AMP-activated Protein Kinase Mediates Carotid Body Excitation by Hypoxia*

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Early detection of an O2 deficit in the bloodstream is essential to initiate corrective changes in the breathing pattern of mammals. Carotid bodies serve an essential role in this respect; their type I cells depolarize when O2 levels fall, causing voltage-gated Ca2+ entry. Subsequent neurosecretion elicits increased afferent chemosensory fiber discharge to induce appropriate changes in respiratory function (1). Although depolarization of type I cells by hypoxia is known to arise from K+ channel inhibition, the identity of the signaling pathway has been contested, and the coupling mechanism is unknown (2). We tested the hypothesis that AMP-activated protein kinase (AMPK) is the effector of hypoxic chemotransduction. AMPK is co-localized at the plasma membrane of type I cells with O2-sensitive K+ channels. In isolated type I cells, activation of AMPK using 5-aminoimidazole-4-carboxamide riboside (AICAR) inhibited O2-sensitive K+ currents (carried by large conductance Ca2+-activated BKCa channels) and TASK (tandem pore, acid-sensing potassium channel)-like channels, leading to plasma membrane depolarization, Ca2+ influx, and increased chemosensory fiber discharge. Conversely, the AMPK antagonist compound C reversed the effects of hypoxia and AICAR on type I cell and carotid body activation. These results suggest that AMPK activation is both sufficient and necessary for the effects of hypoxia. Furthermore, AMPK activation inhibited currents carried by recombinant BKCa channels, whereas purified AMPK phosphorylated the α subunit of the channel in immunoprecipitates, an effect that was stimulated by AMP and inhibited by compound C. Our findings demonstrate a central role for AMPK in stimulus-response coupling by hypoxia and identify for the first time a link between metabolic stress and ion channel regulation in an O2-sensing system.

Chronic and intermittent deficits in O2 supply to the body precipitate a variety of pathologies including dementia (3) and pulmonary hypertension (4). To develop effective therapies, it is necessary to understand the homeostatic mechanisms that monitor O2 supply to the body and elicit corrective changes in respiratory and circulatory function to maintain O2 levels. O2-sensitive ion channels, which were first identified in the carotid body type I cell, play a pivotal role in this respect and have now been reported in a diverse range of highly specialized O2-sensing tissues (5). Within the carotid body, clusters of type I cells lie in presynaptic contact with afferent sensory fibers, whose discharge increases in proportion to the degree of systemic arterial O2 deficit, providing information concerning blood O2 levels to the central respiratory centers (1, 2). This occurs subsequent to hypoxic inhibition of type I cell K+ channels, membrane depolarization, voltage-gated Ca2+ influx (6), and consequent neurotransmitter release. For many years, there has existed compelling evidence that mitochondria serve an important role in O2 sensing by type I cells (2, 7). Indeed, mitochondrial inhibitors can both mimic the cellular effects of hypoxia and occlude type I cell O2 sensitivity (8, 9). These effects, and the downstream cellular responses, are manifest at relatively high tissue O2 levels, which would not be limiting in other, non-specialized cells (10). Thus, type I cell metabolism may be the key to the mechanism by which hypoxia is coupled to neurosecretion. We therefore investigated the potential role in this process of AMPK, a key sensor of metabolic stress that is universally expressed in eukaryotes. When cells are exposed to metabolic stresses, a rise in the cellular ADP/ATP ratio is partly compensated by the adenylate kinase reaction, which precipitates a much larger increase in the AMP/ATP ratio, although the fall in ATP may be negligible (11, 12). AMPK is activated via multiple mechanisms that make the system acutely sensitive to relatively small changes in the AMP/ATP ratio (13). Although it was originally proposed to regulate energy balance within single cells (14), it has recently become clear that AMPK also performs this function at the level of the whole animal, through its regulation of hypothalamic neurons that drive food intake and by its ability to stimulate energy expenditure in peripheral tissues (15). The hypothesis that AMPK also underpins the response of other physiological systems to stimuli that affect metabolism, e.g. excitation of the vascular smooth muscle, neuronal firing, and cardiac function (16).}

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6 The abbreviations used are: AMPK, adenosine monophosphate-activated protein kinase; TASK, tandem pore, acid-sensing potassium channel; BKCa, large conductance voltage and Ca2+-activated potassium channel; AICAR, 5-aminoimidazole-4-carboxamide riboside; pO2, partial pressure of oxygen; PBS, phosphate-buffered saline.
carotid body by hypoxia (16), has therefore become attractive. This is particularly so given that our previous study established that physiologically relevant levels of hypoxia increased the AMP/ATP ratio in O₂-sensing cells, leading to concomitant activation of AMPK activity that was primarily associated with heterotrimeric containing the AMPKα catalytic subunit isoform (16). Furthermore, this study suggested that activation of AMPK by 5-aminoimidazole-4-carboxamide riboside (AICAR) induces transmembrane calcium influx into carotid body type I cells and thereby increases afferent fiber discharge from the isolated carotid body (16). However, we have yet to determine whether this is achieved by the same mechanisms that are engaged by hypoxia. Here we demonstrate that AMPK elicits carotid body activation by mimicking precisely the effects of hypoxia on carotid body type I cells and that inhibition of AMPK reverses these effects.

**EXPERIMENTAL PROCEDURES**

All experiments were performed under the United Kingdom Animals (Scientific Procedures) Act 1986.

**Carotid Body Type I Cell Isolation**—Neonatal rats (10–21 days) were killed, and carotid bodies were rapidly removed and placed in ice-cold, oxygenated phosphate-buffered saline without Ca²⁺ or Mg²⁺ (Sigma). These were then enzymically dissociated and cultured as described previously (9).

**HEK 293 Cell Culture**—HEK 293 cells that express human brain αβ-BKCa channels (17) were cultured as described previously (18). The co-expressed α and β subunits were KCNMA1 (GenBank™ accession number U11717) and KCNMB1 (GenBank accession number U42600), respectively.

**Immunocytochemistry**—Type I cells were fixed, immunostained, and visualized as described previously (16). The primary antibodies were selective for the AMPKα subunit (1:500, sheep polyclonal, D. G. Hardie, Dundee, Scotland, raised against peptide residues 995–1113 of human brain BKα). Images were deconvolved and analyzed off-line via Softworx (Applied Precision) and Volocity (Improvision).

**Carotid Body Electrophysiology**—Recordings were made using the amphotericin perforated-patch configuration of the whole-cell patch clamp technique. Data were acquired using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) controlled by Clampex 6.0 or Fetchex 6.0 software (Axon Instruments). Currents were digitized at 6.6 kHz and filtered at 1–2 kHz, and membrane potential recordings were digitized at 0.1 kHz and filtered at 1 kHz. Experiments were performed at 35–36 °C. Cells were bathed in solution A of composition (in mM): 4.5 KCl; 140 NaCl; 2.5 CaCl₂; 1 MgCl₂; 11 glucose; 20 HEPES, adjusted to pH 7.4. Pipette solution was (in mM): 5 K₂SO₄; 30 KCl; 5 MgCl₂; 1 EGTA; 10 glucose; 20 HEPES, adjusted to pH 7.2. Pipette solution contained amphotericin B (240 μg ml⁻¹, Sigma). Liquid junction potential was ~6 mV. Results were not leak-subtracted, seal resistance was typically >5 gigaohms, access resistance was typically 15–20 megaohms and was not compensated for, and holding currents were typically less than 5 pA. All data were analyzed using Clampfit and Fetchan programs (pClamp6, Axon Instruments).

**Ca²⁺ Imaging**—Cells were incubated for 30 min (22–24 °C, dark) in a Ca²⁺-free solution (in mM): 4.5 KCl; 140 NaCl; 1 MgCl₂; 11 glucose; 1 EGTA; 20 HEPES, adjusted to pH 7.4, containing 5 μM FURA-2AM (Molecular Probes), and then allowed to equilibrate for 20 min in solution A (35–36 °C). Hypoxic solutions were generated by gassing solution A with 100% N₂, PO₂ was monitored using an ISO2 oxygen meter (World Precision Instruments, Stevenage, UK). Changes in [Ca²⁺]i were monitored using alternate excitation wavelengths of 340 and 380 nm (F₃₄₀ and F₃₈₀ 20-ms exposure). Emitted fluorescence was recorded as described previously (16) at 0.05 Hz with background subtraction carried out online.

**Isolated Carotid Body**—Rats were anesthetized with 1–4% halothane in O₂, and carotid bodies with attached carotid sinus nerve were excised as described previously (19). Extracellular recordings of single or few-fiber afferent fiber action potentials were recorded on videotape and sampled digitally via Spike2 software (Cambridge Electronic Design, Cambridge, UK) for analysis of discharge frequency.

**Co-immunoprecipitation**—Cells were harvested in phosphate-buffered saline (PBS) and centrifuged at 1000 × g after which the pellet was homogenized in 1 ml of ice-cold buffer A (50 mm Tris-HCl (pH 7.4), 140 mm KCl, 1 mm EGTA, 1 mm MgCl₂, 1 mm phenylmethanesulfonyl fluoride, and 50 μg/ml aprotinin). This was followed by centrifugation (1000 × g, 5 min, 4 °C). The pellet was then solubilized in 0.5 ml of ice-cold buffer B (5 mm Tris-HCl (pH 7.4), 500 mm NaCl, 1.5% v/v Triton X-100, with 1 mm phenylmethylsulfonyl fluoride and 50 μg/ml aprotinin). Antibodies (either anti-BKα subunits (5 μg) or anti-AMPKα subunits (6 μg), as used for immunocytochemistry) were added to solubilized suspensions and incubated overnight at 4 °C before the addition of 50 μl of protein G beads. Following further incubation (>4 h, 4 °C, with rotation), the beads were pelleted at 7000 × g (5 min) and washed three times in 1 ml of ice-cold buffer C (5 mm Tris-HCl (pH 7.4), 20 mm NaCl, 0.5% v/v Triton X-100, with 1 mm phenylmethylsulfonyl fluoride and 50 μg/ml aprotinin). Pelleted immunoprecipitates were taken up into 50 μl of sample buffer (52.5 mm Tris-HCl (pH 6.8), 10% v/v glycerol, 0.2% w/v SDS 5% v/v β-mercaptoethanol, 0.02% bromphenol
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blue), heated for 3 min at 100 °C, and loaded onto 10% (w/v) SDS-polyacrylamide gels and separated at 100 V for 1 h. Proteins were transferred to polyvinylidene difluoride membrane at 250 mA for 1 h, and membranes were blocked overnight with 10% (w/v) nonfat powdered milk in PBS at 4 °C. After washing membranes three times in PBS/0.05% Tween 20, they were incubated with anti-AMPKα1 antibody (1:500) or anti-BK<sub>Ca</sub>α antibody (1:250) in PBS/0.5% nonfat powdered milk for 1 h at 21 °C. Membranes were then washed extensively with PBS before incubation with anti-mouse or anti-sheep peroxidase-linked secondary antibody (1:10,000 in PBS, Pierce) for 1 h. Membranes were then washed with PBS/0.05% Tween 20, they were incubated with anti-AMPKα1 antibody (1:50) or anti-BK<sub>Ca</sub>α antibody (1:250) in PBS/0.5% nonfat powdered milk for 2 h at 4 °C followed by centrifugation (230 × g, 5 min). BK<sub>Ca</sub>α was subsequently immunoprecipitated from the supernatant using 20 μg of anti-BK<sub>Ca</sub>α antibody (BD Transduction Laboratories) prebound to 40 μl of protein G-Sepharose. After 2 h at 4 °C, the precipitate was collected by centrifugation (230 × g, 5 min). The immunoprecipitate was washed five times in lysis buffer containing 1 M NaCl followed by five times washing in lysis buffer without NaCl.

Phosphorylation of BK<sub>Ca</sub>α Subunit Using Purified Rat Liver AMPK—Immunopurified BK<sub>Ca</sub>α was washed six times in assay buffer (50 mM sodium-Hepes, pH 7.2, 1 mM dithiothreitol, 0.02% (w/v) Brij-35) containing 1 M NaCl followed by eight washes in assay buffer without NaCl. The immune complexes were incubated for 30 min at 30 °C with 4.5 μg/ml recombinant PP1γ in assay buffer. They were washed five times in assay buffer containing 1 M NaCl followed by five further washes in assay buffer. They were then incubated for 30 min at 30 °C with 5 mM MgCl<sub>2</sub> and 200 μM [γ<sup>32</sup>P]ATP (300 cpm/pmol) in 50 mM sodium-Hepes, pH 7.2, 1 mM dithiothreitol, 0.02% (w/v) Brij-35, with or without purified rat liver AMPK (Hawley et al. (34)) and with or without AMP (200 μM) or compound C (100 μM). The reactions were stopped by the addition of lithium dodecyl sulphate loading dye and reducing agent (Invitrogen). The samples were analyzed by SDS-PAGE using the 3–8% gradient Tris acetate system (Invitrogen) and gels stained with Colloidal Blue staining kits (Invitrogen). Destained gels were dried and subjected to autoradiography. The BK<sub>Ca</sub>α band was excised, and the incorporation of [<sup>32</sup>P]phosphate was determined by Cerenkov counting. The amount of BK<sub>Ca</sub>α subunit was estimated by running known amounts of bovine serum albumin on the same gel, comparing intensities of Coomassie Blue-stained bands by densitometry and assuming that the same amount of dye bound per μg of protein.

Identification of the BK<sub>Ca</sub> α Subunit by Mass Spectrometry—A gel slice was excised and washed successively for 15 min in water, CH<sub>3</sub>CN + 100 mM NH<sub>4</sub>HCO<sub>3</sub> (50:50 v/v), and finally in 20 mM NH<sub>4</sub>HCO<sub>3</sub>/CH<sub>3</sub>CN (50:50 v/v). The gel slice was dehydrated in water + CH<sub>3</sub>CN (50:50 v/v) for 5 min. The supernatant was dried in a SpeedVac concentrator and resuspended in 20 μl of 12.5 μg/ml trypsin (Roche Applied Science, modified sequencing grade) in 20 mM NH<sub>4</sub>HCO<sub>3</sub>/0.1% n-octylglucoside. Digestion occurred overnight at 30 °C on a shaking platform. An equal volume of CH<sub>3</sub>CN was then added to the digest and incubated for 30 min at 30 °C on a shaking platform. An aliquot (10%) of the peptide digest was then analyzed by tandem matrix-assisted laser desorption/ionization-time of flight tandem mass spectrometry (Applied Biosystems 4700 Proteomics Analyzer). The mass spectrometry and tandem mass spectrometry data generated were used to search the Celera Discovery System human data base (Applied Biosystems) using the Mascot search algorithm (35). This confirmed the identity of the 125-kDa band as the BK<sub>Ca</sub>α subunit (SwissProt accession number KCNMA1).

Statistics—All data are presented as means ± S.E. of the mean. Statistical significance was assessed using two-tailed Bonferroni-Dunn post hoc test (in vitro carotid body) or Student’s paired or unpaired t test (all other data) as appropriate. p < 0.05 was considered significant.

RESULTS

Consistent with the hypothesis that AMPK serves a key role in O<sub>2</sub> sensing by the carotid body, we found that in isolated carotid body type I cells, the AMPKα1 catalytic subunit isoform and O<sub>2</sub>-sensitive BK<sub>Ca</sub> channels co-localize at the plasma membrane (Fig. 1, A, panels i–iv). Thus, 75.1 ± 4.4% of the total AMPKα1 staining was located within 1 μm of the type I cell plasma membrane (n = 14), where it co-localized with O<sub>2</sub>-sensitive BK<sub>Ca</sub> channels (21), with 71.0 ± 2.6% of staining for the pore-forming BK<sub>Ca</sub>α subunit coinciding with that for the AMPKα1 catalytic subunit isoform (Pearson’s correlation 0.65 ± 0.05, n = 5; Fig. 1, A, panels iii and iv). Furthermore, BK<sub>Ca</sub> channel currents were selectively inhibited in acutely isolated type I cells by 5-aminoimidazole-4-carboxamide riboside (AICAR), a nucleoside that is taken up by cells and metabolized to the AMP mimic, ZMP, thus activating AMPK in the absence of ATP depletion (22). Analysis of current-voltage relationships for macroscopic K<sup>+</sup> currents showed that AICAR (1 mM, 10 min) reversibly inhibited voltage-sensitive K<sup>+</sup> currents throughout their activation range, e.g. by 40.0 ± 12.2% during a voltage step from −80 to +30 mV (Fig. 1B; n = 5, p = 0.041). Selective blockade of BK<sub>Ca</sub> channels by iberiotoxin (100 nM) inhibited type I cell macroscopic K<sup>+</sup> currents by 60.3 ± 6.8% at +30 mV (n = 7, p = 0.0069) and, in the presence of the toxin, AICAR had no further inhibitory effect on residual voltage-gated K<sup>+</sup> currents (Fig. 1C; n = 3). Rat carotid body type I cells also express O<sub>2</sub>-sensitive, voltage-independent leak K<sup>+</sup> channels (23) in addition to BK<sub>Ca</sub> channels, although the molecular identity of these TASK-like channels is not known. AICAR also inhibited this Ba<sup>2+</sup>-sensitive, voltage-independent leak con-
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FIGURE 1. AMPKα1 and BKCa subunits co-localize and activation of AMPK inhibits O2-sensitive K+ currents in type I cells. A, panel i, bright field image of a type I cell. panel ii, Z-section with immunostaining for AMPKα1 and nuclear staining by 4′,6-diamidino-2-phenylindole (DAPI). panel iii, Z-section with immunostaining for BKCa subunit and DAPI. panel iv, three-dimensional reconstruction showing AMPKα1 and BKCa co-localization. B, AICAR inhibits macroscopic current. Data are mean ± S.E. The insets are example currents ± AICAR. C, AICAR inhibits the iberiotoxin (IBTX)-sensitive current. Data are mean ± S.E. The insets are example currents, control, +IBTX, and +IBTX and AICAR. D, Ba2+ and AICAR inhibit leak K+ currents. E, AICAR does not inhibit residual currents in the continued presence of Ba2+.

ductance (measured during voltage ramps from −100 mV to −40 mV) by 33.0 ± 2.3%, from 258 ± 24 pS to 171 ± 11 pS (Fig. 1D; n = 4, p = 0.008). Furthermore, in the continued presence of Ba2+, AICAR did not significantly inhibit the residual currents (Fig. 1E; n = 5). These data indicate that AMPK activation, like hypoxia (21, 23), selectively inhibits both the O2-sensing BKCa currents and TASK-like leak K+ currents in rat carotid body type I cells, without affecting O2-insensitive delayed rectifier K+ currents.

Inhibition of the O2-sensing K+ currents led to reversible depolarization of type I cells by 12.6 ± 0.9 mV (Fig. 2A; n = 6, p = 0.0055) within 12.6 ± 1.9 min of application of AICAR (n = 6). The magnitude of depolarization was equivalent to that seen in response to hypoxia (24). As expected, AMPK activation triggered voltage-gated Ca2+ influx into carotid body type I cells as monitored by the Ca2+ indicator dye Fura-2 (Fig. 2B). Thus, AICAR reversibly increased the Fura-2 fluorescence ratio (F340/F380) from 0.62 ± 0.01 to a peak of 1.71 ± 0.23 (n = 7, p = 0.0028), and this was reversibly inhibited by 84.0 ± 9.3% by Ni2+ (5 mM, n = 7, p = 0.0037), an inhibitor of voltage-gated Ca2+ channels. Likewise, hypoxia (14–21 mm Hg) increased the Fura-2 fluorescence ratio from 0.67 ± 0.02 to 2.5 ± 0.17 (n = 5). Most significantly, the AMPK antagonist compound C (25) (40 μM) reversibly inhibited the increase in intracellular Ca2+ concentration in response to hypoxia by 65.1 ± 9.8% (Fig. 2C; n = 5, p = 0.00097) and to AICAR by 70.7 ± 4.2% (Fig. 2D; n = 7, p = 0.0004). In marked contrast, compound C (40 μM) had little effect on voltage-gated Ca2+ influx in response to type I cell depolarization by K+ (50 mM), the Fura-2 fluorescence ratio increasing from 0.59 ± 0.008 to 2.87 ± 0.23 in its absence and from 0.58 ± 0.01 to 2.22 ± 0.20 in its presence (Fig. 2E, n = 5). Thus, compound C blocked carotid body type I cell excitation in response to hypoxia without affecting basal levels of intracellular Ca2+ concentration, but it did not block membrane depolarization in response to interventions that reduce the outward K+ conductance, nor did it block voltage-gated Ca2+ entry per se.

Recordings from type I cells are not necessarily a reliable indicator of a functional response at the level of the intact carotid body (26, 27). We therefore investigated the role of AMPK in mediating afferent chemosensory fiber discharge from the carotid body in vitro. Consistent with our findings on isolated carotid body type I cells, 1 mM AICAR caused a 7-fold increase in spontaneous, single afferent fiber discharge frequency (Fig. 3A). AICAR at 100 or 300 μM was without effect upon basal discharge. The excitatory effect of AICAR at 1 mM was significantly inhibited by around 50% after preincubation with compound C (40 μM) for 10–20 min (Fig. 3A). After 30–40 min of preincubation with compound C, basal discharge was reduced to essentially zero, and the response to AICAR was abolished. Basal carotid body single afferent fiber discharge frequency was also inhibited by preincubation with compound C. At 10–20 min, the degree of inhibition was around 50% (Fig. 3C). Despite a reduction in basal discharge following sustained
incubation with compound C, a hypoxic response was retained, albeit reduced by about 90% at 70 min. That this was not simply due to “run down” of the preparation was confirmed by control studies, which showed that the hypoxia response was largely retained for up to 120 min following repeated stimulation trials. The mean data are shown in Fig. 3D. Thus, AMPK activation mediates a large component of carotid body type I cell activation by hypoxia, and this is sufficient to trigger an increase in afferent chemosensory fiber discharge that can be substantially blocked by compound C.

We next sought to determine whether inhibition by AMPK of O2-sensing K⁺ currents was mediated by direct phosphorylation of channel proteins, by examining the regulation of human BKCa channels stably expressed in HEK293 cells (17). Analysis of voltage ramps between −100 mV and +100 mV showed that, as in carotid body type I cells, AMPK activation by AICAR (1 mM, 10 min) inhibited BKCa channel currents throughout their current-voltage range; e.g. by 74.4% at +30 mV, from 46.1 ± 8.7 pA pF⁻¹ to 11.8 ± 1.9 pA pF⁻¹ (n = 20, p = 0.00057). Inhibition of BKCa channel currents by AICAR was also reversed to 85.5% of control, i.e. to 39.4 ± 4.4 pA pF⁻¹, by compound C (Fig. 4A; n = 20). Unlike in type I cells, there was no specific co-localization of the α1 subunit of AMPK with the BKCaα subunit at the plasma membrane (not shown), and the two proteins also failed to co-immunoprecipitate (Fig. 4B). Thus, AMPK-dependent regulation of BKCa channels can occur without coupling of these proteins at the molecular level and without precise targeting of AMPK to the plasma membrane protein. It seems likely, therefore, that channel regulation may result from phosphorylation of the BKCa channel protein by AMPK in a manner that may be further facilitated by targeting of AMPK to the plasma membrane in carotid body type I cells. Consistent with this view, when immunoprecipitates made using anti-BKCaα antibodies from HEK293 cells were incubated with [γ-32P]ATP and AMPK purified from rat liver (a mixture of the α1β1γ1 and α2β1γ1 heterotrimers), we obtained phosphorylation of the pore-forming BKCaα subunit in the presence of AMPK (Fig. 4C). This phosphorylation was stimulated by AMP and inhibited by compound C (Fig. 4D). The identity of this phosphorylated band as BKCaα was confirmed by mass spectrometry of tryptic peptides. Estimates of the stoichiometry of phosphorylation showed that this was substantial, reaching 0.7–0.9 mole/mole of α subunit within 30 min using 5 units/ml AMPK.

**DISCUSSION**

In the present investigation, we have addressed the molecular mechanism by which AMPK effects carotid body excitation by hypoxia. In doing so, we have shown that AMPK activation by
AICAR precisely mimics the effects of hypoxia on the two key O2-sensitive K⁺ channels in rat type I cells, i.e. BKCa channels and TASK-like channels. Moreover, we show that activation by hypoxia and AICAR of carotid body type I cells and of the carotid body itself is inhibited, if not abolished, by the AMPK inhibitor, compound C. AICAR is not known to activate any protein kinases other than AMPK, so the finding that compound C reverses carotid body type I cell activation induced by AICAR, but not that induced by depolarization, provides strong support for the view that its effects are mediated by inhibition of the AMPK pathway.

Although AICAR does not directly bind to adenosine receptors, it is known to compete with adenosine for the adenosine nucleoside transporter (28) and could therefore increase the extracellular adenosine concentration by competing with released adenosine for uptake into cells. As carotid body afferent discharge is increased by adenosine in a concentration-dependent manner (29, 30), there was a possibility that the effect of AICAR could have been mediated through this mechanism. However, this would only be apparent in the whole carotid body preparation where adenosine might build up in the synaptic cleft and would not occur in isolated, superfused carotid body type I cells. In a separate series of experiments, we showed that the non-selective adenosine receptor antagonist, 8-P-(sulphophenyl)-theophylline (300 μM), when applied prior to AICAR, reduced the afferent chemosensory response to AICAR in carotid body preparations by 53 ± 12%. Given that the AMPK antagonist, compound C, significantly inhibited AICAR-induced increases in afferent fiber discharge and that hypoxia elicits carotid body activation by releasing a variety of neurotransmitters including adenosine (31), our findings are consistent with the view that a substantial proportion of the response to AICAR in the whole organ was mediated via AMPK activation, and only a small proportion can be attributed to inhibition of adenosine uptake.

Thus, our results suggest that activation of AMPK is necessary as well as sufficient for carotid body activation by hypoxia. We also provide a plausible mechanism for the inhibitory effects on the BKCa channels by showing that AMPK directly phosphorylates the pore-forming α subunit in immunoprecipitates. As expected for an effect of AMPK, phosphorylation was stimulated by AMP and inhibited by compound C, and the stoichiometry approached 1 mole of phosphate/mole of α subunit, showing that the extent of phosphorylation was significant. We propose, therefore, that the AMPK system acts as the primary metabolic sensor as well as the effector of Ca2⁺ response to hypoxia in carotid body type I cells. This mechanism can unite the previous mitochondrial and membrane hypotheses for chemotransduction by hypoxia (2).

Recently, O2 sensing by BKCa channels has been proposed to arise via O2-dependent CO production from the closely associated enzyme, heme oxygenase-2 (HO-2 (32)). Clearly, when this enzyme is active (i.e. in the presence of its substrates, including O2), channel activity is markedly enhanced due to CO production by HO-2. However, the requirement of this mechanism of channel regulation for O2 sensing in the intact carotid body has not been evaluated. Furthermore, the importance of HO-2 in O2 sensing has more recently been questioned since BKCa channels have been shown to be suppressed by hypoxia in excised membrane patches (33). Collectively, these data suggest that there exist multiple means by which hypoxia might inhibit BKCa channels. However, unlike our proposed mechanism, other mechanisms cannot account for findings that inhibitors of mitochondrial oxidative phosphorylation prevent carotid body activation by hypoxia (8, 9). Furthermore, no other mechanism has been so comprehensively tested with respect to its

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7. S. A. Pearson and P. Kumar, unpublished data.
involvement in O₂ sensing at the level of ion channel activity, membrane potential, [Ca²⁺], regulation, and afferent nerve discharge. On the basis of our present data, therefore, we propose that AMPK is the physiologically relevant mediator of O₂ sensing by the carotid body. Furthermore, we propose that the targeting of AMPKα1 complexes to the plasma membrane of carotid body type 1 cells, the acute sensitivity of AMPK to changes in the AMP/ATP ratio, and a reliance of these cells on mitochondrial oxidative phosphorylation for ATP production underpin the activation of type 1 cells by relatively small, physiological changes in arterial pO₂. Thus, in addition to its ubiquitous role in regulating metabolism in response to metabolic stress at the cellular level, the AMPK signaling pathway has become adapted to mediate the responses of physiological systems that are key to meeting the metabolic needs of the whole body, including hypoxia-response coupling in O₂-sensing cells.

REFERENCES

1. Gonzalez, C., Almaraz, L., Obeso, A., and Bigual, R. (1994) Physiol. Rev. 74, 829–898
2. Prabhakar, N. R. (2006) Exp. Physiol. 91, 17–23
3. Desmond, D. W., Moroney, J. T., Sano, M., and Stern, Y. (2002) Stroke 33, 2254–2260
4. Voelkel, N. F. (1986) Am. Rev. Respir. Dis. 133, 1186–1195
5. Lopez-Barneo, J., del Toro, R., Levitsky, K. L., Chiara, M. D., and Ortega-Saenz, P. (2004) J. Appl. Physiol. 96, 1187–1195; discussion 1170–1182
6. Weir, E. K., Lopez-Barneo, J., Buckler, K. J., and Archer, S. L. (2005) N. Engl. J. Med. 353, 2042–2055
7. Mills, E., and Jobiss, F. F. (1972) J. Neurophysiol. 35, 405–428
8. Mulligan, E., Lahari, S., and Storey, B. T. (1981) J. Appl. Physiology 51, 438–446
9. Wyatt, C. N., and Buckler, K. J. (2004) J. Physiol. (Lond.) 556, 175–191
10. Duchen, M. R., and Biscoe, T. J. (1992) J. Physiol. (Lond.) 450, 13–31
11. Wyatt, C. N., Kumar, P., Aley, P., Peers, C., Hardie, D. G., and Evans, A. M. (2006) Adv. Exp. Med. Biol. 580, 191–196; discussion 351–199
12. Hardie, D. G. (2004) J. Cell Sci. 117, 5479–5487
13. Hardie, D. G., Salt, I. P., Hawley, S. A., and Davies, S. P. (1999) Biochem. J. 338, 717–722
14. Hardie, D. G., and Carling, D. (1997) Eur. J. Biochem. 246, 259–273
15. Kahn, B. B., Alquier, T., Carling, D., and Hardie, D. G. (2005) Cell Metab. 1, 15–25
16. Evans, A. M., Mustard, K. J., Wyatt, C. N., Peers, C., Dipp, M., Kumar, P., Kinnear, N. P., and Hardie, D. G. (2005) J. Biol. Chem. 280, 41504–41511
17. Ahring, P. K., Strobaek, D., Christophersen, P., Olesen, S. P., and Johansen, T. E. (1997) FEBS Lett. 415, 67–70
18. Lewis, A., Peers, C., Ashford, M. L., and Kemp, P. J. (2002) J. Physiol. (Lond.) 540, 771–780
19. Pepper, D. R., Landauer, R. C., and Kumar, P. (1995) J. Physiol. (Lond.) 485, 531–541
20. Hartness, M. E., Brazier, S. P., Peers, C., Bateson, A. N., Ashford, M. L., and Kemp, P. J. (2003) J. Biol. Chem. 278, 51422–51432
21. Peers, C. (1990) Neurosci. Lett. 119, 253–256
22. Corton, J. M., Gillespie, J. G., Hawley, S. A., and Hardie, D. G. (1995) Eur. J. Biochem. 229, 558–565
23. Buckler, K. J. (1997) J. Physiol. (Lond.) 498, 649–662
24. Wyatt, C. N., and Peers, C. (1995) J. Physiol. (Lond.) 483, 559–565
25. Zhou, G., Myers, R., Li, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M. F., Goodyear, L. J., and Moller, D. E. (2001) J. Clin. Investig. 108, 1167–1174
26. Donnelly, D. F. (1999) Respir. Physiol. 115, 151–160
27. Donnelly, D. F. (1996) J. Appl. Physiol. 81, 657–664
28. Gadalla, A. E., Pearson, T., Currie, A. J., Dale, N., Hawley, S. A., Sheehan, M., Hirst, W., Michel, A. D., Randall, A., Hardie, D. G., and Frenguelli, B. G. (2004) J. Neurochem. 88, 1272–1282
29. McQueen, D. S., and Ribeiro, J. A. (1981) Br. J. Pharmacol. 74, 129–136
30. Vanders, C., Conway, A. F., Landauer, R. C., and Kumar, P. (1999) J. Physiol. (Lond.) 515, 419–429
31. Conde, S. V., and Monteiro, E. C. (2006) Adv. Exp. Med. Biol. 580, 179–184; discussion 351–179
32. Williams, S. E., Wootton, P., Mason, H. S., Bould, J., Iles, D. E., Riccardi, D., Peers, C., and Kemp, P. J. (2004) Science 306, 2093–2097
33. McCartney, C. E., McClafferty, H., Huibant, J. M., Rowan, E. G., Shipston, M. J., and Rowe, I. C. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 17870–17876
34. Hawley, S. A., Davison, M., Woods, A., Davies, S. P., Beri, R. K., Carling, D., and Hardie, D. G. (1996) J. Biol. Chem. 271, 27879–27887
35. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999) Electrophoresis 20, 3551–3567