Supporting Information

Singh et al. 10.1073/pnas.1302028110

SI Materials and Methods

Antibodies. Primary antibodies were against the following: large conductance Ca^{2+}- and voltage-activated K^+ channel, BK_{Ca} (polyclonal antibody, pAb; APC021, lots AN05 and AN12, Alomone Labs), c-Myc epitope (monoclonal Ab, mAb, ab69258; and pAb, ab87854, AbCam), and voltage-dependent anion channel 1, VDAC1 (mAb, Ab14734, Abcam). Fluorophore-conjugated secondary antibodies for Western blots were the following: Alexa Fluor 680 goat anti-rabbit (A-21109, Invitrogen) and IRDye 800CW goat anti-mouse (926-32210, LI-COR) IgGs, and for immunochemistry were: Alexa Fluor 488 (A-11008, Molecular Probes) and Atto 647N (50185, Sigma) goat anti-mouse IgGs, and Atto 647N goat anti-rabbit IgG (15048, Active Motif).

Isolation of Ventricular Myocytes from Left Ventricle. Animals were injected intraperitoneally with heparin (200 IU/kg) and 20 min later they were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Hearts were then harvested and instantaneously arrested in ice-cold PBS (KCl 2 mM, KH_2PO_4 1.5 mM, NaCl 138 mM, Na_2HPO_4 8.1 mM) to remove excess blood. Hearts were transferred to ice-cold Tyrode's solution [NaCl 130 mM, KCl 5.4 mM, $MgCl_2$ 1 mM, Na_2HPO_4 0.6 mM, Glucose 10 mM, Taurine 5 mM, 2,3-butanedione monoxime 10 mM, and Hepes 10 mM, pH 7.4, oxygenated with 95% (vol/vol) O_2-5% (vol/vol) CO_2], and mounted on a modified Langendorff apparatus at a constant pressure of 80 cm H_2O. After 5 min of perfusion at 37 °C with Tyrode's solution, the heart was perfused for 10 min with Tyrode's solution containing 186 U/mL Collagenase Type-2 and 0.5 U/mL Protease Type-XIV, and then washed for 5 min with a high K^+ buffer (KB) [KCl 25 mM, KH_2PO_4 10 mM, $MgSO_4$ 2 mM, Glucose 20 mM, Taurine 20 mM, Creatine 5 mM, K-Glutamate 100 mM, Aspartic acid 10 mM, EGTA 0.5 mM, Hepes 5 mM, and 1% (wt/vol) BSA, pH 7.2 oxygenated with 95% O_2-5% (vol/vol) CO_2]. For rat hearts, the enzyme-Tyrode solution was perfused for ~22 min and contained 372 U/mL Collagenase Type-2 and 1.0 U/mL Protease Type-XIV, while washing with KB was for 15 min. After washing, the left ventricle was cut into pieces in KB to dissociate cells. Isolated ventriculocytes were filtered (100-μm strainer), and centrifuged 2 min at 1,000 × *g* for further use.

Mitochondria Isolation. Mouse or rat left ventricles, isolated mouse left ventriculocytes (WT or BK_{Ca} channel gene knockout, *Kcnma1*$^{-/-}$) or mouse whole brain (WT) were used. Briefly, finely minced tissue or isolated cardiomyocytes were homogenized in isolation buffer A (70 mM sucrose, 210 mM mannitol, 1 mM EDTA-Na_2, and 50 mM Tris·HCl, pH 7.4) using a Potter-Elvejem homogenizer (10 rapid strokes). The homogenate was centrifuged at 1,300 × *g* for 3 min. The supernatant was carefully collected and centrifuged at 10,000 × *g* for 10 min. The pellet containing crude mitochondria was saved and resuspended in 55 μL of isolation buffer A. The crude mitochondria preparation was gently overlaid on 3 mL of 30% (vol/vol) Percoll solution in buffer B (250 mM sucrose, 10 mM Hepes, 1 mM EDTA-Na_2, pH 7.4). The sample was centrifuged at 50,000 × *g* for 45 min at 4 °C. After ultracentrifugation, three clear layers of mitochondria were observed, M1, M2, and M3, characterized previously (1). Purified M3 fraction was collected and centrifuged at 12,000 × *g* for 5 min. M3 mitochondrial pellets were washed twice with 1 mL isolation buffer A and resuspended in 100 μL of the same buffer.

Electron Microscopy. Percoll-purified mitochondria were imaged by electron microscopy to confirm the integrity and purity of our preparation. The mitochondrial pellet (M3) from WT mouse was fixed in 2.5% (wt/vol) glutaraldehyde (Fluka) in buffer A for 2 h at room temperature and then stored in the same solution at 4 °C overnight. The pellet was then washed with PBS, postfixed in 2% (wt/vol) osmium tetroxide for 2 h at room temperature. Pelleted mitochondria were dehydrated in a graded alcohol series and embedded in Eponate 12 medium. The blocks were then cured at 60 °C for 48 h, and sections (70 nm) were cut with an RMC ultramicrotome, and mounted on Formvar-coated grids. The sections were double-stained with uranyl acetate and lead citrate, and examined with a 100CX JEOL transmission electron microscope.

Western Blot Analysis. Mouse brain or Percoll-purified mitochondria from mouse left ventriculocytes were treated with lysis buffer [Tris·HCl 50 mM, NaCl 150 mM, EDTA-Na_2 1 mM, EGTA-Na_4 1 mM, Na_3VO_4 1 mM, NaF 1 mM, 1% (vol/vol) Nonidet P-40, 0.5% (wt/vol) Na-deoxycholate and 0.1% (wt/vol) SDS, pH 7.4] containing protease inhibitors (1 tablet/50 mL; Roche), and incubated 1 h at 4 °C with shaking. Samples were centrifuged 5 min at 10,000 × *g* and the supernatants were collected as lysate. Proteins (50 μg/lane) were separated on 4–20% SDS/PAGE and transferred to nitrocellulose membranes. Loading was corroborated with Ponceau S staining. Nitrocellulose membranes were blocked with 5% (wt/vol) milk in TBS (NaCl 150 mM, Tris·HCl 20 mM, pH 7.4) at room temperature for 60 min and incubated overnight with anti-BK_{Ca} pAb (2 μg/mL). Membranes were washed thrice with TBS and incubated with 0.01 μg/mL secondary Abs for 60 min at room temperature. After extensive washing, membranes were visualized using Odyssey Imaging System (Li-Cor).

Systematic Exon Scanning. Messenger RNA (mRNA) was purified from isolated ventriculocytes using the oligotex mRNA spin-column protocol (Qiagen). The quality of the mRNA samples was analyzed by gel electrophoresis and RNA samples with visibly twofold of 28S/18S ratio were used. cDNA was prepared using Omniscript Reverse Transcription kit (Qiagen), 2 μg of mRNA and oligo-dT or 3′-UTR primers targeting BK_{Ca} mRNAs (Table S2). cDNAs were analyzed by PCR with gene-specific primer pairs (Table S2) to identify 200–400 bp of the predicted or constitutive BK_{Ca} exons (National Center for Biotechnology Information Gene ID 16531). All of the primer pairs were directed to different exons and had introns inbetween. The thermal cycling conditions comprised an initial denaturation step at 95 °C for 15 min and 35 cycles of 94 °C for 60 s, 58 °C for 60 s, 72 °C for 60 s, and a final step at 72 °C for 10 min. PCR products were separated on 2% (wt/vol) agarose gels, DNA bands were excised, eluted, and commercially sequenced.

Quantification of Cardiac *Kcnma1* Transcripts. Total RNA was purified from isolated ventriculocytes or left ventricle using TRIzol reagent (Invitrogen). Purified RNA was digested for 10 min at room temperature with RNase-free DNase and further cleaned-up with RNeasy mini kit (Qiagen). Cleaned-up RNA (2 μg) was reverse-transcribed with Omniscript Reverse Transcription (RT) kit (Qiagen) using oligodT primer to obtain 20 μL of reaction product. The reverse transcriptase was inactivated by heating at 95 °C for 5 min. Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad), 1.6 μL of RT reaction product, and

300 nM primer pairs (Table S3) in a 20-μL reaction. Primer pairs flanked an intron to control contamination from genomic DNA. The thermal cycling conditions comprised an initial denaturation step at 95 °C for 3 min, and 35 cycles of 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s. As controls, we used mock cDNA (no-reverse transcriptase), H_2O instead of template, and primers to amplify β-actin. All samples were run in duplicates. Threshold cycle values (Cq) were measured at a relative fluorescence of ~100. Standard curves (threshold cycle vs. log of DNA concentration) were constructed amplifying known concentrations of BK_{Ca}, BK_{Ca}-SV27 (BK_{Ca} containing a 27-aa splice insert), BK_{Ca} containing the 57-aa splice insert of the stress-axis regulated exon (BK_{Ca}-STREX), and BK_{Ca} containing a 50-aa C-terminal splice insert (BK_{Ca}-DEC) (Fig. S2) linearized plasmids. The efficiency of the primers was obtained from the slope of standard curves according to percent efficiency = $(10^{-1/\text{slope}} - 1) \times 100$, which was 94.2 ± 1.8% for total BK_{Ca}, 94.9 ± 2.9% for SV27, 97.9 ±1.3% for STREX, and 99.8 ± 0.3% for DEC ($n = 3$ for each primer pair). Clear single peaks at their melting temperature in the first derivative of fluorescence (dF/dT) versus temperature plot (melting curve) and a clear band at the expected size in agarose gels confirmed the amplification of specific products. Absolute concentrations of total BK_{Ca} and splice inserts were calculated by extrapolation from the linear fit of the respective standard curves and by correcting for primer efficiency. In ventricle samples, absolute concentrations were also corrected for the background signal obtained without RT which was ~0.01%. Percent expression was obtained by normalizing absolute concentrations of each splice insert to that of total BK_{Ca}.

Cardiomyocyte Transduction and Culture. N terminus c-Myc–tagged *insertless* BK_{Ca}, BK_{Ca}-SV27, and BK_{Ca}-DEC were cloned into pAd-DEST Gateway vector (Invitrogen). A silent mutation at position 1787 (TTA to TCA) was introduced to remove a PacI digestion site using as forward primer: CCG AGA GAG CCG TAT ATT AAT CAA TCC TGG, and as reverse primer: CCA GGA TTG ATT AAT ATA CGG CTC TCT CGG. Primers for cloning were for *insertless* BK_{Ca} and BK_{Ca} containing SV27: forward primer, GGGG ACA AGT TTG TAC AAA AAA GCA GGC TCC ACC ATG GGC GCC GAG GAG CAG AAG and reverse primer, GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCA AAG CCG CTC TTC CTG CAC GTA C; and for BK_{Ca} containing DEC: forward primer, same as for *insertless* BK_{Ca} and reverse primer, GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCA ACA TTC ATC TTC AAC TTC TCT GAT TGG.

Freshly isolated rat ventriculocytes were resuspended in 10 mL Ca^{2+} solution (NaCl 120 mM, KCl 5.4 mM, $MgSO_4$ 1.2 mM, NaH_2PO_4 20 mM, Glucose 5.6 mM, BDM 10 mM, Taurine 5M, $CaCl_2$ 0.125 mM, and 5 mg/mL BSA, pH 7.2). Cells were allowed to sediment by gravitation for 10 min on ice. Supernatant was discarded and cells were resuspended in 10 mL Ca^{2+} solution with 0.25 mM $CaCl_2$ and incubated for 10 min on ice. This step was repeated with 0.5 mM and 0.75 mM $CaCl_2$. Cardiomyocytes were then resuspended in MEM containing 2.5% (vol/vol) FBS and washed three times with the same solution for 10 min each under sterile conditions. Cardiomyocytes were seeded onto coverslips (previously coated with 1 mg/mL poly-L-lysine for 60 min at 37 °C followed by 10 μg/mL laminin for 60 min) and washed with PBS for 5 min at room temperature. Cardiomyocytes were allowed to settle for 1 h at 37 °C. Media was replaced with FBS-free MEM. After 3 h, media was aspirated and FBS-free MEM (1 mL per 35-mm Petri dish) containing adenovirus (10 μl of 1×10^6 pfu/mL strength) was carefully added. Cells were incubated at 37 °C for 60 min and infection media was replaced with pre-warmed FBS-free MEM. After 60 min, MEM containing 2.5% (vol/vol) FBS was added. Cells were cultured for a maximum of 36 h at 37 °C prior fixation.

Immunolabeling. Coverslips (0.17-mm thickness) were coated with poly-L-lysine [0.1% (vol/vol) in PBS] for seeding isolated mitochondria and cardiomyocytes. All of the biological samples preloaded with Mitotracker red CMXRos were kept in the dark on ice to prevent photobleaching.

In general, samples were washed three times with ice-cold PBS for 5 min each followed by fixation with 4% (wt/vol) paraformaldehyde in PBS for 10 min at room temperature. Samples were permeabilized with 0.5% (vol/vol) Triton-X 100 in PBS for 10 min at room temperature and blocked for nonspecific binding with 10% (vol/vol) normal goat serum in 0.2% (vol/vol) Triton-X 100/PBS for 15 min at room temperature. Samples were labeled overnight at 4 °C with specific antibodies diluted in PBS containing 1% (vol/vol) normal goat serum (NGS; Jackson ImmunoResearch) and 0.2% (vol/vol) Triton-X 100. After washing three times with PBS containing 0.2% (vol/vol) Triton-X 100, samples were incubated at room temperature for 60 min with corresponding secondary antibody conjugates Atto 647N (1 μg/mL anti-mouse and anti-rabbit IgGs) or Alexa-488 (2 μg/mL anti-mouse IgG) in 1% (wt/vol) NGS, 0.2% (vol/vol) Triton-X 100 in PBS. Samples were mounted for confocal microscopy with ProLongGold (Invitrogen). For stimulation emission depletion (STED) microscopy, mitochondrial preparations were successively incubated with 10%, 25%, 50%, and 97% (vol/vol) 2,2′-Thiodiethanol (TDE; Sigma-Aldrich) solutions in PBS (pH 7.5) for 5 min each at room temperature (2). Coverslips were sealed with nail polish. Confocal images in Figs. 2, 5, and 6 were acquired with an Olympus confocal microscope using a 60× oil immersion objective with 1.42 NA (PlanAppoN) at a scanning resolution of 0.0575 μm/pixel. STED and confocal images in Fig. 3 were acquired at 3.5 nm/pixel with a custom-made confocal-STED system using an oil immersion objective (HCX PL APO CS 100×/1.40–0.70 oil; Leica). Sequential images were acquired for the same field; acquisition of the confocal image preceded that of the STED image as described earlier (1).

Adult mouse ventriculocytes. Freshly dissociated ventriculocytes were incubated with 200 nM Mitotracker red CMXRos (Invitrogen) for 10 min at 37 °C and rested for 2 h at 4 °C to facilitate their attachment to coverslips. After loading, cells were immediately transferred onto ice for immunolabeling. Cells were fixed, labeled with 2 μg/mL wheat germ agglutinin (under non-permeabilized conditions), and then permeabilized to label with anti-BK_{Ca} pAb (2 μg/mL).

Adult rat ventriculocytes. Culture media was aspirated from cells transduced with adenovirus, and MEM containing 200 nM Mitotracker was added for 10 min. Cells were kept in an incubator at 37 °C. Cells were then kept on ice for immunolabeling with anti–c-Myc Ab (1 μg/mL) prior or after permeabilization.

Cardiac mitochondria. Isolated mitochondria were incubated with 500 nM Mitotracker for 60 min at 4 °C on a rotator shaker to label mitochondria. After loading, mitochondria were seeded onto poly-L-lysine coated coverslips for 2 h at 4 °C, fixed, permeabilized, and labeled. Primary antibodies were anti-BK_{Ca} (pAb, 2 μg/mL), and anti-VDAC1 (mAb, 0.2 μg/mL).

Image Analysis. Confocal images of isolated mitochondria and cardiomyocytes were median filtered (median intensities of 64 × 64-pixel and 32 × 32-pixel squares centered at the target pixel were subtracted from the target pixel, respectively) and analyzed using custom built software to determine the protein proximity index (PPI) as described previously (3, 4).

Mitoplast Preparation and BK_{Ca} Immunopurification for LC/MS/MS. Percoll-purified mitochondria (M3 fraction) from one rat left ventricle or from one mouse brain were resuspended in hypotonic buffer (Hepes 5 mM, EDTA 2 mM, pH 7.2 adjusted with KOH) and incubated 10 min on an orbital shaker. The sample was then centrifuged 5 min at 10,000 × *g*. The mitoplast pellet was resuspended in isotonic buffer (KCl 150 mM, Hepes 5 mM, pH 7.2

adjusted with KOH), centrifuged 5 min at 10,000 × *g* and resuspended in lysis buffer. Samples were incubated 60 min at 4 °C and centrifuged 5 min at 10,000 × *g*. The supernatant (lysate) was precleared with 50 μL of protein A/G Sepharose beads for 60 min and incubated overnight at 4 °C with beads saturated with anti-BK_{Ca} pAb [5 μg anti-BK_{Ca} pAb and 25 μl of protein A/G Sepharose beads were incubated for 60 min at 4 °C and washed with coimmunoprecipitation buffer containing NaCl 150 mM, Tris·HCl 50 mM, EDTA-Na_2 5 mM, Hepes 10 mM, 0.1% (vol/vol) Nonidet P-40, 0.25% (vol/vol) Na-deoxycholate, pH 7.4]. Proteins were eluted with SDS sample buffer for 60 min at 37 °C and separated using 4–20% 1D SDS/PAGE followed by staining with SYPRO Ruby overnight at room temperature. For each LC/MS/MS run, gel plugs from 10 independent immunopurifications were excised from the whole gel lane (~10–300 kDa), trypsin digested, eluted (5), and combined to enrich proteins' constituent peptides. For brain mitoplasts, gel plugs from a single immunopurification were used.

Protein Identification by LC/MS/MS. LC/MS/MS was performed at the University of California at Los Angeles W. M. Keck Proteomic Center using a Thermo LTQ-Orbitrap XL mass spectrometer equipped with an Eksigent NanoLiquid chromatography-1D plus system and an Eksigent autosampler. Proteolytic digests were loaded onto a 35 mm length, 100 μm ID C18 pre-Trap column (CVC Microtech) and washed for 10 min with 100% Buffer A [2% (vol/vol) acetonitrile, 0.1% formic acid in H_2O] at a flow rate of 5 μL/min. The peptides were then separated on a 15 cm ProteoPep IntegraFrit column (New Objective) using a flow rate of 300 nL/min and an increasing gradient of Buffer B [98% (vol/vol) acetonitrile and 0.1% formic acid in H_2O]. The following elution gradient was used: 0–15 min 0–30% (vol/vol) Buffer B, 15–20 min 30–80% (vol/vol) Buffer B and 20–22 min 80% (vol/vol) Buffer B in H_2O. The column was then reequilibrated for 13 min with Buffer A. The eluting analytes were sprayed in positive mode into the LTQ-Orbitrap MS using electrospray ionization voltage of 2,300 V, capillary voltage of 45 V, tube lens of 130 V, and capillary temperature of 200 °C. Information dependent acquisition was performed where the 6 most intense ions were selected in the *m/z* range of 300–1,600 using a 60 K resolution FTMS scan and subjecting them to MS-MS using broadband collision induced dissociation of normalized collision energy of 35 and LTQ detection. Peaks were excluded from further MS-MS for a period of 60 s.

The resulting MS/MS spectra were searched against Swiss-Prot database using the MASCOT Daemon search engine (Matrix Science). The following search parameters were used: peptide tolerance, ± 10 ppm; MS/MS tolerance, ± 0.3 Da; maximum missed cleavages, 2; fixed modifications, carboxymethyl (C); and variable modifications, deamidization (ND) and oxidation (M). The criteria for having a protein identified within a particular band is that there needs to be a minimum of two unique peptides that are ranked as number 1 and have ion scores with a $P < 0.05$.

Langendorff Preparation. Three-month-old male mice (WT and *Kcnma1*$^{-/-}$) were injected intraperitoneally with heparin (200 IU/kg) to prevent blood coagulation. After 20 min, animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Hearts were rapidly harvested and arrested in cold (4 °C) Krebs Henseleit bicarbonate buffer (KH): 11.1 mM glucose, 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 2 mM $CaCl_2$ at pH 7.4. The heart was cleaned to remove excess fat and noncardiac tissue. The aorta was rapidly cannulated, and the heart was retrograde-perfused with KH solution at a constant rate of 3 mL/min and maintained in air at 37 °C in the water-jacketed chamber of a Langendorff apparatus. The buffer was constantly bubbled with 95% (vol/vol) O_2 and 5% (vol/vol) CO_2 at 37 °C. After 20 min of equilibration, hearts were preconditioned by perfusion with KH solution supplemented with 10 μM NS1619 or DMSO (0.01%) for 10 min, global normothermic ischemia was then performed by stopping the perfusion flow for 18 min and immersing the heart in KH solution at 37 °C in the same chamber. At the end of 18 min, the KH solution surrounding the heart was drained and the heart was reperfused again with KH solution for 60 min at 37 °C.

Heart Function. Heart function was recorded during the entire experimental duration. Left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP) and heart rate (HR) were recorded with a pressure transducer (1.4F Millar SPR-671, Power Lab; AD Instruments), which was directly inserted in the LV through a left atrial incision that exposed the mitral annulus. The LV developed pressure (LVDP) and the rate pressure product (RPP) were calculated according to: LVDP = LVSP – LVEDP, and RPP = HR × LVDP. The maximum rate of the LV contraction velocity (dP/dt_{max}) and the maximum isovolumetric rate of relaxation ($-dP/dt_{min}$) were calculated directly from the recordings (LabChart5.5; ADInstruments).

Myocardium Infarct Size. Myocardial infarction was assessed by staining heart sections with 2,3,5-triphenyltetrazolium chloride (TTC). At the end of reperfusion, hearts were cut into four transverse sections parallel to the atrioventricular groove. Heart slices were incubated for 10 min in 1% (wt/vol) TTC in Tris·HCl (pH = 7.4) at 37 °C followed by fixation with 4% (wt/vol) paraformaldehyde. This staining procedure differentiates the infarcted (pale) from the viable (brick red) myocardial tissue. The slices were photographed using digital microscopic imaging. The area of necrosis was quantified by computerized planimetry with Adobe Photoshop. Total area of necrosis was calculated, and expressed as the percentage of the total left ventricular area.

Mitochondrial Calcium Retention Capacity. Crude mitochondria were isolated at 10 min of preischemic reperfusion as described above. Free Ca^{2+} concentration outside the mitochondria was continuously recorded using calcium green-5N (Invitrogen) and an AMINCO-Bowman Series 2 (AB2) spectrofluorometer. Excitation and emission wavelengths were set at 500 and 530 nm, respectively. Measurements were performed at 28 °C under constant stirring. The recording buffer was: 150 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, 5 mM succinic acid and 20 mM Tris·HCl, pH 7.4 supplemented with 0.5 μM calcium green 5-N. Baseline fluorescence was recorded using recording buffer alone. Mitochondria (0.5 mg protein) were then added to the 2-mL spectrofluorometer cuvette and mitochondrial Ca^{2+} uptake was recorded for 90 s. After this period, $CaCl_2$ pulses (40 nmol) were applied every 60 s until a massive release of Ca^{2+} was induced, presumably because of the opening of the permeability transition pore. Recordings were normalized to the maximum fluorescence prior mitochondria addition (ΔF/F). The Ca^{2+} retention capacity was defined as the amount of Ca^{2+} required to trigger the massive Ca^{2+} release and was expressed as nmol of $CaCl_2$/mg mitochondria protein (6).

1. Singh H, et al. (2012) Visualization and quantification of cardiac mitochondrial protein clusters with STED microscopy. *Mitochondrion* 12(2):230–236.
2. Staudt T, Lang MC, Medda R, Engelhardt J, Hell SW (2007) 2,2′-thiodiethanol: A new water soluble mounting medium for high resolution optical microscopy. *Microsc Res Tech* 70(1):1–9.
3. Li M, et al. (2010) Thromboxane A2 receptor and MaxiK-channel intimate interaction supports channel trans-inhibition independent of G-protein activation. *Proc Natl Acad Sci USA* 107(44):19096–19101.
4. Wu Y, et al. (2010) Quantitative determination of spatial protein-protein correlations in fluorescence confocal microscopy. *Biophys J* 98(3):493–504.

5. Singh H, Warburton S, Vondriska TM, Khakh BS (2009) Proteomics to identify proteins interacting with P2X2 ligand-gated cation channels. *J Vis Exp* 1178(27):pii, 1178.

6. Bopassa JC, Eghbali M, Toro L, Stefani E (2010) A novel estrogen receptor GPER inhibits mitochondria permeability transition pore opening and protects the heart against ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 298(1):H16–H23.

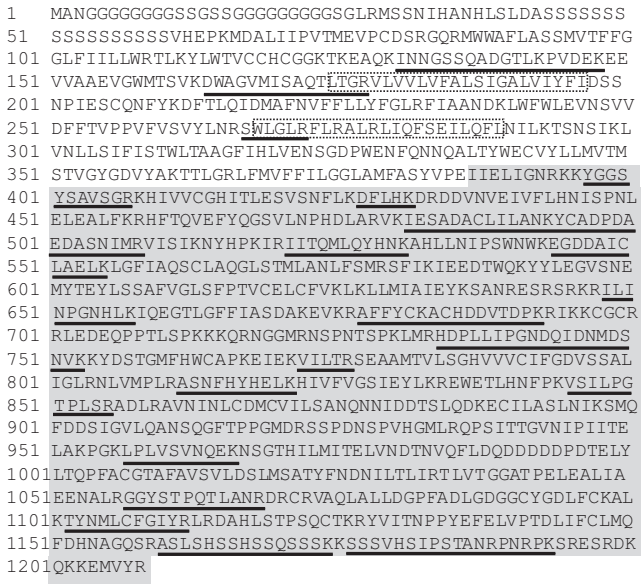

Fig. S1. Peptides identified by LC/MS/MS analysis of brain mitoplasts. Amino acid sequence of BK_{Ca} showing peptides identified by LC/MS/MS. Twenty-one unique peptides (underlined) were identified in the N terminus, transmembrane domains (dashed squares), and C terminus (gray shade).

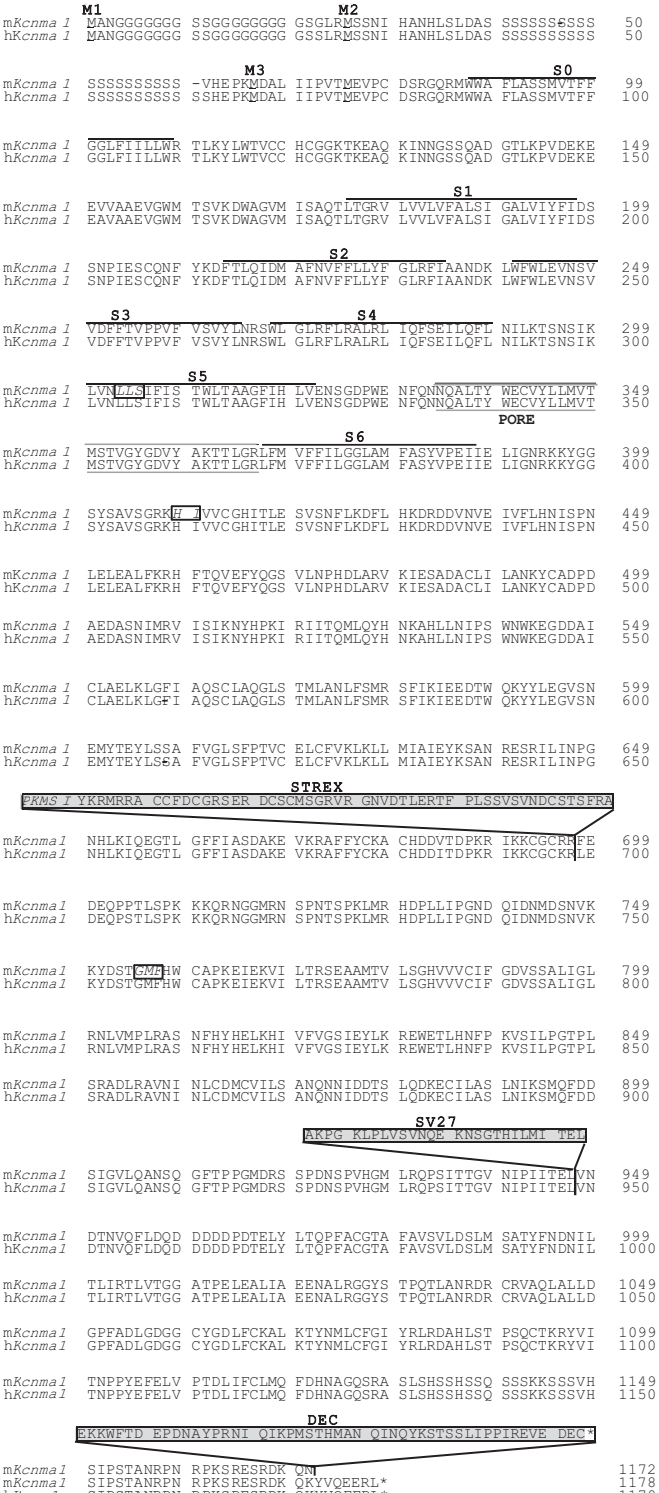

Fig. S2. Alignment of mitochondrial BK_{Ca} with human BK_{Ca}. Sequence alignment with constitutive human BK_{Ca} shows that identified mouse cardiac BK_{Ca} is identical to the human isoform. The boxed residues in italics were undefined in our sequences. Sequences of three alternatively spliced exons were identified (boxed), STREX, SV27, and DEC in the cardiac BK_{Ca}. Transmembrane domains (S0–S6) and pore region are also highlighted. M1, M2, and M3 mark three possible start sites.

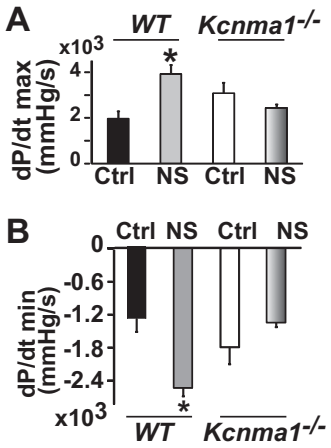

Fig. S3. NS1619 beneficial effect on heart function is lost in *Kcnma1*$^{-/-}$ mice. Mean maximum rate of rise, dP/d*t* max (*A*) and maximum rate of fall, dP/d*t* min (*B*) in hearts from WT and *Kcnma1*$^{-/-}$ mice. Values were obtained by calculating the mean of the last 10 min of reperfusion in each condition. NS1619 significantly improved the two parameters in WT but not in *Kcnma1*$^{-/-}$ mice. Ischemia-reperfusion protocol was as in Fig. 7*A*. *$P < 0.05$ vs. control (Ctrl).

Table S1. MitoBK$_{Ca}$ peptides identified by LC/MS/MS in ventriculocytes and in brain mitoplasts

Peptide	Exp#	Score	Exon	Excised band (kDa)	M_r (exp)	M_r (calc)
Ventriculocytes mitoplasts						
YGGSYSAVSGR	1	31	9	125–150	1102.5032	1102.5043
NLVMPLR	1	9	21	125–150	857.4794	857.4793
VSILPGTPLSR	1	7	21,22	125–150	1218.6258	1218.6258
LPLVSVNQEK	1	17	SV27	125–150	1125.6416	1125.6394
GGYSTPQTLANR	1	28	25	125–150	1263.6188	1263.6208
YGGSYSAVSGR	2	81	9	125–150	1102.5074	1102.5043
IESADACLILANK	2	33	12	125–150	1416.7256	1416.7282
ILINPGNHLK	2	9	17	125–150	1117.6588	1117.6608
NSPNTSPKLMR	2	6	19	125–150	1245.5978	1245.6023
NLVMPLR	2	18	21	125–150	857.4750	857.4793
TLVTGGATPELEALIAEENALR	2	43	25	125–150	2267.2034	2267.2009
GGYSTPQTLANR	2	30	25	125–150	1263.6162	1263.6207
ASLSHSSHSSQSSSK	2	24	27	125–150	1515.6852	1515.6913
IESADACLILANK	3	16	12	100–125	1416.7234	1416.7282
NLVMPLR	3	16	21	100–125	857.4784	857.4793
GGYSTPQTLANR	3	35	25	100–125	1263.6232	1263.6208
YGGSYSAVSGR	3	13	9	125–150	1102.4988	1102.5043
IESADACLILANK	3	5	12	125–150	1416.7270	1416.7282
AHLLNIPSWNWK	3	7	14	125–150	1479.7438	1479.7510
NLVMPLR	3	26	21	125–150	857.4798	857.4793
VSILPGTPLSR	3	20	21,22	125–150	1138.6710	1138.6710
Brain mitoplasts						
YGGSYSAVSGR	1	19	9	125–150	1102.4972	1102.5043
IITQMLQYHNK	1	8	13	125–150	1403.7361	1403.7231
VILTR	1	11	20,21	125–150	600.3968	600.3959
VSILPGTPLSR	1	20	21,22	125–150	1138.6670	1138.6710
LPLVSVNQEK	1	28	SV27	125–150	1125.6372	1125.6394
GGYSTPQTLANR	1	26	25	125–150	1263.6230	1263.6208
INNGSSQADGTLKPVDEK	2	27	2	100–125	1872.9103	1872.9065
YGGSYSAVSGR	2	77	9	100–125	1102.5052	1102.5043
YCADPDAEDASNIMR	2	60	12	100–125	1742.6880	1742.6876
ILINPGNHLK	2	11	17	100–125	1117.6630	1117.6608
AFFYCK	2	13	18	100–125	1102.5052	1102.5043
VILTR	2	8	20,21	100–125	600.3968	600.3959
VSILPGTPLSR	2	33	21,22	100–125	1138.6742	1138.6710
LPLVSVNQEK	2	30	SV27	100–125	1125.6422	1125.6394
GGYSTPQTLANR	2	29	25	100–125	1263.6222	1263.6208
YCADPDAEDASNIMR	2	9	12	125–150	1742.6862	1742.6876
LPLVSVNQEK	2	31	SV27	125–150	1125.6404	1125.6394
ASNFHYHELKH	3	20	21	125–150	1138.6710	1138.6710
TYNMLCFGIYR	3	33	26	100–125	1244.5894	1244.5938
ASLSHSSHSSQSSSK	3	48	27	100–125	1452.6556	1452.6530
SSSVHSIPSTANRPNRPKS	3	26	27	100–125	1572.7072	1572.7128
DWAGVMISAQTLTGR	3	23	2	125–150	1655.7756	1655.7617
SWLGLR	3	16	5,6	125–150	730.4146	730.4126
DFHLHK	3	2	10	125–150	658.3442	658.3439
IESADACLILANK	3	38	12	125–150	1416.7278	1416.7282
EGDDAICLAELK	3	50	14	125–150	1332.6202	1332.6231
ACHDDVTDPK	3	5	18	125–150	1156.4844	1156.4819
HDPLLIPGNDQIDNMDS	3	36	20	125–150	1997.9648	1997.9728
LPLVSVNQEK	3	31	SV27	125–150	1125.6430	1125.6393

Exp#, experiment number; M_r (exp), experimental mass,; M_r (calc), theoretical mass. Peptides with score of "zero" are not listed.

Table S2. Exon scanning primers

Primer	Sequence
Exon1-3 Fwd	5′ GGT CTT AGA ATG AGC AGC AAT ATC CAC GCG 3′
Exon1-3 Rev	5′ CTA GCA TGT TAG ACC CTC TGC TGA ATG CTG 3′
Exon3-9 Fwd	5′ CAG CAT TCA GCA GAG GGT CTA ACA TGC TAG 3′
Exon3-9 Rev	5′ ACT TCC AGC CAG AAC CAC AGC TTA TCG TTG 3′
Exon9-13 Fwd	5′ CAA CGA TAA GCT GTG GTT CTG GCT GGA AGT 3′
Exon9-13 Rev	5′ CTG CAG TCA GCC ACG TGC TGA TAA ATA TGG 3′
Exon13-15 Fwd	5′ CCA TAT TTA TCA GCA CGT GGC TGA CTG CAG 3′
Exon13-15 Rev	5′ TTT CTT CCA CTA ACC GCG CTA TAG GAG C 3′
Exon15-19 Fwd	5′ GCT CCT ATA GCG CGG TTA GTG GAA GAA A 3′
Exon15-19 Rev	5′ CTT ATT GGC AAG GAT CAG GCA TGC ATC TGC 3′
Exon19-21 Fwd	5′ GCA GAT GCA TGC CTG ATC CTT GCC AAT AAG 3′
Exon19-21 Rev	5′ GAG AAG AGA TTG GCA AGC ATT GTG GAG AGG 3′
Exon21-26 Fwd	5′ CCT CTC CAC AAT GCT TGC CAA TCT CTT CTC 3′
Exon21-26 Rev	5′ ACC TTC TTG GAT CTT AAG GTG GTT CCC AGG 3′
Exon26-29 Fwd	5′ CCT GGG AAC CAC CTT AAG ATC CAA GAA GGT 3′
Exon26-29 Rev	5′ GGA AGG TTC TCT CAA GGG TGT CCA CGT TA 3′
Exon29-32 Fwd	5′ TAA CGT GGA CAC CCT TGA GAG AAC CTT CC 3′
Exon29-32 Rev	5′ ACT TTC TCA ATC TCC TTG GGT GCA CAC CAG 3′
Exon32-34 Fwd	5′ CTG GTG TGC ACC CAA GGA GAT TGA GAA AGT 3′
Exon32-34 Rev	5′ CAG CCC TTA AAT CAG CCC GAC TTA ATG GTG 3′
Exon34-36 Fwd	5′ CAC CAT TAA GTC GGG CTG ATT TAA GGG CTG 3′
Exon34-36 Rev	5′ GAT TGA CTG ATA CCA AAG GCA ACT TGC CCG 3′
Exon36-38 Fwd	5′ CGG GCA AGT TGC CTT TGG TAT CAG TCA ATC 3′
Exon36-38 Rev	5′ CGA AGT GCA TTC TCC TCA GCT ATT AGA GCC 3′
Exon38-40A Fwd	5′ GGC TCT AAT AGC TGA GGA GAA TGC ACT TCG 3′
Exon38-40A Rev	5′ ACT GCA TCA GGC AGA AGA TCA GGT CTG TTG 3′
Exon40A-40B Fwd	5′ CAA CAG ACC TGA TCT TCT GCC TGA TGC AGT 3′
Exon40A-40B Rev	5′ GTG GCT ACA CAT ATC AAA GCC GCT CTT CC 3′
Exon40A-41 Fwd	5′ GTG CAA CAA GGA TGA CTA GAA TGG GCC A 3′
Exon40A-41 Rev	5′ TGG CCC ATT CTA GTC ATC CTT GTT GCA C 3′
Exon40B Fwd	5′ GGA AGA GCG GCT TTG ATA TGT GTA GCC AC 3′
Exon40A-41A Rev	5′ GTT AGC CAT GTG GGT ACT CAT GGG CTT GA 3′
Exon40A-41B Rev	5′ GGT CTC CTG GGA GTC AAC ATT CAT CTT C 3′
BK_{Ca} 3′ UTR	5′ CCA CAT GCA CAC TAT CAT ACA TAT GC 3′
BK_{Ca} DEC 3′ UTR	5′ CCA CCA GCT GAT TCA GCA TGT AAG AGG AAG 3′

Exon numbers are from the National Center for Biotechnology Information evidence viewer (Gene ID: 16531); www.ncbi.nlm.nih.gov/sutils/evv.cgi?taxid=10090&contig=NT_039606.8&gene=Kcnma1&lid=16531.

Table S3. Quantitative PCR primers

Splice variant	Primer	Sequence	Expected size
Insertless	Forward	5′ CCATTAAGTCGGGCTGATTTAAG 3′	187 bp
	Reverse	5′ CCTTGGGAATTAGCCTGCAAGA 3′	
BK_{Ca}-STREX	Forward	5′ GCCAAGATGTCCATCTACAAG 3′	174 bp
	Reverse	5′ GCACGGAAACTGGTGGAGCAA 3′	
BK_{Ca}-SV27	Forward	5′ CTAAGCCGGGCAAGTTG 3′	164 bp
	Reverse	5′ GAGTCCAGGACACTGACG 3′	
BK_{Ca}-DEC	Forward	5′ GGTTTACAGATGAGCCGGATA 3′	134 bp
	Reverse	5′ CATCTTCAACTTCTCTGATTGG 3′	
β-Actin	Forward	5′ TCCTTCGTTGCCGGTCCACA 3′	225 bp
	Reverse	5′ CCTCTCTTGCTCTGGGCCTCG 3′	