytes, but had no effect in atrial multicellular preparations.

IC$_{50}$ values in isolated cell systems can therefore not be directly translated to those in multicellular preparations. Furthermore, in this study, we were not able to wash out the drug effect. Instead TMCs were used to demonstrate the stability and robustness of the experimental protocol. In addition, expression of SK channels in the atria may be non-uniform. It has been reported that SK2 channel expression is increased locally following burst pacing in the pulmonary vein region of rabbit atria. Furthermore, it was recently shown that SK2 expression, both at mRNA and protein levels, is more abundant in the pulmonary vein region compared with the left atrium in dogs. In the present study, only RAAs have been used, and we can therefore not rule out regional differences. Whether SK channel expression is up- or down-regulated in patients diagnosed with early stages of AF, or whether heterogeneity in expression increases is unknown. In this study, we have focused on patients in either SR or chronic AF, whereas patients diagnosed with recent-onset AF have not been included. Since human
ventricular tissue is very scarce, we were only able to perform a small number of ventricular muscle experiments, reflected by the low number of experiments in the ICAGEN group. Regarding the immuno-

histochemistry and confocal imaging performed in this study, we have not succeeded in addressing co-localization of SK2 protein with cardiac membrane-specific proteins such as caveolin-3.

6. Conclusions
SK channels participate in repolarization of the AP and in controlling the RMP in the human atrium, while these channels seem to have no functional importance in the human ventricle under the conditions used in this study. Interestingly, atrial SK channels are not only active during the termination (Phase 3) of the AP but also during atrial diastole where SK current provides a hyperpolarization of the RMP of 2–4 mV. SK channel inhibition in tissue from chronic AF patients showed little or no effect, probably due to the high degree of remodelling. In summary, SK channel inhibition can exert an antiarrhythmic action by the direct block of SK channels which results in increased APD

and ERP, and the indirect block of Na+

channels, due to the depolarization of RMP, causing reduction of upstroke velocity, further prolongation of ERP, and leading to post-repolarization refractoriness. SK channel inhibition might therefore constitute an interesting antiarrhythmic therapeutic target for patients with recent-onset AF, before SK channel expression is strongly down-regulated due to intensive remodelling.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Acknowledgements
We thank NeuroSearch A/S, Ballerup, Denmark, for their kindness of supplying NS5893 and ICAGEN. The authors thank Göran Duker, Senior Principle Scientist, Astra Zeneca, for supplying selectivity data for SK channels via alpha-actinin2. Supplementary material in KCNN3 results in sudden cardiac death. (Abstract). Heart Rhythm 2012;9:

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