

SUPPLEMENTAL MATERIAL

Cardiac small conductance Ca²⁺-activated K⁺ (SK) channel subunits form heteromultimers *via* the coiled-coil domains in the C-termini of the channels

Dipika Tuteja¹, Sassan Rafizadeh¹, Valeriy Timofeyev¹, Shuyun Wang¹, Zheng Zhang¹,
Ning Li¹, Robertino K Mateo¹, Anil Singapuri¹, Anne A. Knowlton^{1,2}
and Nipavan Chiamvimonvat^{1,2}

¹Division of Cardiovascular Medicine
University of California, Davis

²Department of Veterans Affairs, Northern California Health Care System
Mather, CA

Materials and Methods

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.^{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, MgCl₂ 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% O₂ at 37°C under constant perfusion pressure (60 mmHg) and a flow rate of ~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⁺ solution (mmol/L) (K glutamate 120, KCl 20, MgCl₂ 1, EGTA 0.3, glucose 10 and HEPES 10, pH 7.4 with KOH), and gently triturated 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 Ca²⁺ tolerant atrial and ventricular myocytes with clear striation. For

electrophysiological recordings, cells were used within 8 hours of isolation. All chemicals were purchased from Sigma-Aldrich unless stated otherwise.

Immunofluorescence confocal microscopy

Immunofluorescence confocal microscopy was performed as described previously.¹ Isolated single mouse cardiomyocytes were fixed with 4% formaldehyde in phosphate-buffered solution (PBS, 20–30 min), washed with PBS (3x; 5 min), and permeabilized with 0.4% Triton X-100 (15 min), followed by second washing with PBS (3x; 5 min). Blocking was performed by incubation with 1% bovine serum albumin for 2 hours. Cells were immunostained simultaneously with two SK specific primary antibodies (1:100 dilution) overnight at 4°C. Primary antibodies used were: goat and rabbit anti-SK1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA and Sigma-Aldrich), rabbit anti-SK2 (Sigma-Aldrich) and mouse and rabbit anti-SK3 (Santa Cruz Biotechnology, Inc. and Novus Biologicals, LLC, Littleton, CO) antibodies. Secondary antibodies used for double labeling were Alexa Fluor[®] 488-conjugated (chicken anti-rabbit, donkey anti-mouse), and Alexa Fluor[®] 555-conjugated (chicken anti-rabbit, donkey anti-goat and chicken anti-mouse) antibodies (Invitrogen, Carlsbad, CA, 1:300 dilution, 2 hrs at room temperature). Non-specific labeling was minimized by washing cells with PBS (3x, 5 min). Alexa Fluor[®] 488-conjugated secondary antibody was excited at 488 nm with Argon laser and detected with 505-530 nm band pass filter whereas Alexa 555-conjugated secondary antibody was excited at 543 nm with the HeNe 1 laser and detected with 560 nm long pass filter. To test for the possibility of overlap between Alexa 488 and Alexa 555 signal, single-labeled cells were imaged under identical conditions as those used for dual-labeled probes. Control experiments were performed by pre-incubation of primary antibodies with the respective antigenic peptide (1:5) and also by incubating myocytes with secondary antibodies only, under the same

experimental conditions. Identical settings were used for all specimens. Images were acquired using a confocal LSM 510 microscope (Carl Zeiss, Inc.) and analyzed using the LSM 510 software.

Immunogold-labeled transmission electron microscopy

Mouse hearts were fixed in 4% formaldehyde (Electron Microscopy Sciences (EMS), Hatfield, PA) in 0.1 M 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).

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 μ l of protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) per 500 μ g of soluble proteins were used for pre-clearing the samples for 30 minutes at 4 °C. Beads were spun down at 3,000 \times 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 μ l 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 K_v10 (ERG) and K_v7 (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 μ M) 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 ($I_{\text{K,Ca}}$). Additional inhibitory peptides were generated from the CCD regions of SK1 and SK3 channel subunits as follows: ELQAQQELEARLAALESR and ELNDRSEDLKQIGSLESK 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),⁴ amino acids 395-580 for human cardiac SK2 channel (accession # AY258141.1)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 2×10^5 cells in 500 μ l 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- Δ CCD) in pM and pVP16 vectors were generated. pM-SK2- Δ CCD and pVP16-SK2- Δ CCD 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, Clontech) using previously cloned SK channels from mammalian hearts.⁴ Site-directed mutagenesis was performed using overlapping mutagenic primers containing pore mutations of glycine, tyrosine and glycine (GYG) to alanine (AAA) and QuickChange Lightning 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 μ g/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.¹⁰ 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.

Ca²⁺-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.^{1,11} 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, MgCl₂ 1, glucose 5, and HEPES 10 (pH 7.4 using methane sulfonic acid). Patch pipettes had resistances of 3-6 M Ω when filled with the following solution (in mM): K-glutamate 144, MgCl₂ 1.15, EGTA 5, HEPES 10 and CaCl₂ yielding a free (unchelated) [Ca²⁺_i] of 500 nM or 100 nM where stated, using Calcium Titration Software¹² to calculate free, bound and dissociated [Ca²⁺_i]. SK channel-specific inhibitory or scramble peptides were added in the pipette solution at concentrations of 50 μ M. 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 \pm s.e.m. Statistical comparison was performed using the Student's paired *t*-test with $p < 0.05$ considered significant.

References

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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 μ m. 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 μ m. 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 μ m. (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 μ m.

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.

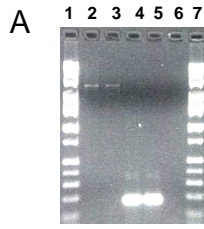
Online Figure IV. Dominant-negative (DN) suppression of SK2 current by SK1-DN, SK2-DN or SK3-DN constructs in tsA201 cells. Whole-cell apamin-sensitive $I_{K,Ca}$ was elicited using a voltage-ramp protocol from +40 to -100 mV with a slope of -180 V/s from a holding potential of -55 mV in tsA201 cells. $I_{K,Ca}$ 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 μ M 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.



B

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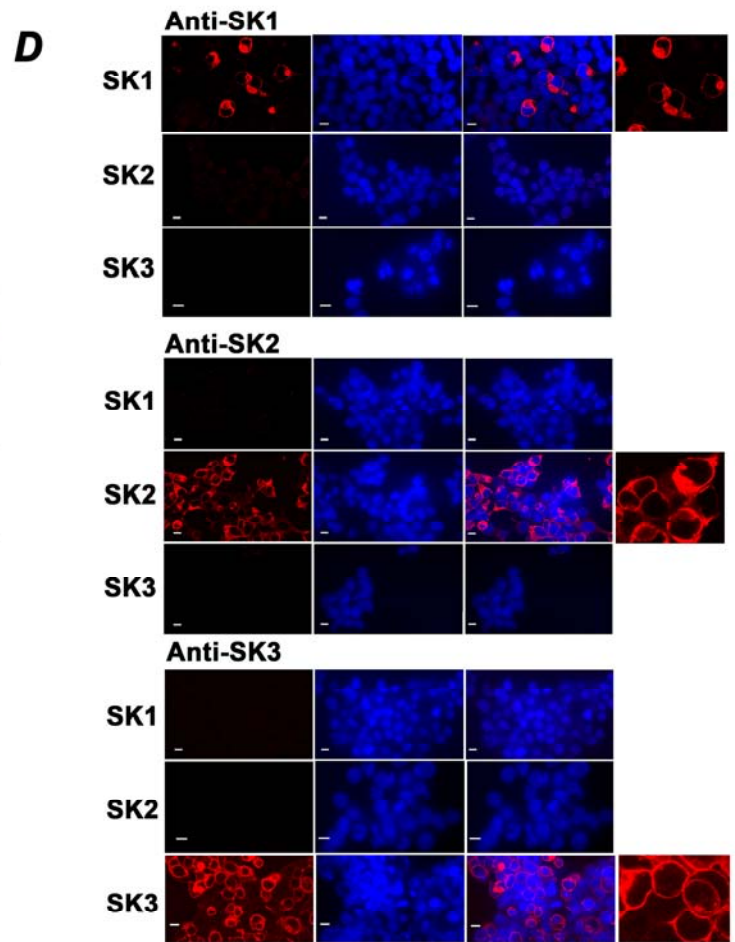
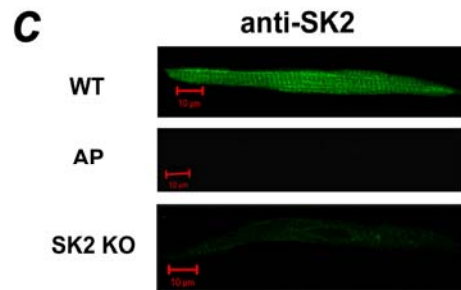
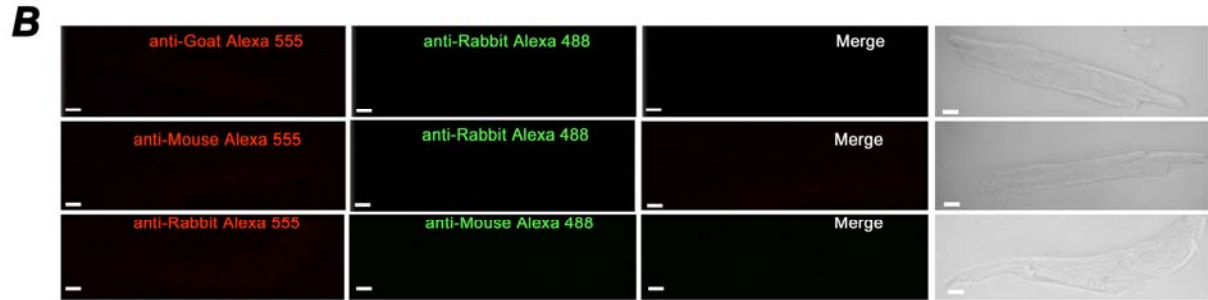
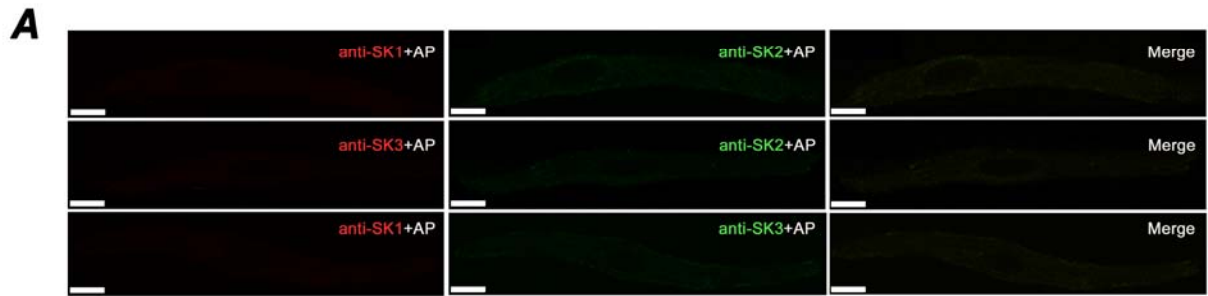
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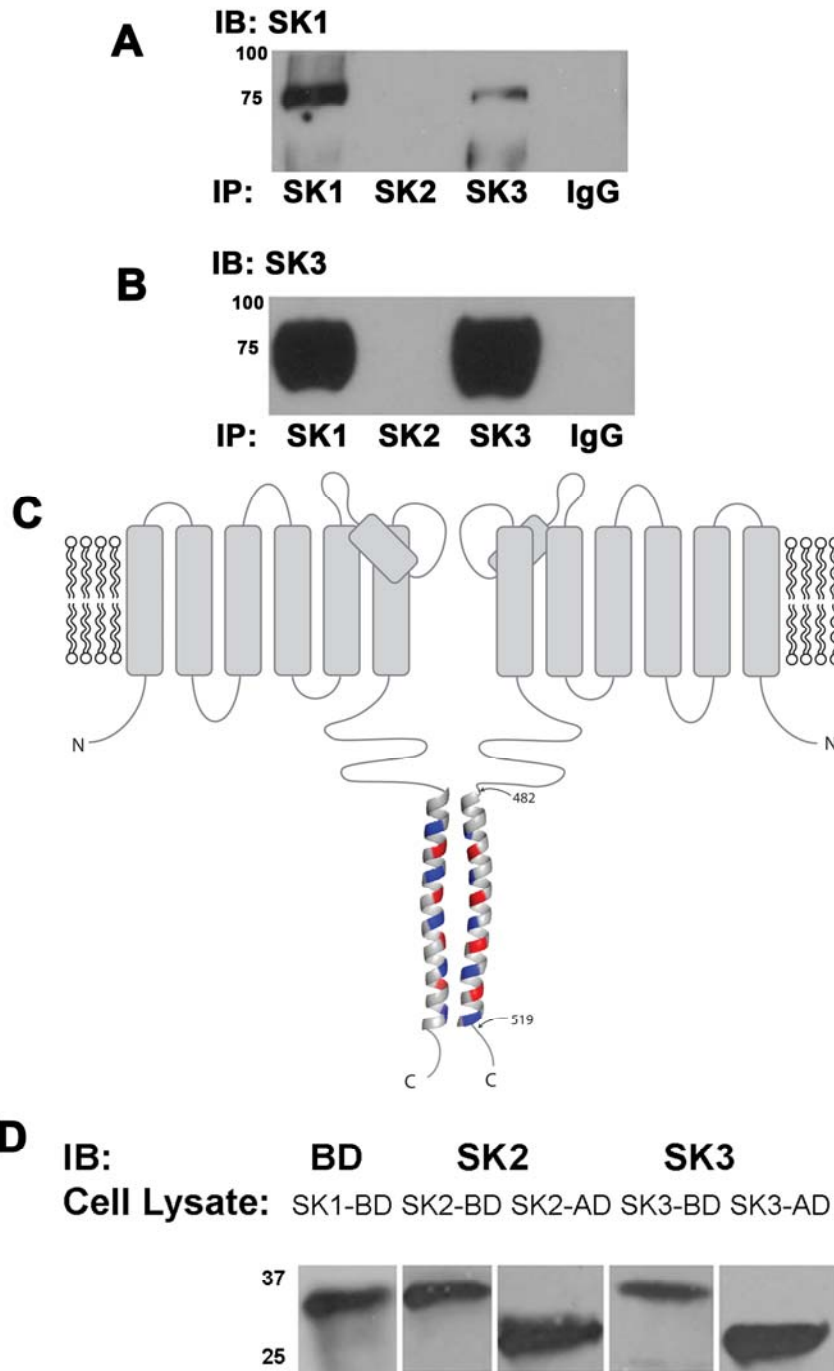
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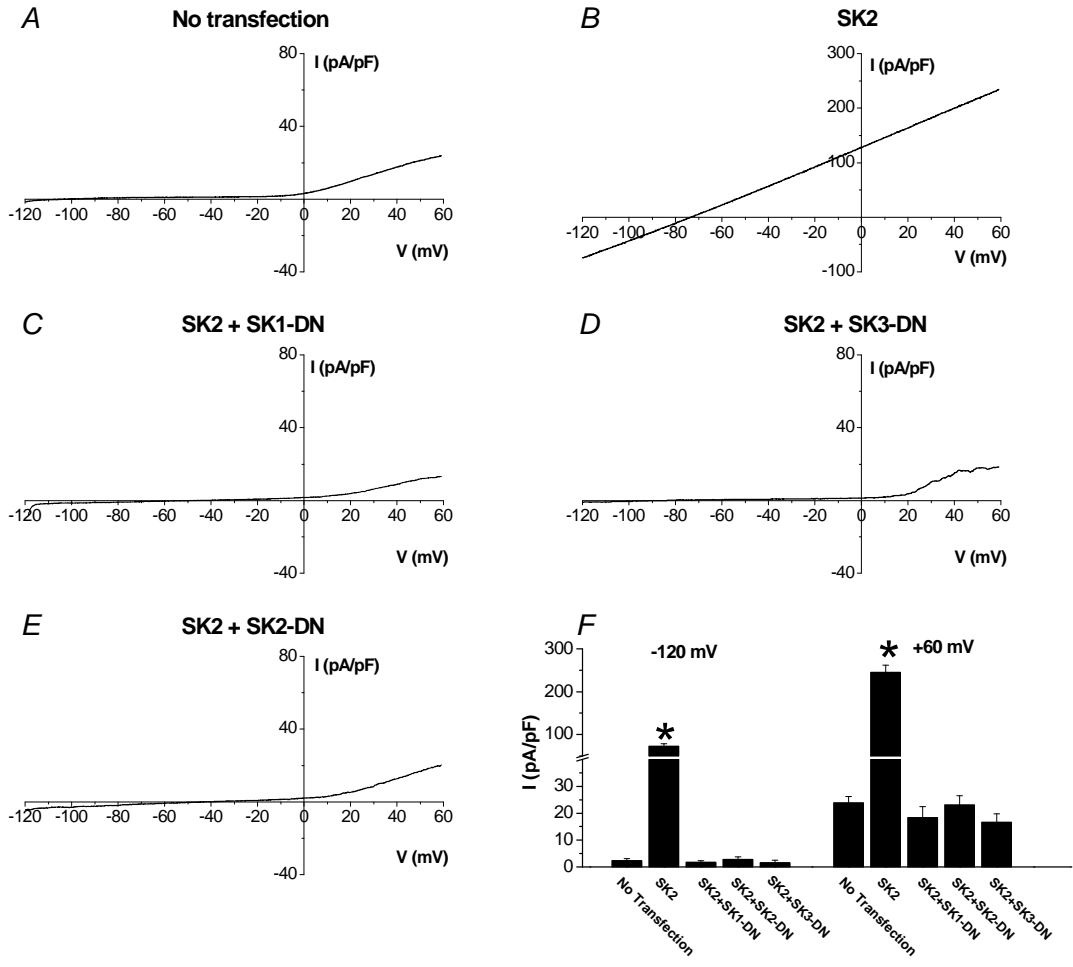
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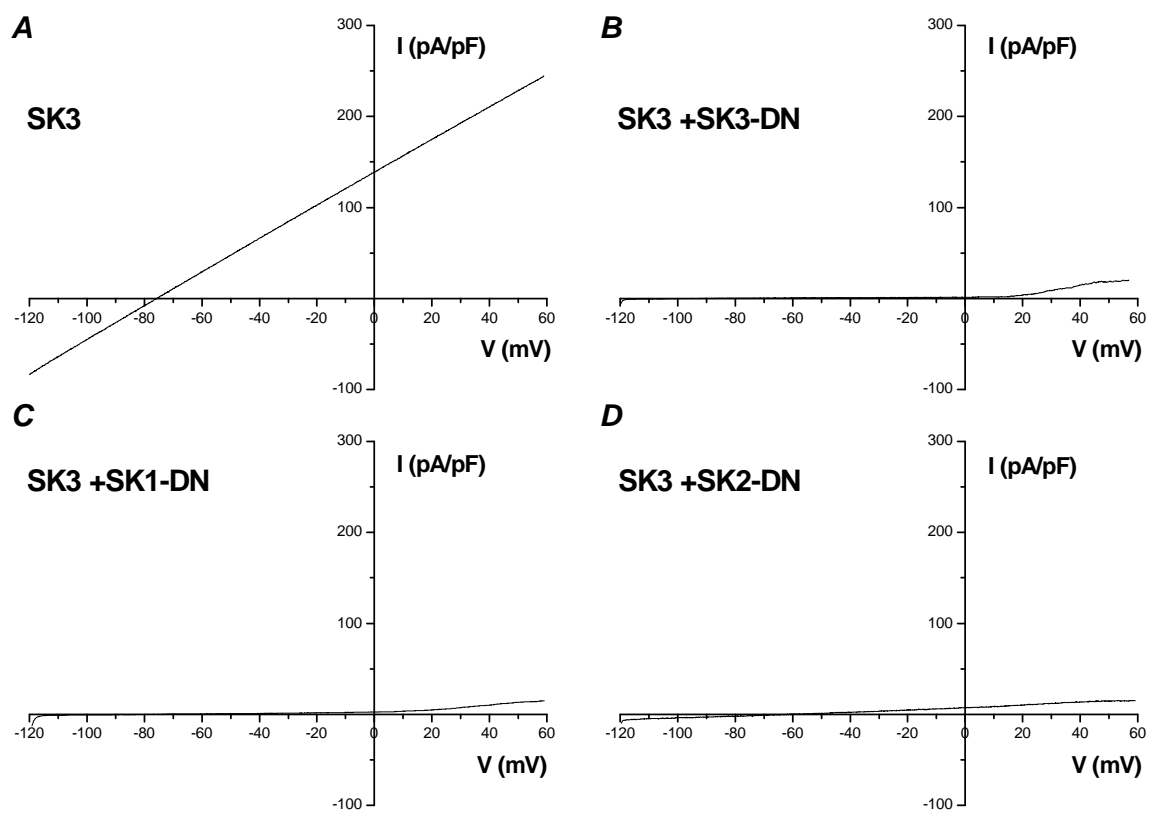
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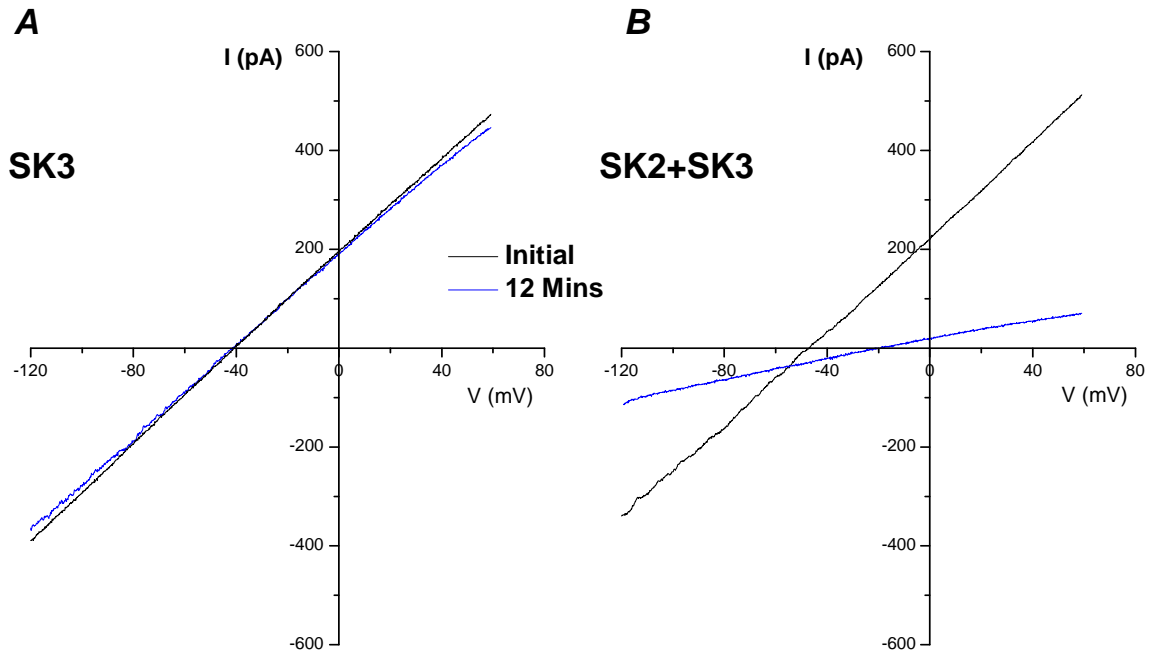
Online Figure III



Online Figure IV



Online Figure V



Online Figure VI