AMPK Dilates Resistance Arteries via Activation of SERCA and BK_{Ca} Channels in Smooth Muscle

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Abstract—The protective effects of 5'-AMP–activated protein kinase (AMPK) on the metabolic syndrome may include direct effects on resistance artery vasomotor function. However, the precise actions of AMPK on microvessels and their potential interaction are largely unknown. Thus, we set to determine the effects of AMPK activation on vascular smooth muscle tone and the underlying mechanisms. Resistance arteries isolated from hamster and mouse exhibited a pronounced endothelium-independent dilation on direct pharmacological AMPK activation by 2 structurally unrelated compounds (PT1 and A769662). The dilation was associated with a decrease of intracellular-free calcium [Ca^{2+}]i in vascular smooth muscle cell. AMPK stimulation induced activation of BK_{Ca} channels as assessed by patch clamp studies in freshly isolated hamster vascular smooth muscle cell and confirmed by direct proof of membrane hyperpolarization in intact arteries. The BK_{Ca} channel blocker iberiotoxin abolished the hyperpolarization but only partially reduced the dilation and did not affect the decrease of [Ca^{2+}]i. By contrast, the sarcoplasmic/endoplasmic Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin largely reduced these effects, whereas combined inhibition of SERCA and BK_{Ca} channels virtually abolished them. AMPK stimulation significantly increased the phosphorylation of the SERCA modulator phospholamban at the regulatory T17 site. Stimulation of smooth muscle AMPK represents a new, potent vasodilator mechanism in resistance vessels. AMPK directly relaxes vascular smooth muscle cell by a decrease of [Ca^{2+}]i. This is achieved by calcium sequestration via SERCA activation, as well as activation of BK_{Ca} channels. There is in part a mutual compensation of both calcium-lowering mechanisms. However, SERCA activation which involves an AMPK-dependent phosphorylation of phospholamban is the predominant mechanism in resistance vessels. (Hypertension. 2015;66:108-116. DOI: 10.1161/HYPERTENSIONAHA.115.05514) Online Data Supplement

Key Words: muscle, smooth, vascular phospholamban vasodilation

The 5'-AMP–activated protein kinase (AMPK) consists of heterotrimeric complexes which are expressed ubiquitously in mammalian cells and in essentially all other eukaryotes. AMPK is considered to work as a cellular fuel sensor promoting catabolic pathways and inhibiting anabolic pathways of cell metabolism. Therefore, its function in liver and skeletal muscle has been studied extensively. However, AMPK has also been shown to have significant effects on the vascular system and blood constituents, including endothelial and smooth muscle cells, as well as platelets and leukocytes. In many arterial vessels of different sizes (from aorta down to small arteries) and from various vascular beds and several species, AMPK activators were reported to induce vasodilation. Conversely, vessels from animals being deficient of subunits of AMPK (mainly α1) show a reduced or even abolished vasodilation. Accordingly, in vivo treatment with partially unspecific AMPK activators has been shown to reduce arterial blood pressure in several studies. This offers the interesting possibility that AMPK stimulators may not only act as potent antidiabetic drugs but also as efficient antihypertensive drugs, particularly in vascular disease states, such as metabolic syndrome.
However, it is still unclear which potential cellular targets of AMPK activation are involved in vasodilation. In the endothelium, several studies report nitric oxide–dependent vasodilation in response to AMPK activation,\textsuperscript{10,12,16} whereas a recent study in small mesenteric vessels exhibiting endothelium specific knockout of the AMPK α\textsubscript{1} subunit suggests a role of AMPK in endothelium-dependent hyperpolarization.\textsuperscript{17} In contrast other studies demonstrated also endothelium-independent, direct effects of AMPK stimuli on vascular smooth muscle at least in large rodent arteries, a dilator mechanism which may be important especially in conditions of impaired endothelial function.\textsuperscript{8,9,11} 

Smooth muscle relaxation, which underlies vasodilation, is principally the result of a reduction in calcium sensitivity of the smooth muscle contractile apparatus or a reduction in the concentration of \([\text{Ca}^{2+}]\).\textsuperscript{18} \([\text{Ca}^{2+}]\) is regulated either by changes of calcium release from intracellular stores or of calcium influx mainly via voltage-dependent calcium channels (\(\text{Ca}_\text{v}\)) which may involve a role of other channels, for example, potassium or chloride channels regulating smooth muscle membrane potential. Alternatively or in addition, \([\text{Ca}^{2+}]\) can be controlled by changing its transport out of the cell or back to intracellular stores, which involves activity changes of the sodium/calcium exchanger,\textsuperscript{19} as well as the sarcoplasmic/endoplasmic \(\text{Ca}^{2+}-\text{ATPase (SERCA)}.\textsuperscript{20} At present, there is limited information as to whether microvascular smooth muscle is directly affected by AMPK and, if so, which of the aforementioned mechanisms play a role and how they interact. We therefore studied the effects of AMPK activation on vascular tone and smooth muscle \([\text{Ca}^{2+}]\) in resistance arteries of hamster and mouse. In different vascular beds, we studied whether stimulation with 2 structurally distant AMPK activators induced changes of vascular tone predominantly via endothelial or smooth muscle mechanisms. We also studied whether AMPK stimulation elicited changes of \([\text{Ca}^{2+}]\) in these vessels and which of the several processes potentially involved in the control of \([\text{Ca}^{2+}]\) was affected by AMPK stimulation. Although our data confirm AMPK stimulation of BK\(_{\text{Ca}}\) channels in microvascular smooth muscle, they show for the first time that the control of \([\text{Ca}^{2+}]\) and vasodilation by AMPK in microvessels predominantly occurs via activation of SERCA in a phospholamban-dependent manner.

**Methods**

Detailed Methods are included in the Online-only Data Supplement.

**Isolation and Cannulation of Resistance-Type Arteries**

All animal care and experimental protocols were conducted in accordance with German federal animal protection laws. Hamster or mouse arteries supplying the gracilis muscle were isolated and processed as previously described.\textsuperscript{21} We verified that the relevant AMPK subunits for pharmacological stimulation were indeed expressed in hamster femoral arteries (Figure S1 in the online-only Data Supplement). Accordingly, these AMPK subunits could also be demonstrated in mouse mesenteric arteries via quantitative polymerase chain reaction (data not shown). Stimulation of AMPK by A76 led to a concentration-dependent decrease in cytosolic calcium along with vasodilation (Figure 1A and 1B) in vessels of both species. Hamster gracilis and mouse mesenteric arteries had comparable EC\textsubscript{50} values for dilation (21.0±3.9 µmol/L, n=5 versus 34.6±6.0 µmol/L, n=6; no significant difference) and calcium decrease (39.4±9.9 µmol/L, n=5 versus 39.8±8.4 µmol/L, n=6).

To exclude nonspecific actions of A76, we studied the effects of a second, structurally unrelated AMPK activator, PT1, that is reported to act via a different mechanism.\textsuperscript{25} PT1 mimicked all of the effects of A76, including vasodilation, potassium channel activation, and SERCA stimulation (see below and Figures S2 and S3).

At their highest concentrations, both compounds induced a full dilation of the vessels. Neither vasodilation nor calcium decrease was diminished by removal of the endothelium. Rather, the responses were augmented to some extent (Figure 1C and 1D). Likewise, de-endothelialization neither attenuated the dilation nor the decrease in \([\text{Ca}^{2+}]\) in mouse vessels. In addition, incubation with the nitric oxide synthase inhibitor L-NAME (Nω-nitro- L-arginine methyl ester; 30 µmol/L) and the COX (cyclooxygenase) inhibitor indomethacin (30 µmol/L) revealed no inhibition of the AMPK-mediated dilation and calcium decrease in vessels with intact endothelium. For the sake of comparability with previous experiments from our laboratory, all further studies used cyclooxygenase and nitric oxide synthase inhibition during AMPK stimulation.

**Western Blot of Whole Arteries**

For the detection of phospholamban and SERCA expression, mouse and hamster femoral arteries, as well as mouse mesenteric arteries, were snap-frozen in precooled 15% trichloroacetic acid in acetone and processed as described.\textsuperscript{23}

**Isolation of Vascular Smooth Muscle Cells and Patch Clamp Measurements**

To investigate changes in macroscopic potassium currents caused by AMPK in freshly isolated vascular smooth muscle cells (VSMCs), arteries supplying the gracilis muscle of hamster were isolated and digested enzymatically as described before.\textsuperscript{24} The perforated patch clamp technique was used to maintain the integrity of cytoplasmic components like soluble second messengers. Membrane potentials from VSMCs were recorded using the perforated-patch clamp configuration.

**Statistics**

Statistical tests comprised 2-way ANOVA plus Tukey’s test, 1-way ANOVA plus Holm–Sidak method, paired t test, t test, and Kruskal–Wallis test followed by Dunn’s multiple comparison method whenever applicable. For descriptive reasons, all values are presented as means together with the SEM. Differences were considered statistically significant when the error probability was <0.05.

**Results**

**AMPK-Mediated, Endothelium-Independent Vasodilation Is Associated With a Decrease in \([\text{Ca}^{2+}]\)**

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AMPK Effects in VSMC Involve Activation of BK$_{Ca}$ Channels and Associated Hyperpolarization

Membrane potential measurements were performed to examine the possibility of voltage changes because of potassium channel activation accounting for the decrease in calcium and the associated vasodilation.

Again, the results obtained for resting membrane potential in isolated hamster VSMC were nearly identical to those in intact mouse arteries (−33.8±3.6 mV, n=8 versus −33.3±2.3 mV, n=22). A 50-µmol/L A76 induced compound C-sensitive hyperpolarization in isolated hamster VSMC (−51.7±3.2 mV, n=8). Likewise, 30-µmol/L A76 induced iberiotoxin-sensitive hyperpolarization of preconstricted, cannulated murine vessels (−43.4±1.5 mV, n=14; Figure 2).

To further clarify the role of potassium channels in AMPK-induced dilation, we performed perforated patch clamp analysis of hamster VSMC. AMPK activation by A76 showed a concentration-dependent increase in outward current during voltage-clamp measurements with voltage steps and was also observed at the resting membrane potential. The outward current was sensitive to inhibition by paxilline and iberiotoxin (Figure 2H), suggesting the involvement of BK$_{Ca}$ potassium channels. Furthermore, the AMPK inhibitor compound C (dorsomorphin), although not a completely specific inhibitor of AMPK,26 ablated the outward current induced by A76 (Figure 2J). Similar results were obtained with PT1 (Figure S3). Both experimental series were consistent with an essential role of AMPK in BK$_{Ca}$ potassium channel activation.

No Changes by BK$_{Ca}$ Channel Inhibitors on AMPK-Induced Effects in Intact Vessels

Despite the full inhibition of A76-induced hyperpolarization (Figure 2D), paxilline and iberiotoxin showed only minor inhibitory effects on AMPK-mediated dilation and did not affect the calcium decreases induced by A76 (Figure 3; A76+IbTx). In agreement with this, BK$_{Ca}$ gene-deficient mice showed no impairment of the A76-induced vasodilation and the associated calcium decrease (Figure S10). Patch clamp measurements in isolated VSMC from these BK$_{Ca}$ gene-deficient mice showed indeed no outward current on AMPK activation (Figure 2A–2C).

Significant Impairment of Calcium Decreases and Associated Dilation by SERCA Inhibition

We conducted another series of concentration response curves with AMPK stimulators in intact vessels to study the effects of the SERCA inhibitor thapsigargin (1 µmol/L), with or without iberiotoxin or paxilline. Application of thapsigargin to vessels preconstricted with norepinephrine gradually augmented VSMC cytosolic Ca$^{2+}$ throughout the incubation time (2 minutes) suggesting ongoing store depletion (not shown) but did not change the tone of the vessels significantly. In sharp contrast to results obtained with sole BK$_{Ca}$ inhibition, pretreatment with thapsigargin alone substantially and significantly reduced the dilatory and calcium-decreasing effects of AMPK stimulation. The effects of thapsigargin could be reproduced with a second SERCA inhibitor, cyclopiazonic

Figure 1. 5′-AMP–activated protein kinase (AMPK)-mediated, endothelium-independent vasodilation paralleled by a decrease in [Ca$^{2+}$]. Representative example of an original recording of the effects of A769662 (A76, 1–100 µmol/L, gray) versus time control (black) on diameter (A) and intracellular calcium (B). Arrowheads indicate application of A76 at increasing concentrations or sham solution. The small resistance artery preconstricted with 0.3 µmol/L norepinephrine (NE) shows potent vasodilation associated with a decrease of [Ca$^{2+}$] when AMPK was stimulated. The application of the Ca$_{V}$-channel blocker nifedipine (5 µmol/L) elicited no further dilation and only minor additional calcium decrease. Microvessels showed dose-dependent and endothelium-independent vasodilation (C) associated with a decrease of [Ca$^{2+}$] (D) when exposed to A76 in concentrations reaching from 10$^{-6}$ to 3×10$^{-4}$ mol/L (n=4–5). Note the stable calcium signal of the time controls in comparison with the decrease in the AMPK-stimulated vessels. *P<0.05, intact endothelium vs without endothelium, 2-way ANOVA, Tukey.
acid (10 µmol/L, not shown). Combinations of thapsigargin with iberiotoxin or paxilline virtually abrogated the calcium decrease (Figure 3, A76+TG+IbTx).

Increased SERCA Activity on AMPK Stimulation

To further address the issue of an effect of AMPK on SERCA in intact vessels, we indirectly assessed smooth muscle sarcoplasmic reticulum calcium content by measuring the calcium transient on application of caffeine in hamster and mouse vessels (1 and 10 mmol/L, respectively).27 The transients were recorded after 5 minutes (hamster) and 20 minutes (mouse) in calcium-free buffer to avoid any confounding effects of channel-related influx. We assessed the release of sarcoplasmic reticulum calcium after caffeine application from the relative height of the calcium peak after caffeine treatment. Pretreatment with A76 increased calcium transients in response to caffeine in both species significantly (Figure 4).

In a separate set of experiments in hamster vessels, voltage-gated calcium channels were blocked by nifedipine and again caffeine was applied in the absence or presence of A76. Pretreatment with A76 enhanced the nifedipine-mediated basal [Ca2+]i reduction to a greater extent than under control conditions (Figure S6), suggesting stimulated activity of [Ca2+]i lowering systems because of AMPK activation.
Increased Phosphorylation of the SERCA Modulator Phospholamban in Microvascular Smooth Muscle After AMPK Stimulation

Because phospholamban is an important modulator of SERCA activity in many tissues, we studied its expression and potential changes of its phosphorylation state in mouse microvessels. Quantitative polymerase chain reaction and Western blot experiments showed mRNA and protein expression of phospholamban in small mesenteric resistance arteries (Figure S8). The protein was expressed in much smaller amounts than observed in cardiac muscle tissue (not shown) so that several vessel segments had to be pooled. After incubation with submaximal dilator doses of the AMPK activator A76, the phosphorylation of phospholamban at the regulatory T17 site (Figure 5; Figure S9) was significantly increased in vessels from both vascular regions.

Discussion

In this study, we report that AMPK can directly relax vascular smooth muscle of resistance vessels of 2 different species and vascular regions, respectively, by reducing \([\text{Ca}^{2+}]\). The AMPK stimulation targets 2 potent calcium-reducing mechanisms, BKCa channels and, as a novel target, SERCA.

In contrast to other studies, direct AMPK stimulation by A769662 and by PT1 did not result in an endothelium-dependent vasomotor response. Our finding of an...
AMPK-mediated, but endothelium-independent dilation is, however, in line with studies involving large arteries. The lack of an endothelium-dependent dilation as observed here does not exclude a role of AMPK in endothelial vasomotor control. Rather it may be explained by a preferential action of A769662 on vascular smooth muscle. Most studies reporting AMPK-induced endothelium-dependent dilation have been performed using the unspecific stimulator AICAR (5-aminimidazole 4-carboxamide 1-β-d-ribofuranoside) which seems to be rather ineffective in vascular smooth muscle when compared with other tissues. There are no comparable data for A769662 with regard to endothelial versus smooth muscle sensitivity to this compound. Interestingly, 2 studies on AMPK effects in cultured endothelial cells in which A769662 was used as AMPK stimulator report 3- to 5-fold higher A769662 concentrations than used in our study which might suggest that relatively high concentrations are necessary for endothelial AMPK stimulation. A recent study demonstrating loss of endothelium-dependent dilation by hyperpolarization (EDH-dependent dilation) after endothelium-specific knockout of the α1 subunit does not contradict our results either, because in these experiments only the endothelium was stimulated (with acetylcholine) but not the smooth muscle. Our studies indicate for the first time that microvessels can be dilated via AMPK stimulation directly in smooth muscle and this finding may be important for treatment strategies in cardiovascular diseases being associated with loss or severe impairment of endothelial function.

AMPK can induce smooth muscle relaxation directly by a decrease of calcium or of calcium sensitivity. Although ongoing studies in our laboratory indicate (slowly incipient) effects of AMPK on calcium sensitivity in resistance arteries as well, we focused in the present study on the mechanisms of calcium decrease. We identified 2 underlying mechanisms: increased SERCA activity as a result of enhanced phospholamban phosphorylation and, although to our surprise functionally less effective, smooth muscle hyperpolarization induced by stimulation of BKCa channels.

Dilation as well as [Ca2+] decrease was observed as common responses to 2 structurally unrelated AMPK stimulators. We have confirmed the expression of the AMPK sub-units which are required for the stimulator effects of A76 and PT1. We did not study the effect of acute pharmacological AMPK inhibition on vascular responses to A76 and PT1 because the best known AMPK inhibitor, compound C, has not only limited selectivity for AMPK but also elicited an acute and almost maximal vasodilation by itself, thus precluding any assessment of AMPK-mediated vasodilator effects in the present setting. Nevertheless, compound C did block the effects of both AMPK stimulators on BKCa channels in freshly isolated smooth muscle cells, thereby further supporting that this effect was AMPK-dependent.

The smooth muscle hyperpolarization in the intact arterial segments studied here, as well as in isolated smooth muscle, was in good accordance with the findings of Weston et al. in rat and mouse mesenteric arteries being consistent with an AMPK-induced activation of BKCa channels. This is further substantiated by the full inhibitory effects of iberiotoxin on the A76-induced hyperpolarization. In addition to pharmacological inhibition of BKCa channels, we also studied BKCa knockout mice. Accordingly, in our patch clamp studies both PT1 and A76 elicited outward currents whose magnitudes were characteristic for BKCa channels and were sensitive to paxilline and iberiotoxin. Furthermore, the strikingly similar effects of A76 on dilatation (and calcium decreases) in BKCa knockout mice yield an independent piece of evidence that the effects cannot be explained by BKCa channel activation alone. We did not study the mechanisms which lead to channel activation in detail because it has been shown already in other cells that AMPK can directly phosphorylate BKCa channels and increase their conductance, depending on the specific splice variant expressed by the tissue. It is however conceivable that additionally or alternatively, AMPK could sensitize ryanodine receptors thereby increasing the amount and frequency of calcium sparks leading to BKCa channel activation and spontaneous transient outward currents indicative of BKCa channel activation. The latter could not be studied in the isolated cells under the experimental conditions chosen. Nevertheless, A76-induced augmentation of calcium transients at sufficiently high-caffeine concentrations to deplete the stores completely (10 mmol/L) argue against mere sensitization of ryanodine receptors.

Surprisingly, treatment of intact resistance arteries with the BKCa channel blockers iberiotoxin or paxilline only...
partially reduced the dilation in response to AMPK stimulation and did not affect the \([\text{Ca}^{2+}]\), decrease, in spite of fully blocking the A76-induced hyperpolarization. This points toward a simultaneously acting second calcium-reducing mechanism. The existence of such a potent calcium-lowering mechanism potentially acting in parallel to membrane hyperpolarization effects may be 1 explanation for the surprisingly mild vascular phenotype of BKs, knockout mice. This is in line with our observation of a fully preserved A76 effect in vessels of these animals. Only when we pharmacologically inhibited SERCA, the effect of BKs channel activation became visible although it did not reduce calcium to the same extent as SERCA did. Thapsigargin has been shown to reduce VSMC spark frequency which may explain why BKs channel activation could not fully compensate for SERCA inhibition.

SERCA is a well-known and potent modulator of smooth muscle \([\text{Ca}^{2+}]\). Further, the activity of SERCA2b, which is the major isoenzyme in VSMC, can increase several fold on stimulation. Accordingly, in isolated smooth muscle cells, SERCA activity could substantially reduce \([\text{Ca}^{2+}]\), even when the membrane was mildly permeabilized, clearly indicating a considerable calcium scavenging capacity of the endoplasmic reticulum. In this study, we show for the first time that AMPK stimulation rapidly induced SERCA-dependent calcium-lowering mechanisms to an extent that potently diminished VSMC \([\text{Ca}^{2+}]\), even in the presence of continuous calcium influx via Ca, channels. Several lines of evidence let us conclude that the \([\text{Ca}^{2+}]\), lowering effect of AMPK stimulation is dependent on SERCA: (1) The effects of AMPK stimulators were greatly reduced after selective inhibition of SERCA with thapsigargin or cyclopiazonic acid. This occurred under conditions where the SERCA inhibition did not induce a further increase in tone which may have compromised the interpretation. (2) Caffeine induced higher calcium transients in cells pretreated with an AMPK stimulator than in control cells, suggesting that the stores were more filled, probably because of a higher SERCA activity. This was observed in the absence of extracellular calcium, which excludes involvement of an altered store-operated calcium entry. (3) The \([\text{Ca}^{2+}]\), decrease after the caffeine-induced calcium transient was more pronounced in cells pretreated with an AMPK stimulator, again suggesting a more efficient removal of \([\text{Ca}^{2+}]\), into internal stores. In line with this interpretation, we also observed a stronger decrease of \([\text{Ca}^{2+}]\) immediately after blockade of \(\text{Ca}_\text{s}\) channels when an AMPK stimulator was present. (4) We were able to demonstrate an enhanced phospholamban phosphorylation at the regulatory Thr17 site in isolated mesenteric and femoral arteries of mice on AMPK stimulation. This phosphorylation has been shown to diminish SERCA and may therefore explain the stimulatory effect of AMPK on SERCA. Nonetheless, these findings do not exclude additional mechanisms that may be involved in AMPK-mediated stimulation of SERCA. Because the phospholamban phosphorylation was studied in mouse arteries only, a caveat has to be made with respect to the phospholamban-dependent regulation of SERCA activity in hamster vessels (which was not directly shown).

A reduced SERCA activity in association with higher basal levels of \([\text{Ca}^{2+}]\), has been described before in cultured endothelial and smooth muscle cells of AMPK-\(\alpha_\text{1}\)-deficient mice: However, this impaired calcium handling was not ascribed to direct, AMPK-related SERCA inhibition. Rather it was assumed that an increased formation of reactive oxygen species and associated endoplasmic reticulum calcium spillover to the cytosol, both mediated by endoplasmic reticulum stress, occurred in the chronic absence of AMPK. This influence of ROS on SERCA seems to be chronic and irreversible in nature, whereas the acute effects of AMPK stimulators on SERCA that we describe were found to be quickly reversible.

**Perspectives**

Small arteries and arterioles play a significant role in modifying peripheral resistance and thereby blood pressure. Our results demonstrate the ability of AMPK activators to lower \([\text{Ca}^{2+}]\), at the level of microvascular smooth muscle in this kind of vessels. AMPK exerts its effects via 2 independent mechanisms allowing redundant control of calcium homeostasis. Under the conditions studied here, the prevailing mechanism is an AMPK-dependent activation of SERCA which involves phosphorylation of phospholamban (Figure 6). The parallel stimulation of \(\text{BK}_\text{s}\) channels becomes important in cases of impaired SERCA function. We therefore propose AMPK as a potential new target for the therapeutic induction of vasodilation, especially under conditions of impaired endothelial function as found in hypertension or metabolic syndrome.

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Disclosures

None.

References


**Novelty and Significance**

**What Is New?**

We describe for the first time that in smooth muscle of intact resistance vessels, 5′-AMP–activated protein kinase (AMPK) regulates intracellular-free calcium by activating 2 different, potentially redundant mechanisms. Our findings are consistent across species borders. The prevailing mechanism is an AMPK-dependent sarcoplasmic/endoplasmic Ca2+-ATPase (SERCA) activation which involves phosphorylation of its regulator, phospholamban. At the same time, AMPK stimulation also induces activation of BKCa. Although both mechanisms converge on [Ca2+]i decrease and vasodilation, SERCA stimulation alone was sufficient to exert a substantial decrease of [Ca2+]i, and full dilation of intact resistance vessels.

**What Is Relevant?**

We identified 2 major mechanisms by which AMPK directly relaxes vascular smooth muscle, including a potent activation of SERCA via its regulator phospholamban, introducing AMPK as a novel versatile player in the control of [Ca2+]i, in microvessels.

**Summary**

We identified 2 major mechanisms by which AMPK directly relaxes vascular smooth muscle, including a potent activation of SERCA via its regulator phospholamban, introducing AMPK as a novel versatile player in the control of [Ca2+]i, in microvessels.
AMPK Dilates Resistance Arteries via Activation of SERCA and BK$_{Ca}$/ Channels in Smooth Muscle

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ONLINE DATA SUPPLEMENT

AMPK relaxes resistance arteries via activation of SERCA and BK\textsubscript{Ca} channels in smooth muscle.

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Supplemental Methods

\textit{Isolation and cannulation of resistance-type arteries.} All animal care and experimental protocols were conducted in accordance with German federal animal protection laws. Arteries supplying the gracilis muscle were isolated from male Golden Syrian Hamsters (Janvier, Le Genest-Saint-Isle, France) as described before \textsuperscript{1}. Male C57BL6/N mice were
purchased from Charles River Laboratories (Sulzfeld, Germany). Mesenteric of gracilis artery segments were isolated using identical procedures.

**Calcium and diameter registration.** The isolated arteries were treated as published before. 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 45 mmHg. The smooth muscle layer was loaded with the calcium indicator Fura-2-AM (2 μM, LifeTechnologies, Carlsbad, CA, USA) over an incubation period of 2 h. The measured Fura signals were corrected for background fluorescence (as measured at the end of each experiment after quenching with 8 mM MnCl₂). Simultaneously with the measurement of [Ca²⁺], vascular diameters were recorded by videomicroscopy (Hasotec, Rostock, Germany).

As shown by us before, these vessels develop little myogenic tone (about 10% of resting diameters) at 45 mmHg transmural pressure. Nifedipine was always used to achieve a minimal calcium level and maximal diameter in pre-constricted vessels. The difference between maximum values (diameter: nifedipine, calcium: norepinephrine) and minimum values (diameter: norepinephrine, calcium: nifedipine) was used to normalize the calcium and diameter changes. Nifedipine in the concentrations used at 5 μM always usually induced a full dilation and minimal calcium unless this was already reached with the highest concentration of A76. Maximal diameters were close to the initial resting diameters. Maximal diameter and minimal calcium levels were not different between (A76-) treated and control vessels.

Table S1 summarizes the absolute diameters of hamster vessels under various conditions (basal, 0.3 μM norepinephrine, 0.3 μM norepinephrine + 100 μM A76, 5 μM nifedipine) to allow evaluation of relative changes.

Mouse vessels required slightly higher concentrations than hamster vessels to achieve the same defined minimal degree of pre-constriction (30 %). Therefore, for mouse vessels 1 μM
norepinephrine was used whereas 0.3 µM norepinephrine were sufficient in hamster vessels. Norepinephrine was chosen as a pre-constrictor because it produced a stable tone and calcium signal in the time control experiments.

Upon addition of substances to the organ bath, small, transient elevations of [Ca$$^{2+}$$] were observed. These were not studied further since they (1) occurred under any condition studied, i.e. also in time controls and (2) did not lead to any changes in tone of the apparently maximally constricted arteries.

In experiments involving deendothelialization, an air bubble (1 ml) was passed through the lumen to destroy the endothelium. Absence of functional endothelium was verified by the blunted dilator response to 3 µM acetylcholine in preconstricted arteries (300 nM norepinephrine) which had been present prior to deendothelialization.

**Western blot of whole arteries.** For the detection of phospholamban (PLB) phosphorylation, several small arteries of a murine mesenterium or both femoral arteries (male mice, C57BL6J, Charles River, Sulzfeld, Germany) were isolated, cannulated on one side and flushed free from intraluminal blood. The length of each artery was assessed via a custom-made ruler that was placed underneath the organ bath. Subsequently, the glass cannula was broken and the artery was transferred to a cell culture dish filled with MOPS buffer. The samples were divided into 2 groups of approximately equal length. Then they were transferred to fresh MOPS buffer, heated to 37 ºC and stimulated with 1 µM norepinephrine for 5 minutes followed by 5 minutes of norepinephrine plus 0.03 % DMSO or norepinephrine plus 30 µM A76. After that, the arteries were snap-frozen in pre-cooled 15 % trichloroacetic acid (TCA) in acetone and processed as previously described 3 with the additional use of phosphatase inhibitors NaF (500 µM) and Na$_3$VO$_4$ (1 mM) in the lysis buffer. After lysis, the samples were sonicated (3 pulses for 10 seconds). For the detection of AMPK subunit expression, hamster femoral arteries were placed in lysis buffer and repeatedly deep-frozen in dry ice and subsequently scraped with a cannula. The isolated proteins were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE),
transferred onto a nitrocellulose membrane (AMPK detection) or 0.2 µm PVDF membrane (phospholamban detection), fixed by incubation with 0.5 % PonceauS and incubated with the primary antibody overnight in a shaker at 4 °C. After that step, the membrane was washed with Tris-buffered saline and tween (TBST) buffer and incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody. Finally, bound secondary antibody was visualized by HRP-mediated luminol oxidation (AppliChem, Darmstadt, Germany) which was detected with a CCD camera. Anti-AMPK α1 and α2 antibodies were kindly provided by Beate Fißlthaler (Goethe University, Frankfurt a. M., Germany), anti-SERCA 2 (atp2a2), anti-AMPK β1 and β2 antibodies were purchased from New England Biolabs (Frankfurt a. M., Germany), anti-GAPDH antibody from Merck Millipore (Billerica, MA, USA) and anti-phospholamban and phospho-phospholamban (pT17) antibodies from Badrilla (Leeds, UK).

Isolation of vascular smooth muscle cells and patch clamp measurements.

To investigate changes in macroscopic potassium currents caused by AMPK in freshly isolated vascular smooth muscle cells (VSMCs), arteries supplying the gracilis muscle of hamster or mouse were isolated and digested enzymatically as described before. The perforated patch clamp technique was used to maintain the integrity of cytoplasmic components like soluble second messengers. In a cell culture dish mounted to a low volume chamber, adherent VSMCs were superfused with the extracellular solution (in mM): NaCl 135, KCl 5, CaCl2 1, MgCl2 1, glucose 10, HEPES 10, adjusted to pH 7.4 with NaOH. The tip of this patch electrode was first filled with the amphotericin B-free solution (in mM: KCl 30, K-aspartat 110; MgCl2 1, EGTA 0.1 and HEPES 10, titrated to pH 7.2 with NaOH) by aspiration to a column length of 500 µm. Then the pipette was backfilled with the same solution additionally containing 300 µg ml⁻¹ water-soluble amphotericin B (Sigma-Aldrich, Deisenhofen, Germany). The osmolarity of all solutions was controlled with vapor osmometer Vapro 5520 (Wescor, Logan, USA). The liquid junction potential was +13.8 mV and offset corrections were made by the Patch Master software (v2.52, HEKA, Lambrecht, Germany). The perforation started shortly after seal formation and reached a steady-state level within 1–
2 min. Patch pipettes of resistances of 2.5 to 4.0 MΩ caused series resistances of 6 to 11 MΩ. The values of pipette and cell membrane capacitance were estimated with the Patch Master software and corrected automatically. Series resistance compensation (70–80%) was used to limit voltage errors to less than 5 mV. Only cells with minimal leakage currents were used. Data of perforated-patch recordings were acquired at a frequency of 10 kHz after filtering at 1.67 kHz with an EPC10 patch clamp amplifier (HEKA, Lambrecht, Germany) using the Patch Master software. The cells were depolarized for 800 ms from a holding potential of -80 to +40 mV or to potentials between -70 and +40 mV (with increments of 10 mV). Membrane potentials from VSMCs were recorded using the perforated-patch clamp configuration.

_Determination of mRNA levels in isolated arteries._ cDNA was isolated from murine mesenteric arteries as previously described. Quantitative PCR was performed on a StepOne machine (LifeTechnologies, Carlsbad, CA, USA) using the SYBR-Green reagent (Power SYBR Green, LifeTechnologies, Carlsbad, CA, USA) with normalization of the target genes to the geometric mean of hprt1 (hypoxanthine phosphoribosyltransferase 1), ywhaz (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide) and sdha (succinate dehydrogenase complex, subunit A). PCR primer sequences were
tgagctttcctgcgtaacag (plb_for), tggtaagagaaagataaagtga (plb_rev),
tcgacagtcaattctcagag (atp2a2_for), cagggaaggtcagtatgc (atp2a2_rev),
tcctcggaagtgaaggt (prkaa1_for), gaatctctgcctggtgagt (prkaa1_rev),
tctcttctcagacgctttt (hprt1_for), cctgtgtcatcatcgttaatc (hprt1_rev),
taaaggtcaagggcgttc (ywhaz_for),
caccacagcagatgc (ywhaz_rev),
cctctgacatcgatact (sdha_for) and tcttctcagacctgttta (sdha_rev).

_Measurement of membrane potential of VSMC in intact arteries._ The AgCl counter electrode was connected to the organ bath solution via an agar bridge (3% in 3M KCl). Cannulated murine mesenteric arteries were constricted by norepinephrine (1 µM). Subsequently, the arteries were relaxed by application of A769662 (30 µM) followed by
application of iberiotoxin (100 nM). In another series of experiments, thapsigargin (1 µM) was applied after pre-constriction, followed by 30 µM A76. The arteries were impaled with glass microelectrodes (tip size <1 µm, resistance 60-100 MΩ) that had been backfilled with 3 M KCl and mounted on a micromanipulator unit (Scientifica, Uckfield, UK). Membrane potential was recorded with a pre-amplifier (BA-01X, Npi Electronic GmbH, Tamm, Germany) connected to a data-acquisition system (LabChart, ADInstruments, Bella Vista, Australia). Criteria for valid registrations of membrane potential were sharp negative deflection of the voltage upon impalement and sharp positive deflection upon retraction of the microelectrode.

**Drugs and buffer solutions.** The MOPS (3-morpholinopropanesulfone acid) -buffered salt solution used in the experiments contained (in mM): CaCl$_2$ 3.0, EDTA (ethylenediaminetetraacetic acid) 0.02, glucose 5.0, KCl 4.7, MgSO$_4$ 1.17, MOPS 3.0, NaCl 145, NaH$_2$PO$_4$ 1.2, pyruvate 2.0. Norepinephrine was purchased from Aventis, indomethacin from Fluka, nifedipine and Nω-Nitro-L-arginine methyl ester (L-NAME) and manganese (II) chloride tetrahydrate from Sigma Aldrich (Seelze, Germany), thapsigargin, PT1, iberiotoxin and paxilline from Tocris (Bristol, UK). A769662 (A76) was a generous gift from D. Grahame Hardie (University of Dundee, Scotland, UK) and later on purchased from Tocris or Adooq Bioscience (Irvine, USA).

**Supplemental results**

**Expression of AMPK subunits targeted by the AMPK stimulators.** The AMPK activators used are reported to activate AMPK either by binding between the kinase and auto-inhibitory domains of the α1 or α2 subunits (PT1)$^7$ or between the carbohydrate-binding module of the β1 subunit and the N-lobe of the kinase domain (A769662, A76)$^8$-10. Western blots from hamster arteries were therefore performed to confirm the expression of these subunits. The blots yielded signals consistent with the expression of α1, α2, β1 and β2 subunits of AMPK (Fig. S1).

**Effects of PT1 as second AMPK activator mimic those of A769662.** To further substantiate that the effects of A76 were not related to unspecific activities we used PT1 as a
second activator of the AMPK. PT1 elicited, like A76, a dose-dependent vasodilation which was not mediated by the endothelium (see Fig. S2).

In freshly isolated smooth muscle cells of the resistance arteries, PT1, like A76, activated \( \text{BK}_{\text{Ca}} \) channels as derived from magnitude of stimulated currents (Fig. S3A) and from the effect of \( \text{BK}_{\text{Ca}} \) inhibitors (Fig. S4B). Again, the inhibitor of AMPK, compound C, virtually abolished the electrophysiological effects of PT1 (Fig. S3B).

**PT1 interferes with the aquisition of Fura-2 calcium signals.** In a Fura-2 containing cell-free system with a constant calcium concentration, we observed a concentration-dependent depression of the Fura-2 signal which occurred instantly after addition of PT1 to the solution suggesting a direct optical interference (Fig. S4). Since the Fura-2-specific fluorescence emission at the excitation wavelengths (340 and 380) were affected differently, PT1 induced an apparent increase of calcium concentration though the calcium concentration of the solution did not change. In intact vessels in which \([\text{Ca}^{2+}]\) had been “clamped” by depolarization with high extracellular potassium, treatment with the highest concentration of PT1 (30 µM) was also associated with an increase of the 340/380 ratio which however occurred with some measurable delay, probably reflecting the diffusion time of PT1 into the cells. This occurred as an apparent increase of \([\text{Ca}^{2+}]\), upon PT1 treatment. In contrast, in vessels which could still respond with a cytosolic decrease of calcium, the Fura-2 F340/F380 ratio did not increase to the same extent as in the “Ca\(^{2+}\) clamped” vessels indicating a decrease of \([\text{Ca}^{2+}]\), similar as observed with A76, which was however partly overcome by the optic interference of PT1 with the Fura-2 signal. (Fig. S4). We therefore noted a calcium decrease qualitatively but we did not consider it appropriate to quantify the calcium changes within the vascular smooth muscle since a correction of the optical effect would have required determination of the intracellular PT1 concentration.

**AMPK enhances nifedipine-mediated calcium decrease.** To further study the effects of the AMPK stimulator A76 on SERCA activity in a functional manner, we tested whether the calcium decreases in response to the \( \text{CaV} \) channel blocker nifedipine were enhanced in the
presence of the AMPK stimulator A76. Initially, the vessels were stimulated with 
norepinephrine (NE) to assess their calcium reactivity. After washout of NE, nifedipine with 
and without A76 was added to the organ bath and the resulting decrease of calcium was 
studied. Consistent with a higher activity of a second, probably SERCA mediated calcium 
removing mechanism, the calcium decrease after blockade of the calcium influx by nifedipine 
was significantly enhanced (Fig. S6).

**Phospholamban primary sequence matches the AMPK recognition sequence.** In search 
for the mechanism by which AMPK could induce an increase of SERCA-dependent calcium 
decrease, we analysed whether the SERCA modulator phospholamban contained 
sequences which could be directly phosphorylated by AMPK. The sequence around T17 
turned out to be a reasonably good fit to the AMPK recognition motif, with a hydrophobic 
residue (Ile) at P-5 and basic residues (Arg) at P-3 and P-4, although the residue at P+4 in 
phospholamban is proline and a residue with a bulky hydrophobic side chain is preferred\(^\text{11}\) 
(Fig. S7). However, we did discover an increased phospholamban phosphorylation at T17 
despite the mismatch at the terminal amino acid (see main manuscript).

**Supplemental References**

1. Bolz SS, Pieperhoff S, De Wit C, Pohl U. Intact endothelial and smooth muscle 
   function in small resistance arteries after 48 h in vessel culture. *Am J Physiol Heart 

2. Bolz SS, de Wit C, Pohl U. Endothelium-derived hyperpolarizing factor but not NO 
   reduces smooth muscle Ca\(^{2+}\) during acetylcholine-induced dilation of microvessels. 

3. Lubomirov LT, Reimann K, Metzler D, Hasse V, Stehle R, Ito M, Hartshorne DJ, 
   Gagov H, Pfister G, Schubert R. Urocortin-induced decrease in Ca\(^{2+}\) sensitivity of


Supplemental Tables:

<table>
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<th>Treatment</th>
<th>Mean diameter ± SEM, n (steady state)</th>
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<td>0.3 µM norepinephrine (NE)</td>
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<td>0.3 µM + 100 µM A769662</td>
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<tr>
<td>5 µM nifedipine</td>
<td>228.5 ± 6.9 µm, n=55</td>
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*Table S1: Mean diameters of hamster vessels in response to various stimuli.*
Supplemental figures

**Fig. S1:** Expression of AMPK subunits targeted by the AMPK stimulators. Western blots of two different vessels confirming the expression of all AMPK α (upper and middle lane) and β subunits (lower lane) in 2 hamster femoral arteries (FA).

**Fig. S2:** The application of PT1 (3*10^{-7} to 3*10^{-5} M) also caused dose-dependent and endothelium-independent vasodilation (n=3-4 per group). The dilatations were normalized to the maximal possible dilator responses of the pre-constricted vessels to nifedipine (*=P<0.05, intact endothelium vs. without endothelium, Two Way ANOVA, Tukey).
Fig. S3: Effect of PT1-treatment on the electrophysiological properties of smooth muscle cells freshly isolated from hamster resistance arteries. A: Typical patch clamp experiment showing the effect of PT1 on the current magnitude at depolarizing steps from -80 mV to +40 mV. Corresponding tracings and columns are coded with identical grey shades. B: Effects of PT1 (50 µM) on the current density determined at +40 mV in isolated smooth muscle cells. 100 µM paxilline and 30 µM compound C significantly reduced the outward current densities in response to PT1, suggesting that the effect was due to an AMPK-mediated activation of BK<sub>Ca</sub> channels.
**Fig. S4:** A: Application of increasing concentrations of PT1 to a cell-free organ bath with constant calcium concentration resulted in concentration-dependent signal depression of Fura-2. B: The signal at 380 nm excitation was more depressed, yielding an apparent increase in calcium concentration (increase in F340/F380 ratio).

**Fig. S5:** Application of PT1 to an artery at clamped intracellular calcium concentration (3 mM) increased the apparent Fura-2 F340/F380 ratio compared to control situation (pre). In a non-clamped situation (time control, PT1 at the very right), the ratio elevation was virtually absent, suggesting a decrease of [Ca^{2+}] in these vessels.
Fig. S6: A, B: Representative original registrations of intracellular calcium \([\text{Ca}^{2+}]_i\). Nifedipine (Nif, 5 µM) was applied alone (A) or in combination with A76 (30 µM, B). The resulting reduction in \([\text{Ca}^{2+}]_i\), was calculated as percent of the maximum calcium reduction under calcium-free conditions. In both cases, the previous calcium response to norepinephrine (300 nM) is shown for comparison. C: Upon blockade of extracellular calcium influx by nifedipine (5 µM), AMPK stimulation with 30 µM A76 caused a significantly stronger \([\text{Ca}^{2+}]_i\) reduction. \((n=7-9, *=P<0.05)\).
Fig. S7: Alignment of AMPK recognition sequence with the phospholamban amino acid (AA) sequence and comparison with the required AA sequence for the phosphorylation of either of the two regulatory phosphorylation sites S\textsubscript{16} and T\textsubscript{17}. \( \phi \) denotes hydrophobic AA, B denotes basic AA and X denotes any AA. Red pi (\( \pi \)) indicates mismatch by a small chain AA in the phospholamban primary sequence (A or P). Bold S and T indicate regulatory phosphorylation site S\textsubscript{16} or T\textsubscript{17}. AMPK recognition sequence taken from ref. \textsuperscript{12} and PLB AA sequence taken from ref. \textsuperscript{13}. 

<table>
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<th>AMPK recognition sequence</th>
<th>PLB AA sequence</th>
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**Fig. S8:** A: Relative mRNA levels in mouse mesenteric arteries show comparably low quantities for phospholamban (pln) in contrast to SERCA 2 (atp2a2). For comparison with mRNA levels of an already confirmed transcript AMPK alpha 1 (prkaa1) was also amplified (n=3 qPCR reactions from 3 different cDNA isolates). B: Representative Western blot from 2 hamster femoral arteries (FA) which confirms the expression of SERCA2.
**Fig. S9: Increased phosphorylation of the SERCA modulator phospholamban in mesenteric arteries after AMPK stimulation.**

*Fig. S9: Increased phosphorylation of the SERCA modulator phospholamban in mesenteric arteries after AMPK stimulation.* A: Representative Western blot from pooled small mesenteric arteries showing phosphorylation of phospholamban at threonine 17 (pT17-PLB, 24 kDa) in pooled arteries stimulated with norepinephrine and treated either with 30 µM A76 (A76) or with solvent only (0.03 % DMSO, Ctrl). A76 increased PLB phosphorylation. Heart tissue ("Heart") stimulated with isoprenaline (ISO) was used as a positive control. B: T17 phosphorylation in A76-stimulated arteries was virtually doubled in comparison to vehicle-treated control (n=3, *=P<0.05, paired t-test).
Fig. S10: Little difference between BK$_{\text{ca}}$ KO mice and WT littermates. Mesenteric arteries from BK$_{\text{ca}}$ KO mice showed no impairment in dilation in response to A76. Rather, dilation was significantly better at 30 µM A76. Likewise, the decrease in [Ca$^{2+}$], was unaffected (n=4 each,*=P<0.05, Two Way ANOVA, Tukey).