SUPPLEMENTAL MATERIALS

Genetic ablation of $Ca_V3.2$ channels enhances the arterial myogenic response by modulating the RyR-BK_{Ca} axis

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Running Title: $Ca_V3.2$ counterbalances myogenic constriction

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DETAILED METHODS

Animal procedures

Animal procedures were approved by the Animal Care and Use Committee at the University of Calgary and Loma Linda University. Briefly, male or female C57BL/6J (wild-type) and $Ca_V3.2$ knockout ($Ca_V3.2^{-/-}$) mice (2–4 months old, obtained from Jackson Laboratories) were asphyxiated in a CO_2 chamber. The mesentery was carefully removed and placed in cold phosphate-buffered saline solution (pH 7.4) containing (in mM): 138 NaCl, 3 KCl, 10 Na_2HPO_4, 2 NaH_2PO_4, 5 glucose, 0.1 $CaCl_2$ and 0.1 $MgSO_4$. Third and fourth order mesenteric arteries were dissected out of surrounding tissues and cut into 2–3 mm segments.

Vessel myography and membrane potential (V_M) measurement

Mesenteric arteries were mounted in an arteriograph and superfused with physiological saline solution (PSS; 37°C; pH 7.4; 21% O_2, 5% CO_2, balance N_2) containing (in mM): 119 NaCl, 4.7 KCl, 20 $NaHCO_3$, 1.1 KH_2PO_4, 1.2 $MgSO_4$, 1.6 $CaCl_2$ and 10 glucose. To limit the endothelial influence, air bubbles were passed through the lumen for 1–2 min. Arteries were equilibrated at 15 mmHg and contractile responsiveness assessed by briefly applying 60 mM KCl. Following equilibration, intravascular pressure was incrementally elevated from 20 to 100 mmHg and external diameter monitored. Maximal diameter was assessed in Ca^{2+}-free PSS (zero Ca^{2+} + 2 mM EGTA). Percentage myogenic tone was calculated as follows: % Myogenic tone=100*(D_0–D)/D_0; where D is external diameter under control conditions (Ca^{2+} PSS) or treated conditions, and D_0 is external diameter in Ca^{2+}-free PSS. Smooth muscle membrane potential (V_M) was ascertained as previously published[1] by inserting a glass microelectrode backfilled with 1 M KCl (tip resistance ~120-150 MΩ) into the vessel wall while pressurized at 60 mmHg. Criteria for successful impalement included: 1) a sharp negative V_M deflection upon insertion; 2) a stable V_M for ≥1 min after entry; and 3) a sharp return to baseline upon electrode removal.

Quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from mouse C57BL/6 or $Ca_V3.2^{-/-}$ mesenteric arteries using the RNeasy plus micro kit (Qiagen) following manufacturer's recommendations. Reverse transcription was performed with 20 ng per sample of total RNA using the Quantitect reverse transcription kit (Qiagen). For the negative control groups, all components except the reverse transcriptase were included in the reaction mixtures. Real-Time PCR using intron-spanning primer sequences was performed using the Kapa SYBR Fast Universal qPCR Kit (Kapa Biosystems). Mouse beta-actin gene was utilized as the reference gene. Control reactions and those containing cDNA from arteries were performed with 1 ng of template per reaction. The running protocol extended to 45 cycles consisting of 95°C for 5 s, 55°C for 10 s and 72°C for 10 s using an Eppendorf Realplex 4 Mastercycler. PCR specificity was checked by dissociation curve analysis, and assay validation was confirmed by testing serial dilutions of pooled template cDNAs suggesting a linear dynamic range of 50-0.05 ng template and yielded percent efficiencies ranging from 85-95%. No template controls yielded no detectable fluorescence. Expression levels of the various genes of interest in arteries from $Ca_V3.2^{-}$

$^{/-}$ relative to C57BL/6 mice were determined using the relative expression software tool (REST) version 2.0.13.[2]

Quantitative PCR primer sequences and validation parameters.

Target	Primers	Amplicon	Efficiency (%)	Linear Dynamic Range ng template (Cq values)	Cq values ± SEM C57BL/6, *n*=3 $Ca_V3.2^{-/-}$, *n*=3
$Ca_V1.2$	ATTCGACGTGAAGGCACTG GAGTTCAGGACCACCTGGAG	90	95	50-0.05 (23.41-33.75)	33.22±0.43 31.93±0.24
$Ca_V3.1$	TCCTGGTCAATACCCTCAGC GAGGCTGGTGAAGACGATGT	98	85	50-0.005 (24.09-39.29)	38.98±0.21 36.68±1.05
$BK_{Ca}\alpha$	TCTCAGCATTGGTGCCCTCGTAAT GTAGAGGAGGAAGAACACGTTGAA	127	92	33-0.0033 (22.48-36.48)	34.56±0.06 32.30±0.14
RyR2	CTGAGCTGGCATTCAAGGAC AGCCTTCTTGTAAGCCACAG	144	88	50-0.005 (21.78-36.15)	35.11±0.50 33.47±0.02
ACTB	ACTGTCGAGTCGCGTCCA GCAGCGATATCGTCATCCAT	100	88	50-0.0005 (20.61-39.35)	31.15±0.43 28.71±0.59

Isolation of arterial smooth muscle cells

As previously described[3], arterial segments were placed in an isolation medium containing (in mM): 60 NaCl, 80 Na-glutamate, 5 KCl, 2 $MgCl_2$, 10 glucose and 10 HEPES with 1 mg/ml bovine serum albumin (pH 7.4, 37 °C, 10 min). Vessels were then exposed to a two-step digestion process: 1) 13 min incubation in isolation medium containing 0.5 mg/ml papain and 1.5 mg/ml dithioerythritol; and 2) 10 minutes incubation in isolation medium containing 100 μM Ca^{2+}, 0.7 mg/ml type F collagenase and 0.4 mg/ml type H collagenase. Following incubation, tissues were washed repeatedly with ice-cold isolation medium and triturated with a fire-polished pipette. Liberated cells were stored in ice-cold isolation medium for use within ~6 hr.

Electrophysiological recordings

Conventional patch-clamp electrophysiology was used to monitor whole-cell voltage-gated Ca^{2+} channel currents in isolated smooth muscle cells. [3] Recording electrodes (5-8 MΩ) were pulled from borosilicate glass microcapillary tubes using a micropipette puller, and backfilled with pipette solution (in mM): 135 CsCl, 5 Mg-ATP, 10 HEPES, and 10 EGTA (pH 7.2). Cells were voltage-clamped and equilibrated in bath solution (in mM): 110 NaCl, 1 CsCl, 10 $BaCl_2$, 1.2 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.4). A 1 M NaCl–agar salt bridge between the reference electrode and the bath solution was used to minimize offset potentials. To record whole-cell Ba^{2+} currents, isolated cells held at -60 mV were exposed to a pre-pulse (-90 mV, 200 ms) and then test pulses (-50 to 40 mV, 10 mV intervals, 300 ms).

Perforated patch-clamp electrophysiology was used to measure spontaneous transient outward K^+ currents (STOCs) in freshly isolated smooth muscle cells.[1] The bath solution contained (in mM): 134 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES (pH 7.4). The pipette solution contained (in mM): 110 K aspartate, 30 KCl, 10 NaCl, 2 $MgCl_2$, 10 HEPES, and 0.05 EGTA (pH 7.2) with 200 µg/ml amphotericin B. Currents were recorded while cells were held at -40 or -20 mV. STOC analysis was performed using Clampfit 10.3; threshold for detection was set to be ~3 times the BK_{Ca} single channel conductance. Whole-cell currents were recorded using an Axopatch 200B patch-clamp amplifier, filtered at 1 kHz, digitized at 5 kHz, and were stored on a computer for offline analysis. Whole-cell capacitance averaged ~13 pF and all experiments were performed at room temperature.

Ca^{2+} spark measurement

Ca^{2+} sparks were recorded in mouse arterial myocytes loaded with the Ca^{2+} sensitive dye Fluo-4 AM and using a Zeiss LSM 710 NLO laser scanning confocal imaging workstation on an inverted microscope platform (Zeiss Axio Observer Z1).[1] Fluo-4 AM was dissolved in DMSO and added from a 1 mM stock to the arterial suspension at a final concentration of 10 µM, along with 0.1% pluronic F127 for 1-1.5 hr at room temperature in the dark in balanced salt solution. Arterial segments were then washed (30 min) to allow dye esterification and then cut into linear strips. Arterial segments were pinned to Sylgard blocks and placed in an open bath imaging chamber mounted on the confocal imaging stage. Arteries were illuminated at 488 nm with a krypton argon laser, and emitted light was collected using a photomultiplier tube. Line scans were imaged at 529 fps with the emission signal recorded at 493-622 nm. The acquisition period for Ca^{2+} spark recordings was 18.9 s and the resultant pixel size ranged from 0.0148 to 0.0911 µm per pixel. To ensure that sparks within the cell were imaged, the pinhole was adjusted to provide an imaging depth of 2.5 µm, this is roughly equivalent to 50% the width of the cell based on morphological examination of live preparations. Analysis was performed to characterize the percentage of scans with Ca^{2+} sparks (% firing), Ca^{2+} spark frequency, amplitude and spatiotemporal characteristics using SparkLAB 4.2.1. Threshold for spark detection was 3.2 times the standard deviation of the background noise above mean background. Prior to analysis, background fluorescence was subtracted from each image assuming homogeneous background levels in each cell.

Intravascular catheterization

Male C57BL/6 or $Ca_V3.2^{-/-}$ mice were anesthetized with intraperitoneal injection of ketamine (150 mg/kg) and xylazine (10 mg/kg). Anesthesia was extended by additional ketamine (10 mg/kg) as required. Anesthesia adequacy was verified by the abolition of the withdrawal and blink reflexes. A small neck incision was made and the left common carotid artery was ligated and catheterized using an arterial cannula. Similarly, a small incision was made to expose and catheterize the right jugular vein for intravenous drug administration. Changes in mean arterial pressure (ΔMAP) were monitored using a data acquisition system (ACQKnowledge). Mice were sacrificed at the end of the experiment using a high dose of anesthetic.

Statistical analysis

Data are expressed as means±S.E.M., and *n* indicates the number of cells, arteries or mice. Where appropriate, paired/unpaired *t*-tests or one way ANOVA were performed to compare the effects of a given condition/treatment on arterial diameter, or whole-cell current. *P values ≤ 0.05 were considered statistically significant. Averaged current-voltage relationships were fit to the following Peak Gaussian function: $I(V)=I_{max}*\exp[-0.5\{(V-V_{max})/b\}^2]$; where I_{max} is peak current (I), V_{max} is V at I_{max}, and b is the slope of the distribution.

Solutions and Chemicals

Drugs, enzymes and buffer reagents were purchased from Sigma-Aldrich, unless otherwise mentioned.

SUPPLEMENTAL REFERENCES

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3. Harraz OF, Welsh DG. Protein kinase A regulation of T-type Ca^{2+} channels in rat cerebral arterial smooth muscle. J Cell Sci. 2013:126:2944-2954.