SUPPLEMENT MATERIAL I

METHODS

Cell isolation and culture

Smooth muscle cells from the superior cerebellar and posterior cerebral arteries of a male guinea pig (DH, ~500 g, euthanised by sodium pentobarbital overdose in accordance with the Animal (Scientific Procedures) Act UK 1986) were enzymatically isolated by incubation in isolation buffer (55 mM NaCl, 80 mM Na-glutamate, 6 mM KCl, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, 0.2 mM EDTA and 0.1 mM CaCl₂, pH 7.3, 34.5°C) plus 1.24 mg ml⁻¹ BSA, 2.2 mg ml⁻¹ type F collagenase and 1 mg ml⁻¹ hyaluronidase for 14 min, followed by a second incubation in isolation buffer plus 1.24 mg ml⁻¹ BSA, 1.7 mg ml⁻¹ papain and 0.7 mg ml⁻¹ dithioerythritol for 14 min. Tissue was washed 3 times in isolation buffer and then 3 times in the same solution without BSA. Individual myoctes were released by gentle trituration. Cells were stored at 4°C for use within 6 h or were diluted 1:9 in 50:50 Ham F-12 and Waymouth MB752 media supplemented with 10% FBS, 0.1 U/ml penicillin and 100 μg/ml streptomycin and grown on 13 mm coverslips in 24-well plates maintained at 37°C in humidified air (5% CO₂).

Intact artery preparation and culture

Sections of arteries were cannulated in an arteriograph (Living Systems Instrumentation, St. Albans, VT, USA) containing oxygenated Krebs solution (118.4 mM NaCl, 25 mM NaHCO₃, 1.13 mM NaH₂PO₄, 4.7 mM KCl, 11.2 mM glucose, 2.7 mM CaCl₂, 1.2 mM MgCl₂, bubbled with 95% O₂/5% CO₂) and then allowed to equilibrate in the dark in oxygenated Krebs solution plus 100 nM tetramethylrhodamine ethyl ester perchlorate (TMRE) at 36°C for 30 min at an intraluminal pressure of 20 mmHg. Intraluminal pressure was then raised to 40 mmHg for the duration of the experiment. All intact artery experiments were at 36°C and Krebs bubbled with 95% O₂/5% CO₂ throughout. In other experiments, sections of arteries 1-2 mm in length were placed in culture media as above and maintained at 37°C in humidified air (5% CO₂) for up to 4 days prior to cannulation. Pressurisation, loading with TMRE and imaging as for fresh arteries. For myogenic tone measurement, sections of arteries were allowed to equilibrate with 10 μM Fura2-AM for 2-3 hr at room temperature in incubation buffer (without BSA or enzymes) prior to
cannulation and pressurisation in oxygenated Krebs solution in a custom-made arteriograph.

**Fluorescence imaging of cells and intact arteries**

Freshly-isolated cells were loaded with 100 nM TMRE in isolation buffer (20 min, 22°C) or mitotracker green (100 nM for 15 min) then settled onto a coverslip before being washed into extracellular solution (80 mM Na glutamate, 40 mM NaCl, 20 mM TEA-Cl, 1.1 mM MgCl₂, 3 mM CaCl₂, 10 mM HEPES, 30 mM glucose, 100 nM TMRE, pH 7.4). Prior to imaging cells grown in culture conditions on 13 mm diameter coverslips, media was replaced with isolation buffer plus 100 nM TMRE (20 min, 37°C). A coverslip was then removed and placed cell-side uppermost on top of a 24 x 50 mm No. 0 coverslip, which was then attached to an open imaging chamber filled with extracellular solution.

Cells and arteries were imaged on an inverted epifluorescence microscope (Nikon TE2000U) with a 100x 1.3 NA S-Fluor oil objective plus internal 1.5x convertor lens. The output of a xenon arc lamp monochromator system (PTI Inc, 560 ± 5 nm) guided via an optical light guide, through a field stop diaphragm and a ND4 filter before being reflected off a long-pass dichroic mirror (Chroma) reflective from 550-570 nm and transmissive >580 nm to illuminate the field. Emitted light was collected through the objective, dichroic and a barrier filter and imaged by a Photometrics Cascade 512B camera (Roper Scientific) controlled by EasyRatio pro software (PTI Inc)¹,². Images were acquired at 10 or 30 Hz (100 or 30 ms exposure) for 90 s only (to minimise photo-toxicity) unless otherwise indicated. Imaging experiments on cells were carried out at room temperature and 37°C (using a microscope stage top incubator (TokaiHit INUB-ONICS-F1)).

**Image Analysis**

Images were analyzed using either Metamorph 7.1.3 (Molecular Devices), ImagePro 7..0 (MediaCybernetics) or the MotionStudio³ package. In the case of analysis with Metamorph, images were background subtracted and smoothed using a 9 frame rolling average. The number of moving mitochondria observed over 60 s within a full-frame image stack was manually counted and corrected for mitochondrial area or percentage of intact artery image that was in focus. For intact arteries, an estimate of the number of cells containing mobile mitochondria was also
MotionStudio measures the motion tracks of mitochondria to sub-pixel accuracy (typically ~10 nm) using an image correlation based approach. Briefly, individual mitochondria are selected in the first frame of a video sequence and the organelle image is used by the software to create a template. Image correlation is then applied to find the location of this template within the next frame and the template image then updated to be that of the object in the second frame. The template finding and updating process is repeated for all frames to build up a track of particle positions. Refreshing the template image in each frame accommodates gradual changes in the image of the mitochondria, caused by morphological changes, changes in pointing direction of non circular objects and photobleaching. Further analysis was performed including measuring the instantaneous speed and the time-variant displacement of the mitochondria. Displacement was defined as the absolute distance between a mitochondria's position at a given time and its location in the first frame. The instantaneous speed of the mitochondria was computed by measuring the rate of change of position over 1 second intervals. This interval was chosen as one long enough to smooth the effects of measurement noise but short enough to accurately determine the speed without averaging out brief, directed motion events.

**Measurement of mitochondrial area**

Cerebral artery smooth muscle cells maintained in culture for 4 days in the presence of 10 μM Mdivi-1 or vehicle control (0.1% DMSO) were loaded with 100 nM TMRE (20 min, 37°C) and imaged as above. Regions of contiguous mitochondria were defined as regions displaying synchronous increases or decreases in TMRE fluorescence. Images were analyzed using Metamorph, background was subtracted and images smoothed using a 9 frame rolling average. Transient localised differences were highlighted by subtracting a duplicate image stack that was offset by 5 frames (and hence 0.5 s), applying either an inclusive threshold (to detect increases) or exclusive threshold (to detect decreases) and measuring the area of localised change.

**Western blotting**

Intact arteries were frozen in liquid N₂ immediately following dissection or after maintenance in culture. 6 vessels were pooled for each timepoint, homogenised in 50 μl CellLyticM solution (Sigma) using Eppendorf-tube pestles, which were then rinsed with an additional 30 μl solution. Homogenates were incubated at room
temperature for 15 min before centrifugation at 13,000 rpm at 4 °C for 20 min. Supernatants were immediately frozen at -20 °C or mixed with 4x reducing sample buffer and separated by standard SDS-PAGE on a 12% gel, transferred to nitrocellulose membrane then blocked with 5% BSA for 1 h, incubated with primary antibodies: monoclonal mouse-anti-mitofusin-2 (ab56889, Abcam, 1:500), polyclonal rabbit-anti-PCNA (ab2426, Abcam, 1:500), monoclonal mouse-anti-mitochondrial cytochrome oxidase IV (ab, Abcam, 1:1000), or polyclonal rabbit-anti-GAPDH (ab9485, Abcam, 1:500) overnight at 4 °C or at room temperature for 1 h; incubated with 1:80,000 HRP-conjugated anti-mouse or anti-rabbit secondary antibody for 1 h at room temperature then visualized using ECL.

**Immunocytochemistry**

Arteries were cannulated, pressurised to 40 mmHg and fixed in 10% formal basic solution for at least 30 min prior to removal to individual 1.5 ml tubes. Whilst in the 1.5 ml tubes the arteries were permeabilised with 0.5% Triton X-100 and blocked with 2% BSA for 1 hr before incubation with rabbit anti-PCNA (ab2426, Abcam, 1:50) overnight at 4°C and then anti-rabbit-Alexa 488 (Invitrogen, 1:100) plus mouse-anti- α-actin-Cy3 conjugate (Sigma, 1:100) for 1 h prior to mounting the arteries in Vectashield containing DAPI. Fluorescence images were captured on a Leica SP5 confocal inverted microscope with 40x oil objective, no digital zoom applied and constant illumination and gain settings throughout. Images were analyzed using Metamorph 7.1.3 to select smooth muscle α-actin positive regions and measure total PCNA staining within these regions.

**[^3H]-Thymidine incorporation assay**

Cells were grown to ~70% confluency in 12 well plates, quiesced for 24 h in media containing 0.1% FBS then returned to media containing 10% FBS for 24 h.[^3H]-Thymidine (9.25 kBq) was added to each well for the final 6 h of this 24 h treatment period. Cells were washed with ice cold PBS followed by 10% trichloroacetic acid then by 10% sodium dodecyl sulfate in 0.2 M sodium hydroxide and radioactivity was quantified by liquid scintillation (Packard 1500 Tri-carb or ScintSafe3).
FACS analysis

Cells were grown to ~70% confluency in 6 well plates, harvested, resuspended in PBS and fixed in ice-cold 70% ethanol (added dropwise while vortexing to ensure proper fixation of cells and prevent clumping) at 4°C overnight. Cells were then washed with PBS, incubated with 50 μg/ml RNase A for 1 h at 37°C and stained with 40 μg/ml propidium iodide. Cell cycle profiles were acquired by flow cytometry (BD FACSDiva software; BD Biosciences) and analyzed using the software FlowJo.

Statistical analyses

Other than Figures 5a and 6, a parametric distribution of raw data around mean values was confirmed prior to statistical testing. Mean values ± standard error (s.e.) are shown (unless otherwise indicated) and were analysed using unpaired Student’s t-tests or, for more than 2 groups, ANOVA with Games-Howell post-hoc comparison of variance (for samples with unequal variance). α was set to 0.05, P values < 0.05 were considered significant. The median mitochondrial areas with and without Mdivi-1 treatment were compared using a Mann-Whitney U-test. The normalised data in Figure 6 was analysed using Kruskal-Wallis tests with P values adjusted for multiple testing. t-test were carried out in Microcal Origin v6.0, with Bonferroni considerations applied for cell cycle data, all other tests in SPSS 19 for Windows.

Chemicals, drugs and enzymes

Papain was from Worthington; TMRE, mitotracker green, F-12 and Waymouths’ media and FBS from Invitrogen; 100x Pen/Strep from PAA Cell Culture Company; Mdivi-1 was from Enzo Life Sciences. Collagenase, hyaluronidase, BSA and all other chemicals were from Sigma.

References

2. McCarron JG, Olson ML, Currie S, Wright AJ, Anderson KI, Girkin JM. Elevations of intracellular calcium reflect normal voltage-dependent behavior, and not constitutive activity, of voltage-dependent calcium channels in

