Does AMP-activated Protein Kinase Couple Inhibition of Mitochondrial Oxidative Phosphorylation by Hypoxia to Calcium Signaling in O₂-sensing Cells?*

A. Mark Evans†, Kirsteen J. W. Mustard‡, Christopher N. Wyatt§, Chris Peers‡, Michelle Dipp‡, Prem Kumar¶, Nicholas P. Kinneas*, and D. Grahame Hardie†

From the †Division of Biomedical Sciences, School of Biology, Bute Building, University of St. Andrews, St. Andrews, Fife KY16 9TS, United Kingdom, ‡Division of Molecular Physiology, School of Life Sciences, Wellcome Trust Biocentre, University of Dundee, Dow Street, DD1 5EH, United Kingdom, §Institute for Cardiovascular Research, University of Leeds, Leeds LS2 9JT, United Kingdom, and ¶Department of Physiology, The Medical School, University of Birmingham, Birmingham B15 2TT, United Kingdom

Specialized O₂-sensing cells exhibit a particularly low threshold to regulation by O₂ supply and function to maintain arterial pO₂ within physiological limits. For example, hypoxic pulmonary vasoconstriction optimizes ventilation-perfusion matching in the lung, whereas carotid body excitation elicits corrective cardio-respiratory reflexes. It is generally accepted that relatively mild hypoxia inhibits mitochondrial oxidative phosphorylation in O₂-sensing cells, thereby mediating, in part, cell activation. However, the mechanism by which this process couples to Ca²⁺ signaling remains elusive, and investigation of previous hypotheses has generated contradictory data and failed to unite the field. We propose that a rise in the cellular AMP/ATP ratio activates AMP-activated protein kinase and thereby evokes Ca²⁺ signals in O₂-sensing cells. Co-immunoprecipitation identified three possible AMP-activated protein kinase subunit isoform combinations in pulmonary arterial myocytes, with α1β2γ1 predominant. Furthermore, their tissue-specific distribution suggested that the AMP-activated protein kinase-α1 catalytic isoform may contribute, via amplification of the metabolic signal, to the pulmonary selectivity required for hypoxic pulmonary vasoconstriction. Immunocytochemistry showed AMP-activated protein kinase-α1 to be located throughout the cytoplasm of pulmonary arterial myocytes. In contrast, it was targeted to the plasma membrane in carotid body glomus cells. Consistent with these observations and the effects of hypoxia, stimulation of AMP-activated protein kinase by phenformin or 5-aminoimidazole-4-carboxamide-riboside elicited discrete Ca²⁺ signaling mechanisms in each cell type, namely cyclic ADP-ribose-dependent Ca²⁺ mobilization from the sarcoplasmic reticulum via ryanodine receptors in pulmonary arterial myocytes and transmembrane Ca²⁺ influx into carotid body glomus cells. Thus, metabolic sensing by AMP-activated protein kinase may mediate chemotransduction by hypoxia.

Spaviaized O₂-sensing cells within the body have evolved as vital homeostatic mechanisms that monitor O₂ supply and alter respiratory and circulatory function, as well as the capacity of the blood to transport O₂. By these means, arterial pO₂ is maintained within physiological limits. Two key systems involved are the pulmonary arteries and the carotid body. Constriction of pulmonary arteries by hypoxia optimizes ventilation-perfusion matching in the lung (1), whereas carotid body excitation by hypoxia initiates corrective changes in breathing patterns via increased sensory afferent discharge to the brain stem (2). Although O₂-sensitive mechanisms independent of mitochondria may also play a role (3–5), it is generally accepted that relatively mild hypoxia inhibits mitochondrial oxidative phosphorylation and that this underpins, at least in part, cell activation (2, 6–10). Despite this consensus, the mechanism by which inhibition of mitochondrial oxidative phosphorylation couples to discrete cell-specific Ca²⁺ signaling mechanisms has remained elusive. Recently, the field has focused on the possible role of the cellular energy status (ATP) (7, 11), reduced redox couples (12), and reactive oxygen species (13–17), respectively, but extensive investigation of these hypotheses has delivered conflicting data and failed to unite the field since its inception in 1930 (18, 19). However, Sylvester and colleagues (11) have suggested that previous assessment of the role of the energy state may have been limited by the lack of knowledge of the identity of the energy variable that might signal the response.

Recently, the AMP-activated protein kinase (AMPK)² cascade has come to prominence as a sensor of metabolic stress that appears to be ubiquitous throughout eukaryotes (20, 21). AMPK is activated by many different metabolic stresses, including heat shock and metabolic poisons in hepatocytes (22), exercise in skeletal muscle (23), and ischemia and hypoxia in the heart (24). AMPK complexes are heterotrimers comprising a catalytic α subunit and regulatory β and γ subunits (20), which monitor the cellular AMP/ATP ratio as an index of metabolic stress (20). Through the action of ademylate kinase, any increase in the cellular AMP/ATP ratio is converted into an increase in the AMP/ATP ratio (25). Binding of AMP to two sites in the γ subunits triggers activation of the kinase via phosphorylation of the α subunit at Thr-172, an effect antagonized by high concentrations of ATP (26, 27). This phosphorylation is catalyzed by upstream kinases (AMPK kinases), the major form of which is a complex between the tumor suppressor kinase, LKB1, and

---

* This work was supported by The Wellcome Trust Grant 070772 and Biotechnology and Biological Sciences Research Council Grant 01/A/S/07453 (to A. M. E.) and by a contract for an Integrated Project from the European Commission (LSHM-CT-2004-005272) and the pharmaceutical companies supporting the Division of Signal Transduction Therapy Unit at Dundee (AstraZeneca, Boehringer-Ingleheim, GlaxoSmithKline, Merck & Co., Inc., Merck KGaA, and Pfizer) (to D. G. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Div. of Biomedical Sciences, School of Biology, Bute Bldg., University of St. Andrews, St. Andrews, Fife KY16 9TS, UK. Tel.: 44-1334-463579; Fax: 44-1334-463600; E-mail: ame3@st-and.ac.uk.

‡ The abbreviations used are: AMPK, AMP-activated protein kinase; AICAR, 5-aminoimidazole-4-carboxamide-riboside; MOPS, 4-morpholinepropanesulfonic acid; ACC, acetyl-CoA carboxylase; T, torr; HPV, hypoxic pulmonary vasoconstriction; cADPR, cyclic ADP-ribose; SR, sarcoplasmic reticulum.
two accessory subunits, STRAD and MO25 (28–30). Upon activation, AMPK serves to maintain ATP levels by activating catabolic pathways and by inhibiting non-essential ATP-consuming processes.

The primary targets for AMPK had previously been presumed to be mainly involved in energy metabolism, but it is now recognized that AMPK can also target non-metabolic processes (20). Given that inhibition of mitochondrial oxidative phosphorylation by hypoxia would be expected to promote a rise in the AMP/ATP ratio (20), we considered the proposal (31) that AMPK activation may mediate, in part, pulmonary artery constriction and carotid body excitation by hypoxia. The findings of the present investigation support this proposal.

MATERIALS AND METHODS

Cell Isolation—All experiments were performed under the United Kingdom Animals (Scientific Procedures) Act 1986. Pulmonary arteries were excised from male Wistar rats (150–300 g) after cervical dislocation and then placed in physiological salt solution A (PSS-A) (mM: 130 NaCl, 5.2 KCl, 1 MgCl2, 1.7 CaCl2, 10 glucose, 10 Hepes, pH 7.4). Arteries were incubated overnight (4 °C) in Ca2+-free PSS-A with 0.05 mg ml−1 papain and 1 mg ml−1 bovine serum albumin. Thereafter 0.2 mM 1,4-dithio-DL-threitol was added (22 °C, 1 h), the tissue washed three times in Ca2+-free PSS-A without enzyme, and smooth muscle cells were isolated by trituration and stored at 4 °C (32).

Carotid bodies were excised from 9–12-day-old rats (anesthetized by 4% halothane), placed in ice-cold phosphate-buffered saline containing collagenase (0.05% w/v), trypsin (0.025% w/v), and 50 μM Ca2+, incubated at 37 °C (30 min), centrifuged (200 × g, 5 min, 4 °C), and resuspended in Ham’s F-12 culture medium (84 units liter−1, 1 % penicillin, 100 μM streptomycin, 1 mg ml−1 bovine serum albumin). Thereafter 0.2 mM 1,4-dithio-DL-threitol was added (22 °C, 1 h), the tissue washed three times in Ca2+-free PSS-A without enzyme, and smooth muscle cells were isolated by trituration and stored at 4 °C (32).}

Western Blots and AMPK Activities—AMPK subunit protein expression was analyzed using precast 4–12% Bis-Tris gels in MOPS buffer. Acetyl-CoA carboxylase (ACC) phosphorylation and total ACC protein levels were analyzed using precast 3–8% Tris acetate gels in Tris acetate buffer. Proteins were transferred to nitrocellulose membranes using an Xcell II blot module and probed with antibodies against AMPK subunits (34). ACC phosphorylation and total ACC protein were measured via Western Blots and AMPK Activities (35).

Measurement of the AMP/ATP and ADP/ATP Ratio—Adenine nucleotide content of arterial smooth muscle lysates was determined by capillary electrophoresis (36).

Immunocytochemistry—Cells were placed onto poly-d-lysine-coated coverslips, fixed using ice-cold methanol (15 min), permeabilized by three 5-min washes with 0.3% Triton X-100 in phosphate-buffered saline (pH 7.4), washed three times for 5 min each in blocking solution (1% bovine serum albumin, 4% goat serum, and 0.3% Triton X-100 in phosphate-buffered saline), and incubated overnight at 4 °C with antibodies against the AMPK α1 subunit (1:500). Coverslips were washed four times with blocking solution and incubated (1 h, 22 °C, in the dark) with fluorescein isothiocyanate-conjugated secondary antibodies (1:200; excitation 490 nm, emission 518 nm), washed five times with phosphate-buffered saline, and attached to slides by mountant (2.4 g Mowiol 4–88, 6 g of glycerol, 2 ml of 0.2 M Tris-HCl, pH 8.5, 2.5% 1,4-diazabicyclo(2.2.2.) octane) with 4’,6-diamidino-2-phenylindole (1 μg/ml; excitation 358 nm, emission 461 nm). For controls, the primary antibody was omitted. Images were acquired using a Deltavision microscope system (Applied Precision) on an Olympus IX70 microscope using a 60×, 1.4 numerical aperture, oil immersion objective, and Photometric CH300 charge-coupled device camera. Single or multiple Z sections (0.2 μm) were taken through a cell. Images were deconvolved and analyzed off-line via Softworx software (Applied Precision).

Ca2+ Imaging—Intracellular Ca2+ concentration was reported by Fura-2 fluorescence ratio (F340/F380 excitation; emission 510 nm). Emitted fluorescence was recorded at 22 °C, with a sampling frequency of 0.05 Hz, to avoid photobleaching during long recording periods using a Hamamatsu 4880 charge-coupled device camera via a Zeiss Fluar (pulmonary artery smooth muscle cells) or a Nikon Fluar (carotid body glomus cells) 40×, 1.3 numerical aperture, oil immersion lens and Leica DMIRBE microscope. Background subtraction was performed on-line. Analysis was via Openlab (Improvement) (32). Pharmacological agents were applied extracellularly by a microperfusion system via a flow pipe positioned close to the cell under investigation, as described previously (32).

Autofluorescence Measurements—Cells were excited by a Blue diode laser (pinhole 114.4 μm; 25% power) via a Leica 63×, 1.2 numerical aperture, water immersion objective on a Leica SP2 confocal system. Cell auto fluorescence was recorded (0.05 Hz, 22 °C) as the average of 8 Z scans. Acquisition and analysis was by Leica LCS 3D Physiology.

Tension Recording—Records were from third order pulmonary artery branches (internal diameter 300–400 μm; 2–3 mm in length) via small vessel myographs (AM10; Cambustion Biological, Cambridge, UK) as described previously (32). Experimental chambers were filled with PSS-B (in mM: 118 NaCl, 4 KCl, 1 MgSO4, 1.2 NaH2PO4, 24 NaHCO3, 2 CaCl2, 2 MgCl2, 5.6 glucose, pH 7.4) at 37 °C, and bubbled with 75% N2, 20% O2, 5% CO2 (normoxia, 150–160 Torr) or 93% N2, 2% O2, 5% CO2 (hypoxia, 16–21 Torr) via a gas-mixing flowmeter (Columbus Instruments).

Isolated Carotid Body—Rats were anesthetized with 1–4% halothane in O2 (Pepper et al. (37)), killed, and exsanguinated. Left and right carotid bifurcations were identified and removed, pinned on Sylgard (184) in a 0.2-ml chamber and perfused (3 ml min−1) with PSS-C (mM; 125 NaCl, 3 KCl 1.25 NaH2PO4, 5 NaSO4, 1.3 MgSO4, 24 NaHCO3, 2.4 CaCl2, 10 glucose, pH 7.4) at 37 ± 1 °C. Perfusion was equilibrated to 40 Torr pCO2 and 400 Torr pO2 via precision flow valves (Cole–Palmer). The sinus nerve was sectioned at the junction with the glossopharyngeal nerve, extracellular recordings of afferent fiber spike activity recorded on video tape, and action potentials sampled digitally (37) via LabVIEW software (National Instruments Co.).

Drugs and Chemicals—Secondary antibodies used were from Strat-ech and Molecular Probes. Gels and buffers were from Invitrogen. Phenformin, ryanodine, 8-bromo-cyclic AMP-riboside (8-bromo-cADPR), bovine fetal serum albumin, dithiothreitol, collagenase, trypsin, insulin, penicillin, poly-d-lysine, papain, and caffeine were from Sigma. Fura-2/AM was from Molecular Probes. 5-aminomidazole-4-carboxamide riboside (AICAR) was from Calbiochem. Stock solutions of ryanodine and 8-bromo-cADPR were in Me2SO, and minimum dilution 1:1000 in PSS was without effect on preparations.
AMPK and Ca^{2+} Signaling in O_{2}-sensing Cells

FIGURE 1. AMPK subunit isoforms in pulmonary versus systemic arterial smooth muscle and AMPK activation in pulmonary arterial smooth muscle by hypoxia. A, Western blot of AMPK-α1, -α2, -β1, -β2, -γ1, and -γ3 expression. Lanes 1 and 2, pulmonary arterial smooth muscle; lane 3, rat liver. B, AMPK activity immunoprecipitated from pulmonary arterial smooth muscle with anti-α1, -α2, -γ1, -γ2, and -γ3 antibodies. C, AMPK activity immunoprecipitated from pulmonary and systemic arterial smooth muscle with anti-α1 and -α2 antibodies. D, activation of AMPK in pulmonary arterial smooth muscle by switching from normoxia (154–160 T, 1 h) to hypoxia (16–21 T, 1 h). E, Western blot of ACC phosphorylation in response to a switch from normoxia (154–160 T) to hypoxia (16–21 T). Upper panels, blots; lower panels, mean ± S.D. (n = 3, 32 arteries from ≥8 animals).

RESULTS

The Tissue-specific Distribution of the AMPK-α1 Isozyme May Afford the Pulmonary Selectivity Required of a Mediator of Hypoxic Pulmonary Vasoconstriction.—To determine whether or not AMPK activates O_{2}-sensing cells in response to hypoxia, we first focused on pulmonary arterial smooth muscle. Hypoxic pulmonary vasoconstriction (HPV) is the critical and distinguishing characteristic of pulmonary arteries (1). In marked contrast, systemic arteries dilate in response to hypoxia to match tissue perfusion to metabolism (38). Thus, a mediator of hypoxic pulmonary vasoconstriction should offer some degree of pulmonary selectivity. We assessed, therefore, the relative activities of the various isoforms of the catalytic α subunit and regulatory β and γ subunits that comprise AMPK in pulmonary and systemic arterial smooth muscle.

Western blot analysis in combination with co-immunoprecipitation analysis of pulmonary arterial smooth muscle lysates identified the presence of the α1, α2, β2, γ1, and γ3 subunits of AMPK (Fig. 1, A and B). Our anti-γ2 antibodies were not sufficiently specific in Western blotting to confirm the presence of the γ2 subunit isoform. However, they did not cross-react with anti-α1 or -α2 antibodies, and immunoprecipitate kinase assays revealed that γ2 accounted for 40% and γ1 for 60% of the total AMPK activity in pulmonary arterial smooth muscle lysates, with γ3 accounting for an insignificant fraction (n = 3, 32 arteries, 8 animals) (Fig. 1B). Furthermore, immunoprecipitated kinase assays using anti-α1 and -α2 antibodies showed that α1 accounted for 80–90% and α2 only 10–20% of the total catalytic activity in smooth muscle lysates from second and third order branches of the pulmonary arterial tree (n = 3, 32 arteries, 8 animals) (Fig. 1C). The predominant AMPK subunit composition in small pulmonary arterial smooth muscle is likely, therefore, to be α1β2γ1, although other combinations are probably present (i.e. α1β2γ2, α2β2γ1, and α2β2γ2). This contrasted markedly with the activities in the main pulmonary artery that feeds the lung and systemic (mesenteric) arteries, where the activity of the α1 isoform became progressively lower, while that of the α2 isoform was similar in each tissue type (Fig. 1C). Thus, AMPK-α1 activity is inversely related to pulmonary artery diameter, as is the magnitude of pulmonary artery constriction by hypoxia (39) and, significantly, the enzymes for the synthesis and metabolism of cADPR, a Ca^{2+}-mobilizing messenger (40, 41) that has been identified as a primary mediator of HPV (42, 43). Most importantly, perhaps, the AMPK-α1 activity was much higher in second and third order branches of the pulmonary arterial tree when compared with systemic (mesenteric) arteries (Fig. 1C), which dilate rather than constrict in response to hypoxia (38). Once more, this correlated with the distribution of the enzyme activities for the synthesis and metabolism of cADPR. Thus, the differential arterial distribution of AMPK-α1 catalytic activity, together with that of the enzymes for the synthesis and metabolism of cADPR, could provide via signal amplification the degree of pulmonary selectivity required for HPV, the critical and distinguishing characteristic of pulmonary arteries (1).

Hypoxia Elicits an Increase in the AMP/ATP Ratio and Concomitant Activation of AMPK in Pulmonary Arterial Smooth Muscle.—Previous studies (25) have shown that, through the action of adenylate kinase, any increase in the cellular ADP/ATP ratio is converted into a rise in the AMP/ATP ratio leading to consequent activation of AMPK (28–30). Consistent with these findings, capillary electrophoresis analysis on pulmonary arterial smooth muscle lysates (32 arteries, 8 animals) showed that the AMP/ATP ratio rose from 0.040 under normoxia (155–160 T, 2 h) to 0.083 under hypoxic conditions (16–21 T, 1 h; following 1 h equilibration under normoxia), whereas the ADP/ATP ratio rose from 0.183 to 0.259. This suggests, as one would expect (20, 21), that the adenylate kinase reaction is close to equilibrium, because the AMP/ATP ratio varies approximately as the square of the ADP/ATP ratio. Most significantly, immunoprecipitate kinase assays demonstrated that the rise in the AMP/ATP ratio was associated with a concomitant, 2-fold increase in AMPK activity (n = 3, 32 arteries, 8 animals) (Fig. 1D) and phosphorylation of a classical AMPK substrate, acetyl-CoA carboxylase (n = 3, 32 arteries, 8 animals) (Fig. 1E, ACC).

Hypoxia activated both AMPK-α1 and AMPK-α2 catalytic activity in pulmonary arterial smooth muscle (Fig. 1D). However, consistent with AMPK-α1 playing a prominent role in regulating pulmonary arterial smooth muscle function, hypoxia increased AMPK-α1 activity to a greater extent than it increased AMPK-α2 activity (Fig. 1D).

AMPK Activation Initiates cADPR-dependent Ca^{2+} Release from Ryanodine-sensitive Sarcoplasmic Reticulum (SR) Stores in Pulmonary Arterial Smooth Muscle Cells.—Our previous studies have established that cADPR-dependent Ca^{2+} release from smooth muscle SR stores via ryanodine receptors is required for the full expression of HPV (42, 43). Consistent with a role for AMPK in mediating such a response, immunocytochemistry showed the AMPK-α1 catalytic subunit isoform to be distributed throughout the cytoplasm in pulmonary arterial smooth muscle cells, with little staining associated with the plasma membrane (Fig. 2A). Thus, we examined the possibility that AMPK activation, similar to hypoxia, might mobilize SR Ca^{2+} stores in a cADPR-dependent manner. To this end, we employed the now classical method of studying AMPK activation. This requires the use of two drugs, phenformin (10 mM) and AICAR (1 mM), each of which activate AMPK via discrete mechanisms. Phenformin (a drug formerly used in the treatment of type 2 diabetes) inhibits Complex I of the mitochondrial respiratory chain (44, 45) and thereby activates AMPK by increasing the cellular AMP/
AMPK and Ca\(^{2+}\) Signaling in O\(_2\)-sensing Cells

ATP ratio (60). By contrast, AICAR is metabolized to yield the AMP mimic ZMP (AICAR monophosphate) and thereby selectively activates AMPK without affecting the cellular AMP/ATP ratio (45, 46).

Consistent with the effects of hypoxia, both AICAR and phenformin increased AMPK activity in pulmonary arterial smooth muscle. However, longer incubation times (4 h) were required to allow for adequate penetration of bundles of 32 intact arteries (second and third order branches; \(2 \pm 1\) mm internal diameter) due to the pharmacokinetics of AMPK and phenformin. During this time period, basal AMPK activities declined relative to those reported above; importantly, however, the relative level of AMPK-\(\alpha\) and AMPK-\(\beta\) activities, respectively, remained consistent. Under normoxic conditions, AICAR (1 mM) increased AMPK-\(\alpha\)-associated activity from (mean \(\pm\) S.D.) 0.18 \(\pm\) 0.02 to 0.56 \(\pm\) 0.03 nmol of phosphate incorporated/min/mg protein and AMPK-\(\beta\)-associated activity from 0.043 \(\pm\) 0.005 to 0.12 \(\pm\) 0.005 nmol of phosphate incorporated/min/mg protein (\(n = 3\); 32 arteries, 8 animals). Consistent with AMPK activation by AICAR, ACC phosphorylation in the smooth muscle was also increased, the phosphorylated ACC/ACC ratio measuring 0.44 \(\pm\) 0.14 and 2.38 \(\pm\) 0.02 in the presence and absence of AICAR (1 mM; \(n = 3\); 32 arteries, 8 animals).

Likewise, phenformin (10 mM) increased AMPK-\(\alpha\)-1-associated activity from 0.025 \(\pm\) 0.001 to 0.403 \(\pm\) 0.012 nmol of phosphate incorporated/min/mg protein and AMPK-\(\alpha\)-2-associated activity from 0.0096 \(\pm\) 0.001 to 0.126 \(\pm\) 0.006 nmol of phosphate incorporated/min/mg protein (\(n = 3\); 32 arteries, 8 animals). A concomitant increase in ACC phosphorylation in the smooth muscle was also observed, the phosphorylated ACC/ACC ratio measuring 0.46 \(\pm\) 0.014 and 3.51 \(\pm\) 0.12 in the presence and absence of phenformin (10 mM; \(n = 3\); 32 arteries, 8 animals).

As observed during hypoxia (17), AMPK activation by phenformin (10 mM) was associated with an increase in the NAD(P)H autofluorescence in pulmonary arterial smooth muscle cells (\(n = 24\)) (Fig. 2B). This is consistent with the fact that phenformin, similar to hypoxia (6–10), inhibits mitochondrial oxidative phosphorylation (44, 45). In marked contrast, AICAR (1 mM), which activates AMPK without effect on mitochondrial function (46), had no effect on cellular NAD(P)H autofluorescence (\(n = 12\)) (Fig. 2C). Despite their different modes of action, both phenformin (10 mM) and AICAR (1 mM) induced an increase in intracellular Ca\(^{2+}\) concentration in isolated pulmonary arterial smooth muscle cells at room temperature (20–22°C), as reported by the Fura-2 fluorescence ratio (F340/F380), which increased (mean \(\pm\) S.E.) by 0.09 \(\pm\) 0.02 (\(n = 7\)) and by 0.10 \(\pm\) 0.01 (\(n = 22\)) (respectively, Fig. 2, D and E). In each case, the response, similar to that of hypoxia (32), remained unaffected upon removal of extracellular Ca\(^{2+}\) (Fig. 2, D and E).

Prior blocking of sarcoplasmic reticulum stores by preincubation of cells with ryanodine (10 mM) and caffeine (10 mM) abolished the increase in intracellular Ca\(^{2+}\) concentration produced by 1 mM AICAR (0.024 \(\pm\) 0.016, \(n = 8\)) (Fig. 2, F and H) and 10 mM phenformin (0.024 \(\pm\) 0.006, \(n = 7\)) (Fig. 2H). Most significantly, the SR Ca\(^{2+}\) release evoked due to AMPK activation by AICAR (1 mM) was also abolished (0.016 \(\pm\) 0.01, \(n = 23\)) (Fig. 2, G and H) by blocking the Ca\(^{2+}\)-mobilizing messenger cADPR (40, 41) using 8-bromo-cADPR (100 mM), a selective cADPR antagonist (43). Consistent with this observation, 8-bromo-cADPR also blocked the increase in SR Ca\(^{2+}\) release evoked by phenformin (10 mM; 0.024 \(\pm\) 0.007, \(n = 5\)) (Fig. 2H). Thus, AMPK activation by AICAR or phenformin triggers cADPR-dependent SR Ca\(^{2+}\) release via ryanodine receptors in pulmonary arterial smooth muscle cells, as does hypoxia (32, 42, 43, 47).

**FIGURE 2.** AMPK activation elicits cADPR-dependent SR Ca\(^{2+}\) release in pulmonary arterial smooth muscle. A(i), bright field image of a pulmonary arterial smooth muscle cell. A(ii), Z-section showing staining by antibodies to AMPK-\(\alpha\) (green) and of the nucleus by 4',6-diamidino-2-phenylindole (blue). A(iii), three-dimensional reconstruction. NAD(P)H autofluorescence in isolated pulmonary arterial smooth muscle cells with and without phenformin (10 mM) (B) and AICAR (1 mM) (C). Effect on Fura-2 fluorescence ratio (F340/F380) in isolated pulmonary artery smooth muscle cells of phenformin (10 mM) (D) and AICAR (1 mM) (E) with and without extracellular Ca\(^{2+}\) (+1 mM EGTA). Shown are AICAR after preincubation with caffeine (10 mM) and ryanodine (10 mM) (E) and preincubation with 8-bromo-cADPR (100 mM) (E), H. mean increase in the Fura-2 fluorescence ratio \(\pm\) S.E. (\(n \geq 5\)). Phen., phenformin.
AMPK and Ca$^{2+}$+ Signaling in O$_2$-sensing Cells

![Image](351x26 to 378x38)

**FIGURE 3.** AMPK activation by AICAR replicates hypoxic pulmonary vasoconstriction. Shown are pulmonary artery constriction in response to AMPK activation by AICAR (1 mM) with (left panel) and without (right panel) the endothelium. A, with extracellular Ca$^{2+}$; B, without extracellular Ca$^{2+}$ (+1 mM EGTA). C, after preincubation of pulmonary arteries with caffeine (10 mM) and ryanodine (10 μM). D, after preincubation with 8-bromo-cADPR (300 μM). E, after preincubation with 8-bromo-cADPR (300 μM) and submaximal preconstriction by K$^+$ (20 mM). F, mean constriction ± S.E. (n = 3) of isolated pulmonary artery rings by AICAR (1 mM, left panel) and hypoxia (right panel) under the conditions described for A–E. E, endothelium; RC, ryanodine and caffeine; 8Br, 8-bromo-cADPR.

and B) and hypoxia (16–21 T) (32), respectively, was ablated upon removal of extracellular Ca$^{2+}$ (Fig. 3F). In contrast, constriction mediated by mechanisms intrinsic to the smooth muscle was not, although it is notable that this component of constriction was attenuated in the absence of extracellular Ca$^{2+}$ (Fig. 3, A, B, and F). Thus, maintained smooth muscle constriction by AICAR (1 mM) and hypoxia (16–21 T), respectively, exhibits a dependence (~50%) on transmembrane Ca$^{2+}$ influx. In this respect, it is of major significance that blockade of SR Ca$^{2+}$ stores with caffeine (10 mM) and ryanodine (10 μM) or blockade of cADPR with 8-bromo-cADPR (100 μM) (Fig. 3) completely abolished the constriction of pulmonary arteries, with or without endothelium, by both AICAR (1 mM) (Fig. 3, C, D, and F) and hypoxia (16–21 T) (Fig. 3F). Thus, the partial dependence of smooth muscle constriction on extracellular Ca$^{2+}$ must be due to SR store depletion-activated Ca$^{2+}$ influx/store refilling, which is not mediated by AMPK activation by AICAR nor hypoxia per se. Consistent with this observation, hypoxia has been shown to initiate SR store depletion-activated Ca$^{2+}$ influx indirectly by first mobilizing SR Ca$^{2+}$ stores in pulmonary arterial smooth muscle (48). The aforementioned findings are, therefore, entirely consistent with the characteristics of pulmonary artery constriction by hypoxia and support the view that AMPK activation, similar to hypoxia, mediates pulmonary artery constriction via cADPR-dependent mobilization of smooth muscle SR Ca$^{2+}$ stores via ryanodine receptors (32, 42, 43).

Notably, blockade of cADPR with 8-bromo-cADPR completely abolished the constriction of pulmonary arteries, with or without endothelium, by both AICAR (1 mM) and hypoxia (16–21 T) (Fig. 3F). It was significant, therefore, that preconstriction of pulmonary arteries by K$^+$-induced (20 mM) depolarization restored only the endothelium-dependent component of constriction by AICAR (1 mM) in the continued presence of 8-bromo-cADPR (Fig. 3E). This is consistent with the effects of 8-bromo-cADPR on HPV and reflects the fact that the endothelium-derived vasoconstrictor released by hypoxia does not elicit pulmonary artery constriction in the absence of SR Ca$^{2+}$ release by cADPR (32, 42, 43), because it sensitizes the contractile apparatus to the increase in cytoplasmic Ca$^{2+}$ concentration induced by hypoxia (49). AMPK Activation Elicits Transmembrane Ca$^{2+}$+ Influx into Carotid Body Glomus Cells and Consequent Carotid Body Excitation and Afferent Fiber Discharge—The findings above strongly support our hypothesis that AMPK acts as the primary metabolic sensor in pulmonary arteries and is the primary effector of HPV. If, however, metabolic sensing by AMPK were to be a general mechanism of chemotransduction, then one would expect it to mediate the cell-specific Ca$^{2+}$+ signaling mechanisms observed in other O$_2$-sensing cells. We, therefore, turned our attention to the carotid body glomus cell. These cells are also stimulated by hypoxia, but in this case, excitation is primarily mediated by voltage-gated Ca$^{2+}$+ influx, leading ultimately to neurosecretion (10, 33, 37, 50–52) rather than by Ca$^{2+}$+ release from intracellular stores. Immunocytochemistry showed that, in marked contrast to its cytoplasmic distribution in pulmonary arterial smooth muscle cells (Fig. 2A), the AMPKα1 catalytic subunit isofrom was almost entirely restricted to the plasma membrane of carotid body glomus cells (Fig. 4A). Thus, the spatial localization of AMPK in carotid body glomus cells would be consistent with the regulation of plasma membrane-delimited processes such as voltage-gated Ca$^{2+}$+ influx. Consistent with this proposal and with the effects of hypoxia on carotid body glomus cells (10), AMPK activation by AICAR (1 mM) induced an increase in intracellular Ca$^{2+}$+ concentration in acutely isolated carotid body glomus cells at 22°C, as the Fura-2 fluorescence ratio increased by 0.07 ± 0.02 (n = 11). In marked contrast to our findings on pulmonary arterial smooth muscle, this increase in intracellular Ca$^{2+}$+ concentration was abolished by removal of extracellular Ca$^{2+}$ (n = 6) and attenuated by blockade of transmembrane Ca$^{2+}$+ influx pathways with Cd$^{2+}$ (100 μM; 0.009 ± 0.006, n = 6) (Fig. 4B). Consistent with the effects of AMPK, phenformin (10 mM) increased the fluorescence ratio by 0.272 ± 0.07 (n = 7) in a manner that was reversed by the removal of extracellular Ca$^{2+}$ (0.011 ± 0.012, n = 7) (Fig. 4D). Thus, we can conclude that both phenformin and AICAR, similar to hypoxia, trigger transmembrane Ca$^{2+}$+ influx into isolated carotid body glomus cells.

We then investigated the effect of AICAR alone on sensory afferent discharge from the isolated carotid body in vitro to rule out any possible AMPK-independent actions that might result from inhibition of mitochondrial metabolism by phenformin. Under these conditions and at 37°C, AICAR (1 mM) induced a relatively rapid and reversible increase in single fiber sensory afferent discharge from the isolated carotid body from 0.22 ± 0.03 to 2.8 ± 0.56 spikes s$^{-1}$ (n = 19). This too was abolished by the removal of extracellular Ca$^{2+}$ (0.11 ± 0.03, n = 5) and was attenuated by blockade of transmembrane Ca$^{2+}$+ influx with Cd$^{2+}$ (100 μM; 0.91 ± 0.18, n = 5) (Fig. 4C and D). Thus, AMPK activation...
reproduced the precise excitatory effects of hypoxia on the carotid body (10, 37, 52).

**DISCUSSION**

Previous studies (2, 6–10) have established that hypoxia promotes Ca\(^{2+}\)-dependent pulmonary artery constriction and carotid body sensory afferent discharge, in part, by inhibiting oxidative phosphorylation by mitochondria. Our findings now suggest that this leads to a rise in the cellular AMP/ATP ratio, consequent AMPK activation, and the initiation of cell-specific Ca\(^{2+}\) signaling mechanisms in pulmonary arterial smooth muscle and carotid body glomus cells (Fig. 4E). Thus, the characteristic functional response of each tissue type to AMPK activation mirrors the primary aspects of the observed response to hypoxia: (a) constriction of pulmonary arteries by cADPR-dependent SR Ca\(^{2+}\) release in the smooth muscle cells with a secondary component of constriction driven by the pulmonary arterial endothelium (32, 42, 43) and (b) transmembrane Ca\(^{2+}\) influx into carotid body glomus cells and a consequent increase in afferent fiber discharge (10, 33, 37, 52). We propose, therefore, that AMPK may act as a primary metabolic sensor and effector in O\(_2\)-sensing cells. This novel role for AMPK may therefore unite for the first time the mitochondrial and Ca\(^{2+}\) signaling hypotheses for chemotransduction by hypoxia (18, 19). Before we can be certain of a role for AMPK in this process, however, we need to demonstrate that cell activation by hypoxia can be inhibited by selective blocking of AMPK. This may be achieved once the selective AMPK antagonist Compound C (53, 54) becomes freely available or by the use of siRNA, which has yet to be applied successfully in studies on these wild-type cells.

If confirmed, this process of activation would be exquisitely sensitive to metabolic stress by hypoxia because of the triple mechanism by which AMPK is activated: adenylate kinase converts any rise in the cellular ADP/ATP ratio into a rise in the AMP/ATP ratio (25), whereas AMP binding to the \(\alpha\) subunits of AMPK not only promotes phosphorylation by LKB1 but also inhibits dephosphorylation by protein phos-
AMPK and Ca\textsuperscript{2+} Signaling in O\textsubscript{2}-sensing Cells

phatases and causes allosteric activation of the phosphorylated enzyme (20). Furthermore, a role for AMPK could explain contrary data obtained previously with inhibitors of mitochondrial electron transport and mitochondrial uncouplers (18, 19). Briefly, any pharmaceutical intervention that inhibits ATP production without compromising ATP supply to AMPK would mimic, at least in part, the effects of hypoxia on O\textsubscript{2}-sensing cells (7, 10, 12). By contrast, interventions that both inhibit mitochondrial oxidative phosphorylation to the extent that ATP supply becomes limiting for AMPK would block cell activation by hypoxia and fail to mimic the effects of hypoxia (17, 55). An AMPK-dependent process would also explain the finding that mitochondrial uncouplers and inhibitors of electron transport can elicit carotid body excitation, although having opposite effects on cellular NAD(P)H autofluorescence (8, 10), because in each case, mitochondrial ATP production would be compromised.

We identified a number of possible AMPK subunit isoform combinations in pulmonary arterial smooth muscle (α1β2γ1, α1β2γ2, α2β2γ1, and α2β2γ2), although it seems likely that the α1β2γ1 combination predominates. Most significantly, however, the levels of AMPK-α1 expression were found to be higher in pulmonary than in systemic artery smooth muscle. This differential arterial distribution of the AMPK-α catalytic activity parallels that of the enzymes for the synthesis and metabolism (ADP-ribosyl cyclase/cADPR hydrolase) of cADPR (42), a primary mediator of HPV (42, 43). Via signal amplification, these two processes could combine to provide the degree of pulmonary selectivity required for HPV, which is the critical and distinguishing characteristic of pulmonary arteries (1). The fact that both AMPK-α1 and ADP-ribosyl cyclase/cADPR hydrolase activities (42) are inversely related to pulmonary artery diameter may also explain the inverse relationship between the magnitude of constriction of pulmonary arteries by hypoxia and pulmonary artery diameter (39).

The spatial localization of AMPK-α1 may also be a significant determinant of the response to hypoxia. This catalytic subunit isoform is targeted to the plasma membrane of carotid body glomus cells but is located throughout the cytoplasm in pulmonary artery smooth muscle cells. This is consistent with AMPK regulating a membrane-delimited process such as voltage-gated Ca\textsuperscript{2+} influx in carotid body glomus cells. Thus, both the nature of the AMPK isozyme and its spatial localization may determine the cell-specific nature of the response to metabolic stress. Unfortunately, the size of the carotid body and the presence of other cell types within this organ preclude direct quantification of catalytic activities associated with different AMPK subunit isoforms in the glomus cell.

In summary, our findings are consistent with the proposal (31) that AMPK may act as a primary metabolic sensor and primary effector of cell-specific Ca\textsuperscript{2+} signaling mechanisms in O\textsubscript{2}-sensing cells. This acute O\textsubscript{2}-sensitivity may be conferred by the expression of specific AMPK isoforms in a given cell type, their subcellular distribution, and the reliance of the cell on mitochondrial oxidative phosphorylation for ATP production. Given the ubiquitous nature of AMPK in eukaryotes (25), the ability to target acute O\textsubscript{2}-sensitivity to such specialized cells is likely a relatively early evolutionary development. This is supported by the finding that, in common with its effects on pulmonary arteries, hypoxia also constricts the arteries that feed the gills of the Pacific hagfish and the skin of the amphibian Xenopus laevis (56, 57). It also seems likely, therefore, that AMPK-dependent Ca\textsuperscript{2+} signaling by hypoxia will contribute to the regulation of other systems, for example, neonatal adenomucillar chromaffin cells, where hypoxia initiates catecholamine release required to prepare the newborn lung for breathing (58), hypoxia-induced neuro-excitability that contributes to excito-
