Cardiac Small Conductance Ca\(^{2+}\)-Activated K\(^{+}\) Channel Subunits Form Heteromultimers via the Coiled-Coil Domains in the C Termini of the Channels

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**Rationale:** Ca\(^{2+}\)-activated K\(^{+}\) channels are present in a wide variety of cells. We have previously reported the presence of small conductance Ca\(^{2+}\)-activated K\(^{+}\) (SK or K\(_{Ca}\)) channels in human and mouse cardiac myocytes that contribute functionally toward the shape and duration of cardiac action potentials. Three isoforms of SK channel subunits (SK1, SK2, and SK3) are found to be expressed. Moreover, there is differential expression with more abundant SK channels in the atria and pacemaking tissues compared with the ventricles. SK channels are proposed to be assembled as tetramers similar to other K\(^{+}\) channels, but the molecular determinants driving their subunit interaction and assembly are not defined in cardiac tissues.

**Objective:** To investigate the heteromultimeric formation and the domain necessary for the assembly of 3 SK channel subunits (SK1, SK2, and SK3) into complexes in human and mouse hearts.

**Methods and Results:** Here, we provide evidence to support the formation of heteromultimeric complexes among different SK channel subunits in native cardiac tissues. SK1, SK2, and SK3 subunits contain coiled-coil domains (CCDs) in the C termini. In vitro interaction assay supports the direct interaction between CCDs of the channel subunits. Moreover, specific inhibitory peptides derived from CCDs block the Ca\(^{2+}\)-activated K\(^{+}\) current in atrial myocytes, which is important for cardiac repolarization.

**Conclusions:** The data provide evidence for the formation of heteromultimeric complexes among different SK channel subunits in atrial myocytes. Because SK channels are predominantly expressed in atrial myocytes, specific ligands of the different isoforms of SK channel subunits may offer a unique therapeutic opportunity to directly modify atrial cells without interfering with ventricular myocytes. (Circ Res. 2010;107:851-859.)

Key Words: Ca\(^{2+}\)-activated K\(^{+}\) channels  cardiac myocytes  heteromultimerization  coiled-coil domains

Small conductance Ca\(^{2+}\)-activated K\(^{+}\) (SK or K\(_{Ca}\)) channels belong to a family of Ca\(^{2+}\)-activated K\(^{+}\) channels (K\(_{Ca}\)) that have been reported in a wide variety of cells.\(^1,2\) K\(_{Ca}\) channels represent a highly unique family of K\(^{+}\) channels in that they are directly gated by changes in intracellular Ca\(^{2+}\) concentration and, hence, function to integrate changes in Ca\(^{2+}\) concentration with changes in K\(^{+}\) conductance and membrane potentials. K\(_{Ca}\) channels have been extensively studied in the central and peripheral nervous systems, where they mediate afterhyperpolarizations following action potentials\(^1,3\); however, their functional importance in cardiac tissues is only beginning to be elucidated.\(^4-11\) Indeed, evidence for the existence of SK channels in the heart was first established by our laboratory\(^4,5\) and has since been supported by work from others.\(^6,8\) We have identified several isoforms of SK channel subunits in the heart including SK1, SK2, and SK3 (K\(_{Ca}\)2.1, 2.2, and 2.3) and have documented that SK channels are highly expressed and play important functional roles in atrial myocytes and pacemaking tissues compared with the ventricle.\(^4,5,7,9,10\) Genetic ablation of SK2 channels in a mouse model results in atrial action potential prolongation and atrial arrhythmias.\(^10\) Overexpression of SK2 channels in a transgenic mouse model results in the shortening of the spontaneous action potentials of the atrioventricular node cells and an increase in the firing frequency. On the other hand, ablation of the SK2 channel results in the opposite effects.\(^9\)

SK channels are being increasingly recognized as possible drug targets in diseases such as myotonic muscular dystrophy and sickle cell anemia.\(^12,13\) SK channels are encoded by 3 genes (KCNN1, KCNN2, and KCNN3) and exhibit characteristic sensitivity to the bee venom peptide toxin apamin, with
potency of blocking dependent on the specific isoforms.\textsuperscript{1,2,14} Conceptually, SK channel specificity could be accomplished by homomeric, as well as heteromeric assembly.\textsuperscript{15,16} Indeed, heteromeric coassembly of SK channel subunits has been demonstrated in heterologous expression systems.\textsuperscript{14–18} However, structural determinants of SK channel assembly in heteromeric, as well as homomeric, complexes remain unknown. Here, we provide evidence to support the formation of heteromeric complexes among different SK channel subunits in native cardiac tissues. A combination of molecular, biochemical, and electrophysiological techniques were used to directly determine the structural determinants of the heteromeric complexes. Sequence analyses of SK channel proteins suggest the occurrence of coiled-coil domains (CCDs) in the C termini of these channels. Moreover, we have provided data to support the roles of CCDs in mediating SK channel subunit interaction and assembly in atrial myocytes.

Methods

The human study protocol was approved by the UC Davis Institutional Review Board. All animal care and procedures were approved by the UC Davis Institutional Animal Care and Use Committee. Detailed methods are presented in Online Data Supplement at http://circres.ahajournals.org.

Immunofluorescence Confocal Microscopy and Immunogold-Labeled Transmission Electron Microscopy

Immunofluorescence confocal microscopy and immunogold-labeled transmission electron microscopy (immune-EM) were performed as described previously.\textsuperscript{4,11}

Coimmunoprecipitation and Western Blot Analysis

Human heart tissues were procured from a commercial source (T Cubed Inc). Coimmunoprecipitation (co-IP) and Western blot were performed as described previously.\textsuperscript{7}

Sequence Analysis and Design of Inhibitory Peptides

Coils Version 2.2 program (http://www.ch.embnet.org) was used for the prediction of CCDs from primary amino acid sequence of mouse and human cardiac SK channels.\textsuperscript{19–22} The α-helix structure of CCD2 was confirmed by homology modeling of structural coordinates of SK2 amino acid sequence with those of template models from the following servers: SWISS-MODEL (http://swissmodel.expasy.org/workspace/index.php?func=modeling_simple1) and I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER).\textsuperscript{23}

In Vitro Interaction Assays

Mammalian Two-Hybrid System Assay 2 (Clontech, Palo Alto, Calif) was used for testing the in vitro interactions among SK1, SK2, and SK3 channel subunits. C-terminal fusion constructs of SK1, SK2, and SK3 were generated in PM and pVP16 vectors from amino acids 364 to 537 of mouse cardiac SK1 channel (accession no. AY258143.1),\textsuperscript{3} amino acids 395 to 580 of human cardiac SK2 channel (accession no. AY258141.1)\textsuperscript{9} and amino acids 544 to 737 of human cardiac SK3 channel (accession no. AY258142.1; Online Figure I).

Ca\textsuperscript{2+}-Activated K\textsuperscript{+} Current Recordings

Whole-cell Ca\textsuperscript{2+}-activated K\textsuperscript{+} current (\(I_{\text{K,Ca}}\)) was recorded from freshly isolated atrial myocytes and transfected tsA201 cells using patch-clamp techniques as previously described.\textsuperscript{4,24}

Results

Subcellular Distribution of SK1, SK2, and SK3 Channel Subunits in Isolated Mouse Atrial Myocytes

We have previously documented the existence of SK1, SK2, and SK3 channel subunits in human and mouse hearts.\textsuperscript{4,5} To determine the subcellular distribution of SK1, SK2, and SK3 channel subunits in mouse atrial myocytes, we performed immunofluorescence confocal microscopy using double labeling with anti-SK1, -SK2, and -SK3 antibodies (Figure 1A, 1B, and 1C, respectively). There was overlap in the distribution of the 3 isoforms of SK channel subunits along the Z-lines in atrial myocytes. The degree of overlap in the subcellular distribution was directly quantified using scatter plots, as shown in the right panels of Figure 1A through Figure 1C, demonstrating a high degree of correlation between different SK channel subunits. Merged images are shown at higher magnification in the right panels below the scatter plots in Figure 1A through 1C. Control experiments were performed as presented in the Online Data Supplement, including (1) precubation of anti-SK antibodies with antigenic peptide (Online Figure II, A); (2) secondary antibodies only (Online Figure II, B); (3) staining using homozygous SK2 knockout mice as previously described\textsuperscript{8} (Online Figure II, C); and (4) immunostaining using tsA201 cells transfected with SK1, SK2, and SK3 plasmids (Online Figure II, D). In addition, immuno-EM was performed to further resolve the subcellular localization of the SK channel subunits. Electron microscopic postembedding immunogold labeling demonstrates that SK1, SK2, and SK3 channel subunits are clustered together in mouse atrial myocytes (Figure 1D and 1E).

Identification of Heteromultimeric Complexes of SK1, SK2, and SK3 Channel Subunits in Human and Mouse Myocytes Using Co-IP

To directly determine whether SK1, SK2, and SK3 channel proteins form heteromultimeric complexes in human (Figure 2A through 2C) and mouse (Figure 2D through 2F) cardiac tissues, co-IP was performed. Using anti-SK1 antibody, we coimmunoprecipitated SK1, SK2, and SK3 channel proteins from tissue lysate (Figure 2A through 2F, lanes 1 and 2). For SK1 channel protein, both monomers and dimers were detected as 2 distinct bands. Similarly, in the reverse experiments using anti-SK2 antibody (Figure 2A through 2F, lanes 3 and 4) and
Figure 1. Subcellular distribution of SK1, SK2, and SK3 channel subunits in isolated mouse atrial myocytes using immunohistochemistry and immune-EM. Confocal photomicrographs of single isolated mouse atrial myocytes doubly stained with anti-SK antibodies in different combinations as follows: goat anti-SK1 and rabbit anti-SK2 (A); rabbit anti-SK2 and goat anti-SK3 (B); and rabbit anti-SK1 and mouse anti-SK3 antibodies (C). Scale bars, 5 μm, except in B (10 μm). Merged images are shown in the third row of each panel. Scatter plots in the right panels show high correlation between different SK channel subunits. All pixels in the images have been assigned a position on the scatter plots and are placed according to the intensity of red or green color. In addition, merge images are shown at higher magnification in the right panels. D and E, Electron photomicrographs showing the ultrastructure of mouse atrial myocytes and subcellular distribution of SK1, SK2, and SK2 channel subunits. D, Single immunolabeling for SK1, SK2, and SK3 channel subunits. Left, Images taken under ×3800 magnification with scale bars of 2 μm. Right, Magnification images of areas marked by red boxes in the left panels with scale bars of 200 nm. Arrows indicate gold particles, which are 10, 15, and 5 nm for SK1, SK2, and SK3 staining, respectively. E, Double labeling for SK1 and SK2, as well as SK2 and SK3 channel subunits. For SK1 and SK2 double labeling, 10- and 20-nm gold particle–conjugated secondary antibodies were used to localize SK1 and SK2 subunits, respectively. Note the clustering of SK1 (red arrows) and SK2 (blue arrows) channel subunits as shown by arrows. For SK2 and SK3, 20- and 10-nm gold-conjugated secondary antibodies were used to localize SK2 (blue arrows) and SK3 (red arrows) subunits, respectively. Note SK2 and SK3 channels are closely located, as shown by arrows. F, Negative controls are labeled by gold-conjugated secondary antibodies only and are devoid of any gold particles. M indicates mitochondria; N, nucleus.

Sequence Analysis of SK Channel Subunits Revealed Existence of Coil-Coiled Domains in the C Termini of the Channels

To establish the structural determinants mediating the protein-protein interactions, we analyzed amino acid sequences of SK channel subunits using coiled-coil prediction programs (Coils Version 2.2). Figure 3A shows probabilities of coiled-coil formation, which peaked in the C termini and were very low in the N termini (except for SK3 channel). The probability of forming coiled coils of first CCD (CCD1) from C termini of all 3 SK channel isoforms was lower than that for the second CCD (CCD2). The coiled coil probability of CCD1 of SK2 was almost double of that of SK1 and SK3 and was close to the value of the N-terminal CCD of SK3 channel.

Coiled coils in SK channels followed a continuous stretch of 7-residue sequence repeat (with positions named a through g) containing small hydrophobic residues in the “a” (first) and “d” (fourth) positions. The larger polar residues often occurred at positions “e” and “g” (Figure 3 B). As shown in Figure 3, highlighted hydrophobic residues (eg, valine [V], leucine [L], isoleucine [I]) at “a” and “d” positions would form the hydrophobic core for the interaction, whereas charged residues at “g” and succeeding “e” positions would establish complementary electrostatic interactions (Figure 3B; Online Figure III, C). The alignment of bipartite CCDs of SK channels with CCD domains of 2 other human K+ channels (namely, K<sub>7</sub> and K<sub>10</sub>) is presented in Figure 3B.

anti-SK3 antibody (Figure 2A through 2F, lanes 5 and 6), SK1, SK2, and SK3 channel proteins could be immunoprecipitated from tissue lysate. Specificity of the SK antibodies used was directly tested by preincubating the anti-SK antibodies with their corresponding antigenic peptides before immunoblotting the immunoprecipitated complexes from mouse atrial and ventricular tissue (Figure 2G). Control co-IP experiments were also performed by using the normal rabbit IgG or serum to immunoprecipitate complexes from mouse atrial and ventricular tissues (Figure 2H). The 50-kDa bands correspond to immunoglobulin heavy chain (rabbit host) used in the IP reactions when corresponding antigenic peptides before immunoprecipitation of mouse atrial myocytes were doubly stained with anti-SK antibodies in different combinations as follows: goat anti-SK1 and rabbit anti-SK2 (A); rabbit anti-SK2 and goat anti-SK3 (B); and rabbit anti-SK1 and mouse anti-SK3 antibodies (C). Scale bars, 5 μm, except in B (10 μm). Merged images are shown in the third row of each panel. Scatter plots in the right panels show high correlation between different SK channel subunits. All pixels in the images have been assigned a position on the scatter plots and are placed according to the intensity of red or green color. In addition, merge images are shown at higher magnification in the right panels. D and E, Electron photomicrographs showing the ultrastructure of mouse atrial myocytes and subcellular distribution of SK1, SK2, and SK2 channel subunits. D, Single immunolabeling for SK1, SK2, and SK3 channel subunits. Left, Images taken under ×3800 magnification with scale bars of 2 μm. Right, Magnification images of areas marked by red boxes in the left panels with scale bars of 200 nm. Arrows indicate gold particles, which are 10, 15, and 5 nm for SK1, SK2, and SK3 staining, respectively. E, Double labeling for SK1 and SK2, as well as SK2 and SK3 channel subunits. For SK1 and SK2 double labeling, 10- and 20-nm gold particle–conjugated secondary antibodies were used to localize SK1 and SK2 subunits, respectively. Note the clustering of SK1 (red arrows) and SK2 (blue arrows) channel subunits as shown by arrows. For SK2 and SK3, 20- and 10-nm gold-conjugated secondary antibodies were used to localize SK2 (blue arrows) and SK3 (red arrows) subunits, respectively. Note SK2 and SK3 channels are closely located, as shown by arrows. F, Negative controls are labeled by gold-conjugated secondary antibodies only and are devoid of any gold particles. M indicates mitochondria; N, nucleus.

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teins immunoprecipitated with nonspecific IgG (lane 7, A through F), whereas positive control was performed using tissue homogenates (lanes 8, A through F). Lanes 7 and 8 contained mixtures of atrial and ventricular homogenates. **C** Control experiments were performed using mouse atrial (A) and ventricular (V) tissue homogenates immunoprecipitated with anti-SK antibodies, proteins were eluted, and Western blot analysis (IB) was performed using anti-SK antibodies preincubated with their corresponding antigenic peptide (AP) in a ratio of 1:5. **H**, Additional control experiments were performed using mouse atrial (A) and ventricular (V) tissue homogenates immunoprecipitated with nonspecific normal rabbit anti-IgG antibodies, proteins were eluted, and Western blot analysis (IB) was performed with anti-SK antibodies. In both **A and H**, except for heavy chain band of 50 kDa, no other bands were detected.

**Identification of Interaction Between Different SK Channel Subunits Using In Vitro Interaction Assay**

To further document protein–protein interaction among SK channel subunits, mammalian 2-hybrid system was used. The C-terminal fusion constructs of cardiac SK1, SK2, and SK3 channel subunits harboring the CCDs were subcloned into pM vector containing DNA binding domain and pVP16 vector containing transcription activation domain. C termini of the SK1 to SK3 proteins immediately before the start of calmodulin binding domain (CaMBD), as depicted in Figure 4A, were used. The SK1, SK2, and SK3 C termini in pM and pVP16 vectors were cotransfected, along with secreted alkaline phosphatase vector (pSEAP, containing SEAP reporter gene under the control of a GAL4 responsive element) into tsA201 cells (Online Figure III, D). Alkaline phosphatase activity showed positive interactions between C termini of SK1, SK2, and SK3 with intensity that was significantly higher (* P<0.05) than negative controls (pM+pVP16 empty vectors, pM-SK1/2/3+pVP16 empty vectors, or pM empty vector+pVP16-SK1/2). pM3pVP16+pM3pVP16 were used as the positive control (Figure 4B). The strength of interaction observed between SK1 and SK2, SK2 and SK3, and SK1 and SK3 channel subunits were similar. This pattern was also observed when the 2 different SK channel subunits were reversed in the binding domain and activation domain vector, indicating that interactions were not vector specific (Figure 4B). These results support the observed heteromultimeric complexes of SK channel subunits from the co-IP experiments. Finally, deletion of CCD2 domains from the SK2 C termini (SK2-ΔCCD) resulted in a significant decrease in the interaction of the SK2 C termini with SK1 or SK3 C termini (* P<0.05) supporting the importance of CCDs in the heteromultimerization of the SK channel subunits (Figure 4B).

**Triple Mutations GYG to AAA in SK1 and SK3 Channel Subunits Suppressed Wild-Type SK2 Currents**

In our previously published study, we have shown that triple mutations of the pore signature sequence, glycine, tyrosine, and glycine (GYG) to alanine (AAA) in SK2 channel subunit renders the mutant subunit non conductive.10 Moreover, upon coexpression of the mutant subunit with wild-type (WT) SK2 channel subunit, the mutant subunit leads to dominant-negative (DN) suppression of I_K,Ca. Because SK channel subunits have conserved pore sequence, “GYG,” we set out to mutate pore signature sequence of SK1, as well as SK3 channel subunits by site-directed mutagenesis (GYG to AAA). We tested the DN effect of pore mutants of SK1-AAA and SK3-AAA on WT SK2 currents in transfected tsA201 cells. All transfections were performed in the presence of α-actinin2 based on our previously published data showing an increased SK2 channel expression on cotransfection with α-actinin2 proteins.7 We first documented that the 3 SK pore mutant constructs (SK1/2/3-AAA) could indeed lead to a DN...
functional knockdown of their WT counterparts in tsA201 cells (data shown for WT SK2 and AAA-SK2 only). Next, we tested whether the 3 SK mutant subunits could result in the DN suppression of WT SK2 current (Online Figure IV, B through E). Online Figure IV (B) shows $I_{\text{K,Ca}}$ recorded from tsA201 cells transfected with $\alpha$-actinin2 and SK2 channel. $I_{\text{K,Ca}}$ was significantly suppressed by coexpressing WT SK2 with SK2-AAA, showing DN effect of the SK2 pore mutant subunit (Online Figure IV, E). Similar suppression of the $I_{\text{K,Ca}}$ was observed using SK1 or SK3 pore mutant subunits (Online Figure IV, C and D). Summary data are shown in Online Figure IV (F). Similar data were obtained showing DN suppression of WT SK3 current (Online Figure V).

SK1, SK2, and SK3 CCD Peptides Inhibit $I_{\text{K,Ca}}$ in Atrial Myocytes

To further test the roles of CCD in the multisubunit assembly of SK channels in native cells, we designed a SK2-specific peptide directly targeting the CCD2 region of SK2 channel subunit with the amino acid sequence as underlined in Figure 3B. Inhibition of expressed and endogenous $I_{\text{K,Ca}}$ recorded from tsA201 cells transfected with $\alpha$-actinin2 and SK2 channel. $I_{\text{K,Ca}}$ was recorded using whole-cell patch-clamp techniques 1 minute after establishment of whole-cell configuration (initial) and after 12 minutes. Inclusion of 30 to 50 $\mu$mol/L inhibitory peptide in the pipette solution resulted in nearly complete inhibition of $I_{\text{K,Ca}}$ after 12 minutes of recordings compared with the initial traces (Figure 5B and 5D), with no further reduction after application of apamin (100 pmol/L). In contrast, parallel experiments using the same concentrations of scrambled peptide (Figure 5A and 5C) showed stable current recordings at 12 minutes after the establishment of whole-cell configuration with no “run down” or “run up” of $I_{\text{K,Ca}}$. Apamin (100 pmol/L) was applied to further document the presence of $I_{\text{K,Ca}}$. In addition, SK1- and SK3-specific peptides directly targeting the CCD region of SK1 and SK3 subunits with the amino acid sequence, as shown in Figure 3B, were generated. The SK1 and SK3 inhibitory peptides also resulted in the inhibition of $I_{\text{K,Ca}}$ recorded from isolated atrial myocytes (Figure 5E and 5F). However, the effects were less pronounced as compared with the SK2 inhibitory peptide likely because of the lower expression of SK1 and SK3 subunits in atrial myocytes. Summary data in Figure 5G and 5H show significant inhibition of the apamin-sensitive $I_{\text{K,Ca}}$ quantified at the test potentials of $-120$ and $+60$ mV.

We further documented the specificity of the inhibitory peptide. The SK2 inhibitory peptide failed to suppress the SK3 current expressed in tsA201 cells (Online Figure VI, A). In contrast, the peptide resulted in nearly complete inhibition of $I_{\text{K,Ca}}$ current from SK2 homomultimers (Figure 5B) or
heteromultimers from SK2 and SK3 channel subunits (Online Figure VI, B).

Discussion
In the present study, we provide evidence for the formation of heteromultimerization of SK subunits in human and mouse myocytes. We further establish that there is a direct physical interaction of the C-terminal regions among the 3 SK channel subunits via the CCDs. Additionally, we took advantage of specific inhibitory peptides derived from CCDs to inhibit \( I_{\text{K,Ca}} \) in atrial myocytes. Taken together, the findings using inhibitory peptides, as well as in vitro interaction assay with deletion constructs, implicate the role of the CCDs as mediators of the heteromeric assembly.

Heteromultimeric Complex Formation Among SK Channel Subunits
Functional diversity of ion channels is achieved by several mechanisms, ranging from physical association of \( \alpha \) subunits with accessory \( \beta \) subunits that modulate channel activities to alternative splicing and mRNA editing.\(^{25} \) Moreover, in \( K^+ \) channels where the conductive pore is formed by 4 subunits, functional diversity can be further attained by coassembly of different subunits to generate heteromeric channels with novel biophysical properties.

Coassembly of \( K^+ \) channels is precisely controlled by multiple mechanisms, eg, spatial and temporal coexpression, to prevent wasteful or potentially deleterious combinations from hampering normal cellular functioning.\(^{26} \) Typically, assembly of \( K^+ \) channels into functional homo- and heterotetramers occurs by interaction of tetramerization domains in the N and/or C termini. Homo- and heteromeric interac-

tions are conserved within the gene family.\(^{27} \) Many \( K^+ \) channels form heteromultimers via the C-terminal regions that resemble tetramerizing CCDs.\(^{27} \) Tetramerizing CCDs are continuous stretches of CCDs and have been implicated in the enhancement of tetramer stability and selectivity of multimerization among many \( K^+ \) channels.\(^{27,28} \)

Coiled coils are bundles of intertwined \( \alpha \)-helices constituting structural motif repetitions (abcdefg)\(_n\) that are directly involved in protein–protein interactions.\(^{29} \) CCDs serve as sites for dynamic assembly and disassembly of protein complexes in diverse protein classes, including transcription factors, fibrous proteins, membrane fusion proteins, and motor proteins.\(^{30} \) Because many coiled coil–bearing proteins play crucial roles in different physiological and pathological processes, in addition to the high specificity and reversible nature of the CCD associations, CCDs may represent attractive targets for pharmacological intervention.\(^{31,32} \) Several studies have suggested key roles of cytoplasmic CCDs in ion channel assembly, including those from members of the voltage-gated ion channel superfamily (eg, transient receptor potential ion channels,\(^{33} \) \( K_7^{\text{Ca}} \), and cyclic nucleotide-gated channels).\(^{34} \)

Although functional heteromeric interaction of SK channels has been documented in heterologous systems,\(^{14–18} \) it is not clear whether these are present in native tissues. Our study provided evidence for the physical coexistence and heteromeric associations among SK channel subtypes in native cardiac cells. In addition, our in vitro interaction assays suggested almost similar strengths of interactions among SK channel subtypes. Our results from coexpression of full-length SK2 and DN pore mutants of SK channel subunits further demonstrate the suppression of SK2 current via
heteromeric interaction by coassembly of different SK DN constructs with WT SK2 subunits. Theoretically, the probability of tetrameric WT channel being formed when equal ratio of DN and WT subunits are present is close to 6% \((1/2)^4\). This is validated by our observation that DN mutant subunits resulted in marked suppression of the WT subunits supporting the notion that the presence of even a minimum of 1 DN monomeric subunit resulted in nonfunctional channels. These results provide evidence for the ability of SK channel subunits in forming heteromultimers in addition to the formation of homomultimers as deduced from current recorded from SK1, SK2, or SK3 channel subunits when expressed alone.

**Functional Significance of SK Channel Heteromultimerization**

In cardiac myocytes, SK channels contribute to the late phase of cardiac repolarization, whereas in neurons these channels underlie afterhyperpolarization. New insights into SK channel subtype heteromeric associations are physiologically significant and relevant because SK channel variants (SK2-sh, SK3–1B, SK3–1C) have previously been documented to produce nonfunctional channels as homomers in mammalian cells and can selectively suppress the endogenous SK currents. Other studies have reported existence of truncated isoforms of SK3 channel which trap full-length ion channel proteins intracellularly via DN inhibition of functional channel expression on cell surface. Similarly, our previous studies have documented several different splice variants of SK1 and SK3 channels from human and mouse hearts; their functional significance especially in the context of their roles in subunit interactions remain unclear.

**Molecular Determinants of Subunit Assembly**

Initial reports of SK channel subunit assembly indicate the involvement of the N terminus. Here, our study provides new evidence for additional important molecular determinant involving the CCDs within the C termini of the channels. More than 1 domain from SK channel C terminus has been shown to be responsible for intersubunit interaction and

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**Figure 5. Peptides derived from the SK1, SK2, and SK3 CCD inhibit \(I_{K,\text{Ca}}\) in atrial myocytes.** A voltage-ramp protocol was applied from −120 to +60 mV at a holding potential of −55 mV. \(I_{K,\text{Ca}}\) was recorded immediately after establishment of whole-cell mode (black line), 12 minutes thereafter (blue line), and after application of apamin (100 pmol/L) (red line). A and B, \(I_{K,\text{Ca}}\) density (pA/pF) recorded from tsA201 cells expressing SK2 current using scrambled (A) compared with SK2 (B) inhibitory peptides, respectively. C through D, \(I_{K,\text{Ca}}\) density recorded from mouse atrial myocytes using scrambled (C) compared with SK2 (D), SK1 (E), or SK3 (F) inhibitory peptides, respectively. G and H, Summary data of percentage inhibition of apamin-sensitive \(I_{K,\text{Ca}}\) at −120 and +60 mV from tsA201 cells (G) and atrial myocytes (H), respectively (n=4 to 6). *\(P<0.05\) comparing the inhibitory peptides to scrambled peptide.
trafficking. These include the CaMBD distal to S6 transmembrane domain, responsible for Ca\(^{2+}\)-independent binding of calmodulin.\(^{39}\) Our data provide direct evidence for the functional roles of CCDs in SK channel subtypes in forming heteromultimeric complexes. Taken together, it appears that more than 1 region of the channel are involved in SK channel assembly.

**Importance of CCD in Other K\(^+\) Channels**

C-terminal CCDs of some K\(^+\) channels (K\(_{\text{C},1.1.1, \text{K}}\)) have been shown to not only drive tetrameric assembly but also determine stability and selectivity of multimerization.\(^{27,28}\) It is the compatibility of CCDs that determines the heteromeric associations among channel subunits encoded by different genes.\(^{27}\) Absence of CCD significantly impairs functional channel expression.\(^{40}\) This is indicative of prominent roles of genes.\(^{27}\) Absence of CCD significantly impairs functional channel expression associated with pacemaker activity in the mouse heart. J Physiol. 2005;562:223–234.

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**Disclosures**

None.

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What New Information Does This Article Contribute?

- Small conductance Ca\(^{2+}\)-activated K\(^{-}\) (SK) channels represent a highly unique family of K\(^{-}\) channels because they are gated solely by changes in intracellular Ca\(^{2+}\) concentration and consequently function to integrate changes in Ca\(^{2+}\) concentration with changes in K\(^{-}\) conductance and membrane potentials.
- Several isoforms of SK channel subunits including SK1, SK2, and SK3 have been documented to be expressed in the heart and play important functional roles in atrial myocytes and pacemaking tissues compared with the ventricle.

What Is Known?

- A small conductance Ca\(^{2+}\)-activated K\(^{-}\) channel is a potent dominant-negative regulator of SK currents: implications in schizophrenia. Mol Psychiatry. 2003;8:524–535, 460.

 Novelty and Significance

Small conductance SK channels have recently been documented in human and mouse cardiac myocytes that contribute importantly toward cardiac action potential profiles. Three isoforms of SK channel subunits (SK1, SK2, and SK3) have been demonstrated in the heart. The channels are more prominently expressed in atrial and pacemaking tissues compared with the ventricles. Significance of the channels is underscored by the findings that SK2 channels may play a role in atrial fibrillation. The present study demonstrates the heteromultimerization of different SK channel subunits in human and mouse atrial myocytes. Moreover, the study provides evidence for the direct interaction between the coiled-coil domains in the C termini of the different SK subunits. Disruption of the coiled-coil domain interaction results in a significant decrease in the Ca\(^{2+}\)-activated K\(^{-}\) current in atrial myocytes, which is important for cardiac repolarization. Formation of heteromeric channels provides an increase in functional diversity for K\(^{-}\) channels. Moreover, different isoforms of SK channels may represent therapeutic targets to directly modify atrial cells without interfering with ventricular myocytes. Thus, new knowledge into the structure and function of SK channels is important not only from a fundamental viewpoint but may have significant therapeutic implications in cardiac arrhythmias.
Cardiac Small Conductance Ca\(^{2+}\)-Activated K\(^+\) Channel Subunits Form Heteromultimers via the Coiled-Coil Domains in the C Termini of the Channels

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SUPPLEMENTAL MATERIAL

Cardiac small conductance Ca\(^{2+}\)-activated K\(^+\) (SK) channel subunits form heteromultimers via the coiled-coil domains in the C-termini of the channels

Dipika Tuteja\(^1\), Sassan Rafizadeh\(^1\), Valeriy Timofeyev\(^1\), Shuyun Wang\(^1\), Zheng Zhang\(^1\), Ning Li\(^1\), Robertino K Mateo\(^1\), Anil Singapuri\(^1\), Anne A. Knowlton\(^1,2\)
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The human study protocol was approved by the University of California, Davis, Institutional Review Board. All animal care and procedures were approved by the University of California, Davis, Institutional Animal Care and Use Committee.

Isolation of adult mouse atrial myocytes

Adult atrial myocytes were isolated from wild-type (8–10 weeks) C57Bl/6J mice as described previously.\textsuperscript{1,2} In brief, mice were injected with 0.1 ml heparin (1000 units/ml) and after 5 minutes anesthetized with intraperitoneal sodium pentobarbital (80 mg/kg). Hearts were quickly removed and placed in ice-cold Tyrode’s solution (mmol/L) (NaCl 140, KCl 5.4, MgCl\textsubscript{2} 1, HEPES 10 and glucose 10; pH 7.4 with NaOH), cannulated under a dissecting microscope and mounted on a Langendorff apparatus. Perfusion of hearts was done with Tyrode’s solution gassed with 100% O\textsubscript{2} at 37°C under constant perfusion pressure (60 mmHg) and a flow rate of \textasciitilde 2 ml/min. After 5 minutes, solution was changed to 30 ml of Tyrode’s solution containing 13 mg collagenase (type 2, 322 units/mg, Worthington Biochemical Corporation, Lakewood, NJ) and 1 mg protease (type XIV, 4.5 units/mg, Sigma-Aldrich, St. Louis, MO). Hearts were removed from the Langendorff apparatus after 30-45 minutes of enzyme perfusion. Atrial or ventricular tissues were collected in high-K\textsuperscript{+} solution (mmol/L) (K glutamate 120, KCl 20, MgCl\textsubscript{2} 1, EGTA 0.3, glucose 10 and HEPES 10, pH 7.4 with KOH), and gently titurated with pipettes for 3 minutes. Cells were allowed to rest for 15 minutes, and 2 hours before use in immunofluorescence and electrophysiology experiments, respectively. This isolation procedure yielded 60-80\% of Ca\textsuperscript{2+} tolerant atrial and ventricular myocytes with clear striation. For
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Mouse hearts were fixed in 4% formaldehyde (Electron Microscopy Sciences (EMS), Hatfield, PA) in 0.1 M in Sorenson phosphate buffer (pH 7.2, EMS) for more than one hour. The tissue was rinsed in buffer and incubated for 30 minutes at room temperature in 0.1 M Sorenson phosphate buffer containing 0.1% tannic acid. Other procedures were described previously.³ Immunolabeling was performed by placing the grids on drops of primary antibody solution for overnight at 4°C and rinsing six times for 5 min in PBS containing 1% BSA. Grids were then incubated on drops of gold-conjugated secondary antibody solution for 1 hour at room temperature. Grids were then rinsed three times for 5 minutes in PBS. Finally, the grids were washed for 5 minutes in deionized water before being allowed to air dry. Negative controls included grids labeled with secondary antibody alone. Grids were visualized on a transmission electron microscope (PHILIPS CM120H).

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Briefly, 50 μl of protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) per 500 μg of soluble proteins were used for pre-clearing the samples for 30 minutes at 4 °C. Beads were spun down at 3,000 x g for 1 minute at 4 °C and supernatant was incubated overnight with rabbit anti-SK1, anti-SK2 and anti-SK3 antibodies (Sigma-Aldrich) at 4 °C on a rocker. 50 μl of protein A/G plus-agarose beads were added to the mixture and incubated for 1-2 hours with shaking at 4 °C. Beads were washed three times by centrifugation at 3000 rpm for 1 minute, re-suspended in SDS-PAGE sample buffer and boiled for 5 minutes. Immunoblots were performed as described previously.4 Electrophoresis samples were transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) and detected with rabbit anti-SK1, anti-SK2 or anti-SK3 antibodies (1:200 dilution, Sigma-Aldrich). Anti-rabbit HRP-conjugated secondary antibodies (1:2000, GE Healthcare Ltd) were used and signal was detected by enhanced chemiluminescence using Super Signal West Pico Kit (Thermo Fisher Scientific, Inc.).

**Sequence analysis and design of inhibitory peptides**

Coils Version 2.2 program (http://www.ch.embnet.org) was used for the prediction of CCDs from primary amino acid sequence of mouse and human cardiac SK channels.5 We also confirmed the presence of the CCDs by other programs including Marcoil,6 Paircoil2,7 MultiCoils.8 C-terminal parts of the SK channel sequences harboring high coiled-coil probability were also compared with CCDs of other K⁺ channels known to form heteromultimers, i.e., human Kv10 (ERG) and Kv7 (KCNQ) channels. The α-helix structure of CCD2 of SK2 channel was confirmed by homology modeling of structural coordinates at servers: Swiss-model (http://swissmodel.expasy.org/workspace/index.php?func=modelling_simple1) and I-TASSER (http://zhang.bioinformatics.ku.edu/I-TASSER).9 CCD2 models of SK2 were analyzed using PyMol software. Sequence from high coiled-coil probability score in the C-
terminal region of SK2 was used to design inhibitory peptide. Inhibitory peptide (DLNERSEDFEKRIVTLETK) and its corresponding scramble peptide (DEKDIELESFNRTKVRETL) were synthesized (Pi Proteomics, LLC) and used in patch-clamp studies (30-50 μM) in freshly isolated mouse atrial cardiomyocytes, and pIRES2-EGFP-SK2 & α-actinin2 transfected tsA201 cells. The experiments were designed to directly test the functional roles of the identified CCD on Ca\textsuperscript{2+}-activated K\textsuperscript{+} current ($I_{KCa}$). Additional inhibitory peptides were generated from the CCD regions of SK1 and SK3 channel subunits as follows: ELQAQQEELAALR and ELNREDLQEIKQSGSKE for SK1 and SK3 channels, respectively.

**In vitro interaction assays**

Mammalian Two-Hybrid System Assay 2 (Clontech, Palo Alto, CA) was used for testing the *in vitro* interactions between SK1, 2 & 3 channels. C-termini fusion constructs of SK1-3 were generated in pM & pVP16 vectors encompassing amino acids 364-537 for mouse cardiac SK1 channel (accession # AY258143.1),\textsuperscript{4} amino acids 395-580 for human cardiac SK2 channel (accession # AY258141.1)\textsuperscript{1} and amino acid 544-737 for human cardiac SK3 channel (accession # AY258142.1, see Fig. S1). tsA201 cells were transfected using Lipofectamine 2000 (Invitrogen) with different combinations of pM-SK1/2/3 & pVP16-1/2/3, respectively, along with a reporter vector (pG5SEAP) and a control vector (pMetLuc2). Cells were grown at 37°C with 5% CO\textsubscript{2}, 10% serum and were seeded at 2 X 10\textsuperscript{5} cells in 500μl medium per well in 24 well plates. The reporter vector encoded secreted alkaline phosphatase (SEAP), an enzyme that enables sampling of cell culture without cell lysis while the control vector encoded secreted luciferase and enabled the monitoring of sample-to-sample transfection efficiency variations. Luciferase activity was measured from samples using Ready-To-Glow Secreted Luciferase
Reporter System (Clontech, Mountain View, CA). SEAP activity was measured 48-72 hours after transfection using BD Great EscAPE SEAP Chemiluminescence Detection Kit (Clontech).

To directly test the roles of CCDs in the heteromultimerization of SK channels, deletion of CCD2 domain from the SK2 C termini (SK2-ΔCCD) in pM and pVP16 vectors were generated. pM-SK2-ΔCCD and pVP16-SK2-ΔCCD together with pVP16-SK1/3 or pM-SK1/3, respectively, were used for in vitro interaction assays. Negative control experiments include pM+pVP16 empty vectors, pM-SK1/2/3+pVP16 empty vector or pM empty vector+pVP16-SK1/2/3. pM3pVP16+pM3pVP16 were used as the positive control. Experiments were performed in triplicates for each combination. Statistical analysis was performed using ANOVA on test samples with respect to the positive controls.

**DNA constructs, cell culture and transfection**

Full-length mouse SK1, human SK2 and human SK3 cDNA were subcloned in pIRES2-EGFP expression vector (5.3 kb, Clonetech) using previously cloned SK channels from mammalian hearts. Site-directed mutagenesis was performed using overlapping mutagenic primers containing pore mutations of glycine, tyrosine and glycine (GYG) to alanine (AAA) and QuickChange Lightening Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). SK1, 2, 3-AAA pore mutant clones were sequenced to confirm the presence of the desired mutations.

tsA201 cells were cultured in DMEM high glucose media (Invitrogen) with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and maintained at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were plated at a density of 1-2 x 10⁵ on coverslips placed in 35 mm dishes. Both wild-type (WT) and pore mutant (AAA) constructs of SK channels along with full-length α-actinin2 clone were transfected in different combinations using Lipofectamine 2000 (Invitrogen) for functional studies using whole-cell patch-clamp.
techniques. The rationale for using α-actinin2 co-expression is that our previous study has demonstrated that α-actinin2 co-expression leads to an increase in the SK current.\textsuperscript{10} Plasmid combinations (α-actinin2, pIRE2-EGFP-SK2, and pIRE2-EGFP-SK1, 2 or 3-AAA constructs) were used in a 1:1 ratio (by weight). Plasmid with EGFP alone was used as control. Transfected tsA201 cells were used for $I_{K,\text{Ca}}$ recordings 48 hours post transfection.

**Ca\textsuperscript{2+}-activated K\textsuperscript{+} current ($I_{K,\text{Ca}}$) recordings**

Whole-cell $I_{K,\text{Ca}}$ was recorded from freshly isolated atrial myocytes and transfected tsA201 cells using voltage-ramp protocol and patch-clamp techniques as previously described.\textsuperscript{1,11} Whole-cell current records were filtered at 2 kHz and sampled at 10 kHz. Cells were superfused in a standard bath solution containing (in mM): N-methylglucamine (NMG) 140, KCl 4, MgCl$_2$ 1, glucose 5, and HEPES 10 (pH 7.4 using methane sulfonic acid). Patch pipettes had resistances of 3-6 MΩ when filled with the following solution (in mM): K-glutamate 144, MgCl$_2$ 1.15, EGTA 5, HEPES 10 and CaCl$_2$ yielding a free (unchelated) [Ca$^{2+}$]$_i$ of 500 nM or 100 nM where stated, using Calcium Titration Software\textsuperscript{12} to calculate free, bound and dissociated [Ca$^{2+}$]$_i$. SK channel-specific inhibitory or scramble peptides were added in the pipette solution at concentrations of 50 μM. The pH was adjusted to 7.25 using KOH. Tips of the patch pipettes were filled with solution without the peptides while the solution with peptides was backfilled into the patch pipettes. Cells were allowed to stabilize for one minute after whole-cell configuration was obtained before the start of the experiments. $I_{K,\text{Ca}}$ was recorded over a period of 12-20 minutes while the peptides slowly diffused into the cells. Protocols for patch-clamp experiments and data analysis were established using custom-written software.

All experiments were performed using 3 M KCl agar bridges to minimize changes in liquid junction potentials. Liquid junction potentials were measured as described previously.\textsuperscript{13}
Pooled data are presented as means ± s.e.m. Statistical comparison was performed using the Student's paired t-test with p < 0.05 considered significant.

References


**Figure legends**

**Online Figure I.** Cloning and sequence of human atrial SK3 (haSK3) used in the study. (A) Representative agarose gel of RT-PCR amplified products from total RNA isolated from two different human atrial samples (Lanes 2 and 3) using primers designed from published human myometrium SK3 (hmSK3, Accession no. AY049734). Lanes 4 & 5 represent RT-PCR amplification of cDNA from the two different samples of human atria using human GAPDH primers. Lane 6 is a negative control (PCR amplified without RT). Lanes 1 and 7 are Hi-Lo
(B) Amino acid sequence alignment (ClustalW) of deduced SK3 protein sequence from human atrium compared to human myometrium SK3. Six predicted trans-membrane domains (S1-S6) and pore (P) are highlighted in yellow. Highlighted red stretch in P region represents $K^+$ selective filter GYG. If, Isoform; ha, human atrium; hmm, human myometrium; CaMBD, calmodulin binding domain in the C terminus. Numbers on right represent length of predicted protein in terms of amino acid residues, while on the left roman numerals denote isoform nomenclature. Dashes represent Gaps in the sequence alignment.

**Online Figure II.** Confocal photomicrographs of single isolated mouse atrial myocytes doubly stained with (A) anti-SK1, 2 or 3 antibodies pre-incubated with the corresponding antigenic peptides (AP) in different combinations as follows: (Upper Panels) goat anti-SK1 and rabbit anti-SK2, (Middle Panels) rabbit anti-SK2 and mouse anti-SK3, and (Lower Panels) rabbit anti-SK1 and mouse anti-SK3 antibodies. Immunofluorescence labeling was performed by treatment with fluorophore labeled secondary antibodies as in Figure 1. Scale bars are 10 μm. Merged images are shown on the right of each panel. (B) Additional control experiments using secondary antibodies only in different combinations are as follows: (Upper Panels) donkey anti-goat Alexa Fluor 555 and chicken anti-rabbit Alexa Fluor 488, (Middle Panels) donkey anti-mouse Alexa Fluor 555 and chicken anti-rabbit Alexa Fluor 488, and (Lower Panels) chicken anti-rabbit Alexa Fluor 555 and donkey anti-mouse Alexa 488. Scale bars are 10 μm. Merged images are shown in the third column of each panel. Fourth column of each panel shows phase contrast images of the cardiomyocytes. (C) Representative confocal photomicrographs of single isolated mouse atrial myocytes stained with anti-SK2 antibody as follows: (Upper Panel) from wild-type (WT) mice, (Middle Panel) from wild-type mice but the antibody was pre-treated with antigenic peptide and (Lower Panel) from homozygous SK2 knockout (SK2 KO) mice. Scale bars are 10 μm. (D)
tsA201 cells were co-transfected with pIRES-EGFP-SK1, 2 or 3 expression vectors plus pcDNA3-α-actinin2 plasmid labeled as SK1, 2 or 3, respectively. Lack of cross-reactivity between different anti-SK antibodies were demonstrated using immunofluorescence confocal microscopy and anti-SK specific primary antibodies and the corresponding Alexa fluor-labeled secondary antibodies as in Fig 1. Middle panels are DAPI stain for nuclei followed by merged images. The right most panels show the staining at higher magnification. Scale bars are 10 μm.

**Online Figure III.** Cardiac tissue homogenates from homozygous SK2 knockout mice were immunoprecipitated (IP) with anti-SK1, SK2 or SK3 antibodies. Proteins were eluted and Western blot analysis (IB) was performed using anti-SK1 antibody in (A) and anti-SK3 antibody in (B). Anti-SK2 antibody failed to immunoprecipitate SK1 channel protein in (A) or SK3 channel protein in (B). Negative control was performed by immunoblotting of eluted proteins immunoprecipitated with non-specific IgG (Lane 4 in A and B). (C) Representation of coiled-coil interaction/assembly domain model from two subunits of SK channels. “a” and “d” coiled-coil positions are indicated and color coded as blue and red, respectively. (D) tsA201 cells were transfected with pM-SK1 (SK1-BD), pM-SK2 (SK2-BD), pVP16-SK2 (SK2-AD), pM-SK3 (SK3-BD), pVP16-SK2 (SK2-AD). Cell lysates were used for Western blot (IB) with anti-GAL4 DNA-BD, anti-SK2, and anti-SK3 antibodies.

**Online Figure IV. Dominant-negative (DN) suppression of SK2 current by SK1-DN, SK2-DN or SK3-DN constructs in tsA201 cells.** Whole-cell apamin-sensitive \(I_{K, Ca}\) was elicited using a voltage-ramp protocol from +40 to -100 mV with a slope of -180 V/s from a holding potential of -55 mV in tsA201 cells. \(I_{K, Ca}\) density was obtained by normalizing the current to the cell capacitance. (A) non-transfected cells, (B) cells transfected with pIRES2-EGFP-SK2 plasmid encoding full-length human SK2 channel plus pcDNA3-α-actinin2 plasmid, (C,D,E) cells
transfected with pIRES2-EGFP-SK2 and pcDNA3-α-actinin2 plasmids plus SK1-DN, SK2-DN or SK3-DN constructs, respectively. (F) Summary data of $I_{K, Ca}$ density at the test potentials of -120 and +60 mV illustrating DN effects on $I_{K, Ca}$ from SK1-DN, SK2-DN or SK3-DN constructs (*p<0.05, n = 6-12 cells for each group).

**Online Figure V.** Dominant-negative (DN) suppression of SK3 current by SK1-DN, SK2-DN or SK3-DN constructs in tsA201 cells. Whole-cell $I_{K, Ca}$ was elicited using a voltage-ramp protocol from +40 to -100 mV with a slope of -180 V/s from a holding potential of -55 mV in tsA201 cells. $I_{K, Ca}$ density was obtained by normalizing the current to the cell capacitance. (A) cells transfected with pIRES2-EGFP-SK3 plasmid encoding full-length human SK3 channel plus pcDNA3-α-actinin2 plasmid, (B,C,D) cells transfected with pIRES2-EGFP-SK3 and pcDNA3-α-actinin2 plasmids plus SK3-DN, SK1-DN or SK2-DN constructs, respectively.

**Online Figure VI.** (A, B) Peptide derived from the SK2 CCD inhibits whole-cell $I_{K, Ca}$ in tsA201 cells co-expressing SK2 and SK3 subunits but not SK3 subunit alone. (A) Whole-cell $I_{K, Ca}$ was recorded from tsA201 cells co-transfected with pIRES2-EGFP-SK3 and pcDNA3-α-actinin2 plasmids. A voltage-ramp protocol was applied from -120 to +60 mV at a holding potential of -55 mV. $I_{K, Ca}$ current was recorded immediately after establishment of whole-cell mode (black line), 12 minutes thereafter (blue line). (B) Similar experiments were performed in from tsA201 cells co-transfected with pIRES2-EGFP-SK2, pIRES2-EGFP-SK3 and pcDNA3-α-actinin2 plasmids. Inclusion of 50 μM inhibitory peptide in the pipette solution resulted in nearly complete inhibition of $I_{K, Ca}$ after 12 minutes of recordings compared to the initial traces in cells transfected with both SK2 and SK3 subunits. In contrast, parallel experiments using the same concentrations of inhibitory peptide in cells transfected with SK3 alone showed stable current recordings at 12 minutes after the establishment of whole-cell configuration with no “run down”
or “run up” of $I_{K,ca}$. Similar data were obtained in a total of 6 cells. Experiments were performed in a blinded fashion in which the experimentators had no knowledge of the compositions of the channels expressed.
Online Figure II
Online Figure III

A
IB: SK1
IP: SK1 SK2 SK3 IgG

B
IB: SK3
IP: SK1 SK2 SK3 IgG

C

D
IB: BD SK2 SK3
Cell Lysate: SK1-BD SK2-BD SK2-AD SK3-BD SK3-AD

Online Figure III
Online Figure IV
Online Figure V

Graphs showing the relationship between voltage (V) and current density (I/PF) for different conditions:

A. SK3
B. SK3 + SK3-DN
C. SK3 + SK1-DN
D. SK3 + SK2-DN
Online Figure VI

A

SK3

Initial

12 Mins

B

SK2+SK3

Initial

12 Mins
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**Co-immunoprecipitation and Western blot analysis**

Human heart tissues were procured from a commercial source (T Cubed, Inc.). Protein samples of human and mouse atrial and ventricular tissues were prepared separately. Protease inhibitors (8 μg/ml calpain I & II, leupeptin, 0.1 mg/ml benzamidine, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 10 mg/ml PMSF) were included in the lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM MgCl₂, 1% NP-40, pH 7.4. Heart tissues were homogenized in ice-cold lysis buffer and solubilized. Non-solubilized material was removed by centrifugation at 1,000 x g for 5 minutes at 4 °C. Supernatant was collected for co-immunoprecipitation analysis.
Briefly, 50 μl of protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) per 500 μg of soluble proteins were used for pre-clearing the samples for 30 minutes at 4 °C. Beads were spun down at 3,000 x g for 1 minute at 4 °C and supernatant was incubated overnight with rabbit anti-SK1, anti-SK2 and anti-SK3 antibodies (Sigma-Aldrich) at 4 °C on a rocker. 50 μl of protein A/G plus-agarose beads were added to the mixture and incubated for 1-2 hours with shaking at 4 °C. Beads were washed three times by centrifugation at 3000 rpm for 1 minute, re-suspended in SDS-PAGE sample buffer and boiled for 5 minutes. Immunoblots were performed as described previously. Electrophoresis samples were transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) and detected with rabbit anti-SK1, anti-SK2 or anti-SK3 antibodies (1:200 dilution, Sigma-Aldrich). Anti-rabbit HRP-conjugated secondary antibodies (1:2000, GE healthcare Ltd) were used and signal was detected by enhanced chemiluminescence using Super Signal West Pico Kit (Thermo Fisher Scientific, Inc.).

Sequence analysis and design of inhibitory peptides

Coils Version 2.2 program (http://www.ch.embnet.org) was used for the prediction of CCDs from primary amino acid sequence of mouse and human cardiac SK channels. We also confirmed the presence of the CCDs by other programs including Marcoil, Paircoil2, MultiCoils. C-terminal parts of the SK channel sequences harboring high coiled-coil probability were also compared with CCDs of other K^+ channels known to form heteromultimers, i.e., human Kᵥ10 (ERG) and Kᵥ7 (KCNQ) channels. The α-helix structure of CCD2 of SK2 channel was confirmed by homology modeling of structural coordinates at servers: Swiss-model (http://swissmodel.expasy.org/workspace/index.php?func=modelling_simple1) and I-TASSER (http://zhang.bioinformatics.ku.edu/I-TASSER). CCD2 models of SK2 were analyzed using PyMol software. Sequence from high coiled-coil probability score in the C-
terminal region of SK2 was used to design inhibitory peptide. Inhibitory peptide (DLNERSEDFEKRIVTLETK) and its corresponding scramble peptide (DEKDIELESFNRTKVRETL) were synthesized (Pi Proteomics, LLC) and used in patch-clamp studies (30-50 μM) in freshly isolated mouse atrial cardiomyocytes, and pIRES2-EGFP-SK2 & α-actinin2 transfected tsA201 cells. The experiments were designed to directly test the functional roles of the identified CCD on Ca\(^{2+}\)-activated K\(^+\) current (\(I_{K,Ca}\)). Additional inhibitory peptides were generated from the CCD regions of SK1 and SK3 channel subunits as follows: ELQAQQEELEAALRSR and ELNDRSEDLEKQIGSLESK for SK1 and SK3 channels, respectively.

**In vitro interaction assays**

Mammalian Two-Hybrid System Assay 2 (Clontech, Palo Alto, CA) was used for testing the *in vitro* interactions between SK1, 2 & 3 channels. C-termini fusion constructs of SK1-3 were generated in pM & pVP16 vectors encompassing amino acids 364-537 for mouse cardiac SK1 channel (accession # AY258143.1), amino acids 395-580 for human cardiac SK2 channel (accession # AY258141.1) and amino acid 544-737 for human cardiac SK3 channel (accession # AY258142.1, see Fig. S1). tsA201 cells were transfected using Lipofectamine 2000 (Invitrogen) with different combinations of pM-SK1/2/3 & pVP16-1/2/3, respectively, along with a reporter vector (pG5SEAP) and a control vector (pMetLuc2). Cells were grown at 37°C with 5% CO\(_2\), 10% serum and were seeded at 2 X 10\(^5\) cells in 500μl medium per well in 24 well plates. The reporter vector encoded secreted alkaline phosphatase (SEAP), an enzyme that enables sampling of cell culture without cell lysis while the control vector encoded secreted luciferase and enabled the monitoring of sample-to-sample transfection efficiency variations. Luciferase activity was measured from samples using Ready-To-Glow Secreted Luciferase
Reporter System (Clontech, Mountain View, CA). SEAP activity was measured 48-72 hours after transfection using BD Great EscAPe SEAP Chemiluminescence Detection Kit (Clontech). To directly test the roles of CCDs in the heteromultimerization of SK channels, deletion of CCD2 domain from the SK2 C termini (SK2-ΔCCD) in pM and pVP16 vectors were generated. pM-SK2-ΔCCD and pVP16-SK2-ΔCCD together with pVP16-SK1/3 or pM-SK1/3, respectively, were used for in vitro interaction assays. Negative control experiments include pM+pVP16 empty vectors, pM-SK1/2/3+pVP16 empty vector or pM empty vector+pVP16-SK1/2/3. pM3pVP16+pM3pVP16 were used as the positive control. Experiments were performed in triplicates for each combination. Statistical analysis was performed using ANOVA on test samples with respect to the positive controls.

DNA constructs, cell culture and transfection

Full-length mouse SK1, human SK2 and human SK3 cDNA were subcloned in pIRES2-EGFP expression vector (5.3 kb, Clonetech) using previously cloned SK channels from mammalian hearts. Site-directed mutagenesis was performed using overlapping mutagenic primers containing pore mutations of glycine, tyrosine and glycine (GYG) to alanine (AAA) and QuickChange Lightening Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). SK1, 2, 3-AAA pore mutant clones were sequenced to confirm the presence of the desired mutations.

tsA201 cells were cultured in DMEM high glucose media (Invitrogen) with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and maintained at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were plated at a density of 1-2 x 10⁵ on coverslips placed in 35 mm dishes. Both wild-type (WT) and pore mutant (AAA) constructs of SK channels along with full-length α-actinin2 clone were transfected in different combinations using Lipofectamine 2000 (Invitrogen) for functional studies using whole-cell patch-clamp
techniques. The rationale for using α-actinin2 co-expression is that our previous study has demonstrated that α-actinin2 co-expression leads to an increase in the SK current. Plasmid combinations (α-actinin2, pIRES2-EGFP-SK2, and pIRES2-EGFP-SK1, 2 or 3-AAA constructs) were used in a 1:1 ratio (by weight). Plasmid with EGFP alone was used as control. Transfected tsA201 cells were used for \( I_{K,Ca} \) recordings 48 hours post transfection.

**Ca\(^{2+}\)-activated K\(^+\) current (\( I_{K,Ca} \)) recordings**

Whole-cell \( I_{K,Ca} \) was recorded from freshly isolated atrial myocytes and transfected tsA201 cells using voltage-ramp protocol and patch-clamp techniques as previously described.\(^1\)\(^\text{,}11\) Whole-cell current records were filtered at 2 kHz and sampled at 10 kHz. Cells were superfused in a standard bath solution containing (in mM): N-methylglucamine (NMG) 140, KCl 4, MgCl\(_2\) 1, glucose 5, and HEPES 10 (pH 7.4 using methane sulfonic acid). Patch pipettes had resistances of 3-6 MΩ when filled with the following solution (in mM): K-glutamate 144, MgCl\(_2\) 1.15, EGTA 5, HEPES 10 and CaCl\(_2\) yielding a free (unchelated) \([Ca^{2+}]_{i}\) of 500 nM or 100 nM where stated, using Calcium Titration Software\(^12\) to calculate free, bound and dissociated \([Ca^{2+}]_{i}\). SK channel-specific inhibitory or scramble peptides were added in the pipette solution at concentrations of 50 μM. The pH was adjusted to 7.25 using KOH. Tips of the patch pipettes were filled with solution without the peptides while the solution with peptides was backfilled into the patch pipettes. Cells were allowed to stabilize for one minute after whole-cell configuration was obtained before the start of the experiments. \( I_{K,Ca} \) was recorded over a period of 12-20 minutes while the peptides slowly diffused into the cells. Protocols for patch-clamp experiments and data analysis were established using custom-written software.

All experiments were performed using 3 M KCl agar bridges to minimize changes in liquid junction potentials. Liquid junction potentials were measured as described previously.\(^13\)
Pooled data are presented as means ± s.e.m. Statistical comparison was performed using the Student's paired t-test with p < 0.05 considered significant.

References


**Figure legends**

**Online Figure I.** Cloning and sequence of human atrial SK3 (haSK3) used in the study. (A) Representative agarose gel of RT-PCR amplified products from total RNA isolated from two different human atrial samples (Lanes 2 and 3) using primers designed from published human myometrium SK3 (hmSK3, Accession no. AY049734). Lanes 4 & 5 represent RT-PCR amplification of cDNA from the two different samples of human atria using human GAPDH primers. Lane 6 is a negative control (PCR amplified without RT). Lanes 1 and 7 are Hi-Lo
ladders (Bionexus). (B) Amino acid sequence alignment (ClustalW) of deduced SK3 protein sequence from human atrium compared to human myometrium SK3. Six predicted trans-membrane domains (S1-S6) and pore (P) are highlighted in yellow. Highlighted red stretch in P region represents K⁺ selective filter GYG. If, Isoform; ha, human atrium; hmm, human myometrium; CaMBD, calmodulin binding domain in the C terminus. Numbers on right represent length of predicted protein in terms of amino acid residues, while on the left roman numerals denote isoform nomenclature. Dashes represent Gaps in the sequence alignment.

**Online Figure II.** Confocal photomicrographs of single isolated mouse atrial myocytes doubly stained with (A) anti-SK1, 2 or 3 antibodies pre-incubated with the corresponding antigenic peptides (AP) in different combinations as follows: (Upper Panels) goat anti-SK1 and rabbit anti-SK2, (Middle Panels) rabbit anti-SK2 and mouse anti-SK3, and (Lower Panels) rabbit anti-SK1 and mouse anti-SK3 antibodies. Immunofluorescence labeling was performed by treatment with fluorophore labeled secondary antibodies as in Figure 1. Scale bars are 10 µm. Merged images are shown on the right of each panel. (B) Additional control experiments using secondary antibodies only in different combinations are as follows: (Upper Panels) donkey anti-goat Alexa Fluor 555 and chicken anti-rabbit Alexa Fluor 488, (Middle Panels) donkey anti-mouse Alexa Fluor 555 and chicken anti-rabbit Alexa Fluor 488, and (Lower Panels) chicken anti-rabbit Alexa Fluor 555 and donkey anti-mouse Alexa 488. Scale bars are 10 µm. Merged images are shown in the third column of each panel. Fourth column of each panel shows phase contrast images of the cardiomyocytes. (C) Representative confocal photomicrographs of single isolated mouse atrial myocytes stained with anti-SK2 antibody as follows: (Upper Panel) from wild-type (WT) mice, (Middle Panel) from wild-type mice but the antibody was pre-treated with antigenic peptide and (Lower Panel) from homozygous SK2 knockout (SK2 KO) mice. Scale bars are 10 µm. (D)
tsA201 cells were co-transfected with pIRES-EGFP-SK1, 2 or 3 expression vectors plus pcDNA3-α-actinin2 plasmid labeled as SK1, 2 or 3, respectively. Lack of cross-reactivity between different anti-SK antibodies were demonstrated using immunofluorescence confocal microscopy and anti-SK specific primary antibodies and the corresponding Alexa fluor-labeled secondary antibodies as in Fig 1. Middle panels are DAPI stain for nuclei followed by merged images. The right most panels show the staining at higher magnification. Scale bars are 10 μm.

**Online Figure III.** Cardiac tissue homogenates from homozygous SK2 knockout mice were immunoprecipitated (IP) with anti-SK1, SK2 or SK3 antibodies. Proteins were eluted and Western blot analysis (IB) was performed using anti-SK1 antibody in (A) and anti-SK3 antibody in (B). Anti-SK2 antibody failed to immunoprecipitate SK1 channel protein in (A) or SK3 channel protein in (B). Negative control was performed by immunoblotting of eluted proteins immunoprecipitated with non-specific IgG (Lane 4 in A and B). (C) Representation of coiled-coil interaction/assembly domain model from two subunits of SK channels. “a” and “d” coiled-coil positions are indicated and color coded as blue and red, respectively. (D) tsA201 cells were transfected with pM-SK1 (SK1-BD), pM-SK2 (SK2-BD), pVP16-SK2 (SK2-AD), pM-SK3 (SK3-BD), pVP16-SK2 (SK2-AD). Cell lysates were used for Western blot (IB) with anti-GAL4 DNA-BD, anti-SK2, and anti-SK3 antibodies.

**Online Figure IV. Dominant-negative (DN) suppression of SK2 current by SK1-DN, SK2-DN or SK3-DN constructs in tsA201 cells.** Whole-cell apamin-sensitive $I_{K, Ca}$ was elicited using a voltage-ramp protocol from +40 to -100 mV with a slope of -180 V/s from a holding potential of -55 mV in tsA201 cells. $I_{K, Ca}$ density was obtained by normalizing the current to the cell capacitance. (A) non-transfected cells, (B) cells transfected with pIRES2-EGFP-SK2 plasmid encoding full-length human SK2 channel plus pcDNA3-α-actinin2 plasmid, (C,D,E) cells
transfected with pIRES2-EGFP-SK2 and pcDNA3-α-actinin2 plasmids plus SK1-DN, SK2-DN or SK3-DN constructs, respectively. (F) Summary data of $I_{K,Ca}$ density at the test potentials of -120 and +60 mV illustrating DN effects on $I_{K,Ca}$ from SK1-DN, SK2-DN or SK3-DN constructs (*p<0.05, n = 6-12 cells for each group).

**Online Figure V.** Dominant-negative (DN) suppression of SK3 current by SK1-DN, SK2-DN or SK3-DN constructs in tsA201 cells. Whole-cell $I_{K,Ca}$ was elicited using a voltage-ramp protocol from +40 to -100 mV with a slope of -180 V/s from a holding potential of -55 mV in tsA201 cells. $I_{K,Ca}$ density was obtained by normalizing the current to the cell capacitance. (A) cells transfected with pIRES2-EGFP-SK3 plasmid encoding full-length human SK3 channel plus pcDNA3-α-actinin2 plasmid, (B,C,D) cells transfected with pIRES2-EGFP-SK3 and pcDNA3-α-actinin2 plasmids plus SK3-DN, SK1-DN or SK2-DN constructs, respectively.

**Online Figure VI.** (A, B) Peptide derived from the SK2 CCD inhibits whole-cell $I_{K,Ca}$ in tsA201 cells co-expressing SK2 and SK3 subunits but not SK3 subunit alone. (A) Whole-cell $I_{K,Ca}$ was recorded from tsA201 cells co-transfected with pIRES2-EGFP-SK3 and pcDNA3-α-actinin2 plasmids. A voltage-ramp protocol was applied from -120 to +60 mV at a holding potential of -55 mV. $I_{K,Ca}$ current was recorded immediately after establishment of whole-cell mode (black line), 12 minutes thereafter (blue line). (B) Similar experiments were performed in from tsA201 cells co-transfected with pIRES2-EGFP-SK2, pIRES2-EGFP-SK3 and pcDNA3-α-actinin2 plasmids. Inclusion of 50 μM inhibitory peptide in the pipette solution resulted in nearly complete inhibition of $I_{K,Ca}$ after 12 minutes of recordings compared to the initial traces in cells transfected with both SK2 and SK3 subunits. In contrast, parallel experiments using the same concentrations of inhibitory peptide in cells transfected with SK3 alone showed stable current recordings at 12 minutes after the establishment of whole-cell configuration with no “run down”
or “run up” of $I_{K,ca}$. Similar data were obtained in a total of 6 cells. Experiments were performed in a blinded fashion in which the experimentators had no knowledge of the compositions of the channels expressed.
Online Figure II
Online Figure III

A
IB: SK1
IP: SK1 SK2 SK3 IgG

B
IB: SK3
IP: SK1 SK2 SK3 IgG

C
Cell Lysate: SK1-BD SK2-BD SK2-AD SK3-BD SK3-AD

D
IB: BD SK2 SK3
Cell Lysate: SK1-BD SK2-BD SK2-AD SK3-BD SK3-AD

Online Figure III
Online Figure IV
Online Figure V

A

SK3

I (pA/pF)

V (mV)

B

SK3 +SK3-DN

I (pA/pF)

V (mV)

C

SK3 +SK1-DN

I (pA/pF)

V (mV)

D

SK3 +SK2-DN

I (pA/pF)

V (mV)
Online Figure VI