Supplement data

**Measurement of calcium with Fura-2/AM**

*Fluorescence calcium imaging*

No technique was available to measure intracellular calcium changes in smooth muscle and endothelial cells in the perfused afferent arterioles during contraction. Therefore we developed a high resolution, high speed yet simple imaging system for the perfused afferent arteriole. Rabbits were killed and kidneys removed. Slices (1 mm) were placed in ice cold DMEM with 0.5% BSA and afferent arterioles were dissected under a stereomicroscope. Tubular segments were removed and a cortical afferent arteriole was isolated and cut from the vascular tree. The afferent arteriole was transferred to a thermoregulated chamber mounted on an inverted microscope (Zeiss Axiovert 35, Oberkochen, Germany). The afferent arteriole was mounted and perfused with concentric glass pipettes attached to a motorized track system (Luigs and Neumann, Ratingen, Germany). With a negative pressure inside the holding pipette (tip diameter 35-40 μm) it was possible to suck the arteriole into the pipette and at the same time to perfuse it through the perfusion pipette (tip diameter 7-10 μm). Once the vessels had stabilized for 30 min at 37°C, they were challenged with isoosmotic K⁺ (100 mM) to assure viability.

Optical techniques were used to measure intracellular Ca²⁺ ([Ca²⁺]i) in smooth muscle and endothelial cells using the fluorescent probe Fura-2/AM (Molecular Probes). [Ca²⁺], was measured by using a Ca²⁺ imaging system (TILL Photonics GmbH, Gräfelfing, Germany). Fura-2/AM (8 x 10⁻⁷ M) was applied to the abluminal side of the mounted and perfused afferent arteriole for 45 min (37 °C) followed by 20 min of washout to ensure proper deesterification. Afferent arterioles were
visualized by excitation at 380 nm using the TILL Photonics Polychromator IV and Zeiss X40 (0.75 NA w) objective. With overlay of transmission image it was possible to differentiate endothelial and smooth muscle cells (Fig. S1).

Full frame images were collected at both excitation wave lengths (340 and 380 nm) using a 510 nm long pass filter. The images were collected at a frame rate of 2 Hz during stimulation and 0.5 Hz during washout. The 380 nm image collection was used to simultaneously measure luminal diameter changes. Ratio (R) images were generated by TILLVision Software (TILL Photonics GmbH) and analyzed by tracking smooth muscle or endothelial cells during contraction and relaxation. The ratio images are kept as integrated buffers (x 1000, scaled ratio). Camera offset is 40 pixels. However, secondary threshold was used for background corrections. The vessels were always focused in the midplane. Intracellular free Ca\(^{2+}\) concentration, \([\text{Ca}^{2+}]_i\), was measured according to Grynkiewicz et al. (1986): \([\text{Ca}^{2+}]_i = K_D \cdot \beta \cdot (R - R_{\text{min}})/(R_{\text{max}} - R)\), where \(R_{\text{min}} = 0.1\), \(R_{\text{max}} = 1.6\), and \(K_D = 910\) nM, \(\beta = 2.7\) as determined by standard calibrations in this system.

**External standard calibration**

The fura-2 calcium imaging calibration kit from Molecular Probes was used. The calibration kit contained 1 mL each of 11 pre-diluted 10 mM K\(_2\)EGTA/CaEGTA buffers containing 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 mM CaEGTA (free Ca\(^{2+}\) ranging from 0 µM to 39 µM). All the solutions contained the Ca\(^{2+}\) indicator fura-2 at 50 µM plus 100 mM KCl and 30 mM MOPS, pH 7.2. In addition, 15 µm–diameter polystyrene beads in suspension at 16,000 beads per mL were added. The beads served as coverslip spacers and focusing aids. A twelfth buffer, identical to the 10.0 mM CaEGTA standard but without fura-2, served as a control for background fluorescence. A full spectrum was obtained and \(K_D\) was calculated (Fig. S2). Internal calibration of Fura-2 with ionomycin (Sigma) was tried but was not successful.
Selection of ROI/AOI

A characteristic of images collected with a wide-field fluorescence microscope is that at some level the image becomes blurred. Blurring can arise from two sources: 1) contributions of out-of-focus light to the focal imaging plane and 2) diffraction. Large scale blurring is the result of light that is reflected and/or emitted from objects above and below the focal plane.

At first look, it seems impossible to measure Fura-2 inside moving smooth muscle cells during contraction with a wide-field fluorescence microscope. The cells are to some extent moving in and out of the focal plane. However, in the perfused afferent arteriole it is possible to select a region of interest (ROI) or area of interest (AOI) that stays in the focal plane during contraction (Fig S3). This is true for both smooth muscle and endothelial cells. Defining the geometric relations as in Fig. S3 made it possible to program a macro, where the ROI could trace the moving smooth muscle cell during contraction. It is more precise to follow the edge-midplane, see the kinetic differences in fig. S4. There were no differences between manual measurements (frame by frame) and the macro-measurements.

Contraction of the afferent arteriole is relatively slow (seconds). No pixel-shifts were observed between the F380 image and the F340 image when image grabbing time delays were less than 110 ms.

Legends

Fig. S1
Isolated perfused afferent arterioles. Overlay of transmission and fluorescence image (ex. 380nm) made it possible to distinct between smooth muscle cells and endothelial cells. A: Arteriole with Fura-2 loading of the smooth muscle cells. B, Arteriole where predominantly the endothelial cells
were loaded. C: Arrows indicate the basal membrane separating the smooth muscle cells and endothelial cell layers. The overlay was done by Photoshop CS Software (Adobe Systems Europe Ltd, UK).

**Fig. S2**

Spectral analysis of fura-2 in extracellular low calcium (A, 0.04 μM), high calcium (40 μM) and medium calcium (1.4 μM). D, Spectral response of fura-2 in 0-40 μM CaEGTA buffers using a calcium calibration buffer kit was measured. Calibration curve of fura-2 with calcium calibration buffer kit generated by the protocol descriped. As a double log plot, the Ca$^{2+}$ response of the indicator is linear with the x-intercept being equal to the log of the apparent KD of the indicator (910 nM from this data).

**Fig. S3**

Theoretical selection of region of interest (ROI, red circle) in the perfused afferent arteriole. A-E, different projection planes of the smooth muscle cell layer (SMC-L) during relaxation (A,D), medium contraction (B) and full contraction (C,E) of the afferent arteriole. (F) Scaled (F340/F380) images (0s, 20s, and 60s). Focal plane (FP).

**Fig. S4**

Edge tracking versus window tracking. A-D, Different time captures of ratio images. Red boxes indicate edge-tracking areas, and white boxes indicate areas used for window-tracking. The images correspond to a,b,c,d in figure E, where results from edge-tracking is shown in red, and window-tracking in black.
Movies

**Movie 1** (http://www.medkid.com/science/m1.html)

Addition of 100 mM KCl

*Top;* Transmission movie. Realtime movie length 2 min

*Bottom;* Pseudocolor movies grabbed at F340, F380 and calculated ratio movie. Realtime movie length 1 min.

**Movie 2** (http://www.medkid.com/science/m2.html)

*Left;* Addition of 100 mM KCl. Calculated ratio pseudocolor movie. Realtime movie length 1 min.

Note the ratio increase in smooth muscle cells followed by a ratio increase in endothelial cells.

*Right;* Addition of 1 μM acetylcholine. Calculated ratio pseudocolor movie. Realtime movie length 1 min. Note the rapid ratio increase in endothelial cells but no ratio increase in the smooth muscle cells.
Log $[\text{Ca}^{2+}]$ free (M)

-8.0 -7.5 -7.0 -6.5 -6.0 -5.5 -2.5 -2.0 -1.5 -1.0 -0.5 0.0 0.5 1.0

Log $K_D$

Fura-2

Log [(R-R_{min})/(R_{max}-R) \times \beta]$

D

Figure S2
Figure S3