AMP-activated protein kinase inhibits $K_v$1.5 channel currents of pulmonary arterial myocytes in response to hypoxia and inhibition of mitochondrial oxidative phosphorylation

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Key points

- Progression of hypoxic pulmonary hypertension is thought to be due, in part, to suppression of voltage-gated potassium channels ($K_v$) in pulmonary arterial smooth muscle by hypoxia, although the precise molecular mechanisms have been unclear.
- AMP-activated protein kinase (AMPK) has been proposed to couple inhibition of mitochondrial metabolism by hypoxia to acute hypoxic pulmonary vasoconstriction and progression of pulmonary hypertension.
- Inhibition of complex I of the mitochondrial electron transport chain activated AMPK and inhibited $K_v$1.5 channels in pulmonary arterial myocytes.
- AMPK activation by 5-aminoimidazole-4-carboxamide riboside, A769662 or C13 attenuated $K_v$1.5 currents in pulmonary arterial myocytes, and this effect was non-additive with respect to $K_v$1.5 inhibition by hypoxia and mitochondrial poisons.
- Recombinant AMPK phosphorylated recombinant human $K_v$1.5 channels in cell-free assays, and inhibited $K^+$ currents when introduced into HEK 293 cells stably expressing $K_v$1.5.
- These results suggest that AMPK is the primary mediator of reductions in $K_v$1.5 channels following inhibition of mitochondrial oxidative phosphorylation during hypoxia and by mitochondrial poisons.

Abstract  Progression of hypoxic pulmonary hypertension is thought to be due, in part, to suppression of voltage-gated potassium channels ($K_v$) in pulmonary arterial smooth muscle cells that is mediated by the inhibition of mitochondrial oxidative phosphorylation. We sought to determine the role in this process of the AMP-activated protein kinase (AMPK), which is intimately coupled to mitochondrial function due to its activation by LKB1-dependent phosphorylation in response to increases in the cellular AMP:ATP and/or ADP:ATP ratios. Inhibition of complex I of the mitochondrial electron transport chain using phenformin activated AMPK and inhibited $K_v$ currents in pulmonary arterial myocytes, consistent with previously reported effects of mitochondrial inhibitors. Myocyte $K_v$ currents were also markedly inhibited upon AMPK activation by A769662, 5-aminoimidazole-4-carboxamide riboside and C13 and by intracellular dialysis from a patch-pipette of activated (thiophosphorylated) recombinant AMPK heterotrimers ($\alpha_2\beta_2\gamma_1$ or $\alpha_1\beta_1\gamma_1$). Hypoxia and inhibitors of mitochondrial oxidative phosphorylation reduced AMPK-sensitive $K^+$ currents, which were also blocked by the selective $K_v$1.5 channel inhibitor diphenyl phosphine oxide-1 but unaffected by the presence of the BK$_{Ca}$ channel inhibitor.
channel blocker paxilline. Moreover, recombinant human K\textsubscript{v}1.5 channels were phosphorylated by AMPK in cell-free assays, and K\textsuperscript{+} currents carried by K\textsubscript{v}1.5 stably expressed in HEK 293 cells were inhibited by intracellular dialysis of AMPK heterotrimers and by A769662, the effects of which were blocked by compound C. We conclude that AMPK mediates K\textsubscript{v} channel inhibition by hypoxia in pulmonary arterial myocytes, at least in part, through phosphorylation of K\textsubscript{v}1.5 and/or an associated protein.

(Received 16 December 2015; accepted after revision 26 March 2016; first published online 8 April 2016)

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Abbreviations  AICAR, 5-aminoimidazole-4-carboxamide riboside; AMPK, AMP-activated protein kinase; BK\textsubscript{Ca}, large conductance voltage- and calcium-activated K\textsuperscript{+} channel; DPO-1, diphenyl phosphine oxide-1; HEK 293, human embryonic kidney 293 cells; HPV, hypoxic pulmonary vasoconstriction; K\textsubscript{v}, voltage-gated K\textsuperscript{+} channel; LKB1, liver kinase B1.

Introduction

Hypoxia without hypercapnia induces pulmonary vasoconstriction, and thus assists ventilation–perfusion matching in the lung (von Euler & Liljestrand, 1946). However, hypoxia may trigger pulmonary hypertension when it is widespread, for example during ascent to altitude (Bartsch et al. 2005) or due to disorders such as cystic fibrosis (Lahm et al. 2014). While current therapies have been shown to prolong survival, pulmonary hypertension remains a life-threatening disorder (Lahm et al. 2014) and the precise molecular mechanisms underlying it remain unclear. Therefore, greater understanding is critical to the development of effective therapies.

Initially, hypoxic pulmonary vasoconstriction (HPV) is driven by calcium release via ryanodine receptors from the sarcoplasmic reticulum of pulmonary arterial smooth muscle cells (Dipp et al. 2001), but is also associated with concomitant inhibition of voltage-gated potassium channels (K\textsubscript{v}) (Post et al. 1992; Yuan et al. 1993; Archer et al. 2004). The role of K\textsubscript{v} channel inhibition in acute HPV remains open to debate (Wilson et al. 2002; Wang et al. 2004; Evans et al. 2005; Lu et al. 2008), but it has been proposed that loss of K\textsubscript{v} function contributes to smooth muscle proliferation and thus to the progression of pulmonary hypertension (Sweeney & Yuan, 2000; Moudgil et al. 2006) by promoting cell survival (Ekhterae et al. 2001, 2003).

K\textsubscript{v} current suppression during hypoxia (Post et al. 1992; Yuan et al. 1993; Firth et al. 2008) occurs as a consequence of inhibition of mitochondrial oxidative phosphorylation (Firth et al. 2008, 2009). However, the nature of the signalling pathway that couples mitochondrial function to K\textsubscript{v} channels has been unclear. In this respect, little attention has been paid to the role of the AMP-activated protein kinase (AMPK), although we have previously proposed that it couples inhibition of mitochondrial metabolism by hypoxia to acute HPV (Evans et al. 2005; Evans, 2006) and may also contribute to the progression of pulmonary hypertension (Evans et al. 2005; Evans, 2006; Ibe et al. 2013; Goncharov et al. 2014). AMPK, an energy sensor that acts to maintain cellular energy homeostasis, exists as heterotrimers comprising catalytic α subunits and regulatory β and γ subunits (Hardie, 2014a,b,c). AMPK is coupled to mitochondrial metabolism through changes in the cellular AMP:ATP and ADP:ATP ratios. Binding of AMP to the γ subunit causes a 10-fold increase in AMPK activity by allosteric activation, but a further activation of up to 100-fold can be generated by binding of either AMP or ADP, which promotes phosphorylation and inhibits dephosphorylation of Thr172 on the α subunit; these effects are antagonised by ATP (Gowans et al. 2013; Ross et al. 2016). Thr172 is primarily phosphorylated by the tumour suppressor kinase LKB1 (liver kinase B1), which appears to be constitutively active (Sakamoto et al. 2004), but which phosphorylates AMPK more rapidly when AMP is bound to the γ subunit. In an alternative Ca\textsuperscript{2+}-dependent activation mechanism, the calmodulin-dependent protein kinase CaMKKβ can also phosphorylate Thr172 and hence activate AMPK in an AMP-independent manner (Hardie, 2014a,b,c). The classical role of AMPK is to maintain energy homeostasis under conditions of metabolic stress, by activating catabolic processes that generate ATP and inhibiting non-essential anabolic processes that consume ATP. However, AMPK has also been shown to regulate a wide variety of ion channels and membrane transport proteins (Evans et al. 2009; Lang & Foller, 2014), including K\textsubscript{v}2.1 (Ikematsu et al. 2011), KCa3.1 (Ross et al. 2011), and Kir 2.1 and K\textsubscript{v}7.1 (Lang & Foller, 2014).

Of the various known K\textsubscript{v} channel types, it has been established that both K\textsubscript{v}2.1 and K\textsubscript{v}1.5 contribute to voltage-gated potassium currents in pulmonary arterial myocytes (Smirnov et al. 2002; Archer et al. 2004; Firth et al. 2011; Olschewski et al. 2014). Their relative contributions vary in a manner related to arterial diameter, with the greatest level of K\textsubscript{v}1.5 expression (and contribution to K\textsubscript{v} currents) occurring in myocytes from
near-resistance-sized arteries (Archer et al. 1998, 2004; Smirnov et al. 2002; Moral-Sanz et al. 2011), the response of which to hypoxia is critical to acute increases in pulmonary arterial perfusion pressure. Moreover, selective down-regulation of K_{1.5} has been identified as a hallmark of pulmonary hypertension (Yuan et al. 1998; Michelakis et al. 2002; Bonnet et al. 2006; Remillard et al. 2007; Burg et al. 2010; Morales-Cano et al. 2014). Consistent with this view, overexpression of K_{1.5} enhances apoptosis (Brevnova et al. 2004), while adenoviral expression of a K_{1.5} transgene in vivo reduces pulmonary hypertension and restores HPV (Pozeg et al. 2003).

We show here that AMPK selectively inhibits K_{1.5} in pulmonary arterial myocytes, and also phosphorylates and inhibits recombinant K_{1.5} channels expressed in HEK 293 cells.

Methods

Ethical approval

All experiments were performed under the United Kingdom Animals (Scientific Procedures) Act 1986. The animals used in this study were male Sprague Dawley rats that underwent no experimental procedures as recognised under UK Law. They were killed using a Schedule 1 method for collection of tissues only, which does not require formal ethical approval in the UK. Frozen canine tissue was left over from a previous project where surgical procedures and protocols were approved by the Cleveland Clinic Foundation Institutional Animal Care and Use Committee (Cleveland, OH, USA).

Smooth muscle cell isolation

Resistance pulmonary arteries (<200 μm inner diameter) from male Sprague Dawley rats (250–350 g) were dissected into a physiological bath solution of composition (in mM): NaCl 135, KCl 5, MgCl_2 1, CaCl_2 1, glucose 10, Hepes 10 (pH 7.4). For cell isolation, endothelium denuded arteries were transferred into a nominally calcium-free bath solution containing (in mg ml\(^{-1}\)): 1 papain, 0.8 dithiothreitol and 0.7 BSA. The tissue was incubated in the latter solution for 10 min at 37°C and gently triturated using a fire polished glass pipette to get dispersed pulmonary arterial smooth muscle cells.

Electrophysiological recordings

Pulmonary arterial myocytes or HEK 293 cells that stably expressed K_{1.5} were transferred to a recording chamber and perfused at 1 ml min\(^{-1}\) with bath solution. K\(^{+}\) currents were recorded by whole-cell patch clamp and a pipette solution of the following composition (mM): KCl 140, MgCl_2 1, EGTA 10, Hepes 10, Na_2 ATP 4, Na_2 GTP 0.1 (pH 7.2). Cells were superfused (3 ml min\(^{-1}\)) at 37°C with bath solution steadily bubbled with either room air (normoxia) or 95% N\(_2\)/5% CO\(_2\) [hypoxia, 4.4 ± 0.3% O\(_2\) in the experimental chamber; as measured with an optical oxygen meter (FireStingO2, Pyro Science, Aachen, Germany)]. For some experiments recombinant thiophosphorylated AMPK heterotrimers (α2β2γ1, α1β1γ1 or D157A kinase dead mutant) were added to the pipette solution. K\(_{v}\) currents were assessed by voltage ramps (−100 to +40 mV), single voltage steps (−80 to +40 mV) and by acquisition of full I–V relationships for steady state activation (200 ms steps from −80 to +40 mV in 10 mV increments) or inactivation (2 s inactivation steps from −80 to +40 mV in 10 mV increments, a 10 ms pre-pulse at −80 mV followed by a single voltage step to +60 mV). Current magnitude was normalised to cell capacitance as required. Conductance values (G) were calculated from the equation \(G = I/(V - EK)\), where the Nernst equilibrium potential (EK) was calculated as −89 mV at 37°C. Normalised conductance/voltage profiles for K\(_{v}\) currents were fitted to a single Boltzmann function with the form \(G = G_{\text{max}}/(1 + \exp[-(V - V_{\text{mid}})/k])\), where \(G_{\text{max}}\) is the maximal conductance, \(V_{\text{mid}}\) is the test potential for half-maximal conductance (\(G_{0.5}\)) and \(k\) represents the slope of the activation curve. Patch pipettes had resistances of 4–6 MΩ. Series resistance was compensated for (60–80%) after achieving the whole-cell configuration. Signals were sampled at 10 kHz and low-pass filtered at 2 kHz. Voltage-clamp acquisition and analysis protocols were performed using an Axopatch 200A amplifier/Digidata 1200 interface controlled by Clampex 10.0 software (Molecular Devices, Sunnyvale, CA, USA). Off-line analysis was performed using Clampfit 10.0 (Molecular Devices). Data are expressed as current density (pA pF\(^{-1}\)) or \(I/I_{\text{zero}}\), where \(I_{\text{zero}}\) is the current magnitude recorded at the onset of a given experimental intervention.

Cell culture and transfection

HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. Cells were transfected following the manufacturer’s instructions with 12 μg of pcDNA3.1 encoding an HA-tagged human K_{1.5} (KCNA5) using Fugene 6 (Promega, Madison, WI, USA) and lysed 48 h later.

RT-PCR

Total RNA was isolated from frozen canine tissues and from frozen pelleted HEK 293 cells stably expressing K_{1.5}, using the RNasy Mini Kit (Qiagen, Valencia, CA, USA) as per the manufacturer’s instructions. Reverse transcription PCRs (RT-PCRs) were carried out on 200 ng of total RNA using the One-Step RT-PCR Kit.
(ABM, Richmond, Canada) as recommended in the manual. Then, 20 μl of the 50 μl reaction was run on a 1% agarose gel and visualised using Safe-White (ABM) and a GelDoc equipped with Quantity One software (BioRad, Hercules, CA, USA). K,β primer sequences were as previously published (Platoshyn et al. 2004) and for the reference gene ReadyMade GAPDH primers (Integrated DNA Technologies, Coralville, IA, USA) were used with sequences as follows: GAPDH-For, ACCACAGTCCCCATGCATCAC; GAPDH-Rev, TCCACCACCTGTTGCTGTA. RT-PCR was repeated with 250 ng of template and 35 cycles of PCR after very faint bands were observed in the K,1.5 stable cell line for K,β1 and K,β3. Canine ventricle was also repeated for comparison.

K,1.5 phosphorylation assays

Phosphorylation assays were performed as described previously (Ross et al. 2011) using AMPK purified from bacteria and phosphorylated with CAMKKβ (10 units ml⁻¹) in the presence of 200 μM AMP for 30 min at 30°C.

Expression, purification and activation of bacterial AMPK

These were performed as described previously (Ross et al. 2011).

Isoform-specific AMPK activities

Isoform-specific AMPK activity was determined by immunoprecipitating tissue lysate with antibodies raised against α1 or α2 subunits bound to protein G-Sepharose beads and quantified using the AMARA peptide and [γ⁻3²P]ATP substrates (Cheung et al. 2000).

Statistics

Data are expressed as means ± SEM or means ± SD, as indicated; n represents the number of cells tested from at least four different animals. Statistical analysis was performed using Student’s t test for paired observations or one-way ANOVA followed by a Dunnett’s post hoc test. Differences were considered statistically significant at P < 0.05.

Results

Inhibition of mitochondrial oxidative phosphorylation activates AMPK and reduces K,1.5 current density in pulmonary arterial myocytes

Biguanide drugs such as phenformin inhibit complex I of the mitochondrial respiratory chain (El-Mir et al. 2000; Owen et al. 2000; Evans et al. 2005) and elicit consequent increases in the cellular AMP:ATP ratio and AMPK activation (Hawley et al. 2010). Consistent with this, pre-incubation of second- and third-order pulmonary arteries with phenformin (4 h) increased AMPK-α1-associated activity from (mean ± S.D.) 0.025 ± 0.001 to 0.403 ± 0.012 nmol min⁻¹ mg⁻¹ protein and AMPK-α2-associated activity from 0.0096 ± 0.001 to 0.126 ± 0.006 nmol min⁻¹ mg⁻¹ protein (Fig. 1A; n = 3; 32 arteries, 8 rats). Furthermore, and in accordance with previously reported effects of mitochondrial inhibitors (Firth et al. 2008) and hypoxia (Platoshyn et al. 2001), pre-incubation of acutely isolated pulmonary arterial myocytes with 1 mM phenformin (2–4 h) also caused pronounced reductions in K, current density, from 126 ± 17 pA pF⁻¹ in time-matched controls to 55 ± 6 pA pF⁻¹ at +40 mV (Fig. 1Ca–b; n = 9–11, P < 0.001). Consistent with the effects of phenformin and previous investigations by others (Firth et al. 2008), acute application of antimycin A (1 μM), a rapidly acting inhibitor of complex III, caused equivalent reductions in K, current density, from 131.3 ± 10.4 to 62.6 ± 11.1 pA pF⁻¹ at +40 mV (Fig. 1Da–b; n = 6, P < 0.001). Unless stated, in these and all subsequent experiments on pulmonary arterial myocytes, potassium currents were recorded in the presence of paixiline (1 μM) to block the large conductance voltage- and calcium-activated K⁺ (BKCa) channel.

Given that the contribution to native K, currents of K,2.1 and K,1.5 varies, in a manner related to both the size and the regional location within the lung of the arteries from which myocytes are derived (Smirnov et al. 2002), we assessed the nature of the channels that underpin the K, current within the cells under study. Application of the K,1.5 blocker diphenyl phosphine oxide-1 (DPO-1, 1 μM, Fig. 1B) in the absence of paixiline caused almost total inhibition of the K, currents (96 ± 1% at +40 mV; n = 6, P < 0.0001), consistent with the view that K,1.5 drives the majority of voltage-gated K⁺ currents in myocytes of the near-resistance-sized pulmonary arteries studied here, which contribute most to the increase in pulmonary vascular resistance during hypoxia (Kato & Staub, 1966; Archer et al. 2004).

AMPK activation inhibits K,1.5 currents in pulmonary arterial myocytes

We next assessed the effect on K,1.5 current amplitude of extracellular application of three AMPK activators with distinct mechanisms of action, i.e. A769662, 5-aminoimidazole-4-carboxamide riboside (AICAR) and C13. Analysis of the time course for K,1.5 inhibition at steady-state activation (100 ms at +40 mV) showed the time to onset of effect for A769662 (100 μM), AICAR (1 mM) and C13 (30 μM) to be around 2 min, with apparent
maxima for inhibition achieved after 8–10 min (Fig. 2B). After 10 min, Kᵥ1.5 currents had declined (Fig. 2A) from 144 ± 12 to 101 ± 9 pA pF⁻¹ in the presence of A769662 (n = 14), from 186 ± 23 to 136 ± 17 pA pF⁻¹ in the presence of AICAR (n = 6) and from 164 ± 8 to 104 ± 10 pA pF⁻¹ in the presence of C13 (n = 8). Note, however, that in 3 of 9 cells superfused with AICAR we observed no effect (excluded from analysis).

Surprisingly, we also observed inhibition of Kᵥ1.5 currents upon application of the non-selective AMPK antagonist compound C (30 μM), from 164 ± 62 to 59 ± 24 pA pF⁻¹ (n = 3; Fig. 2C); this confounding effect precluded the use of this agent in further studies on myocytes. Previous studies have shown that compound C has little or no effect on resting pulmonary arterial tone, but inhibits acute HPV in a concentration-dependent manner (Robertson et al. 2008), which must therefore be induced independently of the inhibition by hypoxia of Kᵥ1.5 inhibition (Dipp et al. 2001). It is worth noting that in a screen of 70 protein kinases, at least 10 were

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**Figure 1. Inhibition of mitochondrial oxidative phosphorylation activates AMPK and reduces Kᵥ1.5 current density in pulmonary arterial myocytes**

A, bar chart showing the effect of 10 mM phenformin on the activity of AMPK-α1 and AMPK-α2 containing heterotrimers, as determined by immunoprecipitate kinase assay (n = 3; 32 arteries, 8 rats). B, representative records (200 ms pulses from −80 to +40 mV in 10 mV increments, holding potential −80 mV) in pulmonary arterial myocytes before (control) and after extracellular application of 1 μM DPO-1. C and D, representative records (a) and associated I–V relationships (b; 200 ms depolarization pulses from −80 to +40 mV in 10 mV increments, holding potential −80 mV) from myocytes pre-incubated with 1 mM phenformin versus time-matched controls (C, green, n = 9–11), or before and after 5–8 min extracellular application of 1 μM antimycin A (D, n = 6). *P < 0.05, **P < 0.01 and ***P < 0.001.
inhibited by compound C more potently than AMPK (Bain et al. 2007). Thus, it is not a specific inhibitor of AMPK, a point reinforced by our findings that it markedly attenuates K$_{v}$1.5 function. This should be considered when interpreting outcomes of other cell-based assays that have employed compound C, not least with respect to myocyte proliferation and survival (Ibe et al. 2013).

Importantly, upon equilibration of pulmonary arterial myocytes with AICAR, A769662 and C13, reductions in current density were evident throughout the $I$–$V$ range over which K$_{v}$1.5 currents were activated (Fig. 3A, B). This was confirmed by the fact that A769662 was without effect on residual currents observed following pre-incubation of cells with DPO-1 even in the absence of paxilline (Fig. 3C), current density measuring 10 ± 2 pA pF$^{-1}$ in the presence of DPO-1 alone and 11 ± 3 pA pF$^{-1}$ in the presence of DPO-1 and A769662 (n = 6).

### Intracellular application of active AMPK heterotrimer inhibits K$_{v}$1.5 in pulmonary arterial myocytes

Although A769662, AICAR and C13 activate AMPK by different mechanisms and are therefore unlikely to have the same off-target effects, we also analysed the effect on endogenous K$_{v}$1.5 channel function by bacterially expressed human AMPK heterotrimers ($\alpha$2$\beta2\gamma$1 or $\alpha1\beta1\gamma$1 complexes). These had been thiposphorylated at Thr172 using CaMKK$\beta$ to yield active, recombinant AMPK that is also resistant to phosphatases (Ross et al. 2011). Intracellular dialysis of either of the active $\alpha2\beta2\gamma$1 or $\alpha1\beta1\gamma$1 heterotrimers (Fig. 4A) evoked K$_{v}$1.5 current inhibitions that were similar in magnitude (−33 ± 5% for $\alpha2\beta2\gamma$1 and −36 ± 7% for $\alpha1\beta1\gamma$1 at +40 mV; n = 5−7) to the reductions induced by pharmacological activation of AMPK. Importantly, current inhibition was not observed upon intracellular dialysis of an inactive AMPK heterotrimer [Fig. 4B; $\alpha2\beta2\gamma$1 complex with D157A mutation in $\alpha2$ (Ross et al. 2011)]. Based on the use both of pharmacological activation of endogenous AMPK and of exogenous recombinant AMPK, we can conclude that AMPK mediates, either directly or indirectly, inhibition of K$_{v}$1.5 currents in pulmonary arterial myocytes.

### Hypoxia and mitochondrial inhibitors attenuate K$_{v}$1.5 currents and occlude further current inhibition by AMPK activation

AMPK is intimately coupled to mitochondrial metabolism through changes in the AMP:ATP and ADP:ATP ratios (Gowans et al. 2013), which is evident from the fact that AMPK activity was increased by hypoxia (Evans et al. 2005) and by the mitochondrial inhibitor phenformin (Fig. 1A). To assess whether AMPK acted as a downstream mediator of K$_{v}$1.5 inhibition during hypoxia and inhibition of mitochondrial oxidative phosphorylation, we therefore carried out studies to determine if K$_{v}$1.5 inhibition by these stimuli was additive with respect to that induced by AMPK activators.

Superfusion of pulmonary arterial myocytes with a hypoxic solution (~4% O$_2$, > 10 min) markedly inhibited K$_{v}$1.5 currents, with a maximal reduction achieved after
AMPK activators induce a leftward shift in the I–V relationship for K_{1.5} current activation that is occluded by hypoxia and mitochondrial inhibitors

AMPK activation not only reduced K_{1.5} current density in pulmonary arterial myocytes throughout the I–V range over which K_{1.5} currents were activated, but also induced a significant 12–14 mV hyperpolarizing shift in the activation curve (Fig. 6B, C) analysed as G/G_{max} and fitted to a single Boltzmann function. V_{mid} measured: −17.9 ± 1.2 and −30.5 ± 4.5 mV in the absence and presence of AICAR, respectively; −17.4 ± 1.6 and −30.2 ± 3.5 mV in the absence and presence of A769662; −20.9 ± 2.6 and −39.7 ± 4.6 mV in the absence and presence of C13 (n = 5–7, P < 0.01). To allow for direct comparison of the maximal effect of each of these agents, we also expressed the leftward shift as the net change in V_{mid} (Fig. 6B); ΔV_{mid} was −12.6 ± 4.8, −12.7 ± 2.4 and −18.7 ± 2.3 mV, respectively, for AICAR, A769662 and C13. As previously observed for current inhibition, the leftward shift in the I–V range for K_{1.5} activation that was induced by AMPK activation was non-additive with respect to the effect of hypoxia and prior inhibition of mitochondrial oxidative phosphorylation, ΔV_{mid} for A769662 measuring −4.5 ± 0.9 mV in the presence of hypoxia and −3.7 ± 0.6 mV in the presence of antimycin A.

To explore further the functional significance of a leftward shift in the I–V relationship we assessed the voltage-dependence of both K_{1.5} activation and inactivation in the absence and presence A769662, and
thus determined the effect of AMPK activation on the window current, i.e. the proportion of current at a given potential that is never inactivated. Figure 7 clearly shows that AMPK activation by A769662 induced a leftward shift in K\textsubscript{v1.5} activation and inactivation curves and thus of the window current, lowering the threshold for activation while reducing the available non-inactivating current.

**AMPK phosphorylates K\textsubscript{v1.5} and reduces K\textsuperscript{+} currents carried by recombinant K\textsubscript{v1.5} channels stably expressed in HEK 293 cells**

To determine whether AMPK modulates K\textsubscript{v1.5} channel function directly, we examined the effects of AMPK activation on human K\textsubscript{v1.5} channels stably expressed in HEK 293 cells. Application of A769662 (100 \( \mu \)M) reduced K\textsuperscript{+} currents carried by recombinant human K\textsubscript{v1.5} (Fig. 8A(a)) in a manner that was blocked by the non-selective AMPK inhibitor compound C (40 \( \mu \)M, Fig. 8B). Moreover, intracellular dialysis of active AMPK \( \alpha \beta\beta\gamma\textsubscript{1} \) or \( \alpha\beta\beta\gamma\textsubscript{1} \) heterotrimers also reduced K\textsubscript{v1.5} currents, which remained unaffected in the presence of an inactive (D157A mutant) \( \alpha\beta\beta\gamma\textsubscript{1} \) heterotrimer (Fig. 8A(b-c)). Like pulmonary arterial myocytes, therefore, currents carried by human K\textsubscript{v1.5} expressed in HEK 293 cells were similarly inhibited both by AMPK activators and by intracellular dialysis of recombinant active AMPK heterotrimers (Fig. 8D).

We also examined whether AMPK directly phosphorylates K\textsubscript{v1.5}, using as substrate the human protein immunoprecipitated from these HEK 293 cells.

**Figure 4. Intracellular application of active AMPK heterotrimers inhibits K\textsubscript{v1.5} in pulmonary arterial myocytes**

A, voltage ramp and step protocol recorded at 0 and 10 min after intracellular dialysis of the indicated recombinant, thiophosphorylated active AMPK heterotrimer (\( \alpha\beta\beta\gamma\textsubscript{1} \) or \( \alpha\beta\beta\gamma\textsubscript{1} \)). B, time course for reduction in \( K_{v}\textsubscript{current} \) following intracellular dialysis of either active \( \alpha\beta\beta\gamma\textsubscript{1} \) (thiophosphorylated, 5 \( \mu \)M \textsuperscript{-1}), active \( \alpha\beta\beta\gamma\textsubscript{1} \) (thiophosphorylated, 5 \( \mu \)M \textsuperscript{-1}) or inactive \( \alpha\beta\beta\gamma\textsubscript{1} \) (D157A mutant) AMPK heterotrimer. Results are expressed as mean \( \pm \) SEM, \( n = 5-7 \).
We first treated the immunoprecipitate with recombinant protein phosphatase (PP1γ) to remove endogenous phosphate groups, then phosphorylated with purified rat liver AMPK (a mixture of α1β1γ1 and α2β1γ1 isoforms) and [γ-32P]ATP in the presence and absence of 200 μM AMP. The stoichiometry of phosphorylation was estimated by cutting out and counting the 32P-labelled band and estimating the protein content by comparison with serum albumin standards run on the same gel. We obtained estimates of 0.57 and 0.13 moles of phosphate per mole of protein in the presence and absence of AMP (data not shown). We repeated the experiment using recombinant human α1β1γ1 and α2β2γ1 complexes expressed in bacteria, and obtained stoichiometries of 0.7 and 1.6 moles of phosphate per mole of protein respectively (Fig. 8E, we did this only in the presence of AMP because, for reasons that remain unclear, the bacterially expressed complexes are much less AMP-dependent). While we have not yet determined the number and identity of the sites phosphorylated on Kv,1.5, these results indicate that different AMPK complexes can catalyse a substantial AMP-activated phosphorylation of Kv,1.5 in cell-free assays.

**Discussion**

The present investigation describes, for the first time, evidence that AMPK couples Kv,1.5 channel function (defined by the Kv,1.5 blocker DPO-1) to the inhibition by hypoxia of mitochondrial metabolism in pulmonary arterial myocytes. Consistent with this proposal, inhibition by phenformin or hypoxia of the mitochondrial electron transport chain (El-Mir et al. 2000; Owen et al. 2000) increases NAD(P)H autofluorescence (Evans et al. 2005), activates AMPK and inhibits Kv currents in pulmonary arterial myocytes. That AMPK activation may specifically regulate Kv,1.5 in response to metabolic stresses such as hypoxia gained further support from our findings that AMPK activators that are structurally distinct and have different mechanisms of action, namely A769662, AICAR and C13, all markedly inhibited Kv currents in pulmonary arterial myocytes. A769662, which primarily causes allosteric activation (Goransson et al. 2007; Scott et al. 2014), binds in a site located between the α and β subunits of AMPK (Xiao et al. 2013). AICAR (Corton et al. 1995) and C13

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**Figure 6.** Comparison of the change in current density and voltage–conductance relationships induced by activated AMPK and AMPK activators in pulmonary arterial myocytes. A, bar chart showing mean ± SEM change in current density at the end of each experimental intervention after 6–10 min of DMSO (1:1000), inactive α2β2γ2, hypoxia (~4% O2), 5 μM active α2β2γ2, 5 μM active α1β1γ1, 1 mM AICAR, 100 μM A769662, 30 μM C13, 1 mM phenformin (2–4 h), 1 μM antimycin A, 100 μM A769662 in the presence of hypoxia (~4% O2, > 10 min), phenformin and antimycin A (n = 3–16). B, similar to A but showing the net change in \( V_{\text{rest}} \) of the voltage–conductance plots for 1 mM AICAR, 100 μM A769662, 30 μM C13, 1 μM antimycin A, 100 μM A769662 in the presence of 1 μM antimycin A and 100 μM A769662 in the presence of hypoxia (~4% O2, > 10 min); n = 3–7. C, voltage–conductance plots showing effects of 1 mM AICAR (a), 100 μM A769662 (b), 30 μM C13 (c), 1 μM antimycin A (d), 100 μM A769662 in the presence of 1 μM antimycin A (e) and 100 μM A769662 in the presence of hypoxia (f, ~4% O2, > 10 min). Results are expressed as mean ± SEM, n = 3–7. *P < 0.05, **P < 0.01 and ***P < 0.001.

**Figure 7.** A769662 induces a leftward shift in the activation and inactivation curves of Kv,1.5

Plot shows the voltage–conductance relationship for Kv,1.5 activation and inactivation in the absence (control, black) and presence of 100 μM A769662 (red). Activation is indicated by filled symbols and continuous lines; inactivation is indicated by open symbols and dashed lines. Data points are mean ± SEM (n = 3–6). Curves were obtained by fitting to the sigmoidal Boltzmann equation.
(Gomez-Galeno et al. 2010) act similarly in the sense that they are both taken up into cells and converted to molecules (ZMP and C2, respectively) that bind to the γ subunit, mimicking the effects of AMP. However, C2 is a much more potent activator of AMPK than ZMP and, unlike the latter, does not affect other AMP-sensitive enzymes such as glycogen phosphorylase or fructose-1,6-bisphosphatase (Hunter et al. 2014). Moreover, A769662 is selective for complexes containing the β1 subunit (Scott et al. 2008), while C2 is selective for complexes containing the α1 subunit (Hunter et al. 2014). These data suggest that Kv1.5 current inhibition in pulmonary arterial myocytes may be delivered in whole or in part by AMP-dependent activation of heterotrimers containing α1 and β1. Furthermore, inhibition of Kv1.5 by hypoxia or by pre-incubation with inhibitors of mitochondrial oxidative phosphorylation prevented further current reduction by AMPK activators, suggesting that AMPK may act as the primary regulator of Kv1.5 downstream of inhibition of mitochondrial oxidative phosphorylation during hypoxia. This conclusion is also supported by previous findings that hypoxia activates AMPK (Evans et al. 2005) and achieves maximal AMPK phosphorylation within ~10 min (Ibe et al. 2013).

Crucially, given the possible off-target effects of any pharmacological agents, intracellular dialysis of active and phosphatase-resistant (thiophosphorylated) recombinant AMPK heterotrimers selectively inhibited Kv1.5 currents in –60 mV DMSO A

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pulmonary arterial myocytes, while a kinase-dead AMPK mutant was without effect. Although our results with C13 and A769662 suggest that an endogenous α1β1-containing complex may regulate Kv1.5 in pulmonary arterial myocytes, our results with intracellular dialysis of α1β1γ1 and α2β2γ1 heterotrimers suggest that both are capable of regulating Kv1.5.

It is also interesting to note that AMPK activation not only induced a leftward shift in half maximal activation of Kv currents, as previously reported for effects of mitochondrial inhibitors (Firth et al. 2008), but also a leftward shift in half maximal inactivation and thus reduced the available non-inactivating current, i.e. the window current. The overall effect would be to lower the threshold for Kv1.5 activation and thus increase the threshold for membrane depolarization, while reducing the opposition to membrane depolarization once initiated. These outcomes argue against a role for Kv1.5 inhibition in the initiation, through membrane depolarisation, of acute HPV. This view is supported by the fact that compound C inhibits Kv1.5, but has little or no effect on resting pulmonary arterial tone despite the fact that it inhibits acute HPV in a concentration-dependent manner (Robertson et al. 2008). When considered together, our study therefore provides further support for the view that acute HPV is induced in a manner independent of Kv1.5 inhibition (Dipp et al. 2001; Prieto-Lloret et al. 2015). That aside, our findings suggest that care must be taken when assessing studies that have employed compound C to examine the role of AMPK in pulmonary vascular function, given that the marked attenuation of Kv1.5 by compound C presents an important confounding variable with respect to investigations on myocyte proliferation and the progression of pulmonary hypertension (Ibe et al. 2013). Moreover in a screen of 70 protein kinases, at least 10 were inhibited by compound C more potently than AMPK (Bain et al. 2007). Compound C cannot, therefore, be considered to be a selective inhibitor of AMPK, a point reinforced by our findings.

Our conclusions from studies on native Kv currents in pulmonary arterial myocytes were confirmed by further investigations on the regulation of hKv1.5 stably expressed in HEK 293 cells. Native rat liver AMPK and two combinations (α1β1γ1 and α2β2γ1) of bacterially expressed human AMPK isoforms were found to incorporate near-stoichiometric amounts of phosphate into immunoprecipitated hKv1.5 channels, and with rat liver AMPK this was stimulated by AMP, making it very unlikely that the phosphorylation was catalysed by a contaminating kinase. AMPK activators and recombinant heterotrimers also inhibited currents carried by recombinant Kv1.5 channels in intact HEK 293 cells. This suggests that AMPK may directly regulate the channel protein even though Kv1β1, Kv1β2 and Kv1β3 may be expressed to varying degrees in HEK 293 cells that stably express Kv1.5 (Fig. 9), as has been reported previously (Platoshyn et al. 2004).

Our results support a model in which inhibition of mitochondrial oxidative phosphorylation by hypoxia in pulmonary arterial myocytes triggers AMPK-dependent inhibition of Kv1.5 channels, in line with the observation that co-expression of Kv1.5 and AMPK reduced Kv current and Kv1.5 channel abundance in the cell membrane of oocytes (Mia et al. 2012). Our finding that AMPK phosphorylates and inhibits Kv1.5 is also entirely consistent with previous evidence that AMPK mediates acute HPV (Evans et al. 2005; Robertson et al. 2008), and the proposal that AMPK may also contribute to smooth muscle proliferation and the development of pulmonary arterial hypertension (Ibe et al. 2013; Goncharov et al. 2014). This is evident from the fact that down-regulation of Kv1.5 expression and activity is a hallmark not only of HPV but also of pulmonary hypertension (Yuan et al. 1998; Michelakis et al. 2002; Bonnet et al. 2006; Remillard et al. 2007; Burg et al. 2010; Morales-Cano et al. 2014). This down-regulation may lead to increased survival of smooth muscle cells due to attenuation of K+ channel-dependent apoptosis (Krick et al. 2001; Brevnova et al. 2004; Moudgil et al. 2006), and facilitate the phenotypic switch from a contractile to a proliferative state (Cidad et al. 2012, 2015). Further support for this view may be taken from the finding that over-expression of Kv1.5 enhances apoptosis (Brevnova et al. 2004), while adenoviral transgene expression of Kv1.5 in vivo reduces pulmonary hypertension and restores HPV (Pozeg et al. 2003). Therefore, it is possible that dysfunction of the mitochondrial–AMPK

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**Figure 9. Transcripts for all three Kvβ genes are expressed in the HEK Kv1.5 cell line**

Gel showing RT-PCR amplicons for Kvβ1, Kvβ2, Kvβ3 and the reference gene (RG), GAPDH, from canine brain (CB), canine ventricle (CV) and the HEK/Kv1.5 stable line used in this study. The 500 base pair band of the ladder represents ~1200 ng of DNA (2-Log DNA Ladder, New England Biolabs, Ipswich, MA, USA).
signalling pathway may predispose individuals to hypoxia and other forms of pulmonary arterial hypertension (Bonnet et al. 2006). In this respect, it is interesting to note that single nucleotide polymorphisms (SNPs) in the gene encoding K\(_{\alpha,1.5}\) predispose to pulmonary hypertension and reduce K\(_{\alpha,1.5}\) channel availability in pulmonary arterial myocytes (Remillard et al. 2007), raising the intriguing possibility that this may be due, at least in part, to alterations in AMPK-dependent regulation of K\(_{\alpha,1.5}\).

AMPK phosphorylates target proteins containing a \(\Phi(X,\beta)XXS/TXXX\Phi\) (\(\Phi\), hydrophobic; \(\beta\), basic) recognition motif (Hardie et al. 2016). The protein sequence for K\(_{\alpha,1.5}\) presents 15 serines and 4 threonines susceptible to phosphorylation by serine–threonine kinases (Blom et al. 1999). However, none of these represents good matches to the consensus recognition sites for AMPK (http://scansite3.mit.edu), despite the fact that our studies on \(^{32}\text{P}\) phosphorylation indicate that the immunoprecipitated channel protein might be a direct substrate for AMPK. This raises two distinct possibilities, (1) AMPK recognises non-canonical sites within the K\(_{\alpha,1.5}\) sequence, as has been shown for other proteins (Jones et al. 2005; Chang et al. 2009; Egan et al. 2011) or (2) AMPK phosphorylates one or more associated protein(s), such as the regulatory \(\beta\) subunits. The fact that AMPK phosphorylates and regulates K\(_{\alpha,1.5}\) suggests that its effects are mediated, at least in part, independently of such interactions. Nevertheless, we cannot rule out the possibility that outcomes may be modulated by the \(\beta\) subunits, given that rat pulmonary arterial myocytes express K\(_{\alpha,1.5}\), K\(_{\alpha,\beta}\) and K\(_{\alpha,\beta,3}\) (Platoshyn et al. 2006) and phosphorylation of either K\(_{\alpha,1.5}\) or regulatory K\(_{\alpha,\beta}\) subunits may modulate not only channel gating and inactivation kinetics (Holmes et al. 1996; Williams et al. 2002) but also the sensitivity of K\(_{\alpha,1.5}\) to regulation by K\(_{\alpha,1.5}\) subunits (Kwak et al. 1999; David et al. 2012; Macias et al. 2014). It is equally plausible that AMPK-dependent phosphorylation of K\(_{\alpha,1.5}\) in pulmonary arterial myocytes may alter K\(_{\alpha,1.5}\), \(\alpha\)-K, \(\beta\) interactions, sensitivity to metabolic stress, channel trafficking (Martens et al. 1999; Tipparaju et al. 2012) and/or degradation via ubiquitin ligases (Mia et al. 2012; Andersen et al. 2015). Further studies will be aimed at identifying the AMPK phosphorylation sites on K\(_{\alpha,1.5}\) and on associated \(\beta\) subunits.

In conclusion, we propose that AMPK couples the inhibition of mitochondrial oxidative phosphorylation to K\(_{\alpha,1.5}\) channel inhibition in pulmonary arterial myocytes, which may contribute to the regulation by AMPK of smooth muscle proliferation and thus to the development of pulmonary hypertension. In addition, AMPK-dependent modulation of K\(_{\alpha,1.5}\) channel availability may also contribute to proliferative potential associated with other diseases, such as cancer (Bonnet et al. 2007; Comes et al. 2013; Vallejo-Gracia et al. 2013).

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AMP-activated protein kinase and K\textsubscript{\textalpha}\textalpha\textbeta\textalpha\textbeta channel Kv1.5 in pulmonary artery smooth muscle. Studies were conducted at the Centre for Integrative Physiology (University of Edinburgh, Edinburgh, UK), College of Life Sciences (University of Dundee, Dundee, UK) and Department of Anaesthesiology, Pharmacology and Therapeutics, University of British Columbia (Life Sciences Centre, Vancouver, Canada). All authors revised and approved the final version of the manuscript.

**Funding**

This work was primarily funded by the Wellcome Trust (WT081195MA and WT097726) and the British Heart Foundation (RG/12/14/29885), but was also supported by the Canadian Institute for Health Research and the Heart and Stroke Foundation of Canada.