Material and Methods

Animals and tissue preparation

Transgenic mice ($IK1^{-/-}$ and $IK1^{-/-}/SK3^{T/T}$) and same-strain (c57BL/6) wild-type controls (WT) were bred and housed in the University of South Alabama College of Medicine vivarium. For $IK1^{-/-}/SK3^{T/T}$ mice, the $K_{Ca}2.3$ gene is overexpressed (~3-fold normal) in the absence of doxycycline, and is essentially abolished by 5 days exposure to doxycycline.[1] Mice (equal number male and female, 25-35 g) were euthanized with pentobarbital sodium (50 mg/kg), the abdomen was opened and small intestine collected. All animal procedures were approved by the University of South Alabama Institutional Animal Care and Use Committee, and carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Intestines were quickly removed and placed in cold (~4˚C) HEPES solution.

Confocal Ca^{2+} imaging and analysis

Mesenteric artery segments (~ 300 μm diameter) were dissected from intestine, cut open longitudinally, and pinned down on the surface of small silicone (sylgard) blocks with the endothelium facing up[2] using 14 μm diameter pins. Blocks were incubated at room temperature for 40 minutes in dark with Ca^{2+} indicator loading solution containing Fluo-4 AM (15 μM) and 0.06% Pluronic F-127 in HEPES solution (pH 7.45). After washing, blocks were placed with intima facing down in a glass-bottom chamber (separated 100 μm from glass by two

parallel supporting pins) containing HEPES solution. The chamber was mounted on an inverted microscope fitted with a PerkinElmer spinning disk RS-3 confocal unit. Ca^{2+}-dependent fluorescence (488 nm excitation, 510 nm emission) was measured at 8 frames/sec at 25˚C (20X objective) using Ultraview software. Experiments were performed at room temperature to avoid photobleaching and acute temperature change during drug additions. Images were saved as 16-bit raw data during recording, and later converted into 8-bit TIFF format for offline processing. Only recordings with > 90% of total viewable area in focus were processed and analyzed. Data were processed using a custom algorithm LC_Pro.[3] Implemented as a plug-in with ImageJ software, this software is specifically designed to: 1) detect sites of dynamic Ca^{2+} change above statistical ($p < 0.01$) noise, 2) define regions of interest (ROI; 5 pixel or 1.7 μm diameter) at active sites centers, and 3) analyze average fluorescence intensities at ROIs to determine specific event parameters. Fluorescence data are expressed as F/F_0, where F_0 is determined by a linear regression of base data at each ROI. Net lateral stretch placed on block-mounted vessels (~1.5 times resting width) was adequate to prevent folding of the lamina and is consistent with vascular distention by pressure based on previous experiments; a 300 μm diameter artery, opened and pinned at a width of 1.5 mm, is approximately equivalent to its circumference at 80 mmHg pressure. For drug addition protocols, recorded data segments corresponding to solution exchange (~ 4 seconds) were removed offline to avoid analysis artifact. Figure 1 shows auto-detection and tracking of basal endothelial Ca^{2+} events in an open mesenteric artery.

Reagents, materials and solutions

Reagents and ACh were purchased from Sigma-Aldrich (St. Louis, MO). Fluo-4 AM and Pluronic F-127 were purchased from Invitrogen (Carlsbad, CA). Tungsten wires (for making tiny pins) were purchased from Scientific Instrument Services (Ringoes, NJ). HEPES/bicarbonate-buffered physiological saline solution (PSS) contained (mmol/L): NaCl 130.0; $NaHCO_3$ 14.9; KCl 3.7; KH_2PO_4 1.2; $MgSO_4$ 1.2; $CaCl_2$ 1.6; Glucose 11.0; HEPES 10.0; pH 7.4.

Data Analysis

Data are presented as the mean ± standard errors of the mean and statistical analysis is performed with GraphPad Prism software. For multiple-set analysis, one-way ANOVA was performed followed by individual comparisons using the Tukey's post-hoc test. P values < 0.05 were considered significant. For non-Gaussian event parameters, distributions were modeled using a generalized linear mixed model and subsequent analysis of variance was performed using R statistical analysis software. Animal genotype and experimental replicates were used as model fixed effect and random effect factors, respectively, and implemented using the lme4 package in R. Positively skewed parameter distributions were fit using an inverse Gaussian function, and fixed effects were modeled with a logarithmic link function. Analysis of variance was performed using the ANOVA function for generalized linear mixed models from the "car" package in R. Pair-wise comparisons were made using Tukey's post-hoc test,

implemented in the “multcomp” package of R. P-values less than 0.05 were considered significant.

References

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