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Effects of modulators of AMP-activated protein kinase on TASK-1/3 and intracellular Ca^{2+} concentration in rat carotid body glomus cells

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ABSTRACT

Acute hypoxia depolarizes carotid body chemoreceptor (glomus) cells and elevates intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Recent studies suggest that AMP-activated protein kinase (AMPK) mediates these effects of hypoxia by inhibiting the background K^+ channels such as TASK. Here we studied the effects of modulators of AMPK on TASK activity in cell-attached patches. Activators of AMPK (1 mM AICAR and 0.1–0.5 mM A769662) did not inhibit TASK activity or cause depolarization during acute (10 min) or prolonged (2–3 h) exposure. Hypoxia inhibited TASK activity by ~70% in cells pretreated with AICAR or A769662. Both AICAR and A769662 (15–40 min) failed to increase $[Ca^{2+}]_i$ in glomus cells. Compound C (40 μM), an inhibitor of AMPK, showed no effect on hypoxia-induced inhibition of TASK. AICAR and A769662 phosphorylated AMPKα in PC12 cells, and Compound C blocked the phosphorylation. Our results suggest that AMPK does not affect TASK activity and is not involved in hypoxia-induced elevation of intracellular $[Ca^{2+}]$ in isolated rat carotid body glomus cells.

1. Introduction

In carotid body glomus cells, hypoxia inhibits the outward K^+ current, and thereby causes cell depolarization, Ca^{2+} influx via voltage-dependent Ca^{2+} channels and secretion of transmitters (Ortega-Saenz et al., 2007; Peers et al., 2010; Prabhakar, 2006). The hypoxia-sensitive K^+ current in glomus cells is believed to consist mainly of Kv, BK and TASK (TASK-1, TASK-3 and TASK-1/3) channels, but the signaling pathways by which hypoxia inhibits each of these K^+ channels are not well defined. Several mechanisms for hypoxia-induced inhibition of K^+ current have been proposed, including inhibition of heme-oxygenase-2 (Williams et al., 2004), inhibition of mitochondrial oxidative phosphorylation (Buckler and Vaughan-Jones, 1998; Wyatt and Buckler, 2004), and an undefined rotenone-sensitive pathway (Ortega-Saenz et al., 2003). It may be that different O_2 sensors and signals are involved in the inhibition of specific K^+ channels, but this remains to be determined. The inhibition of mitochondrial oxidative phosphorylation is probably responsible for the hypoxia-induced reduction of TASK, as mitochondrial inhibitors and uncouplers reversibly inhibit these two-pore domain background K^+ channels (Buckler, 2007, 2012; Kim, 2013).

Inhibition of mitochondrial oxidative phosphorylation results in the reduction of ATP production and rise in [ADP]/[ATP] ratio. Adenylate kinase converts ADP to AMP and ATP, which causes an increase in cell [AMP]/[ATP] ratio (Oakhill et al., 2011). Increases in both [ADP]/[ATP] and [AMP]/[ATP] ratios have been shown to stimulate AMP-activated protein kinase (AMPK) to regulate cell energy consumption (Hardie and Carling, 1997; Steinberg and Kemp, 2009). In glomus cells, AICAR, a well-known activator of AMPK, was found to inhibit the outward whole-cell K^+ current sensitive to iberiotoxin, suggesting that BK was a target of AMPK (Wyatt et al., 2007). In the same study, AICAR caused cell membrane depolarization, elevated intracellular calcium concentration ($[Ca^{2+}]_i$) in glomus cells and increased the carotid sinus nerve discharge in carotid body-sinus nerve preparations. AICAR also inhibited a Ba^{2+}-sensitive, voltage-independent K^+ current, suggesting that a background K^+ current was also targeted by AICAR (Wyatt et al., 2007). In support of this finding, AICAR inhibited TASK-3 expressed in HEK293 cells by ~50%, and this inhibition was blocked by Compound C, an inhibitor of AMPK (Dallas et al., 2009). These findings have led to the hypothesis that AMPK mediates the

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hypoxia-induced excitation of glomus cells by inhibition of K^+ channels such as BK and TASK that are both well expressed in glomus cells (Peers et al., 2010; Wyatt et al., 2007).

In the course of our studies to identify the effect of phosphorylation by AMPK on TASK single channel behavior and potential amino acid residues involved, we tested the effect of AICAR on TASK single channel kinetics to confirm its inhibitory action. Our preliminary tests using cell-attached patches showed no effect of AICAR on TASK function in glomus cells or in COS-7 cells expressing TASK-3. As our findings are in direct contradiction to the proposal that AMPK inhibits TASK and mediates the hypoxia-induced excitation of glomus cells, we further investigated the effects of AMPK on hypoxia-induced inhibition of TASK and intracellular $[Ca^{2+}]_i$ in glomus cells. TASK channel activity in cell-attached patches and intracellular $[Ca^{2+}]_i$ were recorded in response to modulators of AMPK. Consistent with our preliminary observation, AMPK activators failed to inhibit TASK, and hypoxia still inhibited TASK and produced strong depolarization even after blockade of AMPK with Compound C. Furthermore, AMPK activators failed to produce depolarization or an increase in $[Ca^{2+}]_i$ in glomus cells. Our results show that AMPK is unlikely to be a signal for hypoxia-induced inhibition of TASK and depolarization in isolated rat glomus cells.

2. Methods

2.1. Cell isolation

Rats (postnatal 14–18 day; Sprague-Dawley) were anesthetized with isoflurane and used according to the animal protocols approved by the Animal Care and Use Committees of Rosalind Franklin University and University of Arkansas for Medical Sciences. The carotid bodies were removed and placed in ice-cold low-Ca^{2+}, low-Mg^{2+} phosphate buffered saline solution (low Ca^{2+}/Mg^{2+}-PBS: 137 mM NaCl, 2.8 mM KCl, 2 mM KH_2PO_4, 0.07 mM $CaCl_2$, 0.05 mM $MgCl_2$, pH 7.4). Each carotid body was cut into 3–4 pieces and placed in a solution containing trypsin (400 μg/mL) and collagenase (400 μg/mL) in low Ca^{2+}/Mg^{2+}-PBS and incubated at 37 °C for 20–25 min. Carotid bodies were gently triturated using a fire polished Pasteur pipette to mechanically dissociate the cells. Growth medium (Ham's F-12, 10% fetal bovine serum, 23 mM glucose, 2 mM L-glutamine, 10k units penicillin/streptomycin, and 300 μg/mL insulin) was added to stop enzyme activity. After brief trituration, the solution containing the digested carotid bodies was centrifuged for 4 min at ~6000 rpm (~2000 × *g*) using a microcentrifuge. Supernatant was removed and warm growth medium added to gently resuspend the pellet. Suspended cells were placed on glass coverslips coated with poly-L-lysine, and incubated at 37 °C in a humidified atmosphere of 95% air–5% CO_2. Cells were used within 6 h.

2.2. Electrophysiological studies

Electrophysiological recording was performed using a patch clamp amplifier (Axopatch 200B, Molecular Devices, Sunnyvale, CA, USA). Patches were formed using borosilicate glass pipettes with 3–5 MΩ tip resistance. The pipette solution contained (mM) 150 KCl, 1 $MgCl_2$, 5 EGTA, 10 glucose and 10 HEPES (pH 7.3), and the bath perfusion solution contained (mM) 117 NaCl, 5 KCl, 23 $NaHCO_3$, 1 $CaCl_2$, 1 $MgCl_2$ and 11 glucose (pH 7.3). Channel current was filtered at 3 kHz using 8-pole Bessel filter (−3 dB; Frequency Devices, Haverhill, MA, USA) and transferred to a computer using the Digidata 1320 interface at a sampling rate of 20 kHz. Single-channel currents were analyzed with the pCLAMP program (Version 10). Channel openings were analyzed to obtain channel activity (NP_o, where N is the number of channels in the patch, and P_o is the open probability of a channel). NP_o was determined from 15 to 30 s of current recordings. Because glomus cells express both ~16-pS (TASK-1) and ~35-pS (TASK-3 and TASK-1/3) channels, analysis was done to detect all three isoforms by setting the open levels as multiples of ~16-pS channel. Single-channel current tracings shown in figures were filtered at 1 kHz. All electrophysiological experiments were performed at ~35 °C.

2.3. $[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was measured by quantitative fluorescence imaging using the calcium-sensitive dye fura-2. Cells plated on the coverslip were incubated with 4 μM fura-2 acetoxymethyl ester (fura-2 AM; Molecular Probes) for 30 min at 37 °C. Fura-2 fluorescence emission was measured at 510 nm in response to alternating excitation at 340 and 380 nm. Images were acquired and stored using a NIKON Eclipse TE300 microscope (with 40× oil immersion objective) and CCD (CoolSNAP HQ_2) camera under computer control (MetaFluor: Molecular Devices). For each coverslip, the background light levels were determined and subtracted from each image before measurement of the fluorescence intensity ratio. $[Ca^{2+}]_i$ was determined using the 340 nm/380 nm fluorescence ratio as described previously (Wasicko et al., 1999). Calibration was performed using cell-free solutions (Grynkiewicz et al., 1985). The perfusion solution used for $[Ca^{2+}]$ measurements contained (mM): 118 NaCl, 23 $NaHCO_3$, 3 KCl, 1 $MgCl_2$, 1.2 $CaCl_2$, and 11 glucose.

2.4. Hypoxia studies

Cell-attached patches were formed on glomus cells and perfused with a bicarbonate-buffered solution containing 117 mM NaCl, 5 mM KCl, 23 mM $NaHCO_3$, 1 mM $MgCl_2$ and 11 mM glucose, and bubbled with 5% CO_2/95% air mixture (normoxia) for ~60 min. After steady state channel activity was obtained, the perfusion solution was switched to solution bubbled vigorously (for at least 60 min at 37 °C) with 5% CO_2/95% N_2 mixture (hypoxia) for desired period of time. The pipette solution contained (mM) 150 KCl, 1 $MgCl_2$, 5 EGTA, 10 glucose and 10 HEPES (pH 7.3). The temperature of the perfusion solutions was kept at ~35 °C, and the cells were perfused at a rate of ~2.2 mL/min. O_2 pressure of the solutions was determined using an oxygen meter (ISO2, WPI, Sarasota, USA) that was calibrated to 0% with solution gassed with pure nitrogen for 60 min and to 21% with solution gassed with air for 60 min at 35 °C. The O_2 partial pressure as judged by the reading on the meter for the hypoxic solution inside the recording chamber used in this study was ~2% (range: 15–18 mmHg O_2).

2.5. Western blot analysis

For isolation of total protein, PC12 cells derived from a pheochromocytoma of the rat adrenal medulla were homogenized in a protein extraction solution (PRO-PREP™; iNtRON Biotechnology, Korea) containing 50 mM Tris–Cl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.5% NP-40, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1% EDTA, 1 μM leupeptin, 1 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 0.1 μM aprotinin. The homogenates were incubated for 60 min on ice with intermittent vortexing, and then centrifuged at 13,000 rpm for 20 min at 4 °C. The resulting supernatant was separated on an 8% SDS–polyacrylamide gel and then transferred to a polyvinylidene fluoride (PVDF) membrane for 40 min using a semi-dry transfer (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 3% fat-free dry milk and then incubated with anti-AMPK, anti-phospho (p)-AMPK (1:1000 dilution), and anti-α-tubulin (1:1000 dilution) antibodies. These were followed by incubation with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse