Abstract—Since the first description of the anatomical atrioventricular nodes (AVNs), a large number of studies have provided insights into the heterogeneity of the structure as well as a repertoire of ion channel proteins that govern this complex conduction pathway between the atria and ventricles. These studies have revealed the intricate organization of multiple nodal and nodal-like myocytes contributing to the unique electrophysiology of the AVN in health and diseases. The on the other hand, information regarding the contribution of specific ion channels to the function of the AVN remains incomplete. We reason that the identification of AVN-specific ion channels may provide a more direct and rational design of therapeutic target in the control of AVN conduction in atrial flutter/fibrillation, one of the most common arrhythmias seen clinically. In this study, we took advantage of 2 genetically altered mouse models with overexpression or null mutation of 1 of a small conductance Ca$^{2+}$-activated K$^+$ channel isoform, SK2 channel, and demonstrated robust phenotypes of AVN dysfunction in these experimental models. Overexpression of SK2 channels results in the shortening of the spontaneous action potentials of the AVN cells and an increase in the firing frequency. On the other hand, ablation of the SK2 channel results in the opposite effects on the spontaneous action potentials of the AVN. Furthermore, we directly documented the expression of SK2 channel in mouse AVN using multiple techniques. The new insights may have important implications in providing novel drug targets for the modification of AVN conduction in the treatment of atrial arrhythmias. (Circ Res. 2008;102:465-471.)

Key Words: KCa2.2 channel ■ SK2 channel ■ atrioventricular nodes

The atrioventricular node (AVN) is a highly specialized pacemaking tissue located at the junction of the right atrium and ventricle. Indeed, it is the only electrical connection between atria and ventricles and provides the critical delay between atrial and ventricular contraction to allow for proper atrial emptying before the start of the ventricular contraction. Pharmacological slowing of impulses across AVN is widely used clinically in atrial flutter/fibrillation to ensure physiological ventricular responses in these conditions. Previous studies have identified the roles of several distinct ion channels in the AVN function,1-5 and recent work has begun to assemble an array of ion channel genes in the pacemaking tissues.6 On the other hand, information regarding contribution of specific ion channels to the function of the AVN remains incomplete. We reason that the identification of AVN-specific ion channels may provide a more direct and rational design of therapeutic target in the control of AVN conduction in atrial flutter/fibrillation, one of the most common arrhythmias seen clinically.

Specifically, we have recently identified several isoforms of Ca$^{2+}$-activated K$^+$ channels (KCa) in human and mouse cardiac myocytes that we have shown to be critical in sculpting the duration of the cardiac action potential (AP).7,8 KCa channels are highly expressed in atrial compared with ventricular tissues. Moreover, not only does the current play important functional roles in mouse atrial myocytes, it also contributes significantly to the repolarization process in human atria.7,8 KCa channels are present in a wide variety of cells, where they integrate changes in intracellular Ca$^{2+}$ concentration [Ca$^{2+}$i] with changes in K$^+$ conductance and membrane potential.9,10 KCa channels can be divided into 3 main subfamilies: the large-conductance Ca$^{2+}$- and voltage-activated K$^+$ channels (BK, KCa1), the intermediate-conductance Ca$^{2+}$-activated K$^+$ channels (IK, KCa3), and the small-conductance Ca$^{2+}$-activated K$^+$ channels (SK or KCa2).9-13 SK channels are encoded by at least 3 distinct genes, namely KCNN1 (SK1), KCNN2 (SK2), and KCNN3 (SK3).9,10,13

Here, we directly document the robust expression of SK2 channel in mouse AVN. Moreover, using genetically altered mouse models with overexpression or null mutation of 1 of the KCa isoforms, SK2 channel, we demonstrate significant
changes in the AVN function. The new insights into the functional roles of SK2 channel in AVN may have important implications in providing novel drug targets for the modification of AVN conduction in the treatment of atrial arrhythmias.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

SK2-Null Mutant Mice and Transgenic Mice Overexpressing SK2 Channels
SK2-null mutant (SK2\(^{-/-}\)) mice were generated as described previously. Transgenic mice overexpressing SK2 channels (SK2\(^{+/-}\)) were developed via insertion of a tetracycline-regulatory cassette into the SK2 locus as described previously. Compared with wild-type (WT) littermate mice, in the absence of doxycycline, the SK2 protein and SK2 mRNA are overexpressed in heterozygotes (SK2\(^{+/-}\)) in the absence of doxycycline. SK2-null and transgenic mouse lines were backcrossed more than 7 generations onto the C57Bl/6J background.

AVN Recordings
AVN preparation was prepared as described previously. AVN was recorded from isolated AV nodal preparations using microelectrode techniques with 3 mol/L KCl microelectrodes at 33°C as described previously.

Single AVN cells were isolated from WT and mutant mice as described previously. Whole-cell Ca\(^{2+}\)-activated K\(^+\) current (I\(_{\text{Ca-K}}\)) was recorded from single AVN cells at room temperature using patch-clamp techniques as described previously.

ECG Recordings
ECG recordings were obtained at 33°C using Bioamplifier (BMA 851, CWE Inc, Ardmore, Pa) as described previously.

Immunofluorescence Confocal Microscopy and Immunohistochemistry
Immunofluorescence labeling was performed as described previously. Immunohistochemistry and antibodies used are described in the online data supplement.

Results
SK2\(^{+/-}\) and SK2\(^{-/-}\) Mice Show Evidence of Sinoatrial Node and AVN Dysfunction
We documented robust phenotypes of sinoatrial node (SAN) and AVN dysfunction in the SK2\(^{+/-}\) and SK2\(^{-/-}\) mice. Figure 1A shows ECG recordings from SK2\(^{+/-}\) and SK2\(^{-/-}\) mice compared with WT animals illustrating significant sinus bradycardia with prolongation of the PR intervals in SK2\(^{-/-}\) mice compared with WT animals. In contrast, SK2\(^{+/-}\) mice show significant shortening of the RR and PR intervals. Figure 1B further illustrates examples of ECG recordings in SK2\(^{-/-}\) mice showing complete AV block with AV dissociation. Summary data for PR intervals are shown in Figure 1C (n=10 from each group, *P<0.05). These differences in the RR and PR intervals may represent intrinsic abnormalities in the pacemaking activities in SA and AV nodal cells and/or the His–Purkinje system, respectively, in genetically targeted mice. However, alternative possibilities include altered auto-

Figure 1. Surface ECG recordings from WT, SK2\(^{+/-}\), and SK2\(^{-/-}\) mice. A, Examples of ECG recordings in WT, SK2\(^{+/-}\), and SK2\(^{-/-}\) mice. B, Examples of ECG recordings in SK2\(^{-/-}\) mice showing complete AV block with AV dissociation. C, Summary data for PR intervals in WT, SK2\(^{+/-}\), and SK2\(^{-/-}\) mice in control conditions. There were also significant differences in the RR interval among the 3 groups of animals (135.0\(\pm\)7.8, 109.7\(\pm\)6.1, and 153.8\(\pm\)6.1 ms for WT, SK2\(^{+/-}\), and SK2\(^{-/-}\), respectively). D, Summary data for PR intervals after intraperitoneal injection of atropine and propranolol to abolish autonomic control of the heart (n=6, *P=0.05). There were no significant differences in the percent-age increase in PR intervals after autonomic blockade in WT, SK2\(^{+/-}\), and SK2\(^{-/-}\) mice (D, bottom).
nomic input into the SAN and AVN resulting from the overexpression or deletion of the SK2 channel. To rule out this latter possibility, we recorded ECG in the WT and mutant mice using combined intraperitoneal injection of atropine (1 mg/kg) and propranolol (20 mg/kg) at concentrations shown previously to abolish autonomic control of the heart. Results are presented in Figure 1D. Administration of atropine and propranolol resulted in the prolongation of the PR intervals in the SK2+/+ and SK2−/− mice as well as WT animals. More importantly, significant abnormalities in the AVN in SK2+/+ and SK2−/− animals remain after treatment with atropine and propranolol compared with the WT controls, suggesting that the defects observed are intrinsic to the pacemaking tissues. On the other hand, the prolongation of the PR interval may be related to either AV node and/or the His–Purkinje system. Moreover, it is well known that the specific conductance-mediated influences on spontaneous pacemaker activity are highly rate-dependent. To further assess the effects of SK2 channels on AV nodes at constant rates, we performed in vivo electrophysiologic studies as described in online data supplement. Figure I in the online data supplement shows the prolongation of the AV node refractory period in SK2−/− and SK2+/− compared with WT littermates, consistent with the prolongation of the PR interval as described above.

To further document whether there is significant alteration within the AVN among the different genetically altered mouse models, we evaluated the spontaneous AP characteristics from isolated AV node preparations.

AVNs Isolated From SK2+/+ Mice Show an Increase in the Rate of Firing, Whereas the Opposite Findings Were Observed in SK2−/− Mice

Spontaneous APs were recorded from isolated intact AVN preparation using microelectrode techniques at 33°C to 34°C. Figure 2A shows a photomicrograph of an isolated mouse AVN illustrating the important landmarks used in the identification of the AVN region as described previously. Representative spontaneous APs recorded from the regions within the AVN are shown in Figure 2B comparing WT, SK2+/+, and SK2−/−. Specifically, APs recorded from within the AVN can be identified by the presence of the slow diastolic depolarization and a very slow upstroke of phase 0. SK2+/+ mice show a significant increase in the spontaneous activities of the AVNs compared with age-matched WT controls (Figure 2B and 2C). In contrast, SK2−/− mice show a significant decrease in the firing frequency of the AVNs compared with the age-matched WT controls. Indeed, data obtained from isolated AVN preparations are consistent with the findings from in vivo measurement, as presented in Figure 1, further supporting the notion that the abnormalities observed are intrinsic to the AVN. Moreover, application of apamin (500 pmol/L, a specific blocker of SK2 channel) resulted in a significant decrease in the frequency of firing consistent with data obtained from the SK2−/− mice (see supplemental Figure II).

Figure 2C shows summary data of cycle length (CL) in ms, the maximum diastolic potential, AP amplitude, maximum upstroke velocity (Vmax), rate of diastolic depolarization (DDR), and AP duration at 50% and 80% repolarization (APD50, APD100). Detailed analysis of the spontaneous AP reveals significant changes in the CL, APD, and DDR in the SK2+/+ and SK2−/− compared with the age-matched WT control. Overexpression of the SK2 channel in SK2−/− mice results in a significant shortening of the APD100 compared with WT, whereas APD50 and APD100 were significantly prolonged in SK2−/− mice compared with WT mice. Moreover, SK2+/+ mice show a significant increase in the DDR and a corresponding decrease in the CL. The opposite effects were observed in the SK2 knockout mice. There was also a decrease in Vmax in the SK2−/− mice.
Whole-Cell $I_{K,Ca}$ Recorded From Isolated AVN Cells

To document that there are differences in the expression of the SK2 channels in $SK2^{-/-}$ and $SK2^{+/+}$ mice, we directly assessed the SK2 current density measured as apamin-sensitive $I_{K,Ca}$ in single isolated AVN cells from transgenic and knockout animals compared with age-matched WT controls. Shown in Figure 3A are examples of the whole-cell current density elicited from a holding potential of −55 mV to various voltage steps, as shown at baseline and after application of apamin (500 pmol/L). Apamin-sensitive currents were obtained using digital subtraction and are shown to the right. Summary data in Figure 3B show a significant increase in the apamin-sensitive current density in AVN cells isolated from $SK2^{-/-}$ and a significant decrease in the current density in the $SK2^{+/+}$ mice compared with WT littermates.

Immunohistochemistry and Immunofluorescence Confocal Microscopy

We performed immunofluorescence confocal laser-scanning microscopy using isolated single AVN cells to document the expression of the SK2 channel in $SK2^{+/+}$ and $SK2^{+/-}$ mice. We directly assessed the SK2 current density measured as apamin-sensitive $I_{K,Ca}$ in single isolated AVN cells from transgenic and knockout animals compared with age-matched WT controls. Shown in Figure 3A are examples of the whole-cell current density elicited from a holding potential of −55 mV to various voltage steps, as shown at baseline and after application of apamin (500 pmol/L). Apamin-sensitive currents were obtained using digital subtraction and are shown to the right. Summary data in Figure 3B show a significant increase in the apamin-sensitive current density in AVN cells isolated from $SK2^{-/-}$ and a significant decrease in the current density in the $SK2^{+/-}$ mice compared with WT littermates.

Immunohistochemistry of Histologic Section Through Mouse AVN

Expression of SK2 channel protein in the mouse AVN was further evaluated using immunostaining of cardiac section through AVN. Figure 5A and 5A’ shows photomicrographs of histologic sections of AVN and working myocardium from WT mice at low and high magnification using hematoxylin/eosin. Masson’s trichrome (Figure 5B and B’) was used to stain fibrous tissue to view the AVN, demonstrating compact node cells next to the central fibrous body and the ventricular septum. Expression of SK2 protein in the AVN and working myocardium in WT animals was documented as peroxidase-positive staining (Figure 5C and C’). Absence of SK2 protein expression in the AVN and working myocardium in $SK2^{+/-}$ mice is shown in Figure 5D and 5D’. Treatment with secondary antibody only was used as negative control (Figure 5E and E’).

Figure 3. Whole-cell $I_{K,Ca}$ density elicited using a holding potential of −55 mV in single AVN cells. $I_{K,Ca}$ current density was obtained using the difference current before and after application of apamin (500 pmol/L) normalized to the cell capacitance. A, Representative examples of whole-cell $I_{K,Ca}$ recorded from single isolated AVN cells from WT, $SK2^{+/+}$, and $SK2^{+/-}$ mice. B, Summary data for the current density–voltage relationships of the apamin-sensitive current in AVN cells from WT (11.7±1.3 pF), $SK2^{+/+}$ (10.6±2.0 pF), and $SK2^{+/-}$ mice (13.9±1.6 pF). *P<0.05 (n=6 to 9 cells for each group from 3 set of animals).
Figure 4. Subcellular distribution of SK2 channel in mouse AVN cells. A, Photomicrographs of single isolated AVN cells (a and b). Atrial and ventricular myocytes are shown for comparison in c and d, respectively. B, Photomicrographs of confocal laser-scanning microscopy of AVN cells showing positive immunostaining with anti-Ca,3.1 antibody, followed by anti-rabbit IgG-FITC–conjugated secondary antibody (green) (a), treatment with secondary antibody only as negative control (b), and absence of Ca,3.1 staining in atrial and ventricular myocytes (c and d, respectively). The corresponding differential interference contrast (DIC) images are shown in the right images. C, Photomicrographs of confocal laser-scanning microscopy of immunostaining of AVN cells and atrial and ventricular myocytes with anti-SK2 and anti–α-actinin2 antibodies: double staining with anti-SK2 (green) and anti–α-actinin2 antibodies (red) in an AVN cell (a), preincubation of the anti-SK2 antibody with antigenic peptide (b), treatment with secondary antibodies only (anti-rabbit IgG-FITC–conjugated and anti-mouse IgG–Texas red–conjugated antibodies) as additional negative control (c), and double staining with anti-SK2 (green) and anti–α-actinin2 antibodies (red) in single atrial and ventricular myocytes (d and e, respectively). f, Double staining with anti-SK2 and anti–α-actinin2 antibodies in atrial myocytes from SK2+/− mice used as a negative control showing lack of positive staining in SK2+/− for SK2 channel. Merged images are shown at right. D, Photomicrographs of confocal laser-scanning microscopy as in C, except that anti–NF-160 antibody (red) was used instead of anti–α-actinin2 antibody in AVN cells and atrial and ventricular myocytes. NF-160 was used as an AVN marker. Scale bars=10 μm.

Discussion

The 2 gene-targeted mouse models used in our study with overexpression and knockout of SK2 channel offer us the unique opportunities to directly test the functional roles of SK2 channel in the whole animals and isolated AVN preparations, as well as single isolated AVN cells. Moreover, the 2 mouse models were backcrossed onto the same genetic background, allowing for the direct comparison between these 2 models. Indeed, we documented robust phenotypes of SAN and AVN abnormalities in the 2 mouse models, consistent with a critical role of SK2 channel in the pacemaking activities of both SAN and AVN. Here, we have focused our efforts on the AVN and have documented the existence of SK2 channel in AVN cells using multiple techniques. Overexpression of SK2 channels results in the shortening of the APs of the AVN cells associated with an increase in the firing frequency. On the other hand, SK2 channel knockout results in the opposite effects on the spontaneous APs of the AVN. Specifically, SK2−/− mice show a significant increase in the DDR and a corresponding decrease in the CL. The opposite effects were observed in the SK2 knockout mice. There was also a decrease in V_{max} in the SK2−/− mice, and the firing may result from changes in the Ca^{2+} current. Indeed, we have documented previously that SK2 channels are functionally coupled to L-type Ca^{2+} channels. 26 However, detailed analyses of the Ca^{2+} current in these mouse models are beyond the scope of the present study.

AVN Electrophysiology

Since the first description of the anatomical AVN by Tawara in 1906, 27 a large number of studies have provided insights into the heterogeneity of the structure as well as a repertoire of ion channel proteins that govern this complex conduction pathway between the atria and ventricles. 1–5, 28–32 These studies have revealed the intricate organization of multiple nodal and nodal-like myocytes contributing to the unique electrophysiology of the AVN in health and diseases, including slow conduction and dual pathways of the AVN. Indeed, reentrant tachycardia within the AVN represents the most common type of paroxysmal supraventricular tachycardia in patient population. In addition, previous studies have documented several distinct ion channel proteins that contribute to the pacemaking nature of the AVN as well as distribution of the specific connexin within the AVN structure. 1–6, 30, 31 For example, we and others have documented previously the important roles of Ca,1.3, in addition to Ca,1.2 isoform of the L-type Ca^{2+} channels in pacemaking tissues. 4, 17, 22 In addition, disruption of the gene encoding for Ca,3.1 abolishes T-type Ca^{2+} current in SAN and AVN in mice. On the other hand, no T-type Ca^{2+} current was detected in the atria of the WT or knockout animals. 5

Presence of KCa Channels in the Heart

Even though the diversity of the Ca^{2+}-independent voltage-gated K^{+} channels in cardiac myocytes has been well docu-
Moreover, we have documented the important functional roles of the SK channels in the heart as well as the more prominent expression in atria compared with ventricular tissues.7,8 However, the functional roles of the SK channels in pacemaking tissues have not been investigated to date.

SK channels have been shown to play an important role in setting the tonic firing frequency of neurons.33 Their activation causes membrane hyperpolarization, which inhibits cell firing and limits the frequency of repetitive APs. The increase in intracellular Ca2+ evoked by AP firing decays slowly, allowing SK channel activation to generate a long-lasting hyperpolarization, termed the slow afterhyperpolarization. This spike frequency adaptation protects the cell from the deleterious effects of continuous tetrode activity. In contrast to the hyperpolarization effects of SK channels in neuron, in atrial myocytes, the current contributes markedly toward the late phase of the cardiac repolarization.7,8 Nonetheless, contrary to the atrial myocytes, the maximum diastolic potential in AV nodes is approximately −60 mV, and a significant hyperpolarization may be expected. Therefore, the observed AP shortening with no associated changes in the maximum diastolic potentials in the SK2−/− model may not be entirely attributable to modulation of a Ca2+-activated K+ current. Additional studies are required to further assess possible changes in other ionic currents which may contribute to the above findings.

Pharmacology of SK Channels

The bee venom peptide toxin apamin is an 18-aa peptide with 2 internal disulfide bridges.36 Two residues, an aspartic acid and an asparagine, that reside on opposite sides of the deep pore have been shown to be essential for apamin sensitivity. No other class of K+ channels is blocked by this drug, and among the cloned K+ channels, the residues that endow sensitivity are present at those positions in only SK2 and SK3 channels. These data suggest that apamin is a very specific blocker for SK channels and that the SK channels may represent the sole class of apamin receptors in the body.37 In our voltage-clamp experiments, a relatively low dose of apamin (500 pmol/L) was used to ensure the specificity of the toxin in our study.

Physiological Significance and Implication for Human Diseases

Here, we demonstrate that overexpression of SK2 channel in the heart increases the frequency of firing in pacemaking tissues. Therefore, in cardiovascular system, SK2 channels may serve a distinct role to potentiate the increase in AVN conduction during exercise or heightened sympathetic responses under normal physiologic conditions. On the other hand, an increase in intracellular Ca2+ under certain pathologic conditions may produce profound changes in AVN conduction. For example, during atrial fibrillation, the rapid depolarization may increase intracellular Ca2+ and potentiate the I_{KCa} and AVN conduction. Hence, SK2 channel may represent an attractive target to modulate atrial conduction during atrial arrhythmias.

Limitations

Even though the gene-targeted mouse models offer unique advantage for our study to directly probe the functional roles
of SK2 channel in AVN, we recognize the fact that there are well-documented differences between small animal models and human. Additional studies will need to be performed using large animal models.

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Disclosures

None.

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Functional Roles of a Ca\(^{2+}\)-Activated K\(^+\) Channel in Atrioventricular Nodes
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ALL ANIMAL CARE AND PROCEDURES WERE APPROVED BY THE UNIVERSITY OF CALIFORNIA, DAVIS INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE. ANIMAL USE WAS IN ACCORDANCE WITH NATIONAL INSTITUTES OF HEALTH AND INSTITUTIONAL GUIDELINES.

TRANSGENIC MICE OVER-EX PressING SK2 CHANNEL (SK2 +/-T)

Transgenic mice over-expressing SK2 channels were developed via insertion of a tetracycline-regulatory cassette into the SK2 locus as previously described. Transgenic mouse lines were backcrossed more than seven generations onto the C57Bl/6J background. Compared with wild-type (WT) littermate mice, in the absence of doxycycline, the SK2 protein and SK2 mRNA are over-expressed in heterozygotes (SK2 +/-T) ~10 and 4.5 folds as shown by Western blot and quantitative PCR, respectively. Importantly, the distribution pattern of SK2 expression is unaltered.

All mice were housed and kept on a 12 h light/dark cycle with food and water available ad libitum. Mice were weaned at 3 weeks and genotyped. Although heterozygote SK2 +/-T mice were viable, produced offspring, and were similar in weight and appearance to WT mice, SK2 T/T homozygous mice were not viable without prenatal treatment of doxycycline. For this reason, heterozygous mice were used in our studies. Transgene presence was confirmed using polymerase chain reaction of tail tissue as previously described.

SK2 NULL MUTANT MICE (SK2 +/-Δ mice)

SK2 null mutant mice were generated as previously described. A single loxP site was introduced into the 5'UTR 300 nucleotides 5' of the initiator methionine and a cassette consisting of the neomycin-resistance gene flanked by loxP sites, and the coding sequence for enhanced
GFP was inserted into an EcoRV site in intron 2, ~2.3 kb from the end of exon 2. Recombination at the loxP sites yields an allele that is deleted for SK2 exons 1 and 2. The targeting construct was electroporated into ES cells, and several properly recombined ES cell clones were injected into C57Bl/6 blastocysts. One chimera gave germline transmission of the targeted allele. Crosses between heterozygous-floxed SK2 and the Cre deleter mouse yielded offspring that are heterozygous for the SK2-deleted allele (+/Δ).

**Atrioventricular Node Recordings**

AVN preparation was prepared as previously described. In brief, the animals were anesthetized with pentobarbital (60 mg/kg, i.p.). The heart was excised and placed in Tyrode’s solution at 33°C. The right atrium was separated from the ventricles and then the left atrium. The right atrium was opened under a dissecting microscope to expose the coronary sinus, the triangle of Koch and the interatrial septum. The sinoatrial node (SAN) region was removed. The final preparation which included the entire AVN region and surrounding atrial muscle was pinned down (endocardial surface up) in the recording chamber. AVN region was recognized by its anatomic landmarks (Figure 2A). The strip of the tissue was continuously superfused with Tyrode’s solution contained (mmol/L) NaCl 138, KCl 4, MgCl₂ 1, CaCl₂ 2, NaH₂PO₄ 0.33, glucose 10, and HEPES 10, pH 7.4 with NaOH. Spontaneous APs were recorded from isolated AV nodal preparations using microelectrode techniques with 3 M KCl microelectrodes at 33°C as previously described.

Single AVN cells were isolated from WT and mutant mice as previously described with some modification. Whole-cell Ca²⁺-activated K⁺ current (I₅₋ᵥ, Ca) was recorded from single AVN cells at room temperature using patch-clamp techniques as previously described. The extracellular solution contained (in mmol/L): N-methylglucamine (NMG), 140; KCl, 4; MgCl₂,
1; glucose, 5; and HEPES, 10 (pH 7.4 using HCl). The internal solution consisted of (in mmol/liter): potassium gluconate, 144; MgCl₂, 1.15; EGTA, 5; HEPES, 10; and CaCl₂ yielding a free (unchelated) [Ca²⁺] of 0.5 μmol/L using Calcium Titration Software to calculate free [Ca²⁺], bound and dissociated. The pH was adjusted to 7.25 using KOH. All chemicals were purchased from Sigma Chemical (St. Louis, MO) unless stated otherwise. The cell capacitance was calculated by integrating the area under an uncompensated capacitive-transient elicited by a 20-mV hyperpolarizing pulse from a holding potential of -40 mV. Whole-cell current records were filtered at 2 kHz and sampled at 10 kHz. Liquid junction potentials were measured as previously described and all data were corrected for the liquid junction potentials.

**Electrocardiographic (ECG) Recordings from Transgenic and Knock-out Animals**

ECG recordings were obtained using Bioamplifier (BMA 831, CWE, Incorporated, Ardmore, PA). The animals were anaesthetized with 250 mg kg⁻¹ of tribromoethanol (Avertin) i.p. and placed on a temperature-controlled warming blanket at 33°C. The temperature of the animals was monitored during the recordings using a rectal probe. Four consecutive two-minute epochs of ECG data were obtained from each animal. Signals were low-pass filtered at 0.2 kHz and digitized using Digidata 1200 (Axon Instrument, CA). A total of 100 beats were analyzed from each animal in a blinded fashion.

**Immunofluorescence Confocal Microscopy**

Immunofluorescence labeling was performed as described previously and examined using a Pascal Zeiss confocal laser scanning microscopy. Single isolated AVN cells were blocked with 1% bovine serum albumin in phosphate buffered saline (PBS), incubated overnight at 4°C with primary antibodies. The following primary antibodies were used: (1) anti-SK2 antibody (Sigma, 1:100 dilution), a polyclonal antibody raised in rabbit; (2) anti-neurofilament (NF 160)
antibody (Chemicon, 1:100), a monoclonal antibody; (3) anti-α-actinin2 antibodies (Sigma, 1:800); and (4) anti-Ca\textsubscript{2+} channel antibody developed in rabbit (Sigma, 1:100).

Immunofluorescence labeling for confocal microscopy was performed by treatment with FITC-conjugated goat anti-rabbit antibody (Sigma, 1:250) or Texas red-conjugated donkey anti-mouse antibody (Calbiochem, 1:250). Control experiments were performed by pre-incubation with antigenic peptide or secondary antibody only under the same experimental conditions. Identical settings were used for all specimens.

**Immunohistochemistry of the AVN**

Paraffin sections of mouse hearts (6 μm in thickness) were treated with xylene and ethanol. After hydration, the sections were blocked overnight in 5 % normal goat serum in PBS at 4 ºC, after which the primary antibody (anti-SK2 antibody, 1:200) in PBS containing 5% normal goat serum was added for 30 min. The sections were then treated with biotinylated secondary antibody. After washing for 5 min in PBS, they were incubated in VECTASTAIN ABC reagent (Vector Laboratories) for 30 minutes. The sections were then incubated in peroxidase solution. The sections were rinsed, counterstained and mounted. AVN region was further evaluated in cardiac sections stained with hematoxylin and eosin (H&E) and Masson’s trichrome.

**In vivo electrophysiologic studies in mice**

_in-vivo electrophysiologic studies were performed as previously described.\textsuperscript{14,15} Standard pacing protocols were used to determine the electrophysiologic parameters, including AV nodal refractory periods and AV nodal conduction properties. Each animal underwent an identical pacing and programmed stimulation protocol._
Data Analysis

Curve fits and data analysis was performed using Origin software (MicroCal Inc., Northampton, MA). Where appropriate, pooled data are presented as mean±s.e.m. Statistical comparison was performed using the Student's t-test with p < 0.05 considered significant. Rate of diastolic depolarization (DDR in mV/s) was determined from recordings of the spontaneous AP by determining the first derivative of the diastolic depolarization.

REFERENCES


**Figure Legends**

**Figure S1.** In-vivo EPS in SK2 +/-Δ mice compared to WT animals during atrial extrastimulus (S₂) testing at basic cycle length of 120 ms (S₁S₂ = 120 ms) showing prolongation of AVN effective refractory period (AVNERP) in SK2 +/-Δ mice compared to WT animals. Upper three tracings are surface ECG (Lead I, II and aVF). Lower two tracings are intracardiac electrograms showing atrial and ventricular electrograms with conducted compared to non-conducted premature stimuli at S₁S₂ of 120 ms. Summary data show a significant prolongation of AVNERP of 72 ± 3 and 80 ± 3 ms in SK2 +/-Δ and SK2 Δ/Δ mice at S₁S₁ of 120 ms, respectively compared to WT animals (AVNERP = 65 ± 2 ms, p < 0.05).

**Figure S2.** Examples of spontaneous APs recorded from intact AVNs from WT mice before and after application of apamin (500 pmol/L) showing a significant decrease in the firing frequency
after application of apamin. Summary data show a significant increase in RR interval from 210±12.5 ms at baseline to 408±11.4 ms after application of apamin (*p<0.05, n = 4 cells).
**Figure S1**

**WT**

S1S2 = 60

**SK2 +/-**

S1S2 = 80

S1S2 = 70

S1S2 = 50

Figure S1
Figure S2