Acute hypoxia selectively inhibits KCNA5 channels in pulmonary artery smooth muscle cells

Oleksandr Platoshyn, Elena E. Brevnova, Elyssa D. Burg, Ying Yu, Carmelle V. Remillard, and Jason X.-J. Yuan

Department of Medicine, Division of Pulmonary and Critical Care Medicine, Biomedical Sciences Graduate Program, University of California, San Diego, La Jolla, California

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Platoshyn, Oleksandr, Elena E. Brevnova, Elyssa D. Burg, Ying Yu, Carmelle V. Remillard, and Jason X.-J. Yuan. Acute hypoxia selectively inhibits KCNA5 channels in pulmonary artery smooth muscle cells. Am J Physiol Cell Physiol 290: C907–C916, 2006.—Acute hypoxia causes pulmonary vasoconstriction in part by inhibiting voltage-gated K+ (Kv) channel activity in pulmonary artery smooth muscle cells (PASMC). The hypoxia-mediated decrease in Kv currents [I(Kv)] is selective to PASMC; hypoxia has little effect on I(Kv) in mesenteric artery smooth muscle cells (MASMC). Functional Kv channels are homo- and/or heterotetramers of pore-forming α-subunits and regulatory β-subunits. KCNA5 is a Kv channel α-subunit that forms functional Kv channels in PASMC and regulates resting membrane potential. We have shown that acute hypoxia selectively inhibits I(Kv) through KCNA5 channels in PASMC. Overexpression of the human KCNA5 gene increased I(Kv) and caused membrane hyperpolarization in HEK-293, COS-7, and rat MASMC and PASMC. Acute hypoxia did not affect I(Kv) in KCNA5-transfected HEK-293 and COS-7 cells. However, overexpression of KCNA5 in PASMC conferred its sensitivity to hypoxia. Reduction of PO2 from 145 to 35 mmHg reduced I(Kv) by ~40% in rat transfected with human KCNA5 but had no effect on I(Kv) in KCNA5-transfected rat MASMC (or HEK and COS cells). These results indicate that KCNA5 is an important Kv channel that regulates resting membrane potential and that acute hypoxia selectively reduces KCNA5 channel activity in PASMC relative to MASMC and other cell types. Because Kv channels (including KCNA5) are ubiquitously expressed in PASMC and MASMC, the observation from this study indicates that a hypoxia-sensitive mechanism essential for inhibiting KCNA5 channel activity is exclusively present in PASMC. The divergent effect of hypoxia on I(Kv) in PASMC and MASMC may also be due to different expression levels of KCNA5 channels.

Address for reprint requests and other correspondence: J. X.-J. Yuan, Dept. of Medicine, Univ. of California, San Diego, 9200 Gilman Drive, La Jolla, CA 92039-0725 (e-mail: xiyuan@ucsd.edu).

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HYPOXIC PULMONARY VASOCONSTRICTION is a critical physiological mechanism that directs blood flow away from poorly ventilated regions in the lung to maintain an optimal ventilation-perfusion ratio for maximal oxygenation of the venous blood in the pulmonary artery. One of the potential mechanisms involved in hypoxic pulmonary vasoconstriction is acute hypoxia-mediated inhibition of voltage-gated K+ (Kv) channels in pulmonary artery smooth muscle cells (PASMC) (42, 43, 56). The subsequent membrane depolarization opens voltage-dependent Ca2+ channels (VDCC), increases cytoplasmic free Ca2+ concentration ([Ca2+]cyt), triggers PASMC contraction, and causes hypoxic pulmonary vasoconstriction. The vasocostructive response to hypoxia occurs only in the pulmonary vasculature; hypoxia, or hypoxemia, causes systemic vasodilation in vivo and has little contractile effect on isolated mesenteric arteries in vitro (28, 58). The hypoxia-induced functional inhibition of Kv channels is also selective to PASMC, because hypoxia has little effect on Kv channel activity in systemic artery smooth muscle cells, such as mesenteric artery smooth muscle cells (MASMC) (28, 56).

Functional Kv channels in native cells are either homo- or heterotetramers composed of the pore-forming α-subunits and cytoplasmic regulatory β-subunits (12). In PASMC and systemic (e.g., cerebral, coronary, renal, and mesenteric) arterial smooth muscle cells, multiple Kv channel α- and β-subunits are expressed (2, 6, 49, 50). Therefore, the K+ currents involved in determining the resting membrane potential (Em) are believed to result from activities of these various Kv channels as well as voltage-independent K+ channels (14, 19, 55). Because hypoxia-mediated inhibition of Kv channels was first reported in carotid body (glomus) cells (26), many investigators have attempted to identify the Kv channel subunits that are responsible for sensing changes in oxygen tension (PO2) in PASMC and other oxygen-sensitive tissues and cells (3–8, 19, 22, 25, 26, 28, 35–43, 50, 55–58).

From the use of heterologous transfection systems, we now know that various α-subunit homotetramers (e.g., formed by Kv1.2, Kv2.1, or Kv3.1b) and heterotetramers (e.g., formed by Kv1.2/Kv1.5 or Kv2.1/Kv9.3) as well as αβ-subunit homo- or heterotetramers (e.g., Kv4.2/Kvβ1.2, Kv1.5/Kvβ1.2) are sensitive to hypoxia (22, 38–40). KCNA5 (i.e., Kv1.5) is a pore-forming subunit that forms hetero- or homotetrameric Kv channels in many cell types, including PASMC (4, 6, 8, 23, 44). Normal expression and function of KCNA5 channels in PASMC are necessary for the regulation of resting membrane potential and pulmonary vascular tone (6, 21). In vivo gene transfer of KCNA5 to lung tissues and pulmonary arteries improves pulmonary hemodynamics and induces regression of pulmonary vascular medial hypertrophy in rats with chronic hypoxia-mediated pulmonary hypertension (44), suggesting that enhancing KCNA5 protein expression may represent a potential therapeutic approach for pulmonary arterial hypertension. However, homotetrameric KCNA5 channels have been demonstrated to be insensitive to acute hypoxia in mouse L cells transiently transfected with the KCNA5 gene (22). Therefore, it remains unclear whether homotetrameric KCNA5 channels serve as hypoxia-sensitive Kv channels in PASMC.
In this study we investigated 1) whether acute hypoxia reduces Kv currents \([I_{K(V)}]\) in different cell types (PASMC, MASMC, HEK-293, and COS-7) transiently transfected with the human KCNA5 gene and 2) whether the sensitivity of the homomeric KCNA5 channels to acute hypoxia is a unique property of PASMC or whether hypoxia-mediated effect on KCNA5 channel activity is dependent on the cells in which the KCNA5 gene is transfected. The present study demonstrates that expression of homomeric KCNA5 in various cell types (e.g., rat PASMC, rat MASMC, HEK-293, and COS-7) generates a typical Kv current that is sensitive to acute hypoxia only in rat PASMC, whereas the current (due to overexpressed KCNA5 channels) causes membrane hyperpolarization and is inhibited by 4-aminopyridine (4-AP; a Kv channel blocker) in all cell types. These results suggest that, although expressed ubiquitously in both pulmonary and systemic arterial smooth muscle cells, the KCNA5 channel is a downstream effector used by an exclusive oxygen-sensing mechanism present only in PASMC to reduce Kv currents and cause membrane depolarization during acute hypoxia.

**MATERIALS AND METHODS**

**Cell preparation and culture.** Rat PASMC and MASMC were prepared from pulmonary arteries of male Sprague-Dawley rats (41). Briefly, the isolated pulmonary and mesenteric arteries were incubated for 20 min in Hanks’ balanced salt solution containing 1.5 mg/ml collagenase (Worthington Biochemical, Lakewood, NJ). Adventitia and endothelium were carefully removed after the incubation. The remaining smooth endothelium was digested for 45–50 min with 1.5 mg/ml collagenase and 0.5 mg/ml elastase (Sigma, St. Louis, MO) at 37°C. PASMC were sedimented by centrifugation, resuspended in fresh medium, and plated. HEK-293 (human embryonic kidney epithelial cells) and COS-7 cells (monkey kidney fibroblast-like cells) (ATCC, Manassas, VA), rat PASMC, and rat MASMC were cultured in high-glucose (4.5 g/l) DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (BioFluids, Camarillo, CA) and incubated in 5% CO\(_2\) at 37°C in a humidified atmosphere. The animal use protocol (S990499) and the procedure for isolating and preparing vascular smooth muscle cells from rats were approved by the Institutional Animal Care and Use Committee of the University of California, San Diego.

** Constructs.** In the KCNA5/pBK construct (kindly provided by Dr. M. Tamkun, Colorado State University), the coding sequence of the human KCNA5 gene was subcloned into XhoI and KpnI sites of multiple cloning site (MCS) of the phagemid expression vector pBK-CMV (Stratagene, La Jolla, CA). For electrophysiological experiments, a KCNA5/GFP construct was designed to visualize the transfected cells. In the KCNA5/GFP construct, the coding sequence of the human KCNA5 gene was subcloned into EcoRI and XhoI sites of MCS of the pCMS-EGFP mammalian expression vector (Clontech, Palo Alto, CA). The enhanced green fluorescent protein (EGFP) gene, a red-shifted variant of wild-type GFP from Aequorea victoria, is expressed separately from the gene of interest in the pCMS-EGFP vector and is used as a transfection marker.

**Transfection of KCNA5.** Cells were transiently transfected with the expression constructs using Lipofectamine reagent according to the manufacturer’s instructions. Briefly, cells were first split and then cultured for 24 h. Transfection was performed on 50–80% confluent cells at 37°C in serum-free Opti-MEM I medium (Invitrogen, Carlsbad, CA) with 1.6 μg/ml DNA and 4 μl/ml Lipofectamine reagent. After 5–7 h of exposure to the transfection medium, cells were refed with construct-free serum-containing medium (10% FBS-DMEM) and incubated 12–24 h before experiments. The transfection efficiency was consistently >30% (9).

**Western blot analysis.** Cells were collected in tubes, centrifuged, and washed with cold PBS. Cell pellets were resuspended in 20–100 μl of lysis buffer [1% Triton X-100, 150 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl (pH 7.4)] supplemented with 1× protease inhibitor cocktail (Sigma) and 100 μg/ml PMSF before use and then incubated in the lysis buffer for 30 min on ice. Resulting cell lysates were centrifuged at 14,000 rpm for 15 min, and the insoluble fraction was discarded. The protein concentrations in the supernatant were determined using the Coomassie Plus protein assay (Pierce Biotechnology, Rockford, IL) with BSA as a standard. Proteins were mixed and boiled in SDS-PAGE sample buffer for 2 min. The protein samples separated on 8% SDS-PAGE were transferred onto nitrocellulose membranes by electroblotting in a Mini Trans-Blot cell transfer apparatus (Bio-Rad, Hercules, CA). After incubation for 1 h at 22–24°C in a blocking buffer (0.1% Tween 20 in PBS) containing 5% nonfat dry milk powder, the membranes were incubated with a polyclonal rabbit anti-KCNA5 antibody (Alomone Labs, Jerusalem, Israel) overnight at 4°C. The membranes were then washed with the blocking buffer and incubated with corresponding horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After the unbound antibodies were washed with the blocking buffer, the bound antibodies were detected using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ).

**Electrophysiological measurement.** Whole cell and single-channel K\(^+\) currents were recorded with an Axopatch-1D amplifier and a DigiData 1200 interface (Axon Instruments, Foster City, CA) using patch-clamp techniques. Patch pipettes (2–3 MΩ) were fabricated on an electrode puller (Sutter Instrument, Novato, CA) using borosilicate glass tubes and fire polished on a microforge (Narishige Scientific Instruments, Tokyo, Japan). Command voltage protocols and data acquisition were performed using pCLAMP 8 software (Axon Instruments). All experiments were performed at room temperature (22–24°C). For recording optimal whole cell Kv currents \([I_{K(V)}]\), cells were superfused with a standard extracellular solution containing (in mM) 141 NaCl, 4.7 KCl, 3.0 MgCl\(_2\), 10 HEPES, 1 EGTA, and 10 glucose (pH 7.4). The pipette (intracellular) solution contained (in mM) 135 KCl, 4 MgCl\(_2\), 10 HEPES, 10 EGTA, and 5 Na\(_2\)ATP (pH 7.2). Under whole cell configuration, the resting \(E_{m}\) was measured in all cell types in current-clamp (\(I = 0\)) mode at room temperature.

For cell-attached recording of single-channel Kv currents \([i_{K(V)}]\), the pipette (extracellular) solution was superfused with NaCl solution (350 mM) to equilibrate the cells with extracellular solution, and \(E_{m}\) in cell-attached membrane patches was determined using Fetchan and pStat analysis programs (Axon Instruments). The pipette solution used to measure \(i_{K(V)}\) contained high (135 mM) [K\(^+\)] so that the K\(^+\) equilibrium potential (\(E_K\)) would be close to 0 mV. The current \([i_{K(V)}]\)-voltage relationship results are presented as a function of the current amplitude against the command potential \((E_{com})\) applied to the patched membrane. Because of a negative resting \(E_{m}\), the actual transmembrane potential across the patched membrane \((E_{patch})\) is equal to the difference between the \(E_{com}\) and resting \(E_{m}\) \((E_{patch} = (E_{com} - E_{m})\)). This is why the single channel current-voltage curves do not reverse at \(E_K\) (0 mV) but at potentials equal to \(-E_{m}\).

Green fluorescence emitted at 507 nm was used to visualize the cells transfected with KCNA5/GFP or pCMS-EGFP constructs using an inverted Nikon microscope (Eclipse/TI200) with the TE-FM epifluorescence attachment. The cell images were acquired with an Image Intensifier Tube/Philips 1381 system (Stanford Photonics Electronic Imaging Technologies, Palo Alto, CA).

**Single-cell RT-PCR.** To determine the mRNA expression of exogenous human KCNA5 in rat PASMC transfected with human KCNA5 gene at the single-cell level, we performed multiplex single-cell RT-PCR according to a modified protocol previously described by Comer et al. (16). Briefly, after \(i_{K(V)}\) was recorded, the whole cell was carefully aspirated into a collection pipette, which contained 12 μl of
the pipette solution supplemented with 10 μM DNTP and 0.5 U/μl
RNase inhibitor. The content in the pipette was then expelled im-
mediately into a 0.2-ml PCR tube that contained 8 μl of a solution
composed of 10 mM Tris·HCl, 50 mM KCl, 2.5 mM MgCl2, 10 mM
dithiothreitol, 1.25 mM oligo(dT), 0.5 mM dNTP, and 5 units of AMV
reverse transcriptase XL. The reverse transcription was performed
for 60 min at 42°C. First-round PCR with 45 cycles was then performed
in the same tube with the addition of 80 μl of the premix PCR buffer
containing 10 mM Tris·HCl, 50 mM KCl, 2.5 mM MgCl2, 20 nM of
each sense and antisense primer (first primers) for all the genes of
interest, and 5 units of Taq polymerase (DNA PCR kit; TaKaRa Bio,
Shiga, Japan). Aliquots (2 μl) of the first-round PCR products were
amplified by the second-round PCR with 25–30 cycles, which was
reamplified by the second-round PCR with 25–30 cycles, which was
separately carried out using fully nested gene-specific primers (nested
primers) for each target gene. Second-round PCR-amplified products
were separated on 1.5% agarose gel and visualized with GelStar gel
staining.

RAT-PCR was performed according to protocols described previously
(56). Briefly, normoxic conditions were established in

Electrophoresis Documentation System (Eastman Kodak, Rochester,
NY), were normalized to the net intensity values of the

intensity values of the PCR product bands, measured using a Kodak

polymerase (RNA PCR kit; TaKaRa Bio, Tokyo, Japan). Aliquots (2

values ranging from 140 to 149 mmHg at 24°C. Hypoxia was
established by directly dissolving 0.8 mM sodium dithionite
(Na2S2O4; Sigma), an oxygen scavenger that combines with oxygen
and decreases PO2 in solution, in the extracellular solution to achieve
a PO2 value ranging from 22 to 40 mmHg. An oxygen electrode
(Microelectrodes, Londonderry, NH) was positioned in the cell cham-
ber on the microscope stage to continuously monitor PO2. Na2S2O4
had no effect on Kv channel activity unless accompanied by a
reduction of PO2. Rigorously bubbling the Na2S2O4 (0.8 mM)-con-
taining solution with room air for 20–30 min increased PO2 to ~145
mmHg. Application of the Na2S2O4-containing normoxic solution to
KCN5-transfected cells did not affect the KCN5 currents.

**Statistical analysis.** Data are expressed as means ± SE. Statistical
analysis was performed using unpaired Student’s t-test or ANOVA.
Differences were considered to be significant when P < 0.05.

**RESULTS**

*Overexpression of KCN5 increases I(Kv) and induces mem-
brane hyperpolarization.*** To visibly recognize transfected

cells, we made a GFP-containing construct carrying the human

KCN5 gene (Fig. 1A). We first transfected KCN5 to HEK-

293 cells. KCN5 mRNA (Fig. 1Ba) and protein (Fig. 1Bb)

levels were both markedly increased in HEK-293 cells tran-
siently transfected with KCN5. The time course of KCN5

protein expression showed that the level of the channel protein
dramatically increased 15 h after initial transfection and lasted

for up to 80 h (Fig. 1B, b and c). Overexpression of KCN5

proteins in HEK-293 increased the amplitude of I(Kv) through

KCN5 channels (IKCN5) (Fig. 1C, a and b). The voltage

threshold for activating KCN5 channels was between ~40

and ~50 mV, and IKCN5 showed neither inward rectification

nor inactivation (Fig. 1C, a and b). Furthermore, the resting Em

in KCN5-transfected cells was more negative than in the

Ki.5 (KCN5) (NM_002343)

First Primers

197

195

193

198

292†

274†

244

292†

274†

244

292†

274†

244

292†

274†

244

1,510–1,519

1,627–1,691

1,038–1,057

1,451–1,470

1,595–1,614

1,883–1,902

1,932–951

1,156–1,175

1,564–1,581

1,882–1,898

1,012–1,031

740–759

994–1,013

776–795

1,242–1,261

1,417–1,436

264–283

437–456

841–861

1,018–1,038

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<td>192</td>
<td>5′-GCCAAGAAGCCCTACAGAAGA-3′</td>
<td>2q31</td>
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<td>Kvβ2.1 (KCNB2) (NM_017304)</td>
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<td>5q36</td>
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<tr>
<td>Kvβ3.1 (KCNB3) (NM_031652)</td>
<td>308</td>
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<td>10q24</td>
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<td>β-Actin (NM_031144)</td>
<td>244</td>
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**Human primers sequences**

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<tr>
<td>1,882–1,898</td>
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**Table 1. Oligonucleotide sequences of primers used for RT-PCR**

Accession numbers are from GenBank for the sequences used in designing the primers. *Primers used for the regular RT-PCR experiment shown in Fig. 1. †Primers used for the single-cell RT-PCR experiment shown in Fig. 4.
Fig. 1. Overexpression of the human KCNA5 gene in HEK-293 cells increases whole cell voltage-gated K⁺ (Kv) current (\(I_{K(V)}\)) and causes membrane hyperpolarization. A: map of the construct used to transfected KCNA5. hKCNA5, human KCNA5 gene; EGFP, enhanced green fluorescent protein. B: mRNA (a) and protein (b) expression levels of human KCNA5 channels in wild-type (WT) and HEK-293 cells transiently transfected with human KCNA5. The time course (c) of KCNA5 protein levels is shown in cells immediately before (0 h) or 6, 15, 23, 44, 68, and 72 h after initial transfection. C: representative currents (a), elicited by depolarizing the cells from a holding potential of −70 mV to a series of test potentials ranging from −60 to +60 mV in 20-mV increments, in HEK-293 cells transfected with an empty vector or KCNA5. Summarized current-voltage (I-V) relationship curves (b) and membrane potential (\(E_m\); c) are shown in cells transfected with empty vector (\(n = 12–19\)) or KCNA5 (\(n = 12–23\)). ***P < 0.001 vs. vector.

empty vector-transfected cells (Fig. 1Cc); the membrane hyperpolarization obviously resulted from the increased \(I_{K(CN A5)}\) as a result of overexpressed KCNA5 channels.

In addition to HEK-293 cells, we also were able to efficiently transfect the human KCNA5 to COS-7 cells as well as to rat PASMC and MASMC. Consistent with the results obtained from HEK-293 cells, overexpression of KCNA5 also dramatically increased \(I_{K(V)}\) (Fig. 2A) and caused membrane hyperpolarization (Fig. 2B) in COS-7, rat PASMC, and MASMC.

Inability of acute hypoxia to inhibit homomeric KCNA5 currents in HEK-293 and COS-7 cells. Functional Kv channels are either homogeneous or heterogeneous α₃β₄ tetramers. In HEK-293 and COS-7 cells transiently transfected with KCNA5, \(I_{K(V)}\) was generated mainly by K⁺ efflux through KCNA5 homotetrameric channels. Extracellular application of 4-AP, a Kv channel blocker, significantly and reversibly reduced \(I_{K(CN A5)}\) in HEK-293 and COS-7 cells (Fig. 3, Aa and Ba). However, reducing PO₂ from 154 ± 5 to 32 ± 4 mmHg had little effect on \(I_{K(CN A5)}\) in these cells (Fig. 3, Ab and Bb). These results are consistent with the report that acute hypoxia had a negligible effect on homomeric KCNA5 in other transfection systems (22). Inhibition of KCNA5 channels with 4-AP in KCNA5-transfected cells caused membrane depolarization in all cell types (data not shown), suggesting that overexpressed KCNA5 channels functionally participate in the regulation of resting \(E_m\).

Acute hypoxia selectively inhibits homomeric KCNA5 channels in rat PASMC. It is still unclear how acute hypoxia inhibits Kv channels in oxygen-sensitive cells. Hypoxia may reduce \(I_{K(V)}\) directly by inhibiting Kv channel function (via the pore-forming α- and/or the regulatory β-subunits) (22, 42, 43, 56) and/or indirectly by an intermediate produced via a specific oxygen-sensing mechanism in PASMC (3, 5, 36, 57). In addition, the regulatory Kv channel β-subunits may serve as an oxygen sensor for hypoxia-induced inhibition of Kv channel activity (17, 32). The next set of experiments was designed to examine 1) whether acute hypoxia inhibits homomeric KCNA5 channels and 2) whether the effect of acute hypoxia on \(I_{K(CN A5)}\) is selective to PASMC.

As shown in Fig. 4, transient transfection of the human KCNA5 gene into rat PASMC increased KCNA5 mRNA expression (Fig. 4A) and produced a single-channel current \(I_{K(V)}\) with a conductance of 16 ± 5 pS (\(n = 8\)) (Fig. 4B, a and b). In contrast to HEK-293 and COS-7 cells, acute hypoxia (PO₂ = 35 ± 2 mmHg) significantly reduced \(I_{K(CN A5)}\) in PASMC, and the inhibitory effect was reversible upon restoration of extracellular PO₂ to 145–150 mmHg (Fig. 4Bc). In these experiments, the whole cell KCNA5 currents (\(I_{K(CN A5)}\)) (Fig. 4Bc) and the single-channel KCNA5 currents (\(I_{K(CN A5)}\)) (Fig. 4Ba) were recorded from the same rat PASMC (transfected with human KCNA5 gene) in which the single-cell RT-PCR (Fig. 4A) was conducted to elucidate the high expression level of exogenous (human) KCNA5. The primers used in this experiment were specifically designed for amplifying the human KCNA5 transcript; the primers do not completely match those of rat KCNA5 (Fig. 4C). The sense (20 bp) and antisense (20 bp) primers contained five (25%) and four (20%) nucleotides, respectively, that did not match the corresponding sequence in rat KCNA5 (Fig. 4C, underlined). Therefore, the single-cell RT-PCR products should contain little rat KCNA5 transcripts.

Furthermore, the hypoxia-mediated decrease in \(I_{K(CN A5)}\) was selective to PASMC; acute hypoxia had no effect on \(I_{K(CN A5)}\) in human KCNA5-transfected rat MASMC (Fig. 5), although the amplitude and kinetics of the exogenously transfected KCNA5 channels were comparable in PASMC and MASMC. These results are consistent with our previous report that acute hypoxia selectively reduces native \(I_{K(V)}\) in rat PASMC but not in rat MASMC (56). The selective inhibition of native \(I_{K(V)}\) by acute hypoxia was previously demonstrated by other investigators in canine and rat PASMC and in renal arterial smooth muscle cells (3, 42, 43, 56).
In the aforementioned experiments, hypoxia was established by applying the superfusate containing 0.8 mM Na$_2$S$_2$O$_4$, which reduced PO$_2$ to 22–40 mmHg and stably maintained the low PO$_2$ in the superfusate (in a sealed beaker) for several hours. Application of the hypoxic superfusate (PO$_2$ at 22–40 mmHg) significantly reduced $I_{KCNA5}$ in rat PASMC (but not in rat MASMC, HEK-293, and COS-7 cells) transfected with human $KCNA5$ gene (Figs. 4Bc and 5B). Because we used Na$_2$S$_2$O$_4$ (0.8 mM) to reduce PO$_2$ in the superfusate, we also examined whether Na$_2$S$_2$O$_4$ per se affected KCNA5 channel activity. Constantly bubbling the 0.8 mM Na$_2$S$_2$O$_4$-containing solution with room air for 20–30 min increased the solution’s PO$_2$ to ~145 mmHg. Perfusion of the Na$_2$S$_2$O$_4$-containing normoxic solution through the cell chamber, as shown in Fig. 6A, did not change PO$_2$ in the superfusate applied to cells (which was determined using an oxygen electrode positioned closely to the cells examined). Extracellular application of the Na$_2$S$_2$O$_4$-containing normoxic solution did not alter the amplitude of $I_{KCNA5}$ in rat PASMC transfected with human $KCNA5$ (Fig. 6B). These results indicate that the inhibitory effect of the Na$_2$S$_2$O$_4$-containing hypoxic solution on $I_{KCNA5}$ is due to hypoxia; Na$_2$S$_2$O$_4$ has no effect on Kv channel activity unless accompanied by a reduction in PO$_2$.

In the aforementioned experiments, hypoxia was established by applying the superfusate containing 0.8 mM Na$_2$S$_2$O$_4$, which reduced PO$_2$ to 22–40 mmHg and stably maintained the low PO$_2$ in the superfusate (in a sealed beaker) for several hours. Application of the hypoxic superfusate (PO$_2$ at 22–40 mmHg) significantly reduced $I_{KCNA5}$ in rat PASMC (but not in rat MASMC, HEK-293, and COS-7 cells) transfected with human $KCNA5$ gene (Figs. 4Bc and 5B). Because we used Na$_2$S$_2$O$_4$ (0.8 mM) to reduce PO$_2$ in the superfusate, we also examined whether Na$_2$S$_2$O$_4$ per se affected KCNA5 channel activity. Constantly bubbling the 0.8 mM Na$_2$S$_2$O$_4$-containing solution with room air for 20–30 min increased the solution’s PO$_2$ to ~145 mmHg. Perfusion of the Na$_2$S$_2$O$_4$-containing normoxic solution through the cell chamber, as shown in Fig. 6A, did not change PO$_2$ in the superfusate applied to cells (which was determined using an oxygen electrode positioned closely to the cells examined). Extracellular application of the Na$_2$S$_2$O$_4$-containing normoxic solution did not alter the amplitude of $I_{KCNA5}$ in rat PASMC transfected with human $KCNA5$ (Fig. 6B). These results indicate that the inhibitory effect of the Na$_2$S$_2$O$_4$-containing hypoxic solution on $I_{KCNA5}$ is due to hypoxia; Na$_2$S$_2$O$_4$ has no effect on Kv channel activity unless accompanied by a reduction in PO$_2$.

**Fig. 2.** Overexpression of human $KCNA5$ in COS-7 cells, rat pulmonary artery smooth muscle cells (rPASMC), and rat mesenteric artery smooth muscle cells (rMASMC) increases $I_{K(V)}$ and causes membrane hyperpolarization. A: representative whole cell currents (left), elicited by depolarizing the cells from a holding potential of ~70 mV to a series of test potentials ranging from ~60 to ~60 mV in 20-mV increments, in COS-7 cells (a; n = 10), rPASMC (b; n = 9), and rMASMC (c; n = 9) transiently transfected with empty vector or human $KCNA5$. Summarized $I$-$V$ relationship curves are shown at right for empty vector- or $KCNA5$-transfected cells. B: summarized data showing $E_m$ in WT cells (n = 8–9) and cells transiently transfected with $KCNA5$ (n = 9–10).

**Fig. 3.** Inability of acute hypoxia to reduce the amplitude of $I_{K(V)}$ through KCNA5 channels ($I_{KCNA5}$) in HEK-293 and COS-7 cells. Representative whole cell currents (left), elicited by depolarizing the cells from a holding potential of ~70 mV to a series of potentials ranging from ~60 mV to ~60 mV in 20-mV increments, in $KCNA5$-transfected HEK-293 (A) and COS-7 cells (B) before (control, Cont), during (4-aminopyridine, 4-AP), and after (Wash) extracellular application of 3 mM 4-AP under normoxic conditions (a), as well as before (normal, Nor), during (hypoxia, Hyp) and after (recovery, Rec) PO$_2$ was reduced in the superfusate (b). Normalized conductance-voltage ($g$-$V$) relationship curves (Aa and Ab, right; averaged from multiple cells) were best fitted using the Boltzmann equation. Summarized amplitudes of currents at ~60 mV (Ab and Bb; averaged from n = 6 cells) are shown at right; no significant difference is observed between Nor and Hyp (actual $P$ values are 0.74488 and 0.48802 for Ab and Bb, respectively).
Comparable expression levels of various Kv channel β-subunits in PASMC and MASMC. Cytoplasmic auxiliary Kv channel β-subunits, with more than 45% homology to NADPH oxidase (32), have been proposed as sensors for hypoxia-mediated inhibition of Kv channels in various oxygen-sensitive cells (17, 18, 22, 30, 45). Cotransfection of Kv channel β-subunits with certain α-subunits confers hypoxia sensitivity onto some α-subunit homotetramers (17, 40). The expression level of Kv channel β-subunits is positively proportional to the vasoconstrictive response to hypoxia in small pulmonary arteries.

**Fig. 4.** Acute hypoxia reversibly reduces KCNA5 currents in rPASMC. Single-cell RT-PCR-amplified products are shown for KCNA5 and β-actin (A) and the corresponding single-channel I_{KCNAs} (Ba) in 2 rPASMC (left and right) transiently transfected with the human KCNA5 gene. The single-channel current-voltage (i-V) curves of I_{KCNAs} on cell-attached patches before breaking in (Ba) are shown in Bb. I_{KCNAs} (c), elicited by depolarization from a holding potential of −70 mV to potentials ranging from −60 to +60 mV, is shown in the same cells before (Nor, Po2 = 143–146 mmHg), during (Hyp), and after (Rec) hypoxic challenges (Po2 = 28–43 mmHg). Data are representative of 8 cells. C: alignment of the nested primer sequence (specifically designed for human KCNA5 gene) with the sequences of human (NM_002234) and rat (NM_012972) KCNA5 genes. The antisense primer sequence shown is the reverse complement of the actual antisense primer sequence (5'-TGAGGATAACCAAGACCGAG-3’, nt 994–1013) shown in Table 1. Underlined bold letters indicate the nucleotide variations in the rat KCNA5 gene sequence compared with the nested primer sequence designed for the human KCNA5 gene.

**Fig. 5.** Comparison of acute hypoxia-induced effects on I_{KCNAs} in rPASMC and rMASMC. A: identification of cells transiently transfected with human KCNA5/GFP by green fluorescence. B: representative I_{KCNAs} (left), elicited by depolarizing the cells from a holding potential of −70 mV to a series of potentials ranging from −60 mV to +80 mV in 20-mV increments, in human KCNA5-transfected rPASMC (Ba) or rMASMC (Bb) before (Nor), during (Hyp), and after (Rec) exposure to hypoxia (Po2 = 28–43 mmHg) or before (Cont), during (4-AP), and after (Wash) extracellular application of 3 mM 4-AP. Right: summarized data show current amplitudes at +60 mV (bar graphs) or I-V curves in human KCNA5-transfected rPASMC (n = 6) and rMASMC (n = 6) before, during, and after exposure to hypoxia or before (Cont, ○), during (4-AP, ●), and after (Wash, ▲) extracellular application of 4-AP. ***P < 0.001 vs. Nor and Rec (actual P values are 0.000411 and 0.7432978 for Ba and Bb, respectively).
teries and arterioles compared with large pulmonary arteries (17). To examine whether the selective inhibitory effect of hypoxia on KCNA5 channels in PASMC was related to a potentially higher expression level of Kv channel β-subunits, we compared mRNA expression levels of Kv channel β-subunits (e.g., Kvβ1.1, Kvβ2.1, and Kvβ3.1) between rat PASMC and MASMC, using primers specifically designed for rat Kv channel β-subunits, and between HEK-293 and COS-7 cells, using primers specifically designed for human Kv channel β-subunits.

As shown in Fig. 7A, rat PASMC and MASMC expressed comparable levels of rat Kvβ1.1, Kvβ2.1, and Kvβ3.1 subunits, whereas acute hypoxia reduced $I_{\text{KCNA5}}$ only in PASMC. These data indicate that the differential response of KCNA5 channels to acute hypoxia in rat PASMC and MASMC appears not to result from a high expression level of Kv channel β-subunits in PASMC. In contrast, COS-7 cells expressed higher levels of human Kvβ3.1 but lower levels of human Kvβ1.1 and Kvβ2.1 than HEK-293 cells (Fig. 7B), whereas acute hypoxia had no effect on $I_{\text{KCNA5}}$ in both COS-7 and HEK-293 cells. These data suggest that expression level of Kv channel β-subunits is probably not associated with the hypoxic sensitivity of KCNA5 homotetrameric channels in this study.

It must be emphasized that we used primers specifically designed for human KCNA5 for the RT-PCR experiments in HEK-293 and COS-7 cells.

Fig. 6. Effect of sodium dithionite (Na$_2$S$_2$O$_4$) without reduction of PO$_2$ on $I_{\text{KCNA5}}$ in rat PASMC. A: fractional O$_2$ concentration (FIO$_2$) and O$_2$ tension (PO$_2$) determined using an oxygen electrode positioned in the cell chamber, which was superfused with the Na$_2$S$_2$O$_4$-containing solution continuously bubbled with room air (Na$_2$S$_2$O$_4$ + room air) or with the Na$_2$S$_2$O$_4$-containing solution without room air bubbling (Na$_2$S$_2$O$_4$). Arrow indicates the time when the perfusion pump was turned on. B: representative $I_{\text{KCNA5}}$ (a), elicited by depolarizing the cells from a holding potential of $-70$ mV to a series of potentials ranging from $-60$ mV to $+60$ mV in 20-mV increments, in human KCNA5-transfected rPASMC before (control), during (Na$_2$S$_2$O$_4$ + room air), and after (washout) exposure to Na$_2$S$_2$O$_4$-containing normoxic solution (bubbled with room air). PO$_2$ values shown on the oxygen meter while the currents were recorded are indicated. Summarized data (b) show the I-V curves in human KCNA5-transfected rPASMC ($n=9$) before, during, and after exposure to the Na$_2$S$_2$O$_4$-containing normoxic solution (PO$_2$ = 145–146 mmHg). The $P$ value for the current amplitudes at $+80$ mV in cells superfused with control and Na$_2$S$_2$O$_4$-containing normoxic solution is 0.816699.

Fig. 7. mRNA expression levels of Kv channel β-subunits are comparable in rPASMC and rMASMC. RT-PCR-amplified products for rat (Aa) or human (Ba) Kvβ1.1, Kvβ2.1, and Kvβ3.1 in rPASMC, rMASMC, HEK-293, and COS-7 cells. M, 100-bp DNA ladder. RT-PCR-amplified products of β-actin and GAPDH are shown as controls. Summarized data show the mRNA levels of different rat (Aa; $n=15$) or human (Ba; $n=3$) Kv channel β-subunits in the cells tested. **$P<0.01$ and ***$P<0.001$ vs. HEK-293 cells. The actual $P$ values are 0.43950, 0.71439, and 0.70196 for Kvβ1.1, Kvβ2.1, and Kvβ3.1, respectively, in Aa, and 0.0927, 0.0004381, and 0.001354 for Kvβ1.1, Kvβ2.1, and Kvβ3.1, respectively, in Ba.

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Kv channel β-subunits between HEK-293 and COS-7 cells might be related to the species difference.

DISCUSSION

Acute alveolar hypoxia causes pulmonary vasoconstriction, whereas hypoxia or hypoxemia causes vasodilatation in coronary, renal, and cerebral arteries. Hypoxic pulmonary vasoconstriction is thus a unique property of the pulmonary vasculature, involving multiple mechanisms and cell types (e.g., fibroblasts, smooth muscle cells, and endothelial cells), to maintain an optimal ventilation-perfusion ratio for maximal oxygenation of the venous blood. In vitro experiments have demonstrated that acute hypoxia selectively constricts isolated pulmonary arteries in the presence (20) or absence (29, 58) of endothelial cells and contracts single PASMC (34, 59) but has little effect on isolated systemic (e.g., mesenteric and renal arteries) and single MASMC. The unique oxygen- or hypoxia-sensitive contractile system present in the pulmonary vasculature or PASMC should include at least three components: 1) an oxygen-sensing domain or complex that functions as a receptor, 2) signal transduction cascades that may involve multiple pathways or molecules, and 3) an efficient effector that leads to smooth muscle contraction.

One of the important cellular mechanisms mediating hypoxic pulmonary vasoconstriction is reduction of $I_{K(V)}$ in PASMC exposed to acute hypoxia and subsequent membrane depolarization (3, 22, 31, 35, 37, 42, 43, 56). In this scenario, Kv channels function as an effector to induce the membrane depolarization that triggers Ca$^{2+}$ influx through VDCC, increasing $[\text{Ca}^{2+}]_{\text{cyt}}$ and causing PASMC contraction and pulmonary vasoconstriction. In vitro experiments show that acute hypoxia selectively reduces native $I_{K(V)}$ in PASMC but not in systemic arterial (e.g., mesenteric, renal, and coronary) smooth muscle cells (43, 48, 56). The selectivity of hypoxia-mediated Kv inhibition in PASMC is consistent with the selectivity of hypoxia-induced contraction in PASMC and vasoconstriction in pulmonary arteries (relative to systemic vascular smooth muscle cells and systemic arteries). The question is then whether the Kv channel itself (e.g., KCNA5 channel) is directly or indirectly affected by acute hypoxia in PASMC.

The results from the present study indicate that overexpression of KCNA5, a Kv channel α-subunit ubiquitously expressed in various cell types, including pulmonary and systemic arterial smooth muscle cells, increases $I_{K(V)}$ and causes membrane hyperpolarization in HEK-293, COS-7, rat PASMC, and rat MASMC. A similar role for native KCNA5 channels in regulating resting $E_m$ has been proposed in PASMC (7, 8, 21, 22), portal vein smooth muscle cells (15, 23), and bronchial (1) cerebral artery smooth muscle cells (13). Therefore, KCNA5 is an important Kv channel α-subunit that participates in regulating resting $E_m$. Second, the KCNA5 homotetrameric channels in MASMC, HEK-293, and COS-7 cells transiently transfected with the human KCNA5 gene are not sensitive to acute hypoxia. These observations suggest that 1) KCNA5 is a critical effector for hypoxia to inhibit $I_{K(V)}$ in PASMC and to induce membrane depolarization, thereby increasing $[\text{Ca}^{2+}]_{\text{cyt}}$ and causing PASMC contraction, and 2) the oxygen-sensing mechanism(s) involved in triggering KCNA5 inhibition, which may be different from oxygen-sensing procedures involved in angiogenesis and erythropoiesis, is an intrinsic property of PASMC. Our findings are in accord with those of other groups that acute hypoxia preferentially inhibits oxygen-sensitive Kv channels, particularly KCNA5 and KCNB1, to promote hypoxic pulmonary vasoconstriction (6, 8, 21, 22, 44).

There are two schools of thought with regard to how acute hypoxia inhibits Kv channels in oxygen-sensitive cells. Hypoxia may reduce $I_{K(V)}$ directly, by inhibiting Kv channel function (e.g., via conformational changes of the pore-forming α-subunits and interaction of the regulatory β-subunits with the α-subunits) (22, 42, 43, 56), and/or 2) indirectly, by an intermediate released or synthesized via a specific oxygen-sensing complex in PASMC (e.g., oxygen radicals, oxidizing and reducing molecules, metabolic products and by-products) (3, 5, 36, 57). The regulatory Kv channel β-subunits, which have been implicated as oxygen sensors for hypoxia-induced inhibition of Kv channel activity (17, 32), could contribute to 1) directly modulating Kv channel function (e.g., gating and inactivation kinetics) by direct interaction with the α-subunits and/or 2) indirectly modulating native Kv channel function via their potential enzymatic activity, which could produce an “intermediate” Kv channel inhibitor during acute hypoxia (32, 46).

Given the fact that acute hypoxia selectively causes pulmonary vasoconstriction in vivo and selectively reduces $I_{K(V)}$ in PASMC in vitro, the potential mechanisms involved in hypoxia-mediated Kv channel inhibition have to be specific or intrinsic to PASMC or pulmonary arteries; that is, the “direct or indirect” effects of acute hypoxia must be from 1) a specific intermediate that is synthesized or activated exclusively in PASMC during acute hypoxia, 2) a motif or complex adjacent to the channel protein (or the pore-forming α-subunit) that can be turned on or off in particular in PASMC by acute hypoxia, 3) an oxygen- or hypoxia-sensitive Kv channel α- and/or β-subunit that is distinctly expressed in PASMC (relative to systemic arterial smooth muscle cells), or 4) the redox-sensit$\bar{e}$ amino acid residues (e.g., cysteine, methionine) that are explicitly present in PASMC Kv channels (α- or β-subunit) or modulated particularly by the redox status changes in PASMC but not in MASMC (25). It is unclear, however, whether the tertiary structure of Kv channels is different between PASMC and MASMC and whether Kv channel genes expressed in PASMC have somatic mutations that make the Kv channels more sensitive to hypoxia or redox modulation. Furthermore, to date, qualitative differences in mRNA and protein expression of Kv channel α- and β-subunits determined by RT-PCR and Western blot analyses have not been revealed between pulmonary and systemic arterial smooth muscle cells (e.g., MASMC). Therefore, current knowledge favors the contention that a PASMC-specific intermediate or modulator released or activated during acute hypoxia mediates the hypoxia-induced Kv channel inhibition.

It has been well documented that mitochondria serve as oxygen-sensing intracellular organelles that are responsible for many hypoxia-induced effects (11, 33, 51–53). Although it remains debatable whether mitochondrial metabolism (or metabolic inhibition) compromised by hypoxia is a cause of sensitivity of PASMC to acute hypoxia, the changes in mitochondrial production or release of reactive oxygen species have been implicated in modulating Kv channel activity in PASMC (33). In cardiac and skeletal muscle (47), as well as in the
pulmonary vasculature (10, 24, 54), mitochondrial metabolism or ATP production through oxidative phosphorylation is not markedly affected by hypoxia until the \( P_\text{O}_2 \) drops to an anoxic range (1–2 mmHg) (47), whereas hypoxic pulmonary vasoconstriction occurs at a \( P_\text{O}_2 \) range (e.g., 30–45 mmHg) that does not significantly change the mitochondrial ATP production. In contrast, inhibition of glycolysis using 2-deoxy-o-glucose selectively reduces native \( I_{K(V)} \) in PASMC but not in MASMC (57). These results suggest that metabolic inhibition exerts an inhibitory effect on Kv channel activity, but whether hypoxia-mediated inhibition of Kv channels in PASMC results from compromised mitochondrial metabolism is unclear. Nonetheless, mitochondrial production of activated oxygen radicals (e.g., superoxide) has been demonstrated to be altered during acute hypoxia (33, 53), which then leads to direct or indirect (e.g., via altered cellular redox state) inhibition of Kv channel activity.

It must be emphasized that acute hypoxia causes vasoconstriction only in pulmonary arteries but not in systemic arteries. In other words, the selective contractile effect of acute hypoxia on the pulmonary vasculature requires special attention when searching for potential mechanisms that are involved in hypoxic pulmonary vasoconstriction. Hypoxia may alter various cellular functions (e.g., those relating to angiogenesis, erythropoiesis, adaptation, and acclimatization); however, the mechanism involved in causing hypoxic pulmonary vasoconstriction should show selectivity to PASMC compared with systemic arterial smooth muscle cells.

The data from this study provide evidence that 1) a unique oxygen-sensing or hypoxia-sensitive mechanism exists exclusively in PASMC but not in MASMC, 2) KCNA5 (and other Kv channels) is an important effector Kv channel that responds to hypoxia via the PASMC-specific oxygen-sensing mechanism by causing membrane depolarization, and 3) the expression level of KCNA5 (relative to other types of Kv channel subunits) may contribute to determining the sensitivity of a PASMC to hypoxia for mediating membrane depolarization and increase in \([Ca^{2+}]_\text{cyt}\) (via enhanced activity of VDCC). Whether depletion of \( Ca^{2+} \) from the sarcoplasmic reticulum or disrupted mitochondrial function abolishes the acute hypoxia-induced decrease in \( I_{\text{K(CNA5)}} \) in PASMC remains to be elucidated. Nonetheless, we believe that, among the multiple mechanisms that underlie hypoxic pulmonary vasoconstriction, KCNA5 blockade by a PASMC-specific and oxygen-sensitive cellular process is an underlying cause of the membrane depolarization inherent to hypoxic pulmonary vasoconstriction.

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REFERENCES