The AMP-Related Kinase (AMPK) Induces Ca$^{2+}$-Independent Dilation of Resistance Arteries by Interfering With Actin Filament Formation

Kai Michael Schubert,* Jiehua Qiu,* Stephanie Blodow, Margarethe Wiedenmann, Lubomir T. Lubomirov, Gabriele Pfister, Ulrich Pohl, Holger Schneider

Rationale: Decreasing Ca$^{2+}$ sensitivity of vascular smooth muscle (VSM) allows for vasodilation without lowering of cytosolic Ca$^{2+}$. This may be particularly important in states requiring maintained dilation, such as hypoxia. AMP-related kinase (AMPK) is an important cellular energy sensor in VSM. Regulation of Ca$^{2+}$ sensitivity usually is attributed to myosin light chain phosphatase activity, but findings in non-VSM identified changes in the actin cytoskeleton. The potential role of AMPK in this setting is widely unknown.

Objective: To assess the influence of AMPK on the actin cytoskeleton in VSM of resistance arteries with regard to potential Ca$^{2+}$ desensitization of VSM contractile apparatus.

Methods and Results: AMPK induced a slowly developing dilation at unchanged cytosolic Ca$^{2+}$ levels in potassium chloride–constricted intact arteries isolated from mouse mesenteric tissue. This dilation was not associated with changes in phosphorylation of myosin light chain or of myosin light chain phosphatase regulatory subunit. Using ultracentrifugation and confocal microscopy, we found that AMPK induced depolymerization of F-actin (filamentous actin). Imaging of arteries from LifeAct mice showed F-actin rarefaction in the midcellular portion of VSM. Immunoblotting revealed that this was associated with activation of the actin severing factor cofilin. Coimmunoprecipitation experiments indicated that AMPK leads to the liberation of cofilin from 14-3-3 protein.

Conclusions: AMPK induces actin depolymerization, which reduces vascular tone and the response to vasoconstrictors. Our findings demonstrate a new role of AMPK in the control of actin cytoskeletal dynamics, potentially allowing for long-term dilation of microvessels without substantial changes in cytosolic Ca$^{2+}$.

(Circ Res. 2017;121:149-161. DOI: 10.1161/CIRCRESAHA.116.309962.)

Key Words: 14-3-3 proteins ■ actin cytoskeleton ■ cofilin ■ LifeAct ■ muscle, smooth, vascular ■ vasodilation

Matching oxygen supply and demand of the tissues is achieved by numerous and partly redundant mechanisms controlling smooth muscle tone of resistance vessels, as well as their structure and growth. Rapidly occurring, short-term changes of blood flow are primarily achieved by changes in vascular smooth muscle (VSM) tone. There is now ample evidence that vasoconstriction and vasodilation are mediated by increases or decreases of VSM cytosolic Ca$^{2+}$ ([Ca$^{2+}$]i) levels, ultimately leading to changes in the phosphorylation of myosin light chain (MLC$_{20}$) by virtue of a calcium–calmodulin-dependent MLC kinase (MLCK). An additional mechanism with relevance to the control of vascular tone is regulation of the activity of MLC$_{20}$ phosphatase (MLCP), which can be altered independently of MLCK activity and of changes of [Ca$^{2+}$].

Most therapeutic approaches to affect peripheral resistance in hypertension or inadequate blood supply focus on one of these mechanisms.

However, there is also mounting evidence that additional mechanisms may be involved in the control of smooth muscle tone, especially when longer lasting changes of vascular diameter and tone come into focus. Some of these mechanisms are considered to underly vascular plasticity or short-term remodeling and to be mediated, among other mechanisms, by functional alterations of VSM structure, in particular of the smooth muscle cell (SMC) cytoskeleton. It is involved in the anchoring of the contractile apparatus with the matrix, as well as a dynamic rearrangement of the SMC length and smooth muscle intercellular adhesions. In fact, a complex set of cytoskeletal events can be triggered by classical vasoactive compounds and hormones that seem to play a fundamental role in...
The mechanical response of the muscle tissue.\textsuperscript{5-11} Thus, actin dynamic cytoskeletal processes may contribute to the unique adaptive properties of smooth muscle that enable them to modulate their contractile and mechanical properties to accommodate to changes in mechanical load and to maintain long-term alterations of vascular tone. Growing evidence suggests that the cytoskeletal processes that occur during the contractile activation of SMC may have much in common with the cytoskeletal mechanisms that govern cell motility and migration. With this regard, several reports have put the adenosine monophosphate-activated protein kinase (AMPK) into focus—an enzyme which was originally described as a cellular fuel sensor but has also been shown to have actions on smooth muscle tone and in the establishment of cell polarity.\textsuperscript{12,13} Indeed, Nakano et al\textsuperscript{14} and Yan et al\textsuperscript{15} previously reported that AMPK regulates cell migration by controlling microtubule and actin dynamics. Recent publications have outlined AMPK as an enzyme which causes vasodilation in various vessel beds of different size. AMPK can induce smooth muscle relaxation directly by a decrease of [Ca\textsuperscript{2+}], or of Ca\textsuperscript{2+} sensitivity.\textsuperscript{16-21} In a recent study, we identified AMPK as a new potential upstream kinase of the BK\textsubscript{Ca} (large conductance calcium-activated potassium channel) in microvascular smooth muscle. Nonetheless, there was still an additional, slowly developing vasodilation, which was probably Ca\textsuperscript{2+}-independent because it was still observed after inhibition of SERCA BK\textsubscript{Ca}.\textsuperscript{56}

In view of recent studies, which have implicated AMPK in the regulation of actin cytoskeletal dynamics and reorganization at the plasma membrane,\textsuperscript{22-27} it may be hypothesized that AMPK is inducing Ca\textsuperscript{2+}-independent dilation by reducing actin filament size or number similar as observed during reduction of cell migration. However, AMPK has also been reported to interfere with upstream regulators of the myosin phosphatase and to phosphorylate MLCK, thereby inhibiting its activity.\textsuperscript{18,28} Both mechanisms would also result in Ca\textsuperscript{2+}-independent relaxation of blood vessels. In view of the decisive role of microvessels in remodeling processes and the fact that microvascular regulatory pathways often differ from those in larger vessels, we studied whether and how AMPK could affect actin remodeling processes in microvascular smooth muscle and whether this could reduce microvascular tone in a Ca\textsuperscript{2+}-independent manner.

**Novelty and Significance**

- **What Is Known?**
  - AMPK is a ubiquitous key enzyme preserving cellular energy load.
  - On a systemic level, AMPK facilitates energy supply to tissues by virtue of its potent vasodilator actions in resistance arteries.
  - The vasodilation is initially induced by reducing Ca\textsuperscript{2+} in vascular smooth muscle (VSM) cells, but little is known about its maintenance.

- **What New Information Does This Article Contribute?**
  - Here, we show that AMPK can elicit prolonged vasodilation by remodeling the contractile apparatus of VSM cells.
  - This is achieved by actin depolymerization—an effect that is mediated by activation of the actin severing factor cofilin.

This novel mechanism allows vasodilation by directly reducing F-actin. It renders the control of microvascular smooth muscle tone independent of cytosolic Ca\textsuperscript{2+} changes, which otherwise may affect other vital cell functions. This AMPK-dependent mechanism may represent a novel target for the treatment of hypertension and vasospasm. It assigns a new role to AMPK with potential implications also for cell motility and migration.

**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
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<tr>
<td>AMPK</td>
<td>5' adenosine monophosphate-activated protein kinase</td>
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<tr>
<td>BK\textsubscript{Ca}</td>
<td>large conductance calcium-activated potassium channel</td>
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<tr>
<td>F-actin</td>
<td>filamentous actin</td>
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<td>G-actin</td>
<td>globular actin</td>
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<td>HUASMC</td>
<td>human umbilical artery smooth muscle cells</td>
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<td>MLCK</td>
<td>myosin light chain kinase</td>
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<td>MLCP</td>
<td>myosin light chain phosphatase</td>
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<td>MYPT1</td>
<td>myosin phosphatase targeting subunit 1</td>
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<tr>
<td>PCASMC</td>
<td>porcine coronary artery smooth muscle cells</td>
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<tr>
<td>SERCA</td>
<td>sarco/endoplasmic reticulum Ca\textsuperscript{2+} ATPase</td>
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<td>SMC</td>
<td>smooth muscle cells</td>
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<td>VSM</td>
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**Methods**

**Animals**

Male C57BL/6/N mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Mice expressing LifeAct were kindly provided by Reinhard Fässler (Max Planck Institute of Biochemistry, Martinsried, Germany) and Eloi Montanez (Biomedical Center, Martinsried, Germany).

**Isolation and Processing of Arteries**

Mesenteric artery segments were isolated using procedures that have been described before\textsuperscript{29} and either cannulated and pressurized at 60 mmHg or threaded onto stainless steel wires in a wire myograph (Danish Myo Technologies, Aarhus, Denmark). Pharmacological compounds were added directly into the organ bath. Vascular diameters together with Fura2 F340/F380 signal or vessel force, respectively, were registered as read-out.

**Immunofluorescence**

After stimulation, arteries were fixed, permeabilized, and subsequently stained by incubation with phallolidin, DNAse I or antibodies from the adventitial side. The vessel was then imaged using a confocal microscope (Leica TCS SP5; Leica, Mannheim, Germany).

**Western Blot**

For analysis of phospho-cofilin, phospho-myosin phosphatase targeting subunit 1 (MYPT1) at Thr696 and Thr853, and MLC\textsubscript{20} at Ser19 for analysis of phospho-cofilin, phospho-myosin phosphatase targeting subunit 1 (MYPT1) at Thr696 and Thr853, and MLC\textsubscript{20} at Ser19 and Ser19 in intact vessels, the arteries supplying the small intestine were dissected free from fat and connective tissue, flushed to remove luminal blood, divided into 2 halves, and stimulated with either potassium chloride (KCl) plus 0.03% dimethyl sulfoxide (DMSO) or KCl plus...
30 µmol/L PT1 for 20 minutes, followed by snap-freezing in liquid nitrogen and protein extraction as previously described.16

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.

β-actin/total protein, phospho-cofilin/total cofilin and phospho-LIM domain kinase (LIMK)1/2-total-LIMK2 were detected using near-infrared Western blot.

**Cell Culture**

Human umbilical artery SMC (Lonza, Basel, Switzerland) or porcine coronary artery SMC (PCASMC; Sigma Aldrich, Taufkirchen, Germany) were cultured according to the provider’s instructions.

**AMPK Knockdown**

SiRNA against both AMPK α subunits or control siRNA (Santa Cruz, Heidelberg, Germany) were transfected into PCASMC using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher, Waltham, MA).

**Statistics**

For data presentation and computation of statistical tests, SigmaPlot (Systat, Erkrath, Germany) was used. Statistical tests comprised parametric tests, that is, 2-way ANOVA plus Tukey test or Holm–Sidak method, 1-way ANOVA plus Holm–Sidak method, 1-way repeated measures ANOVA followed by Shapiro–Wilk test, paired t test, and t test when the data were normally distributed and nonparametric tests, that is, signed-rank test, Friedman repeated measures ANOVA on ranks followed by Tukey test wherever appropriate. The exact test procedure used is indicated in the respective figure legends. For descriptive reasons, all values are presented as means±SEM. Differences were considered statistically significant when the error probability was <0.05. Methods are further outlined in detail in the Online Data Supplement.

**Results**

Substantial AMPK-induced dilatation after blockade of BK$_{Ca}$- and SERCA-mediated calcium decrease.

We have demonstrated before that AMPK can dilate smooth muscle by activating BK$_{Ca}$ channels and SERCA simultaneously.16 However, de-endothelialized mouse arteries, preconstricted with 125 mmol/L K$^+$ and pretreated with the SERCA inhibitor thapsigargin still showed a prominent dilation on stimulation with PT1 (Figure 1A). In contrast to the dilations already described,16 these dilations developed slowly and reached a maximum of 59.3±3.3% only after 40 minutes, compared with 4.4±3.8% in time controls. PT1, 27.0±3.9%; Figure 1B).

A similar dilation was observed after incubation of vessel segments with urocortin— a compound which is released from hypoxic myocardium15 and known to activate AMPK16 (Figure 2).

**Reduced Ca$^{2+}$ Sensitivity of VSM Cell After AMPK Activation**

In a separate series of experiments, VSM was depolarized by using high (125 mmol/L) extracellular K$^+$ concentrations. Under these conditions, stepwise increases of extracellular Ca$^{2+}$ concentrations starting from zero Ca$^{2+}$ were followed by respective changes in intracellular Ca$^{2+}$ as measured by Fura2 ratio. Preincubation with the AMPK activators PT1 (Figure 1C and 1D) and A76 (Online Figure II) significantly impaired the constriction induced by the elevations of cytosolic Ca$^{2+}$.

The concentrations of both compounds used were equally effective to induce short-term dilation of norepinephrine preconstricted arteries.16 Wash-out of the AMPK activators partly reversed their inhibitory effect (not shown). The [Ca$^{2+}$], response to the stepwise increase of extracellular Ca$^{2+}$ concentrations was the same under all 3 conditions, suggesting a reduction of Ca$^{2+}$ sensitivity caused by AMPK activation (Figure 1C; Online Figure II).

**Unchanged MLC$_{20}$ and MYPT1 Phosphorylation Status After Preactivation of AMPK Before Constriction**

Single segments of mesenteric arteries constricted with high K$^+$ in the presence or absence of the AMPK stimulator PT1 were snap frozen after 20 minutes of constriction and processed as described. Neither the phosphorylation of MLC$_{20}$ nor the phosphorylation of MYPT1 at T853 were significantly changed compared with time controls (Figure 3A through 3D) in spite of the fact that PT1 had significantly compromised KCl-induced constriction at that time point. Figure 1B depicts the diameter tracings of the same arteries that were then assessed via Western blot. In contrast, the Rho kinase inhibitor Y27632 (Y27; 10 µmol/L), taken as positive control, caused dephosphorylation of T853 (Figure 3D). In addition, we studied α-toxin permeabilized mesenteric and tail arteries and found that Y27 reduced T853 phosphorylation. Phosphorylation of T696 was responsive to Y27 only in tail arteries (Online Figure V), indicating that T853 is the main Rho kinase site in mesenteric arteries. In neither artery type did we observe AMPK-mediated changes in MYPT1-T853 or T696 phosphorylation.

**Increased G-Actin Levels in Intact Arteries by AMPK Activation**

Single cannulated arterial segments were snap frozen after 20-minute incubation with KCl in the presence of PT1 or solvent (DMSO). Arteries pretreated with PT1 showed significantly higher G-actin (globular actin) levels compared with DMSO-treated ones (Figure 4C). A PT1-induced increase of G-actin was also observed in PCASMC. It was, however, abolished after transfection with siRNA against both catalytic subunits of AMPK (Figure 4D and 4F). SiRNA treatment resulted in a significant downregulation of AMPK expression to 25.8±5.5% of control (Figure 4D through 4E).

The AMPK-induced G-actin formation in intact vessels was also studied with optical methods using fluorophore-coupled DNase I and phalloidin to label G-actin and F-actin, respectively. Here, we compared G-actin formation under jasplakinolide—an agent that stimulates actin polymerization and hence maximizes F/G-actin ratio with agents known to affect actin stability. The F/G-actin ratio observed after PT1 was close to the low values induced by latrunculin A—an agent that inhibits actin polymerization. Of note, inhibition
of LIMK with LIMKi3 led to similarly low F/G-actin ratios as PT1 (Figure 4A and 4B). Inhibition of AMPK via compound C yielded comparable ratios as jasplakinolide, despite concomitant stimulation with PT1.

**Decreased Mean Actin Filament Thickness and Filament Network Branching Points in Cultured Human VSM Cell On AMPK Activation**

Confocal images of human umbilical artery SMC revealed that on AMPK activation by PT1, actin filaments were on average significantly thinner by 7.4±1.6% (Figure 5A and 5B). Moreover, AMPK activation increased the average length of fibers between branching points, suggesting reduced ramifications (Figure 5C).

**AMPK-Dependent Rarefaction of F-Actin in Living Arterial VSM Cell In Situ**

Vessels from mice expressing LifeAct	extsuperscript{31} were used to test the effect of AMPK activation on dynamic changes of the actin cytoskeleton. The vessels were kept under isometric conditions in a wire myograph to avoid confounding effects by changes of muscle length. Vessels were subjected to initial activation by high K+. Thereafter, PT1 was added to the organ bath. In the third step, the AMPK inhibitor compound C was added in the continuous presence of PT1. PT1 led to a loss of the LifeAct/F-actin signal in the middle of the cell, an effect that was reversed on addition of compound C. The recovery of the signal in the center part excluded signal decay because of bleaching of LifeAct (Figure 6A through 6C). In accordance with a change of fiber arrangement, the anisotropy of the actin signal increased after PT1 and decreased again after the inhibition of AMPK (Figure 6D). These findings were confirmed in an additional experimental series on pressurized arteries (Online Figure VI). In these arteries, LIMK inhibition again mimicked the augmenting effects of PT1 on anisotropy. Likewise, compound C blunted AMPK-mediated filament anisotropy increase.

**Decreased Cofilin Phosphorylation After AMPK Activation**

In search for a mechanism by which AMPK could cause actin disassembly we conducted a phosphorylation array of proteins...
Coﬁlin. AMPK activation by PT1 (but not control treatment from PCASMC to coimmunoprecipitation for 14-3-3 and cofilin dephosphorylation is known to be inhibited by binding to the adaptor protein 14-3-3, we subjected lysates to 14-3-3 and PCASMC. Western blots showed cofilin dephosphorylation to be reduced by AMPK (not shown). Thus, further studies with a focus on cofilin were performed in pooled mesenteric arteries for the assay. Among others, cofilin phosphorylation was found to be reduced by AMPK (not shown). Thus, further studies with a focus on cofilin were performed in pooled mesenteric arteries and PCASMC. Western blots showed cofilin dephosphorylation after a 35-minute incubation of these arteries with PT1 (Figure 7A). Likewise, immunofluorescence staining of phosphorylated cofilin in pressurized, PT1-treated arteries was reduced as compared with DMSO-treated arteries (Figure 7D). Subsequent experiments showed that the PT1-induced cofilin dephosphorylation was independent of changes in Rho/ROCK (Rho-associated coiled-coil containing kinase) activity (as reﬂected by MYPT1 phosphorylation; Figure 3C and 3D) and the phosphorylation state of LIMK (Online Figure VIII).

AMPK Leads to Liberation of Cofilin From 14-3-3

Because cofilin dephosphorylation is known to be inhibited by binding to the adaptor protein 14-3-3, we subjected lysates from PCASMC to coimmunoprecipitation for 14-3-3 and cofilin. AMPK activation by PT1 (but not control treatment with DMSO) led to a signiﬁcant reduction of 14-3-3-bound cofilin (Online Figure XI). This reduction was abolished after AMPK silencing but not after treatment with control siRNA (Figure 8A and 8B) indicating that AMPK mediated the liberation of cofilin from 14-3-3.

Discussion

In this study, we observed that AMPK activation can cause a substantial dilation of KCl-preconstricted vessels, which is not paralleled by changes of [Ca\textsuperscript{2+}], indicating Ca\textsuperscript{2+} desensitization of VSM. Unexpectedly, this seemed not to be because of an activation of MLCP as reported earlier\cite{16,32} but was associated with an increase in G-actin, as well as thinning and rarefaction of VSM-actin ﬁlaments. Our data are consistent with the view that this AMPK effect involves cofilin activation.

In KCl-preconstricted vessels, subsequent activation of AMPK by means of stimulation with 2 structurally unrelated compounds led to a slow-onset vasodilation, which was not associated with any detectable changes of VSM [Ca\textsuperscript{2+}]. Likewise, in PT1-treated vessels, the same increases in Ca\textsuperscript{2+} elicited a signiﬁcantly attenuated vasoconstriction, indicating that AMPK stimulation resulted in substantial Ca\textsuperscript{2+} desensitization of the contractile apparatus. Preactivation of AMPK also reduced the vessels’ subsequent contractile response to membrane depolarization. Although earlier observations in cultured human and, partly, mouse aortic SMC\cite{18,32} suggest that AMPK is able to reduce Ca\textsuperscript{2+} sensitivity by increasing MLCP activity, we did not ﬁnd any evidence for it in the current experiments. Kinetic analysis of arterial constriction in the presence of an MLCK inhibitor differed from the constriction in the presence of PT1 (Online Figure IV). Though we have not measured MLCK activity directly, these ﬁndings argue against MLCK as a target of AMPK under our experimental conditions. Further and most important, we did not observe a reduction of MLCP\textsubscript{20} phosphorylation, making, thus, unlikely that changes in MLCK or MLCP activity could explain our results.

Based on these ﬁndings, we conclude that the observed Ca\textsuperscript{2+}-independent dilation was caused by a different process, namely actin ﬁlament remodeling. The reasons for the lack of an involvement of MLCP are not clear. Although it is likely that in the other studies reporting MLCP activation [Ca\textsuperscript{2+}], might have changed in response to AMPK activation,\cite{16} we precluded this change in [Ca\textsuperscript{2+}] by maximal depolarization, which was conﬁrmed by the Fura2 measurements. This is of particular importance because [Ca\textsuperscript{2+}], alone can control RhoA activity\cite{33,34} leading to alterations in MYPT1 phosphorylation. Increased MLCP activity as described before\cite{18,32} may have, thus, been the result of confounding effects of the primary AMPK-mediated [Ca\textsuperscript{2+}] decrease leading to subsequent RhoA inactivation.

The actin ﬁlaments of the contractile apparatus, contrary to many textbook sketches, do not seem to represent a merely passive scaffold to permit myosin ﬁlament sliding. Rather, actin polymerization is triggered\cite{35,36} on stimulation with vasoconstrictors—a process which may enable smooth muscle to maintain a shorter cell length. In addition, actin polymerization might allow for better transmission of force to the extracellular matrix via integrins\cite{37} and has also been linked to artery inward remodeling as observed in hypertension.\cite{38} Studies in
nonvascular smooth muscle suggest that recruitment of as little as 10% of total actin is sufficient to exert significant effects on muscle tone.\textsuperscript{37} The aforementioned phenomena have all been reviewed in great detail with regard to muscle contraction.\textsuperscript{10} In the present study, we show for the first time that actin depolymerization can play a functionally relevant role in the process of resistance vessel dilation—thereby showing that the aforementioned associations of vasocostriction and actin polymerization also hold true for the opposite effects. Impaired actin polymerization indeed hampers contractile processes, despite unaltered MLC\textsubscript{20} phosphorylation levels.\textsuperscript{38,39}

Using confocal microscopy and differential ultracentrifugation, we were able to detect significant increases in the G-actin fraction after AMPK activation for 35 minutes. This effect of PT1 was indeed AMPK mediated because pretreatment with siRNA completely abolished the increase in G-actin. Comparison with jasplakinolide and latrunculin A as obtained by our optical measurements of the F/G-actin ratio in intact vessels revealed that AMPK stimulation can decrease the F/G-actin ratio to a similar extent as latrunculin A. This is in line with a previous report from Stone et al\textsuperscript{25} who described that activation of AMPK induces depolymerization of F-actin and consequently an increase of G-actin, thereby hampering migration of cultured VSM cells.

In living VSMC, in the intact vascular wall of LifeAct vessels, we could observe a corresponding loss of central F-actin signal on AMPK activation, which was reversible after AMPK inhibition with compound C. Conversely, Flavahan and coworkers reported an increase of central F-actin during pressure-induced constriction, which is in line with opposite changes of central actin with dilation and constriction. As in our arteries kept under isometric conditions, they reported the highest actin density along the cell borders and a lower density in the cell center in relaxed muscle cells. This subcellular distribution was changed toward increasing central density following myogenic activation.\textsuperscript{40} Both observations challenge the current opinion derived from experiments in cultured cells stating that actin plasticity occurs mainly in the subcortical zone, thereby enhancing force transmission to the matrix environment and anchoring of the contractile apparatus. Rather, our findings suggest that

![Figure 3](http://circres.ahajournals.org/)

**Figure 3.** Adenosine monophosphate-activated protein kinase (AMPK) does not change myosin phosphatase targeting subunit 1 (MYPT)-phosphorylation status. **A**, Representative Coomassie stain of protein gel (upper) and Western blot (lower) from dimethyl sulfoxide (DMSO; control [Ctrl])-treated vs PT1-treated single pressurized arteries. Upper and lower panel are obtained from the same experiment. **B**, 20 kDa myosin light chain (MLC\textsubscript{20}) phosphorylation at Ser 19 was not altered by AMPK (n=9). **C**, Western blots from single isolated pressurized arteries incubated with PT1 or DMSO showed no differences in MYPT1 phosphorylation status (n=10; P=0.695, signed-rank test). **D**, Only ROCK (Rho-associated coiled-coil containing kinase)-inhibition by Y27632 but not PT1 caused a significant reduction of MYPT1-pT853 in isometric vessels (all groups n=4; ***P<0.001 vs PT1; 1-way ANOVA; Bonferroni). MHC indicates myosin heavy chain; and n.s., nonsignificant.
Figure 4. A, Adenosine monophosphate-activated protein kinase (AMPK) activation caused a decrease of filamentous/globular (F/G)-actin levels as detected by immunofluorescence in isolated pressurized arteries. Upper panel shows representative images of 3 µm z stacks in the vascular smooth muscle cell of arteries stained with phalloidin (F-actin, red) and DNase I (G-actin, green). Jasplakinolide (Jasp) and Latrunculin A (Latr) as respective enhancer and inhibitor of actin polymerization were chosen as positive and negative control (Ctrl). PT1 application showed a decrease in the F-actin signal, whereas it increased the G-actin signal, thereby lowering the F/G-actin ratio. All scale bars, 10 µm. B, Quantitative analysis of the images depicted in A. Interestingly, AMPK inhibition by compound C (CC) increased the F/G-ratio by about the same degree as jasplakinolide, whereas LIM domain kinase (LIMK) inhibition by LIMKι3 changed the F/G-ratio to levels comparable with AMPK activation. (Jasp, Latr, PT1, LIMKι n=3; PT1/CC n=5; #P<0.05 vs Jasp; §P<0.05 vs PT1/CC; 1-way ANOVA, Shapiro–Wilk). C, G-actin content in the supernatant of single pressurized arteries is significantly enhanced in response to AMPK activation (n=4; *P<0.05, Mann–Whitney rank-sum test). D and F, These data could be confirmed in cultured porcine coronary artery smooth muscle cells: representative Western blot (D) and quantitative analysis (F) show an increase in G-actin after activation of AMPK in transfection Ctrls. On the contrary, this increase was blunted in AMPK knockdown cells (n=9 each; *P<0.05 vs PT1, 2-way ANOVA, Holm–Sidak). E, AMPK levels in knockdown cells were reduced to 25.8±5.5% of Ctrl (n=18 each; ***P<0.001, t test). GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase.
central, longitudinal actin filaments may be of significance for the regulation of force development in smooth muscle in situ. Our notion of actin reorganization is further supported by determinations of filament anisotropy in VSM cell in situ under isometric conditions. The increased anisotropy in AMPK-activated states is consistent with more parallel organization of the fibers and less branching.

We could further validate the latter finding in cultured VSM. VSM-actin filament network showed significantly less branching points following AMPK stimulation. As a second effect, the actin filament thickness was significantly reduced. Actin filament thickness in turn is regulated by the severing protein cofilin.41 Cofilin also happens to regulate actin filament network branching.42

Cofilin has been implicated in the regulation of VSM contraction via actin polymerization in various studies.43 We focused on cofilin because the ultrastructural appearance of the actin filaments was consistent with cofilin activation41 and cofilin can lead to increased G-actin levels by filament severing.43 Moreover, treatment with an inhibitor of LIMK—the upstream regulator of cofilin—also resulted in increases of G-actin in isolated vessels. Using 3 independent methods, we could show that AMPK induced cofilin S3 dephosphorylation, which was associated with actin depolymerization.

Our findings on the role of cofilin dephosphorylation in VSM relaxation (vasodilation) mirror ample evidence showing increased cofilin phosphorylation in response to contractile VSM stimulation.39,44,45 By contrast, in airway smooth muscle

Figure 5. Human umbilical artery smooth muscle cells (HUASMC) were stimulated, fixed, and stained for α-actin and phalloidin. A, Representative images of α-actin (green) in cultured HUASMC (nuclei; red). Areas with comparable nuclei count were chosen for preprocessing (for algorithm, see methods section) and the filament thickness and intersection density analyzed (normalized by measuring the filament length between intersections). First image, overview; second image, magnification only with α-actin; third image, mask for analysis after preprocessing. B, PT1 significantly decreased fiber thickness compared with DMSO (dimethyl sulfoxide)-treated cells. C, Indirect measurement of branching point density by calculation of filament length between intersections revealed an increase after PT1 incubation suggesting reduced intersections and branching points (n=5; *P<0.05 paired t test). Ctrl indicates control.
ADF (actin depolymerizing factor)/cofilin dephosphorylation was reported to occur during constriction, that is, a condition that goes along with actin polymerization. We cannot explain the conflicting results at present, but differences between VSM and other tissues or the mode of preactivation of the muscle may play a role here. Of note, of 6 studies focusing especially on VSM, 5 reported increased cofilin phosphorylation in response to contractile stimulation. If one can conclude that vasodilation should lead to opposite changes, the majority of these studies are consistent with cofilin dephosphorylation being a relevant factor in controlling cytoskeletal changes, which ultimately lead to vasodilation. However, one vascular study also reported decreased phosphorylation with contraction. The reason for that is not clear at present but may be because of time-, stimulus-, tissue-, or species-dependent variations.

In contrast to our study, AMPK activation has often been linked to cofilin phosphorylation rather than dephosphorylation. Firstly, we suggest that this discrepancy is...
attributable to the fact that none of the discrepant studies was performed on SMC. It has been shown that translational regulation between SMC and non-SMC is controlled by different miRNAs. SMC are the only cell type in which the promoter of miR-143 and miR-145 is expressed and active. These miRNAs not only regulate differentiation to the contractile phenotype, they also control the expression of multiple actin-related proteins, such as Arp2/3 subunit 5, β-actin, γ-actin, and also cofilin 2. The unique influence of these miRNAs on VSM cell actin cytoskeleton has recently been highlighted.

Figure 7. A, Western blots conducted with arterial samples showed a significant dephosphorylation of cofilin in PT1-treated mesenteric arteries and porcine coronary artery smooth muscle cells (PCASMC; n=3 for mesenteric arteries and n=7 for PCASMC; *P<0.05, **P<0.01, paired t test). B, Pressurized arteries that were stained for paxillin (to mark cell borders, green) and for phospho-cofilin (red) showed less phospho-cofilin signal after adenosine monophosphate-activated protein kinase (AMPK) activation with PT1 compared with DMSO (dimethyl sulfoxide)-treated vessels. Paxillin staining confirms sufficient permeabilization in both groups. White asterisks mark cell nuclei. C, Histogram of gray values obtained from regions of interests (yellow rectangles) located in the smooth muscle region to exclude unspecific signal from surrounding adventitia revealed a left shift of the curve (PT1, red; control [Ctrl], gray) and a significant reduction in the mean gray value in PT1 incubated vessels (D). n=4; *P<0.05, Mann–Whitney rank-sum test. All scale bars, 10 µm. GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase.
therefore, propose that only SMC provide the protein machinery necessary for AMPK to induce cofilin dephosphorylation.

Secondly, it should not be neglected that in our experimental setting, we assessed the effect of AMPK activation in vessels preactivated with high extracellular K+ concentrations and, thus, high phosphorylation state of LIMK and cofilin. Under these conditions, it seems plausible that most of cofilin proteins should be in a phosphorylated state because they would otherwise hamper K+-induced vasoconstriction. Starting from such a high phosphorylation level, a dephosphorylation may be seen, which one could not detect whether the phosphorylation level was low.

The reduction of cofilin phosphorylation, which augments the activity of the enzyme, could not be attributed to an inhibitory effect of AMPK on LIMK under our experimental conditions. Alternatively, the phosphorylation of cofilin is reduced by the action of a phosphatase. Our findings suggest that the regulatory influence of AMPK on cofilin phosphorylation is indirect, that is, at least partly exerted via cofilin displacement from 14-3-3. This displacement of cofilin from 14-3-3 clearly involves AMPK because the effect was abolished after silencing of AMPK. Interestingly enough, AMPK has been shown to phosphorylate several 14-3-3 binding partners consistent with a competitive displacement of cofilin. Free cofilin is not anymore protected from dephosphorylation and can subsequently be dephosphorylated, leading to cofilin activation and, ultimately, actin filament severing.

It must be emphasized that additional or alternative mechanisms that potentially contributed to the observed AMPK effects on the actin cytoskeleton have not been studied in detail. For example, it was not studied whether actin linkage to adherens junctions—which is thought to promote intercellular force transmission and smooth muscle contraction—was reduced.

In summary, we show here that AMPK induces sustained vasodilation via smooth muscle Ca²⁺ desensitization, which is because of actin filament disassembly and debranching. One likely pathway occurs via cofilin whose displacement from 14-3-3 leading to subsequent dephosphorylation was shown here to be AMPK dependent.

At present, we can only speculate on the physiological role of AMPK-induced reduction of Ca²⁺ sensitivity. It is important to note that Ca²⁺ as a pivotal second messenger can probably not be kept chronically low to maintain a state of relaxation without at the same time impairing noncontractile Ca²⁺-dependent cell functions. A selective reduction of the calcium sensitivity of the contractile apparatus may preserve other Ca²⁺-dependent cell functions while keeping vessels dilated in tissues, which are particularly vulnerable to ischemic injury, and protect them from spasms. Indeed, hypoxic tissue-derived signals, such as urocortin2 (Figure 2) or adiponectin (found to be increased in

![Figure 8](http://circres.ahajournals.org/)

**Figure 8.** Adenosine monophosphate-activated protein kinase (AMPK) induces displacement of cofilin from 14-3-3 protein.

**A.** Porcine coronary artery smooth muscle cells were either transfected with control (Ctrl) siRNA (si Ctrl) or with siRNA against AMPK (si AMPK). Then both groups were either treated with solvent (dimethyl sulfoxide [DMSO]) or with the AMPK activator PT1. Subsequently, the respective lysates were used for immunoprecipitation with an anti 14-3-3 antibody or Ctrl IgG and the precipitates were subjected to immunoblotting for cofilin and 14-3-3. As shown by the representative blots, activation of AMPK with PT1 led to less cofilin coimmunoprecipitation with 14-3-3. This effect could be blunted by RNA interference (left). In contrast, DMSO treatment did not result in changes between either siRNA group (middle). **Right,** The representative input analysis (7% of IP input protein) refers to the DMSO-treated cells (middle) and clearly shows that equal amounts of protein had been used for IP. **B.** Quantification of cofilin protein bound to 14-3-3 reveals significantly less bound cofilin in the AMPK-activated state (left; siRNA Ctrl-PT1). AMPK silencing with siRNA abolished this effect, whereas DMSO treatment had no effect (right; **P<0.01, paired t test, n=3 each). GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase; and n.s., nonsignificant.
cerebrovascular fluid after hemorrhage\textsuperscript{60}, are known to activate AMPK. In this context, it may not be just coincidence that adiponecin decreases in human cerebrospinal fluid after subarachnoid hemorrhage\textsuperscript{61} at the time point when an increased incidence of vasospasms can be observed. Thus, our findings may form a basis for the establishment of AMPK activation as a therapeutic approach to prevent spasms.

**Sources of Funding**

M.W. was supported by a MD scholarship (Förderprogramm für Forschung und Lehre) granted by the Ludwig-Maximilians-Universität München, Munich, Germany. K.M.S. was enrolled in the Medical Life Science and Technology PhD program of the Technical University of Munich, Germany. J.Q. is a fellow of a European Union Innovative Training Networks project (SmARTer; PITN-GA-2013–606998). This work was funded through grants from the Munich Cluster for Systems Neurology (SyNergy), Munich, Germany, from the German Center for Cardiovascular Research in the Medical Faculty of Cologne (Koeln Fortune).

**Acknowledgments**

We are indebted to Dejana Mokranjac for providing access to an ul-

**Disclosures**

None.

**References**


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Actin and Small Artery Tone: Role of the AMPK
The AMP-Related Kinase (AMPK) Induces Ca^{2+}-Independent Dilation of Resistance Arteries by Interfering With Actin Filament Formation

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Circ Res. 2017;121:149-161; originally published online June 6, 2017;
doi: 10.1161/CIRCRESAHA.116.309962

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Data Supplement (unedited) at:
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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\(^1\).

**[Ca\(^{2+}\)]\(_i\) 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\(^{2+}\) concentrations \([\text{Ca}^{2+}]_i\), vascular outer diameters were recorded by videomicroscopy (Hasotec, Rostock, Germany).

For the construction of Ca\(^{2+}\) sensitivity curves, the arteries were first placed in Ca\(^{2+}\)-free MOPS buffer (\([\text{Ca}^{2+}]_0 = 0\)) 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 \([\text{Ca}^{2+}]_0\). Subsequently, \([\text{Ca}^{2+}]_0\) was elevated stepwise to 0.5, 1, 2 and 3 mM and the resulting changes in \([\text{Ca}^{2+}]_i\) while the related constrictions were recorded at a frequency of 5 Hz\(^2\). 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 \([\text{Ca}^{2+}]_0\) 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 (\([\text{Ca}^{2+}]_0\) protocol) in standard MOPS, followed by a normal potassium (4.7 mM) zero \([\text{Ca}^{2+}]_0\), MOPS, a high potassium (125 mM) zero \([\text{Ca}^{2+}]_0\) (each for five minutes) and eventually constricted with MOPS containing 125 mM potassium and 0.5 mM \([\text{Ca}^{2+}]_0\). 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\(^{2+}\) 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\(^+\) 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\(_3\)VO\(_4\) 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\(^3\). G-actin levels were normalized to GAPDH.
For the detection of pMLC\textsubscript{20}, pMYPT1 (T696), pMYPT1 (T853) and β-actin mesenteric arteries were cannulated, pre-constricted by 125 mM K\textsuperscript{+} at 0.5 or 3 mM extracellular Ca\textsuperscript{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\textsuperscript{4}.

For technical reasons, MLC\textsubscript{20} Ser\textsubscript{19} phosphorylation was expressed relative to actin rather than to MLC\textsubscript{20} total which would have allowed to analyze the relevant ratio of both proteins. To exclude that a change in MLC\textsubscript{20} phosphorylation might be masked if PT1 affected the expression level of MLC\textsubscript{20}, we normalized MLC\textsubscript{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\textsuperscript{2+}] pCa = -log [Ca\textsuperscript{2+}] >8) and ATP-containing buffer (relaxing solution) as described earlier\textsuperscript{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\textsuperscript{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\textsuperscript{2+} MOPS containing either PT1 or DMSO. After that, the arteries were flash frozen in liquid nitrogen and processed as previously described\textsuperscript{4} with the additional use of phosphatase inhibitors NaF (500 µM) and Na\textsubscript{3}VO\textsubscript{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 HUASM and PCASM (HUASMCS: Skeletal Muscle Cell Basal Medium, CC-3161, Lonza plus SmGM BulletKit, C-3182, Lonza; PCASMCS: 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 PCASM 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/cm²) (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. 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 used⁷.

**Drugs and buffer solutions.** The MOPS (3-morpholinopropanesulfone acid) -buffered salt solution used in the experiments contained (in mM): CaCl₂·2H₂O 3.0, EDTA (ethylenediaminetetraacetic acid) 0.02, glucose 5.0, KCl 4.7, MgSO₄·7H₂O 1.17, MOPS 3.0, NaCl 145, NaH₂PO₄·1H₂O 1.2, pyruvate 2.0. In MOPS buffers containing 125 mM KCl and varying concentrations of CaCl₂, concentrations of NaCl were adjusted accordingly so guarantee unchanged overall osmolality. PBS+ (phosphate-buffered saline with divalent cations) contained (in mM): CaCl₂·2H₂O 0.9, glucose 5.6, KCl 5.4, MgSO₄·7H₂O 0.3, MgCl₂·6H₂O 0.3, NaCl 136.9, NaH₂PO₄·1H₂O 0.8, KH₂PO₄ 0.4, NaHCO₃ 3.6. Relaxing solution for α-toxin permeabilized arteries contained (in mM) 20 imidazole, 7.5 Na₂ATP, 10 EGTA, 10 Mg-acetate, 10 creatine phosphate, 31.25 potassium-methanesulfonate, 5 NaN₃, 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 CaCl₂ yielding a pCa (=-log[Ca²⁺]) 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.

**Ca²⁺-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 MLCK (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 measured 7. 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 VIIIIB).

**No changes of LIMK phosphorylation status after AMPK activation.** LIMK is known to be implicated in the regulation of cofilin phosphorylation status\(^9\). 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 Ca$^{2+}$ during acetylcholine-induced dilation of microvessels. 


Supplemental Figures:

**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\textsuperscript{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\textsuperscript{2+} while the intracellular Ca\textsuperscript{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 Ca\(^{2+}\) 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<0.001, 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, MLC20total/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 25kD. 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).