Supplemental Material

The AMP-related kinase induces Ca\(^{2+}\)-independent dilation of resistance arteries by interfering with actin filament formation

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Supplemental Methods

Isolation and cannulation of resistance-type arteries. All animal care and experimental protocols were conducted in accordance with German federal animal protection laws. Male C57BL6/N mice were purchased from Charles River Laboratories (Sulzfeld, Germany). LifeAct mice were kindly provided by Reinhard Fässler (MPI of Biochemistry, Martinsried) and Eloi Montanez (Biomedical Center, Martinsried, Germany). The mice were killed by cervical dislocation, the mesentery was removed and pinned onto a silicon-coated petri dish for subsequent vessel dissection. Fine dissection of mesenteric arteries was performed in ice-cold MOPS buffer. Isolated arteries were then cannulated as described before\(^1\).

\([\text{Ca}^{2+}]\) and diameter registration. The isolated arteries were treated as published before\(^1\). Briefly, the setup was transferred to the stage of a modified inverted microscope (Diaphot 300, Nikon, Düsseldorf, Germany) equipped with a 20x lens (D-APO 20 UV / 340, Olympus) and a video camera (Watec, WAT-902B). The organ bath temperature was slowly raised to 37 °C. The transmural pressure was hydrostatically set to 60 mmHg. The smooth muscle layer was loaded with the Ca\(^{2+}\) indicator Fura2-AM (2 μM, LifeTechnologies, Carlsbad, CA, USA) in an incubation period of 90 minutes. The measured Fura2 signals were corrected for background fluorescence (as measured at the end of each experiment after quenching with 8 mM MnCl\(_2\) ).
Simultaneously with the measurement of cytosolic Ca\textsuperscript{2+} concentrations \([\text{Ca}^2+]_i\), vascular outer diameters were recorded by videomicroscopy (Hasotec, Rostock, Germany).

For the construction of Ca\textsuperscript{2+} sensitivity curves, the arteries were first placed in Ca\textsuperscript{2+}-free MOPS buffer ([Ca\textsuperscript{2+}]_o = 0) with normal extracellular potassium concentration (4.7 mM) at a transmural pressure of 60 mmHg. Then the potassium concentration was elevated to 125 mM at zero [Ca\textsuperscript{2+}]_o. Subsequently, [Ca\textsuperscript{2+}]_o was elevated stepwise to 0.5, 1, 2 and 3 mM and the resulting changes in [Ca\textsuperscript{2+}]_i while the related constrictions were recorded at a frequency of 5 Hz\textsuperscript{2}. Then the whole procedure was repeated again in the presence of AMPK activators (A769662 [A76], 100 µM or PT1, 30 µM). Finally, the activators were washed out for 30 minutes and the [Ca\textsuperscript{2+}]_o elevation was repeated as in step one.

In a further step we investigated the kinetics of the vasodilation caused by PT1 and A76. Therefore mouse arteries were similarly pre-incubated in a stepwise manner ([Ca\textsuperscript{2+}]_o protocol) in standard MOPS, followed by a normal potassium (4.7 mM) zero [Ca\textsuperscript{2+}]_o, MOPS, a high potassium (125 mM) zero [Ca\textsuperscript{2+}]_o (each for five minutes) and eventually constricted with MOPS containing 125 mM potassium and 0.5 mM [Ca\textsuperscript{2+}]_o. Five minutes after the high potassium-induced constriction PT1, A76 or DMSO (vehicle) were added while Fura2 signal and corresponding diameter values were recorded continuously.

**Cytoskeletal phosphoprotein array.** Pig femoral arteries were kindly provided by Rabea Hinkel. After careful dissection of the surrounding connective tissue, arteries were treated with PT1 or DMSO in a sequence of buffers as described for the construction of Ca\textsuperscript{2+} sensitivity curves, flash frozen liquid nitrogen and homogenized with a motor-driven TissueRuptor (Qiagen, Hilden, Germany) in RIPA buffer with the following composition: NaCl 150 mM, Tris-HCl 50 mM at pH = 8, Triton X-100 0.1 %, Na\textsuperscript{+} deoxycholate 0.5 %, SDS 0.1 %, EDTA 5 mM with protease inhibitor cocktail containing: AEBSF 500 µM, antipain 14.7 µM, aprotinin 0.77 µM, leupeptin 10 µM, NaF 500 µM and Na\textsubscript{3}VO\textsubscript{4} 500 µM. Protein purity was assessed by absorption at 280 nm and lysate purity improved as outlined in the manufacturer’s instructions (Antibody Microarray User’s Guide, Full Moon Bio, Sunnyvale, CA, USA). The array was conducted by tebu-bio (Le Perray en Yvelines, France) on behalf of Full Moon BioSystems, Inc.

**Western blot of whole arteries.** For the detection of G-actin small arteries (220 µm) stretched to a length of 3 mm (unloaded 2 mm) were homogenized with a glass pestle (Kimble Chase LLC, Tissue Grinder Micro PKG/6, art. No. 885470-0000) in actin-stabilizing buffer from a commercially available kit (G-actin / F-actin In Vivo Assay Kit Cat. # BK037, Cytoskeleton, Denver, CO, USA), flash frozen in liquid nitrogen and stored until further processed as described elsewhere\textsuperscript{3}. G-actin levels were normalized to GAPDH.
For the detection of pMLC$_{20}$, pMYPT1 (T696), pMYPT1 (T853) and β-actin mesenteric arteries were cannulated, pre-constricted by 125 mM K$^+$ at 0.5 or 3 mM extracellular Ca$^{2+}$ in the absence or presence of 30 µM PT1, snap-frozen in 15 % trichloroacetic acid (TCA) in acetone (w/v), and processed as previously described$^4$.

For technical reasons, MLC$_{20}$ Ser$_{19}$ phosphorylation was expressed relative to actin rather than to MLC$_{20}$ total which would have allowed to analyze the relevant ratio of both proteins. To exclude that a change in MLC$_{20}$ phosphorylation might be masked if PT1 affected the expression level of MLC$_{20}$, we normalized MLC$_{20}$ total to myosin heavy chain from the same gel. This ratio was not affected under our experimental conditions.

Mesenteric and tail arteries were permeabilized with α-toxin (5 U/µl) in a EGTA (free [Ca$^{2+}$] pCa = -log [Ca$^{2+}$] >8) and ATP-containing buffer (relaxing solution) as described earlier$^4$. After permeabilization a submaximal contraction was elicited by incubating the vessels for 20-25 min in contraction solution (pCa 6.99 for mesenteric arteries and pCa 6.1 for tail arteries) in presence of either 30 µM PT1, 10 µM Y27632 or 0.03 % DMSO and fixed in dry ice precooled 15 % TCA/acetone slurry for 3 h. The arteries were then washed with acetone and dried. After homogenization an equal volume from each sample was loaded in the gel slots. The proteins were then transferred to nitrocellulose and incubated with the respective antibodies as in Lubomirov et al$^4$.

To assess cofilin (serine 3) phosphorylation pooled small arteries of a murine mesentery were isolated as a coherent arterial tree originating from the superior mesenteric artery. The superior mesenteric artery was cannulated on one side and thus the whole arterial tree was flushed free from intraluminal blood. Then the sample was subdivided into two groups of approximately equal cumulative artery length. Then they were transferred to fresh MOPS buffer, heated to 37 °C and stimulated in accordance with the aforementioned protocol with 125 mM potassium and 0.5 mM Ca$^{2+}$ MOPS containing either PT1 or DMSO. After that, the arteries were flash frozen in liquid nitrogen and processed as previously described$^4$ with the additional use of phosphatase inhibitors NaF (500 µM) and Na$_3$VO$_4$ (500 µM) in the lysis buffer.

**Near-infrared Western blot.** Lysates from PT1- or A76-treated arteries and PCASMCs were separated via PAGE (#456-1093, Bio-Rad, Munich, Germany) and transferred to a PVDF membrane (#1704156, Bio-Rad, Munich, Germany). The membrane was blocked with 5 % BSA in TBST. Afterwards, it was incubated with REVERT Total Protein Stain from LiCor (LI-COR Biosciences, Bad Homburg, Germany) for 5 minutes, a mixture of anti-phospho-cofilin and anti-total-cofilin or anti-phospho-LIMK1/2 and anti-LIMK2 antibody at 4 °C over night. In an additional step for the beta-actin/whole protein quantification the REVERT Total Protein signal was detected at 700nm with a LiCor Odyssey CLx system. Afterwards, the stain was
removed and the membrane re-incubated with beta-actin antibody at 4 °C over night. After incubation with the primary antibody, each membrane was washed and incubated with a mixture of a goat anti-mouse (IgG IRDye 680RD goat anti mouse) and/or a goat anti-rabbit secondary antibody (IgG IRDye 800CW goat anti rabbit, both LI-COR Biosciences, Bad Homburg, Germany), depending on the source of the primary antibody. After washing the membrane was dried and scanned. The secondary antibodies were detected by excitation at 700 and 800 nm, respectively. Quantification was performed using Image Studio Lite version (LI-COR Biosciences, Bad Homburg, Germany).

**Co-immunoprecipitation.** Cells were grown in 10 cm culture dishes until 80 % confluence. Medium was removed and the cells were kept in MOPS buffer for 1 h to equilibrate. Then they were stimulated for 15 min with MOPS + 0.03 %DMSO or 30 µM PT1 followed by 125 mM KCl plus DMSO/PT1 for 20 min. Cells were then lysed in a buffer of the following composition: 150 mM NaCl 150, 50 mM TrisHCl (pH = 8.0), 1% Triton X-100 in water plus protease and phosphatase inhibitors (AEBSF 500 µM, antipain 14.7 µM, aprotinin 0.77 µM, leupeptin 10 µM, NaF 500 µM, Na₃VO₄ 500 µM). Lysates were incubated over night at 4°C with a polyclonal pan-14-3-3 antibody and with μMACS Protein A MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The mixture was then loaded onto MACS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany) and processed according to the manufacturer's instructions. Lysates, precipitates and flow-through samples were subsequently quantified by Western blot.

**Cell culture and siRNA transfection.** Human Umbilical Artery Smooth Muscle Cells (HUASMCs, Lonza, Verviers, Belgium) or Porcine Coronary Artery Smooth Muscle Cells (PCASMCs, Sigma Aldrich, Deisenhofen, Germany) were cultured according to the provider's instructions in a humidified chamber with ambient conditions of 95 % O₂ and 5 % CO₂. Different smooth muscle cell medium for HUASMC and PCASMC (HUASMC: Skeletal Muscle Cell Basal Medium, CC-3161, Lonza plus SmGM BulletKit, C-3182, Lonza; PCASMC: Porcine smooth muscle cell growth medium, P311_500, Sigma Aldrich)

**AMPK knockdown.** SiRNA against both AMPK α subunits (sc-45312) or control siRNA (sc-37007) (Santa Cruz, Heidelberg, Germany) was transfected into PCASMC cells using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher, Waltham, MA, USA) according to manufacturer's protocol. Cells grown in six well plates were transfected in Opti-MEM medium for 4 hours. Thereafter, 4 ml medium was added on top. After 3 days of culture, cells were stimulated with MOPS buffer plus DMSO or PT1 for 15 minutes and 125 mM KCl plus DMSO or PT1 for 20 minutes. Protein was harvested by adding 150 µl of ice cold RIPA buffer (NaCl 150 mM, Tris/HCl 50 mM pH = 8, Triton X-100 0.1 %, Na deoxycholate 0.5 %,
SDS 0.1 %, EDTA 5 mM) with protease and phosphatase inhibitors in each well and subjected to conventional Western Blot.

**Immunofluorescence.** Cannulated and pressurized or isometric arteries were fixed for one hour with 3.7% formaldehyde. Thereafter, the vessels were permeabilized for 30 minutes with 0.5% Triton X-100, blocked with 1% BSA in phosphate-buffered saline with divalent cations (PBS+) followed by incubation with the respective dyes for 2h or the indicated antibodies overnight. After thorough washing with PBS+, 3 µm transmural image stacks at three areas along the vessel length were obtained in each vessel using Leica TCS SP5 confocal microscope (Leica Microsystems). Images were analyzed in a blinded manner with Fiji ImageJ Software. For F/G-ratio analysis the mean grey values of 5 random cytosolic areas of 3 µm z-stacks (0.3 µm slice distance, maximum intensity projection) of the phallloidin as well as the corresponding DNAseI images were calculated.

Profiles of Life-Act intensity through individual VSMCs in the wire myograph were analyzed according to Flavahan et al.\(^5\). Therefore, time lapse images of the same vessel area (61.51 µm x 61.51 µm) were recorded after stepwise application of high potassium (125 mM, 10 minutes incubation time), PT1 (30 µM, 60 minutes incubation time) and compound C (15 µM, 30 minutes incubation time), respectively. A line perpendicular (y-axis) to the long axis of the VSMC (x-axis) was drawn through individual VSMCs. The edges of the cell were defined as the first and final peaks in relative Life-Act intensity with normalization to the maximum value of each cell. The width of the VSMC was normalized and calculated as the distance between the two peaks with the first peak being defined as 0 and the final peak as 1. Curves were plotted with an additional 0.2 margin of normalized Life-Act intensity distance on either side. Area under the curve (AUC) was determined with a SigmaPlot-Plugin for each cell and used for statistical evaluation via One Way Repeated Measures ANOVA followed by Holm-Sidak method.

To investigate morphological changes in cytoskeletal architecture caused by AMPK HUAMSC were cultured and seated on 8 well µ-slides (Ibidi, Martinsried, Germany) coated with poly-L-lysine (6 µg/cm\(^2\)) (Biochrom, Berlin, Germany) for 24 hours. Adherent HUASMC were incubated either with DMSO, PT1, LIMKi3, jasplakinolide or compound C + PT1 in 125 mM KCl. Afterwards, HUASMC were fixed for 15 minutes in 3.7% formaldehyde, thoroughly washed, permeabilized for 30 minutes in 0.5% Triton X-100, blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline plus divalent cations (PBS+), carefully washed again, incubated with alpha-actin-antibody overnight and stained with a secondary anti-actin antibody and phalloidin on the next day. Cells were then embedded in confocal matrix (Micro Tech Lab, Graz, Austria). Leica TCS SP5 confocal microscope was used to record 3 random
120.6 µm x 120.6 µm images from each well (Leica Microsystems). These images were automatically pre-processed with Fiji ImageJ Software with following algorithm:

Firstly, z-stacks (0.3 µm slice distance, maximum intensity projection) of 3 µm thickness were calculated to correct for filaments of slightly oblique deviation:

run("Z Project...", "projection=[Max Intensity]");

run("Grays");

run("Unsharp Mask...", "radius=4 mask=0.90");

A high-pass filter was applied to the picture to account for uneven illumination at the outskirts of the image

run("Bandpass Filter...", "filter_large=40 filter_small=3 suppress=None tolerance=5 autoscale saturate");

run("Enhance Contrast...", "saturated=5");

getRawStatistics(nPixels, mean, min, max);

t2 = max;

t1 = 1.2*mean;

setThreshold(t1, t2);

setOption("BlackBackground", false);

run("Convert to Mask");

run("Invert");

For the indirect analysis of intersection density by measurement of the mean filament length between intersections a Fiji ImageJ Plugin DiameterJ was used ⁶. Filament thickness was calculated by building the mean of the minors of all particles of the pre-processed images with following characteristics:

size (in microns): 2-infinity (to subtract for background fluorescence)

circularity: 0.0-0.4 (to analyze only particles/ filaments and not round antibody aggregates)

**Image analysis.** The anisotropy index was calculated from the same time lapse Life-Act images. Individual VSMC were encircled and changes in anisotropy followed over time after
application of the aforementioned substances. For the computation of anisotropy, Fiji ImageJ Plugin FibrilTool was used.

**Drugs and buffer solutions.** The MOPS (3-morpholinopropanesulfone acid) -buffered salt solution used in the experiments contained (in mM): CaCl$_2$·2H$_2$O 3.0, EDTA (ethylenediaminetetraacetic acid) 0.02, glucose 5.0, KCl 4.7, MgSO$_4$·7H$_2$O 1.17, MOPS 3.0, NaCl 145, NaH$_2$PO$_4$·1H$_2$O 1.2, pyruvate 2.0. In MOPS buffers containing 125 mM KCl and varying concentrations of CaCl$_2$, concentrations of NaCl were adjusted accordingly so guarantee unchanged overall osmolality. PBS+ (phosphate-buffered saline with divalent cations) contained (in mM): CaCl$_2$·2H$_2$O 0.9, glucose 5.6, KCl 5.4, MgSO$_4$·7H$_2$O 0.3, MgCl$_2$·6H$_2$O 0.3, NaCl 136.9, NaH$_2$PO$_4$·1H$_2$O 0.8, KH$_2$PO$_4$ 0.4, NaHCO$_3$ 3.6. Relaxing solution for α-toxin permeabilized arteries contained (in mM) 20 imidazole, 7.5 Na$_2$ATP, 10 EGTA, 10 Mg-acetate, 10 creatine phosphate, 31.25 potassium-methanesulfonate, 5 NaN$_3$, 0.01 GTP, 0.001 leupeptin, 2 DTT, pH 7.00 at 22°C; submaximal contraction solution contained in addition 2 mM and 6.5 mM CaCl$_2$ yielding a pCa (=-log[Ca$^{2+}$]) of respectively 6.99 (mesenteric) and 6.1 (tail arteries).

Manganese (II) chloride tetrahydrate was purchased from Sigma Aldrich (Deisenhofen, Germany), Calyculin A, latrunculin A, jasplakinolide, LIMKi3, compound C, thapsigargin, PT1, A769662 (A76), ML7 and paxilline from Tocris (Bristol, UK).

**Probes and Antibodies** DNAse I-Alexa 488 and Phalloidin-Alexa546 were purchased from Thermo Fisher Scientific (Waltham, MA, USA), non-conformation specific goat anti-rabbit antibody, anti-pMYPT-Thr696 and anti-pMYPT-Thr853 antibody from Millipore (Darmstadt, Germany), anti-MYPT-Total from BD Transduction Laboratories (San Jose, USA), anti-pMLC20-Ser19 from Rockland (Limerick, USA), conformation specific mouse anti-rabbit antibody, anti-phospho-cofilin and anti-phospho-AMPK from CellSignaling (Danvers, MA, USA), anti-alpha-actin from Sigma Aldrich (Deisenhofen, Germany), anti-beta-actin, anti-total-cofilin, phospho-LIMK1/2 and anti-LIMK2 from abcam (Cambridge, UK), anti-pan-14-3-3 (sc-629) from Santa Cruz (Heidelberg, Germany), anti-paxillin from BD Biosciences (Heidelberg, Germany) and DRAQ5 from Biostatus (Shepshed, UK).

**Supplemental Results:**

**PT1 activates AMPK in cultured cells as well as in isolated intact arteries.** Supplemental Figure I depicts changes in phosphorylation status of AMPK as determined by Western blot from cultured porcine coronary artery smooth muscle cells (PCASMC) and isolated mouse
mesenteric arteries. PT1 lead to an increase in phospho-AMPK levels by a factor of 2 ± 0.3 compared to control in PCASMC and to 4.9 ± 2.2 fold in intact arteries.

**Ca**²⁺-sensitivity is equally reduced in the presence of A769662, an alternative AMPK activator. Vessels were first subjected to a calcium sensitivity protocol. Then the protocol was repeated in the presence of 100 µM A769662 (A76) as a second independent AMPK stimulator. The increase in [Ca²⁺] remained the same (Supplemental Figure II). However, A76 caused a marked decrease in contractile response (A76: constriction to 64.3 ± 1.5 % of initial constriction, n=4 vs. PT1: 27.8 ± 7.5 %, n=5) which was partly reversible after wash-out of this component (not shown).

**AMPK induces Ca²⁺-independent, delayed vasodilation.** To gain insight into the time course of the AMPK-mediated Ca²⁺ desensitization, we added 30 µM PT1 to arteries which were pre-constricted with high potassium concentrations (125 mM) (Supplemental Figure III). The compound caused a delayed (onset after 5 minutes) steadily increasing Ca²⁺-independent vasodilation, reaching 44.4 ± 6.2 % of dilator capacity after 45 min, compared to -3.2 ± 1.4 % in time control.

**PT1 and MLCK inhibitor ML7 affect KCl-induced constriction differently.** In order to test if AMPK causes vasodilation by regulating MLCK, the other major player of MLC-dependent vasodilation, we incubated our arteries with the MLCK inhibitor ML7 (10 µM) before inducing constriction with 125 mM KCl. It took substantially longer to reach a steady state constriction in the arteries treated with ML7 than in the AMPK-activates arteries, suggesting AMPK does not regulate Ca²⁺-independent vasodilation by inhibition of MLCK⁸ (Supplemental Figure IV).

**PT1 neither affects MYPT1-T853 nor -T696 in permeabilized arteries.** In α-toxin permeabilized mesenteric and tail arteries we aimed to circumvent possible confounding effects of membrane ion fluxes (e.g. Ca²⁺) on MYPT1 phosphorylation. Similar as observed in intact arteries, PT1 did not affect the phosphorylation of T853 (Supplemental Figure V). In contrast, Y27 reduced T853 phosphorylation significantly. Whereas T696 phosphorylation was not affected by Y27 in mesenteric arteries, we observed in permeabilized in tail arteries a significant decrease in phosphorylation consistent with our previous report ⁴. These results indicate that T853 is the main Rho kinase phosphorylation site on MYPT1 in mesenteric arteries.

**Anisotropy changes in pressurized arteries.** To confirm the morphological results that we gained in isometric preparations, pressurized arteries were stained with phalloidin and the anisotropy index was measured⁷. Anisotropy changes were virtually reciprocal to the results of the F/G-actin ratio: With PT1 the anisotropy was significantly higher compared to jaspaklinolide but not different from Latrunculin treatment. Moreover, arteries incubated with PT1 in the
presence of the AMPK inhibitor compound C showed a significantly lower anisotropy than PT1 treated arteries (Supplemental Figure VI).

**Cofilin dephosphorylation by AMPK activation in vessels with A76.** In another series of experiments, vessels were pre-constricted for 5 minutes with 125 mM KCl and the stimulated with either 0.1 % DMSO or 100 µM A76 for a total of 2 hours. The vessels were then rapidly frozen in liquid nitrogen, protein was extracted and subjected to Western blot. A76 as second AMPK activator also elicited cofilin dephosphorylation (Supplemental Figure VII).

**Specificity of the 14-3-3 pull-down and band detection.** To investigate the degree of unspecific pull-down of 14-3-3 in the IP an IgG control antibody was used for immunoprecipitation. Samples treated with the control IgG antibody showed 42.9 ± 6.7 % of the protein signal detected using the anti-14-3-3 antibody (Supplemental Figure VIIIB).

**No changes of LIMK phosphorylation status after AMPK activation.** LIMK is known to be implicated in the regulation of cofilin phosphorylation status⁹. To assess whether AMPK changes LIMK phosphorylation and, thus, its activity we performed Western blot for p-LIMK in PCASMC which were either stimulated with 30 µM PT1 or 0.03 % DMSO for 30 minutes. No change of LIMK phosphorylation was detected after the respective time. Moreover, downregulation of AMPK with siRNA had no influence on LIMK phosphorylation status (Supplemental Figure IXB).

**Denominators for normalization in Western blots do not change in response to different treatments.** To test if AMPK stimulation could change the beta-actin/whole protein ratio (the former being used for loading controls) we stimulated isolated arteries either with 30 µM PT1 or 0.03 % DMSO for 30 minutes. Afterwards, the artery samples were homogenized, separated by SDS PAGE and transferred to PVDF membranes. The membranes were then stained for total protein and incubated with an anti-beta actin antibody. No change in whole protein amount as well as in beta-actin/total protein ratio was seen (Supplemental Figure XB).

**Activation of AMPK leads to displacement of cofilin from 14-3-3 protein.** PCASMC were treated with either DMSO (Ctrl) or PT1 in the presence of high KCl concentrations. Immunoprecipitation of the lysates with an anti-14-3-3 antibody showed that more cofilin was bound to 14-3-3 in the control state. This indicates that cofilin is liberated from 14-3-3 by AMPK (Supplemental Figure XI).

**Supplemental References:**

1. Bolz S-S, de Wit C, Pohl U. Endothelium-derived hyperpolarizing factor but not NO
reduces smooth muscle Ca2+ during acetylcholine-induced dilation of microvessels. 


Supplemental Figures:

A

Supplemental Figure I: PT1 activates AMPK. A, left Representative Western blot of porcine coronary artery smooth muscle cells stimulated with MOPS for 15 min followed by 125 mM KCl for 20 min, both supplemented with either 0.03% DMSO (Ctrl) or 30 µM PT1. Right, Pooled mesenteric arteries were stimulated as outlined in the methods section. B, quantification of Western blots from cultured cells. P-AMPK and total AMPK levels were determined in two separate assays which were both normalized to GAPDH. P-AMPK/GAPDH of membrane 1 was then normalized to AMPK/GAPDH of membrane 2 (n=3, *p<0.05, t-test). C, quantification of Western blots from pooled mesenteric arteries (n=3, *p<0.05, t-test).
Supplemental Figure II: A76 as alternative AMPK activator also reduces Ca\(^{2+}\) sensitivity.

Typical experimental trace showing that compared with the same experiment before (grey circles) incubation of arteries with A76 (red circles) caused a significant decrease in contraction (upper panel) in response to increasing concentrations of extracellular Ca\(^{2+}\) while the intracellular Ca\(^{2+}\) increase was not changed (lower panel).
Supplemental Figure III: PT1 as AMPK activator depicts slow-onset vasodilatory kinetics. Shown are the results of the effects on diameter by the AMPK stimulator PT1 (30 µM, red). The respective time controls are depicted in black. Arrows indicate application of PT1 or sham solution. Dilation occurs without Ca²⁺ decrease under these experimental conditions (PT1: n=5, Ctrl: n=4;*p<0.05, t-test of individual AUCs).
Supplemental Figure IV: PT1 does not seem to affect MLCK. All arteries were pre-incubated with Calyculin A to block MLCP function and constricted with high extracellular potassium concentrations in the additional presence of DMSO (control group, grey circles), PT1 (red circles) or ML7 (MLCK inhibitor, blue circles). While constriction kinetics of PT1-treated vessels do not differ from those of the control group, ML7-treated arteries depict substantial delay to reach full constriction. (PT1: n=4, Ctrl: n= 5, ML7: n=3).
Supplemental Figure V: Both MYPT1 phosphorylation sites are unaffected by PT1. A, tail artery MYPT1 T853 phosphorylation at pCa 6.1 was not altered by AMPK activation. However, ROCK inhibition with Y27 significantly reduced T853 phosphorylation (**p<0.01, *p<0.05, One-Way-ANOVA, Bonferroni, Ctrl: n=4, PT1 + Y27: n=8). B, similarly to tail arteries, in mesenteric arteries only Y27 led to dephosphorylation of T853 (** p<0.01, *p<0.05, One-Way-ANOVA, Bonferroni, Ctrl: n=4, PT1: n=6, Y27: n=8). C, also T696 in tail arteries was responsive to ROCK inhibition (*p<0.05, One-Way-ANOVA, Bonferroni, Ctrl: n=4, PT1: n=6, Y27: n=8). D, in contrast, T696 in mesenteric arteries was neither affected by AMPK activation nor by ROCK inhibition, demonstrating tissue selectivity of T696 (n.s.=not significant, One-Way-ANOVA, Ctrl: n=4, PT1: n=7, Y27: n=6).
Supplemental Figure VI: VSM F-actin anisotropy in pressurized arteries. Cannulated arteries were stimulated with KCl in the presence of either jasplakinolide, an F-actin stabilizing agent, latrunculin A, an actin polymerization inhibitor, PT1, PT1 plus AMPK inhibitor compound C (CC) and Limki3, a LIMK inhibitor. Of note, anisotropy values were increased to the same extent by PT1 as by the actin polymerization inhibitor and the LIMK inhibitor. The PT1-induced effect was reversible by parallel inhibition of AMPK. (20-38 observations in 2-4 arteries for each treatment; *p<0.05, Kruskal-Wallis One Way ANOVA on Ranks, Dunn's method).
Supplemental Figure VII: Decrease in cofilin phosphorylation also occurs in response to AMPK activation with A76. A, representative Western blot from mesenteric arteries. B, quantitative analysis of phospho-cofilin/total cofilin ratio in single mesenteric arteries stimulated with either DMSO (Ctrl) or A76 after pre-constriction with 125 mM KCl (n = 3 each, *p<0.05, t-test).
Supplemental Figure VIII: IgG control of the 14-3-3 band: A, representative Western blot from PCASMC. B, quantitative analysis of signal intensities of the 14-3-3 band after IP relative to the signal intensity of 14-3-3 in the IP input. Normalization was performed to control (IP with anti-14-3-3 antibody). (n=6 for IP with Anti-14-3-3 antibody, n=3 for IP with IgG, *p<0.05 Mann-Whitney Rank Sum Test).
Supplemental Figure IX: LIMK phosphorylation is not changed after AMPK activation with PT1 as well as after downregulation of AMPK. A, representative near-infrared Western blot from PCASMC. B, treatment with PT1 as well as AMPK downregulation with siRNA did not cause a change in LIMK phosphorylation. Normalization was performed to control (DMSO-treated samples within each siRNA group). (n = 3 each, t-test).
Supplemental Figure X: Denominators for normalization in Western blots do not change in response to different treatments: A, representative near-infrared Western blot from isolated single arteries. B, treatment with PT1 does not cause a change in the beta actin/total protein ratio (n = 3 each). C, MLC20 total/MHC neither changes in response to AMPK activation nor in response to ROCK inhibition, as determined in isometric arteries (n.s.=not significant, One-Way-ANOVA, time control n=4, PT1 n=3, Y27 n=4)
Supplemental Figure XI: AMPK induces displacement of cofilin from 14-3-3 protein. A, representative Western blot from PCASMC (no siRNA treatment) showing that in PT1-treated cells less cofilin co-immunoprecipitates with 14-3-3 protein. The arrow indicates the 30 kDa band used for quantitative analysis. The enriched lower molecular weight band (red asterisk) in the IP groups was due to non-specific binding of the secondary antibody to denatured IgG light chain at 25kDa. B, Quantification of cofilin protein bound to 14-3-3 reveals significantly less bound cofilin in the AMPK-activated state (**p<0.001, paired t-test, n=3).