Supplemental Material

Detailed Methods:

Dominant-negative peptides

K_V1-C peptide (Figure 1B) consisted of the final 10 amino acids of the C-terminus of K_V1.2α, which represents a PDZ binding motif (VNITKM*LTDV*). It was attached by a spacer (P) to an N-terminus HIV-tat sequence (NH_3-YGRKKRRQRRR) to confer membrane permeability. A fluorescein label was attached to the N-terminus of some peptides for visualization. Two scrambled variations of the peptide (Scm: DVNMTKLVIT; Scm2: TLMKVDTVNI) attached by the same spacer to HIV-tat were used as negative controls (21st Century Biochemicals). For patch-clamp experiments, non-permeable (NP) 10 amino-acid peptides without the HIV-tat (K_V1-C NP and Scm NP) were used (GenScript). Myristoylated PKA inhibitor peptide 14-22 (PKI) was purchased from Tocris. All peptides were prepared as 1 mmol/L aqueous stocks, aliquoted and kept at -20°C until use.

Animals

All protocols complied with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences. Cerebral arteries (CA) were dissected from ten- to fourteen-week-old male Sprague–Dawley rats (Harlan). Rats were euthanized by decapitation under isoflurane anesthesia.

Protein lysate

CA were homogenized in lysis buffer of the following composition (mmol/L): 150 NaCl; 1 EGTA; 1.5 $MgCl_2$; 50 HEPES; 1% Triton X-100; 10% glycerol; pH 7.3 plus protease inhibitor cocktail (Thermo Scientific). Phosphatase inhibitor cocktail (Thermo Scientific) was included when appropriate. A plastic pestle or a tissue homogenizer (Bullet Blender, Next Advance) and 0.9-2.0 mm stainless steel beads were used to homogenize the arteries.

Co-immunoprecipitation

Co-immunoprecipitation (co-IP) was performed as previously described[1] with the Pierce Co-IP Kit (Thermo Scientific) per the manufacturer's instructions with Modified Dulbecco's phosphate buffered saline (PBS) as the binding/wash buffer. Briefly, columns were initially prepared by immobilizing 150 µg of K_V1.2 antibody (Neuromab) on to 50 µL of AminoLink Plus Coupling Resin with a 2 hour incubation. After washing with the supplied coupling buffer and wash solution, the columns were loaded with 125 µg cerebral artery lysate and incubated overnight at 4°C. Dissected CA were incubated with K_V1-C or Scm peptide (10 µmol/L) in PBS for 30 min at 37°C then rinsed in PBS prior to lysing. To preserve the dynamic association of K_V1.2 and PSD95, the centrifugation speed for lysate preparation was reduced to 3000g for co-IP studies (Online Figure VIII-A). Antibody-bound proteins were eluted with 60 µL of elution buffer provided with the kit after incubating for 8 min at room temperature.

Biotinylation

CA dissected from a single rat were incubated with K_V1-C or Scm peptide (10 µmol/L) in PBS for 30 min at 37°C. Subsequently, arteries were centrifuged at 2,300g for 5 min at 4°C, rinsed with chilled PBS and incubated for 1 hour at room temperature in biotin solution (EZ-Link Melamide-PEG-Biotin and Sulfo-NHS-LC-LC-Biotin, 1 mg/mL each, Thermo Scientific) before quenching with 100 mmol/L glycine. An ample amount of avidin beads (Pierce Monomeric Avidin Agarose, Thermo Scientific) were used to separate the biotinylated surface fraction and the cytosolic fraction. The surface fraction was eluted from the avidin beads by heating for 3 min in sodium dodecyl sulfate loading buffer containing 10% 2-mercaptoethanol in near-boiling water.[2]

Western blots

As previously described[1], cerebral arterial protein samples were separated by electrophoresis using 4–15% gradient gels (Invitrogen) and transferred to PVDF membranes. Membranes were blocked in 10% milk or 10% bovine serum albumin (to preserve phosphorylation) and incubated sequentially in primary and secondary antibodies. Primary antibodies were K_V1.2 (1:100, NeuroMab), PSD95 (1:100, NeuroMab) or phospho-PKA substrate (1:1000, Cell Signaling). Antibody against α-actin (Sigma) was used to normalize for loading variability. Secondary antibodies were horseradish peroxidase-linked sheep anti-mouse antibody (GE

Healthcare, 1:3000 dilution) for $K_V1.2$ and PSD95 and donkey anti-rabbit antibody (GE Healthcare, 1:5000 dilution) for phospho-PKA substrate. Immunoreactive bands were detected using enhanced chemi-luminescence reagents (Thermoscientific) along with Hyblot CL film (Denville Scientific) and quantified using ImageJ software (NIH). The density of immunoreactive bands was first normalized to α-actin density for each lane and the resulting values for K_V1-C treatment group were represented as a fraction of Scm treatment. The unglycosylated $K_V1.2\alpha$ band at ~60 kD was proportional to the fully-glycosylated $K_V1.2\alpha$ band at ~75 kD in each sample lane but was not included in the quantification. The lower band is more prominent in lysates prepared from 3000g centrifugation compared to 100,000g spin[3] and also becomes dominant when the sample is heated in 1% SDS as needed for biotinylation (Online Figure VIII-B).

Confocal imaging of fluorescently-labeled peptide

Dissected CA were incubated with fluorescin-labeled K_V1-C or Scm peptide (3 μmol/L) in phosphate buffered saline (PBS) for varying times (0 – 30 min) and then immediately fixed in 4% formaldehyde for 20 min. After a PBS wash, arteries were incubated in 10 μg/mL Alexa350-labeled wheat germ agglutinin (Invitrogen) for 30 min for cell surface counter staining or were mounted directly in Prolong Gold with DAPI (Invitrogen) for nuclear staining and placed on a glass-bottom dish (Mattek) for imaging. The samples were imaged with appropriate filter sets with an Orca EM (Hamamatsu) camera and a 63x 1.4 N.A. oil-immersion objective on an Axiovert 200M microscope (Zeiss) with a CARV II spinning-disk confocal unit (CrEST). Images were acquired and processed with IPLab version 4.04 (BD Biosciences).[1]

Diameter and membrane potential recording in pressurized arteries

Cerebellar arteries were isolated, cannulated and pressurized to 80 mm Hg and equilibrated at 37°C for 1 hour while being superfused with physiological salt solution (PSS) containing (in mmol/L): 119 NaCl, 4.7 KCl, 24 $NaHCO_3$, 1.18 NaH_2PO_4, 1.17 $MgSO_4 \cdot 7H_2O$, 5.5 glucose, 0.03 EDTA, and 1.6 $CaCl_2$ and bubbled a with 7% CO_2 / 93% O_2 gas mixture to obtain pH 7.4. Any arteries that did not develop spontaneous tone after 1 hour equilibration or lacked a vasoconstrictive respond to 60 mM KCl were not used for study. External diameters were automatically measured and recorded by DMTVAS software (DMT). Diameter changes were represented as a percent of the resting diameter before treatment. Drugs were added directly to the superfusate bath away from the artery and allowed to diffuse. Membrane potential (E_m) was measured using glass microelectrodes filled with 3 mol/L KCl fitted over a silver chloride wire mounted on a micromanipulator and connected to a preamplifier (DAGAN). The readings were recorded and analyzed by WinDaq Lite software (DATAQ). A successful measurement of E_m was defined as an abrupt drop in voltage upon impalement, maintenance of a stable value for a minimum of 20 s and an immediate return to baseline upon withdrawal of the microelectrode. Three such measurements were averaged to yield the final value for each E_m recording (Online Figure IX).

Craniectomy and in situ imaging

Rats were anesthetized with isoflurane (2% at 1.0 L/min O_2), mounted in a stereotaxic frame (KOPF) and positioned under a dissecting microscope. Body temperature was monitored with a rectal probe and maintained between 37-38°C with a heating pad. The skull was exposed, cleaned of periosteum, flushed with saline and dried. A 3x7-mm section of bone was cut with a high speed micro-drill and removed from the right parietal plate. The dura mater was retracted and excised using a 30 gauge needle and a micro-knife. A custom-made ported cranial window was fitted to the skull using bone wax, dental acrylic and cyanoacrylate gel. The window was suffused with 37°C PSS bubbled (7% CO_2, 93% O_2) to physiological pH. Middle cerebral artery branches were imaged using an HDR-PJ580 camera (Sony) and analysis was accomplished using an automated script in IPLab.

Patch-Clamp

Patch-clamp recordings were performed in the standard whole-cell configuration on an EPC amplifier (List) and pCLAMP 6 software (Molecular Devices), and data was analyzed with pCLAMP 10. Gigaohm seals and cell access were acquired in a drug-free bath solution composed of (in mmol/L): NaCl 140, KCl 4, $CaCl_2$ 0.1, $MgCl_2$ 1, HEPES 10, glucose 20, pH 7.4 adjusted with NaOH. After initial capacitance recording confirming cell access, bath solution containing 100nmol/L Iberiotoxin was superfused to eliminate any contaminating BK channel currents. The recording electrodes were dipped for 5-10 sec in regular pipette solution consisting of (in mmol/L): K-glutamate 145, $MgCl_2$ 1, HEPES 10, EGTA 4, Na_2ATP 1, pH 7.2 adjusted with KOH and then backfilled with pipette solution containing either 3 μmol/L Scm peptide or 3 μmol/L K_V1-C peptide. Currents

were recorded using a voltage-step protocol that applies 500 ms voltage steps from -70 mV to +58 mV in 8 mV increments before and after superfusion with bath solution containing 100 nmol/L Psora4. Psora4-depedent currents were obtained from digitally subtracting post-Psora4 currents from pre-Psora4 currents. Current densities were calculated by normalizing all currents to the cell capacitance.

Drugs

The following drugs were prepared as stock solutions in DMSO: 5-(4-phenylalkoxypsoralen) (Psora4; Sigma), 10 mmol/L; calyculin-A (CalA; Tocris), 500 μmol/L; okadaic acid (OA; Tocris), 100 μmol/L; and indomethacin (Sigma), 100 mmol/L. Stock solutions for other drugs were prepared in water: Nω-nitro-L-arginine methyl ester (L-NAME; Sigma), 100mmol/L; Linopirdine (Lino; Tocris), 10mmol/L; and $BaCl_2$ (Sigma), 30mmol/L. For pressurized vessel experiments, drugs were diluted first in PSS when appropriate and added directly to the chamber at either 100x or 1000x dilution for the final bath concentrations referred to in the text. The maximum amount of DMSO used in drug delivery (0.6 μL in 1 mL of PSS) was tested as the vehicle control which did not produce significant changes to the diameter (Figure 8B, veh).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Data were analyzed using unpaired *t* tests or when appropriate with one-way ANOVA with Newman-Keuls post-hoc or two-way ANOVA with Bonferroni post-hoc for cranial window measurements. $P<0.05$ was considered statistically significant.

Online Supplement References:

1. Joseph BK, Thakali KM, Pathan AR, Kang E, Rusch NJ, Rhee SW. Postsynaptic density-95 scaffolding of Shaker-type K^+ channels in smooth muscle cells regulates the diameter of cerebral arteries. *J Physiol.* 2011;589:5143-5152.
2. Bannister JP, Adebiyi A, Zhao G, Narayanan D, Thomas CM, Feng JY, Jaggar JH. Smooth muscle cell alpha2delta-1 subunits are essential for vasoregulation by $Ca_V1.2$ channels. *Circ Res.* 2009;105:948-955.
3. Albarwani S, Nemetz LT, Madden JA, Tobin AA, England SK, Pratt PF, Rusch NJ. Voltage-gated K^+ channels in rat small cerebral arteries: molecular identity of the functional channels. *J Physiol.* 2003;551:751-763.

Online Figure Legend

Online Figure I. Representative confocal images of isolated rat CA incubated with 3 μmol/L fluorescein-labeled Scm peptide for 1 min at 37°C. Three layers - adventitia (Ad), smooth muscle (SM), and endothelial cell (EC) – were imaged with DAPI as nuclear counter staining. Individual cVSMCs are vertically wrapping around the cerebral artery circumferentially while endothelial cells run horizontally.

Online Figure II. Representative confocal images of isolated rat CA incubated with 3 μmol/L fluorescein-labeled Scm peptide for 20 min at 37°C. Three layers - adventitia (Ad), smooth muscle (SM), and endothelial cell (EC) – were imaged with DAPI as nuclear counter staining. Individual cVSMCs are vertically wrapping around the cerebral artery circumferentially while endothelial cells run horizontally.

Online Figure III. A) Two different control peptides (Scm and Scm2) do not constrict CA significantly up to 10 μmol/L whereas K_V1-C peptide constricts CA at 3 μmol/L and 10 μmol/L concentrations (n=5 each). Both baseline (base) and peptide-treated (pep) diameters are shown in raw numbers. a, b: significant difference, P<0.05. **B)** KCl-induced constriction is not significantly different between Scm, Scm2, and K_V1-C peptide treatment groups. Replot of 10 μmol/L group from pane A along with diameter recordings after 60 mM KCl application. NS: not significantly different. *: significant difference from Scm and Scm2, P<0.05.

Online Figure IV. K_V1-C peptide disrupts association of PSD95 and K_V1.2 and constricts CA. Data from Figure 3D expressed in actual diameters. Diameter recordings from CA in response to 10 μmol/L peptide and 100 nmol/L of the specific K_V1 channel antagonist Psora4 (n=6 each). a, b: significant difference, P<0.05.

Online Figure V. Average % change in diameter of rat CA in response to 3 μmol/L Scm or K_V1-C peptide with or without pretreatment with 100 μmol/L L-NAME (L) and 10 μmol/L indomethacin (I) (n = 6). a, b: significant difference, P<0.05.

Online Figure VI. A) An example trace of a rat CA constricting in response to 1 μmol/L linopirdine in the presence of 3 μmol/L K_V1-C peptide. **B)** Average % change in diameter of rat CA in response to 1 μmol/L linopirdine (Lino) or 30 μmol/L Ba^{2+} in the absence (Without K_V1-C) or presence (After K_v1-C) of 3 μmol/L K_V1-C peptide (n = 5-9). NS: no statistical significance.

Online Figure VII. Time course of arterial diameter changes in response to K_V1-C peptide administered at either a concentration of 10 μmol/L (n=4) or 30 μmol/L (n=7) through a cranial window. Percent change in diameter from baseline. *: Significant difference from 10 μmol/L, P<0.05.

Online Figure VIII. A) Western blot comparing co-immunoprecipitation (co-IP) of PSD95 and K_V1.2α after a 3000g (3k) or 5000g (5k) centrifugation step during preparation of CA lysate. Lysate from each preparation was used as a control. The elution from a K_V1.2α immunoprecipitation column (K_V1.2 IP) displays a reduction in PSD95 after 5000g spin, suggesting protein dissociation. **B)** Western blot comparing K_V1.2α detection from supernatant of CA lysate after a 3000g (3k) centrifugation, dissolved pellet from 100,000g (100k), and 3000g supernatant boiled in 1% SDS solution (3k SB). Note that ~75 kD band becomes more prevalent in 100k compared to 3k samples whereas boiling (SB) shifts the detected band mostly to ~60kD.

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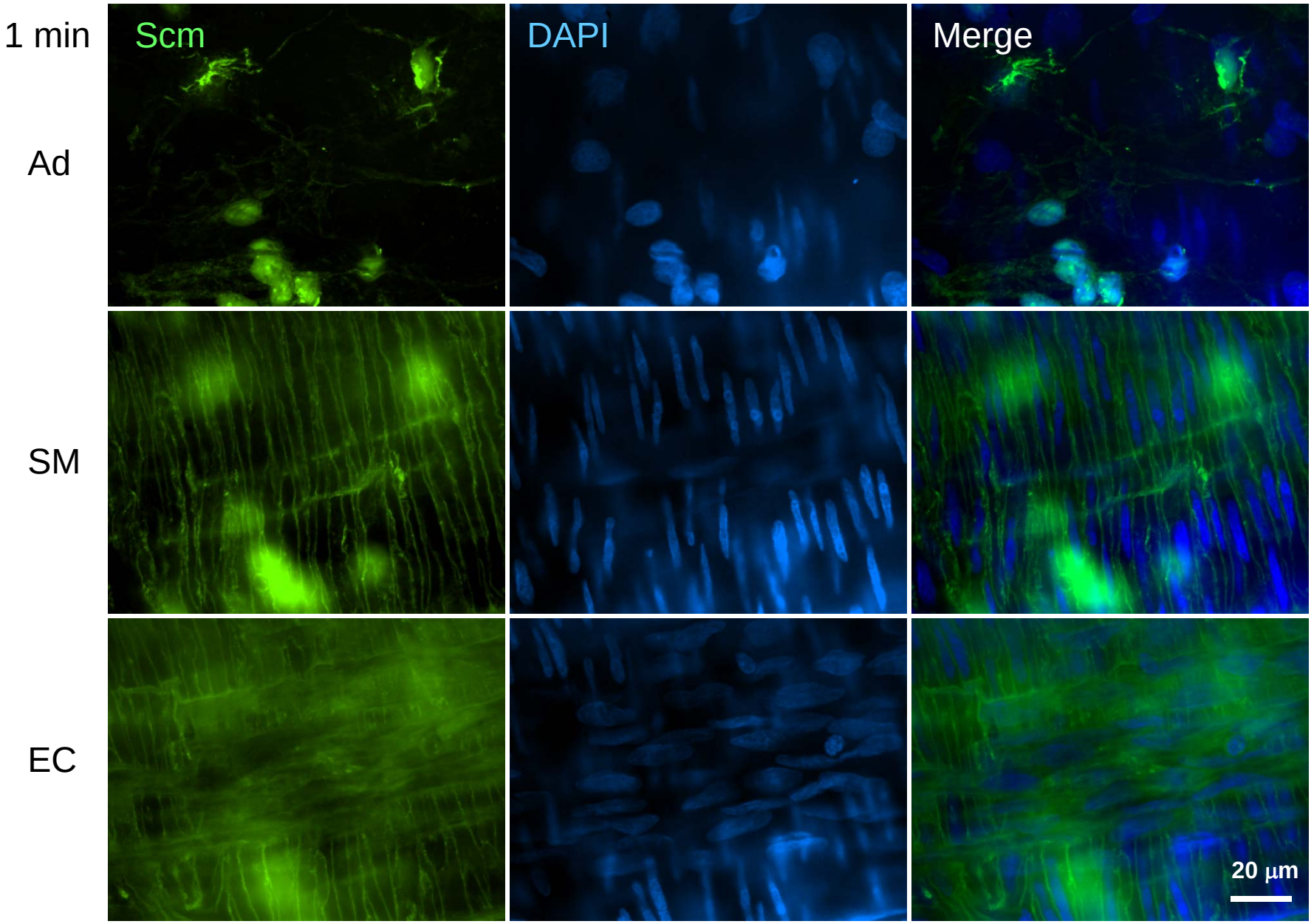

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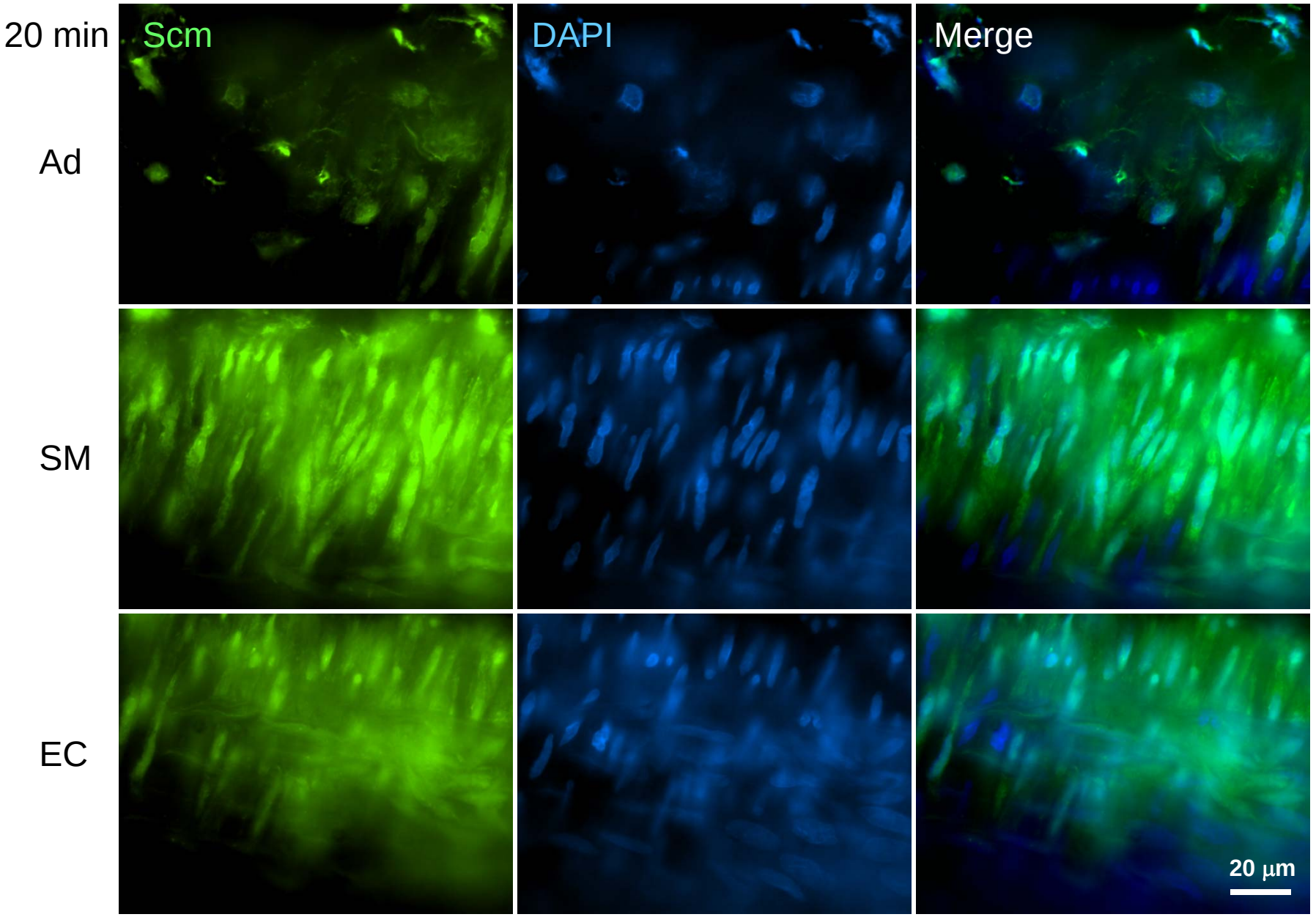

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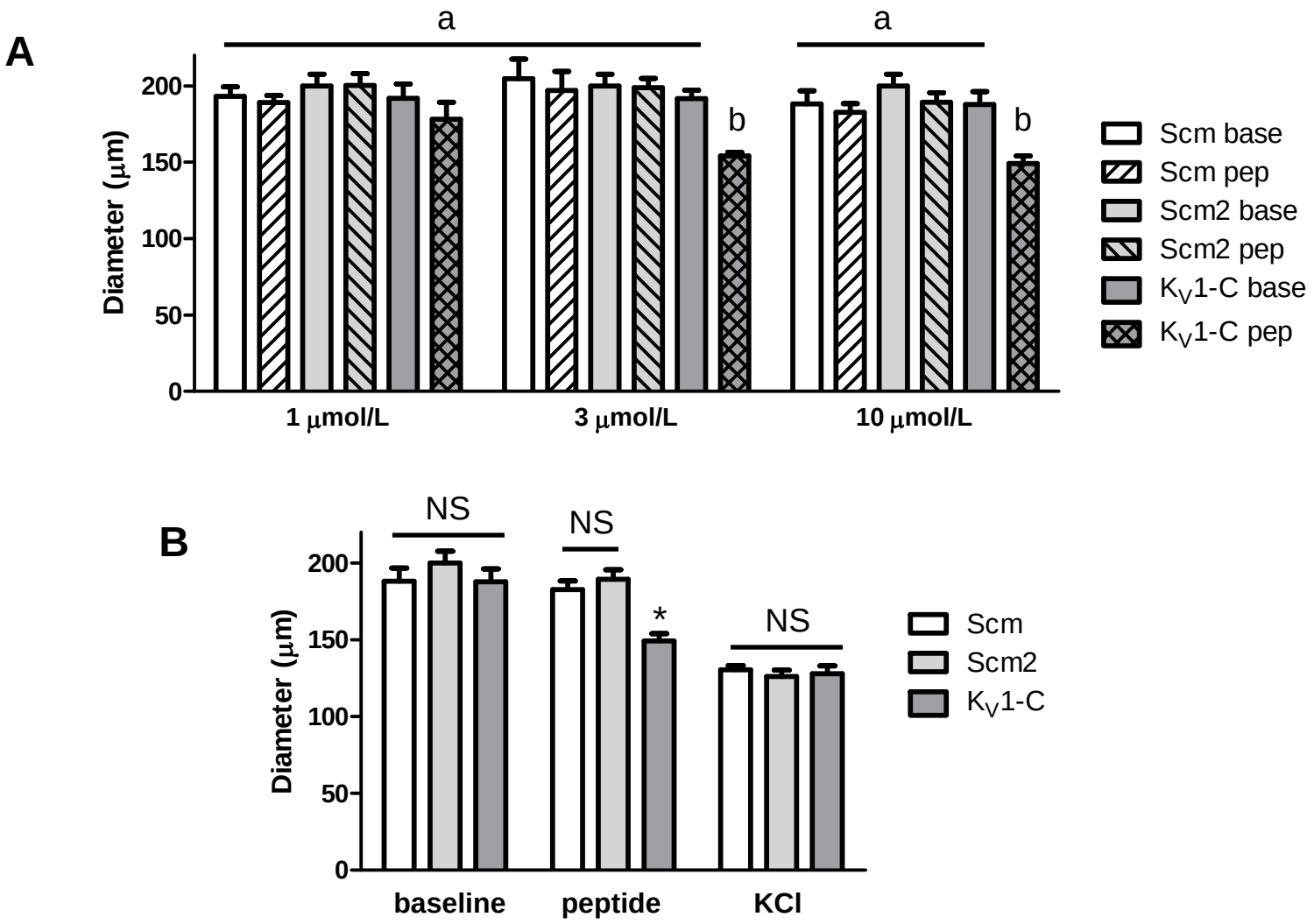

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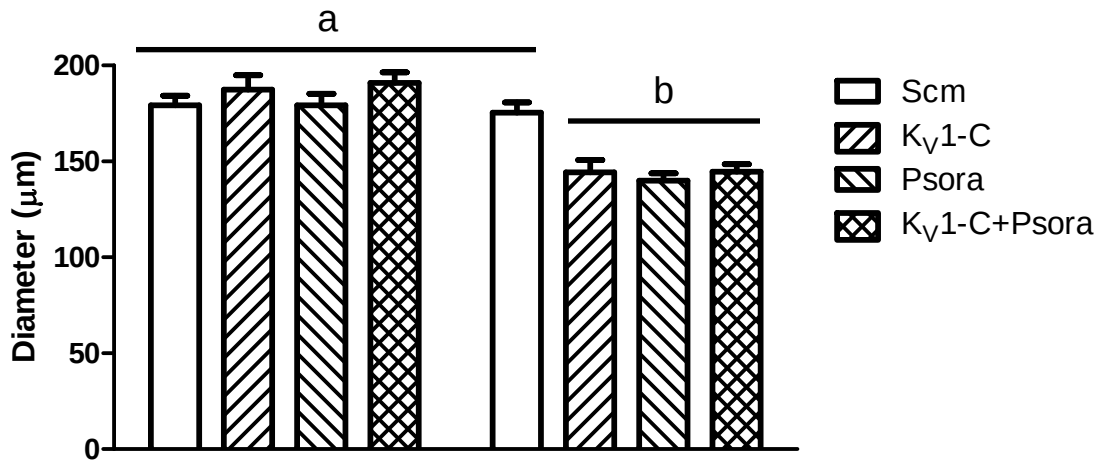

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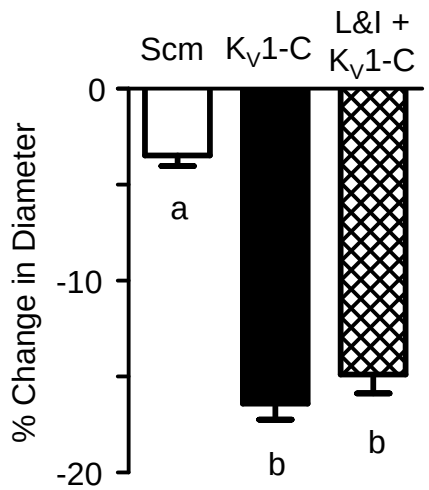

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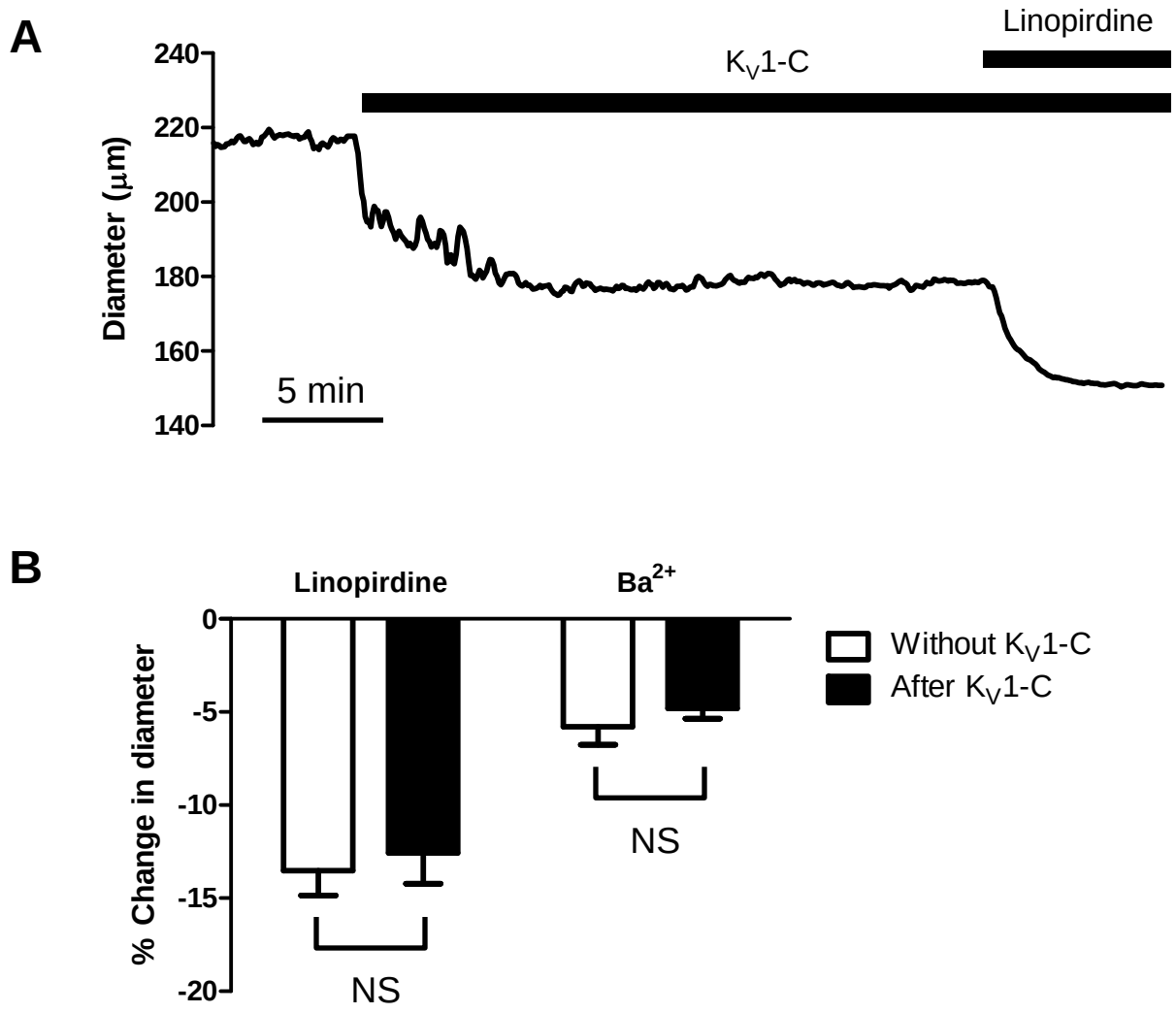

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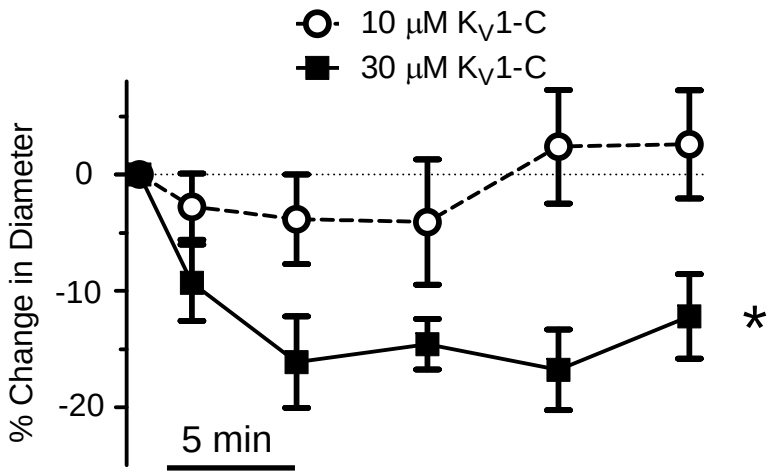

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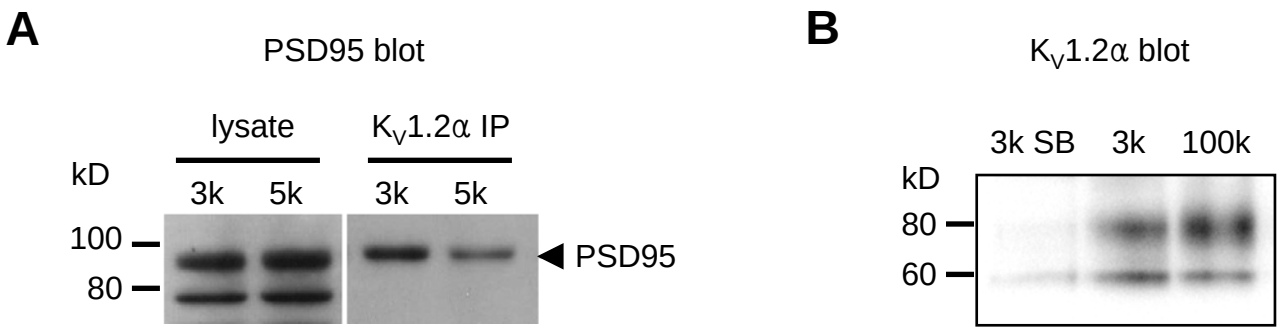

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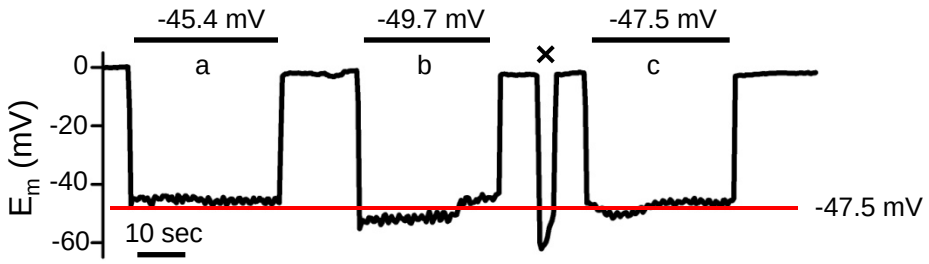

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