BK Channels in Rat and Human Pulmonary Smooth Muscle Cells are $\alpha$-$\beta_1$ Functional Complexes Lacking the Oxygen-Sensitive STREX Insert

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ABSTRACT

A loss of K\textsuperscript{+} efflux in pulmonary arterial smooth muscle cells (PASMCs) contributes to abnormal vasoconstriction and PASMC proliferation during pulmonary hypertension (PH). Activation of high-conductance, Ca\textsuperscript{2+}-activated (BK) channels represents a therapeutic strategy to restore K\textsuperscript{+} efflux to the affected PASMCs. However, the properties of BK channels in PASMCs including sensitivity to BK channel openers (BKCO) are poorly defined. The goal of the present study was to compare the properties of BK channels between PASMCs of normoxic (N) and chronic hypoxic (CH) rats, and then explore key findings in human PASMCs. PCR results revealed that 94.3\% of transcripts encoding BK\textalpha pore proteins in PASMCs from N rats represent splice variants lacking the STREX insert that confers oxygen sensitivity. Subsequent patch-clamp recordings from inside-out (I-O) patches confirmed a dense population of BK channel insensitive to hypoxia. The BK channels were highly activated by intracellular Ca\textsuperscript{2+} and the BKCO lithocholate; these responses require BK\textalpha-\beta\textsubscript{1} subunit coupling. PASMCs of CH rats with established PH exhibited a profound over-abundance of the dominant oxygen-insensitive BK\textalpha variant. Importantly hBK channels in PASMCs from human donor lungs also represented the oxygen-insensitive BK\textalpha variant activated by BKCO. The hBK channels showed significantly enhanced Ca\textsuperscript{2+}-sensitivity compared to rat BK channels. We conclude that rat BK and hBK channels in PASMCs are oxygen-insensitive BK\textalpha-\beta\textsubscript{1} complexes highly sensitivity to Ca\textsuperscript{2+} and the BKCO lithocholate. BK channels are over-expressed in PASMCs of a rat model of PH and may provide an abundant target for BKCOs designed to restore K\textsuperscript{+} efflux.
Pulmonary hypertension (PH) is a severe, progressive disease in which small pulmonary arteries show heightened vasoconstriction, proliferation of the pulmonary arterial smooth muscle cells (PASMCs), and vascular remodeling. Ultimately the increased pulmonary vascular resistance results in right heart failure and death. One feature of PH shared between animal models and human forms of the disease is a loss of voltage-gated K\(^{+}\) (K\(_{V}\)) channels in the PASMCs, which results in membrane depolarization, voltage-dependent Ca\(^{2+}\) influx and vasoconstriction.\(^1,2\) The loss of K\(^{+}\) efflux also facilitates proliferation of PASMCs by inhibiting apoptosis.\(^3\) Thus, a recognized goal for the treatment of PH is to restore K\(^{+}\) efflux to the PASMCs. In proof-of-principle studies, Pozeg et al.\(^4\) achieved a lower pulmonary vascular resistance after using adenoviral gene therapy to transiently restore K\(_{V}\) channel expression to PASMCs of rats with chronic hypoxia (CH)-induced PH. However, a more practical approach to restore K\(^{+}\) efflux to affected PASMCs may be to pharmacologically activate those K\(^{+}\) channels that show persistent and high expression levels in PASMCs during the development of PH rather than trying to restore depleted K\(^{+}\) channel types.\(^5\)

Conceptually, the ideal K\(^{+}\) channel target in PASMCs for pharmacological activation would be: 1) densely expressed in PASMCs during PH and capable of powerful hyperpolarization; 2) available for activation under the conditions of high intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{i}\)]) and depolarization that exist in PASMCs during PH; and 3) insensitive to inhibition by the hypoxic environment that may occur during PH. Considering that K\(_{V}\) channels down-regulate in PASMCs during PH and can be inactivated by hypoxia and [Ca\(^{2+}\)\(_{i}\)],\(^6,7\) they may not be ideal pharmacological targets. In contrast, high-conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK) channels in PASMCs may represent suitable targets for K\(^{+}\) channel activators designed to ameliorate PH. BK channels exhibit a high single-channel conductance (150-250 pS), which generates a strong
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hyperpolarizing K⁺ current. They are active under the conditions of high [Ca²⁺]; and depolarization, which are inherent to PASMCs during PH.¹ Finally, several splice variants of BK channels are oxygen-insensitive and their open-state probability is unaffected by hypoxia.⁸ Unfortunately, the types of BK channel variants in PASMCs are unknown. Similarly, the biological and pharmacological properties of BK channels in PASMCs of preclinical models of PH are poorly defined, and reports disagree on whether the expression of BK channels in PASMCs increases or decreases during experimental PH.⁵,⁹ Finally, the properties of BK channels (hBK) in freshly isolated human PASMCs have not been explored to our knowledge.

Notably, the properties of BK channels in PASMCs from small pulmonary arteries cannot be predicted using findings from other vascular beds, because these properties are highly site-specific largely due to the molecular diversity of channel composition.¹⁰ Although the BK channel pore-forming structure is a tetramer of α-subunits (BKα) encoded by a single gene, alternative splicing creates multiple BKα variants that can co-assemble to form BK channels with variable Ca²⁺-sensitivity.¹¹ Additionally, only some BKα splice variants contain the stress-regulated exon (STREX), which confers oxygen-sensitivity.⁸ The STREX insert was reported to be sparsely expressed in porcine pulmonary arteries,¹² but its prevalence in most arterial beds including the rat and human pulmonary circulations is unknown. Finally, small regulatory β₁-subunits (BKβ₁) can interact in 1:1 stoichiometry with BKα subunits to enhance the Ca²⁺-sensitivity of BK channels.¹³,¹⁴ BKβ₁ subunits also confer sensitivity to certain BK channel openers (BKCOs) including lithocholate (LC), which binds to BKβ₁ subunits to activate BK channels.¹⁵ Thus, LC can be used as a pharmacologic tool to confirm the presence of functional BKβ₁ subunits in BK channel complexes. Notably, BKβ₁ subunits are reported to be functionally
deficient in PASMCs, resulting in low Ca$^{2+}$-sensitivity of pulmonary BK channels and presumed resistance to activation by BKβ$_1$–dependent BKCOs.$^{16}$

The goal of this study was to define the biological and pharmacological properties of BK channels related to their potential as pharmacological targets for PH in PASMCs freshly isolated from small rat and human pulmonary arteries. Initially we characterized BK channels in PASMCs of control rats, and then determined if these properties persisted in PASMCs from CH rats, which are a preclinical animal model used to develop new therapeutics for PH. Finally, we isolated PASMCS from small pulmonary arteries of freshly obtained human lung samples to provide initial information on the properties of human BK (hBK) channels.
METHODS

ANIMALS

Procedures using animals were performed at the University of Arkansas for Medical Sciences as approved by the Institutional Animal Care and Use Committee and in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Age 9- to 13-week-old male Sprague-Dawley rats were anesthetized with isoflurane and euthanized by decapitation. Then the lungs or brain were removed, and submerged in cold physiological saline solution (PSS) consisting of (in mmol/L) 119 NaCl, 24 NaHCO\(_3\), 5.5 glucose, 4.7 KCl, 1.6 CaCl\(_2\), 1.2 NaH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 0.03 EDTA. Arteries were gently removed from adjacent tissue. Second to fourth order intralobar pulmonary arteries with external diameters between 200 µm and 600 µm were used for patch-clamp, quantitative real-time PCR (qPCR), and western blot experiments. Middle and posterior cerebral arteries, the circle of Willis, and the basilar artery were used to obtain cerebral arterial smooth muscle cells (CASMCs) for patch-clamp studies.

Pulmonary hypertension was induced by exposing 9-week-old male Sprague-Dawley rats to 3 weeks of normobaric chronic hypoxia (CH) in BioSpherix (Lacona, NY) environmental chambers. The chambers used a computer-regulated release of nitrogen into the chamber to maintain an inspired O\(_2\) level of 10% to 11%. Immediately following the CH exposure, right ventricular systolic pressure (RVSP) was measured via catheter through the right external jugular vein under isoflurane anesthesia. Hearts were dissected to determine the ratio of the mass of the outer wall of the right ventricle to the mass of the left ventricle plus septum (RV/(LV+S)), a standard index of right ventricular hypertrophy. The CH rats developed pulmonary hypertension (PH) as indicated by an increase of RVSP and the presence of right ventricular hypertrophy after
3 weeks of CH, without a rise in systemic mean arterial pressure (MAP) or heart rate (Supplemental Figure 1). Age-matched normoxic (N) control rats were kept in normal room air (21% O₂) for the same duration of time.

HUMAN LUNG TISSUE

Transplant quality human cadaver lungs were obtained from the National Disease Research Interchange (NDRI) or Arkansas Regional Organ Recovery Agency (ARORA). Lungs were removed by 30 min after cardiac death, placed in University of Wisconsin organ preservation solution, and then shipped to our laboratory on ice. The tissues were used for experiments within 3 days (or snap-frozen in liquid nitrogen for later use in molecular analyses). The use of tissue from deceased organ donors was reviewed by the University of Arkansas for Medical Sciences Institutional Review Board and determined not to be human subjects research as defined by 45 CFR 46.102(f). The lungs were not from donors with known pulmonary hypertension. The following criteria were used in selecting samples: age 15-55; serologies suitable for transplant; no acute disease, bacterial infection or cancer; <10 pack years smoking history; and pO₂>100 mm Hg on inhaled 100% O₂. Asthma, diabetes, hypercholesterolemia, and systemic hypertension were not excluded.

After receipt of donor lungs in the laboratory, the airways were washed with phosphate-buffered saline (pH 7.4) and subsequently inflated with warm low-melting point agarose to identify small airway versus vascular structures. After the agarose had cooled and solidified, small cubes (~1 cm³) of lung tissue were cut and dissected in cold PSS to obtain small pulmonary arteries (≤1 mm diameter). These arteries were used in western blot, qPCR, and patch-clamp analyses.
PREPARATION OF SMOOTH MUSCLE CELLS FOR PATCH-CLAMP EXPERIMENTS

All arterial smooth muscle cell (ASMC) isolations used enzymes from Worthington Biochemical Corporation (Lakewood, NJ) and protocols optimized for different preparations. To obtain ASMCs for patch-clamp experiments, dissected arteries were cut into 1-mm segments, then incubated and gently shaken in a 1.5 mL microcentrifuge tube filled with Ca$^{2+}$-free Tyrode’s (CFT) solution at 37°C, which contained (in mmol/L): 143 NaCl, 5.4 KCl, 1.8 CaCl$_2$, 0.5 MgCl$_2$, 0.33 NaH$_2$PO$_4$, 16.6 glucose, and 5 HEPES, pH adjusted to 7.4 with NaOH. The digestion sequences used to isolate PASMCs from control or normoxic (N) rats included: 20 min in 1.8 mg/mL collagenase, 15 min in 0.5 mg/mL elastase, and another 10 min in 1.8 mg/mL collagenase. Isolation of PASMCs from CH rats required 20 min in 2 mg/mL collagenase, 20 min in 0.5 mg/mL collagenase, 15 min in 2 mg/mL elastase, and 10 min in 0.5 mg/mL collagenase. Lastly, in enzyme-free CFT, the arterial segments were gently triturated with a fire-polished Pasteur pipette for 1 to 2 min to release PASMCs.

To obtain hPASMCs for patch-clamp experiments, human pulmonary arteries (≤1 mm diameter) were cut into 1-mm segments and placed in warm (37°C) enzyme-containing CFT as follows: 20 min in 2 mg/mL collagenase, 20 min in 0.5 mg/mL elastase, 20 min in 2 mg/mL collagenase, and 15 min in 0.5 mg/mL collagenase. Then, in enzyme-free CFT, the arterial segments were gently triturated with a fire-polished Pasteur pipette for 1 to 2 min to release hPASMCs.

A subset of studies compared BK channel properties between rat PASMCs and CASMCs. To isolate CASMCs, rat cerebral arteries were cut into 1-mm segments and placed in warm (37°C) enzyme-containing CFT as follows: 20 min in 1 mg/mL papain, then 20 min in 0.9
mg/mL collagenase and 0.25 mg/mL elastase. Finally, in enzyme-free CFT, the arterial segments were gently triturated with a fire-polished Pasteur pipette for 1 to 2 minutes to release CASMCs. All ASMC suspensions were kept on ice and used in patch-clamp studies the same day.

PATCH-CLAMP EXPERIMENTS

Patch-clamp experiments were performed on ASMCs obtained via enzymatic digestion of freshly dissected pulmonary or cerebral arteries as described above. The inside-out (I-O) patch-clamp configuration was used to record BK channel currents in I-O membrane patches at room temperature. These I-O membrane patches were obtained by sealing cells with a 10-20 MΩ pipette, and then rapidly interfacing the pipette through the bath surface to remove the cell, followed by reintroducing the pipette into the bath. Cells were bathed in a solution that consisted of (in mmol/L): 145 KCl, 10 PIPES, 1 EGTA, 1 MgCl₂, and variable CaCl₂ (range, 10⁻⁸ to 10⁻⁴ mol/L); and was titrated to pH 7.4 using KOH. The amounts of Ca²⁺ required to achieve desired free Ca²⁺ concentrations were calculated using Maxchelator Ca-Mg-ATP-EGTA Calculator v1.0 with constants from NIST database #46 v8 (URL: http://www.stanford.edu/~cpatton/CaMgATPEGTA-NIST.htm). Pipette solution contained (in mmol/L): 145 KCl, 5 HEPES, 1.8 CaCl₂, and 1 MgCl₂; and was titrated to pH 7.4 using KOH. Different patch potentials are indicated in the figure legends. The cell-attached patch-clamp configuration also was used in one series of experiments. For this study, the composition of the bath and pipette solutions were both the same as the pipette solution used for I-O patches.

To achieve a hypoxic environment for patch-clamp studies, the bath solution was bubbled with nitrogen gas in a reservoir to remove dissolved O₂. Then the hypoxic solution was drawn from the reservoir into a glass syringe before immediate, direct infusion into the patch-clamp
chamber to superfuse the cells under study. A MI-730 O$_2$ probe (Microelectrodes, Inc., Bedford, NH) was placed near the outflow port of the patch-clamp chamber to monitor the O$_2$ concentration in the bath. The bath was re-perfused with hypoxic solution as often as necessary to maintain an hypoxic environment (pO$_2$, 26 ± 11 mm Hg; O$_2$, 3.4 ± 1.5%), which was shown earlier to inhibit K$_v$ channels.$^{17,18}$

Patch-clamp studies were performed on an Olympus IMT-2 inverted microscope (Tokyo, Japan) using an L/M-EPC7 amplifier (List Medical, Darmstadt, Germany) and a TL-1 DMA interface (Axon Instruments/Molecular Devices, Sunnyvale, CA). Data were filtered at 1 kHz using a Frequency Devices 902 Low-pass filter (Ottawa, IL) prior to digitization. Traces were recorded using Fetchex version 6. Analysis of single-channel data was performed using Clampfit 10.3.1.5 (Molecular Devices).

WESTERN BLOTTING

Protein lysates were prepared by homogenizing tissues in Thermo Scientific radio-immunoprecipitation assay (RIPA) buffer (Waltham, MA) using stainless steel beads in a Next Advance BBX24B-CE bullet blender (Averill Park, NY). Protein lysates were size-separated on an Invitrogen (Grand Island, NY) 3-8% gradient BIS-TRIS polyacrylamide mini-gel and transferred to a polyvinylidene fluoride (PVDF) membrane for blotting for two hours on ice. The membrane was blocked using 10% dry milk in tris-buffered saline containing 0.1% Tween-20. A monoclonal anti-BK$\alpha$ antibody (75-022, Neuromab; Davis, CA) was used at a dilution of 1:400, and a polyclonal anti-BK$\beta_1$ antibody (APC-036, Alomone Labs; Cambridge, England) was used at a dilution of 1:400. Beta-actin was detected with a monoclonal antibody from Sigma-Aldrich (A5441, St. Louis, MO) at a dilution of 1:5000. Horseradish peroxidase (HRP)-
conjugated secondary antibodies provided chemiluminescent signals, which were detected using X-ray film. After the blots were scanned, densitometry was performed on the images using ImageJ (http://imagej.nih.gov/ij/).

EXPRESSION OF HUMAN BK CHANNELS IN HEK293 CELLS

A plasmid bicistronically expressing a Flag tag (DYKDDDDK) fused to an isoform of human BKα subunit (GenBank U11058.2) and mCherry was a generous gift from Dr. Sarah England (Washington University). A plasmid expressing human BKβ₁ subunit (GenBank NM_004137.3; University of Texas Health Science Center at San Antonio, San Antonio, TX) was provided by Dr. Jerod Denton (Vanderbilt University). HEK293 cells (ATCC) were transfected using the Ca²⁺ phosphate method with 0.2 μg of hBKα plasmid and 0.8 μg of hBKβ₁ plasmid (α+β₁), or with 0.8 μg of negative control plasmid without the hBKβ₁ gene (BKα only). Briefly, HEK293 cells were plated on 35 mm culture dishes in 10% FBS DMEM one day before transfection. A total of 1 μg of DNA plasmid was incubated for 1 min with 100 μL of CaCl₂ and 100 μL of 2x HEPES-buffered solution before the solution was applied to ~50% confluent dishes. Patch-clamp experiments were carried out 24 to 48 hours after transfection. We routinely obtained >80% positive transfection confirmed by red fluorescence.

QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR)

Rat or human pulmonary arteries were snap frozen in liquid nitrogen and pulverized for RNA isolation. Total RNA was isolated using the Qiagen RNeasy Mini Kit (Venlo, Netherlands) according to manufacturer’s instructions. Total RNA was treated with RNase-Free DNase (Qiagen) to remove contaminating DNA. To generate cDNA, 500 ng of total RNA was reverse
transcribed using the Bio-Rad iScript cDNA synthesis kit (Hercules, CA). Separate primer sets for rat (Forward: GAGTCAACATCCATCATC; Reverse: TGTGTCAGGGTCATCATCATC) and human (Forward: GTACGCCATTTAAGTCGGGCT; Reverse: TGCAAGACTCCGATGCTGTC) samples were designed against cDNA sequences corresponding to regions common to all recognized rat or human BKα splice variants, respectively, to detect total BKα transcript. Two additional primer sets, again separately for rat (Forward: TTCATCTACAAGAGAATGAGCCGAGC; Reverse: CACGGAAACTGGTGAGCAATCAT) and human samples (Forward: ACGTGGACACCCTTGAGGAGA; Reverse: TAACAAGGGTCTGACCTCATC), were designed against a region of the cDNA sequence of the rat or human stress-regulated exon (STREX), respectively, to detect the STREX-containing BKα splice variant. Amplification was accomplished using quantitative real-time polymerase chain reaction (qPCR) with iQ SYBR Green Supermix (Bio-Rad) using a CFX96 Touch qPCR detection system (Bio-Rad). Primer sets for rat β-actin (Forward: ATCCTGTGGGATTTCCATGAAACTAC; Reverse: AGGAGCCAGGGCAGTAATCTC), and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Forward: AGGGCTGCTTTTAACTCTGGT; Reverse: CCCCCACTTGATTTTGAGGGA), were used as amplification controls for rat and human samples, respectively. Amplification reactions containing all components except cDNA template were used as negative controls. The relative abundances of human total BKα and human BKα STREX-containing transcripts were estimated by the ΔΔCt method and reported as percentage of total BKα expression. For rat samples, commercially synthesized DNA fragments (GenScript; Piscataway, NJ) containing either rat STREX-containing BKα or rat STREX-lacking BKα were amplified in parallel to quantify absolute transcript copy numbers.
STATISTICAL ANALYSIS

Results are presented as mean ± standard error of the mean. Comparisons between two groups were made using unpaired Student’s t-tests unless noted otherwise. Experiments with more than two groups were analyzed using ANOVA followed by a post-hoc Tukey test; p < 0.05 was regarded as significant. Two levels of statistical significance were used. One asterisk indicates that p ≤ 0.05, and two asterisks indicates that p ≤ 0.01. Data from patch-clamp studies in which the open-state probability (NP₀) of BK channels is plotted as a function of LC concentration exhibited a log-normal distribution; therefore, statistics were performed on the base 10 logarithms of the values, and data are presented on a logarithmic scale. Data generated from patch-clamp studies in which the open-state probability (NP₀) of BK channels was plotted as a function of Ca²⁺ concentration were fitted with variable slope, four parameter sigmoids using GraphPad Prism (GraphPad; La Jolla, CA).
RESULTS

High Ca\(^{2+}\)-sensitivity of BK channels in rat PASMCs infers functional BK\(\alpha\)-\(\beta_1\) complexes. The ability of BK channels to generate a strong hyperpolarizing current depends on their abundance and the degree of functional coupling between BK\(\alpha\) and BK\(\beta_1\) subunits, since BK\(\beta_1\) increases the apparent voltage- and Ca\(^{2+}\)- sensitivity of the channel. In protein lysates from 3\(^{rd}\) and 4\(^{th}\) order rat pulmonary arteries (PA), immunoreactive bands corresponding to the BK\(\alpha\) and \(\beta_1\) subunits were detected using monoclonal anti-BK\(\alpha\) and polyclonal anti-BK\(\beta_1\) antibodies, respectively (Fig. 1A, right lane). The antibodies were verified for target fidelity using protein lysates from HEK293 cells expressing either the human BK\(\alpha\) or the BK\(\beta_1\) subunit (Fig. 1A, left lanes). Notably, rat and human BK channels share the anti-BK\(\alpha\) and anti- BK\(\beta_1\) epitopes. The apparent size of the BK\(\beta_1\) subunit in HEK293 cells is slightly smaller (24 kD) than in rat PA (≥27 kD), which may reflect differences in post-translational modification of BK\(\beta_1\). Meera et al.\(^{20}\) have reported N-glycosylation of the BK\(\beta_1\) subunit in Xenopus laevis oocytes, which results in an apparent 4-kD increase in molecular mass on polyacrylamide gel electrophoresis. Notably, in I-O patches of rat PASMCs, BK channels exhibited a single-channel conductance of 225 ± 4 picosiemens (pS), in agreement with earlier reports.\(^{21,22}\) This value was determined by fitting a linear trend-line to unitary BK current amplitudes obtained at patch potentials between -80 mV and +80 mV (Fig. 1B; n = 4-13).

The Ca\(^{2+}\)-sensitivity of BK channels in different vascular beds is highly variable, and positively correlates to the ratio of BK\(\beta_1\) to BK\(\alpha\) subunits.\(^{21}\) Importantly, BK channels in rat PASMCs were reported to exhibit low Ca\(^{2+}\)-sensitivity possibly related to deficient BK\(\beta_1\).\(^{16}\) However, we only observed BK channels highly sensitive to [Ca\(^{2+}\)]\(_i\) in I-O patches of rat PASMCs. BK channel open-state probability (NP\(_O\)) sharply increased when [Ca\(^{2+}\)]\(_i\) was elevated
from $10^{-6}$ to $10^{-5.5}$ mol/L (Fig. 1D), resulting in a steep Ca$^{2+}$-activation curve (Fig. 1C, solid circles). This curve is nearly identical to that reported for cloned BKα-β1 channels. In contrast, the Ca$^{2+}$-activation curve for BK channels devoid of BKβ1 exhibits a markedly diminished slope and rightward shift. Furthermore, we also compared Ca$^{2+}$-activation curves between BK channels from rat PASMCs and rat CASMCs. The latter BK channels of rat cerebral arteries are known to exhibit high Ca$^{2+}$-sensitivity, a finding we verified in CASMCs by observing their marked activation by stepwise [Ca$^{2+}$]i elevations from $10^{-6}$ to $10^{-5.5}$ mol/L (Fig. 1E). The resulting steep Ca$^{2+}$-activation curve of CASMC BK channels was nearly identical to that of BK channels in PASMCs (Fig. 1C, n = 4-8). Half-maximal Ca$^{2+}$-activation values were -5.90 ± 0.02 log mol/L (PASMCs) and -5.90 ± 0.01 log mol/L (CASMCs), suggesting similarly high Ca$^{2+}$-sensitivities of BKα-β1 channel complexes in both vascular beds.

**Rat PASMCs primarily express oxygen-insensitive STREX$^{-}$ BKα variants.** It is unclear whether BK channels in PASMCs are inactivated by hypoxia, which potentially would render them unavailable to BKCO therapy in some forms of PH characterized by hypoxemia. BKα splice variants that include a 59–amino acid STREX insert in the intracellular carboxy terminus (Fig. 2A) are inhibited by hypoxia, as originally described in mouse anterior pituitary cells. We evaluated the relative percent of STREX insert-containing (STREX$^{+}$) or lacking (STREX$^{-}$) BKα variants in cDNA prepared from rat 3rd to 5th order PA. Using primers designed to amplify the STREX insert, real-time PCR revealed that only 5.7 ± 0.1% of BKα transcripts are STREX$^{+}$ in rat PA, whereas 94.3 ± 0.1% are the STREX$^{-}$ hypoxia-insensitive variant (Fig. 2B; n=7). The functional correlate of this observation was confirmed directly in patch-clamp studies by recording BK channel currents in I-O patches of PASMC in room air (21% O$_2$) and then during hypoxia (3.4 ± 1.5% O$_2$), which was achieved by perfusing the patch-clamp chamber with
recording solution heavily bubbled with nitrogen gas. The NP₀ of BK channels was not significantly different between normoxia and hypoxia, confirming the predominance of a STREX⁻ BK channel variant insensitive to acute hypoxia in rat PASMCs (Figs. 2C, 2D; n = 7). We also exposed BK channels in cell-attached (C-A) patches of rat PASMCs to acute hypoxia in a context in which the cytosolic milieu is preserved. Hypoxia still had no effect on BK channel NP₀ (n = 3) (Supplemental Fig. 2).

**BK channels in rat PASMCs are activated by lithocholate.** The high Ca²⁺-sensitivity of BK channels in rat PASMCs (Fig. 1C) suggests a functionally coupled BKα-β₁ complex. Importantly, this functional coupling is required for channel activation by several classes of BKCOs, including lithocholate (LC), an activator of BK channels that elicits activation via a known binding site on the second transmembrane domain of the BKβ₁ subunit.¹⁵ Lithocholate has been shown to selectively activate β₁-coupled BK channels at concentrations of 15 to 300 μmol/L.²⁴ Here, we used HEK293 cells to express a STREX⁻ BKα variant alone or with BKβ₁, in order to verify that LC activation of BK channels requires both subunits. HEK293 cells co-expressing cloned human BKα and β₁ subunits exhibited a 20.7-fold increase in NP₀ in response to 45 μmol/L LC, whereas cells lacking BKβ₁ subunits showed no change in NP₀ (Figs. 3A, 3B, 3E). This finding confirmed earlier reports that cloned BKβ₁ subunits are required for LC-induced activation of the BK channel.¹⁵, ²⁵ Notably, our patch-clamp studies in HEK293 cells used a lower Ca²⁺ concentration in the bath (10⁻⁶.¹⁷ mol/L) to reduce the high number of cloned BK channels in I-O membrane patches. Thus, the NP₀ response to LC cannot be compared quantitatively between BK channels in HEK293 cells and PASMCs.

To confirm the sensitivity of pulmonary BK channels to LC, I-O patches from rat PASMCs were exposed to LC in bath solution containing 10⁻⁶ mol/L bath Ca²⁺. Addition of 45
µmol/L LC elicited a 19 ± 6-fold increase in NP₀ (Figs. 3C, 3F; n = 11), confirming a functionally coupled BKβ₁ subunit. Lower LC concentrations of 5 and 15 µmol/L resulted in lesser 5 ± 2 and 8 ± 3-fold increases in NP₀, respectively (Fig. 3F; n = 7, 9). The use of higher concentrations (>45 µmol/L) of LC was limited by low drug solubility. The solvent for LC, 0.1% DMSO (Control), did not significantly change the NP₀ of BK channels in PASMCs (Fig. 3F; n = 9) and LC did not alter the pH of the bath solution (data not shown).

We also verified that hypoxia does not prevent LC-induced activation of BK channels in rat PASMCs. Although the vast majority of BK channels in rat PASMCs appear to be STREX-oxygen-insensitive splice variants (Fig. 2), we considered the possibility that hypoxia could disrupt the binding of LC to the BKβ₁ subunit, or alternatively disrupt distal signaling pathways required for BK channel activation. However, the addition of LC (45 µmol/L) to the bath solution caused a pronounced 25 ± 13-fold increase in NP₀ despite the presence of low pO₂ (Figures 3D, 3G; n = 9), showing persistent sensitivity of BK channels to LC under hypoxic conditions. Collectively, these data suggest that BKβ₁-specific pharmacological activators offer the potential to activate the BK channel variant in PASMCs regardless of the presence of hypoxia.

**PASMCs of CH rats express high numbers of BK channels exhibiting normal properties.** The ability of BKCOs to restore K⁺ efflux to PASMCs during PH will rely on the persistent expression of BK channels as the therapeutic target. Thus, our next series of studies evaluated whether BK channels in PASMCs retain normal expression and properties during the development of PH in rats exposed to chronic hypoxia (CH) for 3 weeks. Age-matched rats exposed to a similar duration of normoxia (N) were used as control (separate from the untreated rats used in Figs. 1, 2, and 3). After introduction to N or CH for three weeks, systolic right
ventricular pressures were 22 ± 1 mm Hg (n = 7) and 51 ± 7 mm Hg (n = 4), respectively, similar to findings of other studies. Right ventricular hypertrophy assessed as the ratio of right ventricle/ left ventricle+septum weight was evident in CH rats (0.400 ± 0.071, n = 21) compared to N rats (0.252 ± 0.005, n = 18) (Supplemental Fig. 1B). Systemic mean arterial pressure (MAP) and heart rate were similar between rat groups (Supplemental Figs. 1C, 1D).

After animals were maintained in N or CH for 3 weeks, intralobular PA (2nd to 4th order, 200 - 600 μm diameter) were dissected from isolated lungs and the expression level of the BKα subunit was analyzed by Western blot (Fig. 4A). Each lane was loaded with protein lysate from PA of a single N or CH rat. This analysis revealed that the abundance of BKα subunits was profoundly increased in PA of CH compared to N rats, corresponding to a 3.2 ± 0.5 -fold increase in immunoreactivity (Fig. 4B; n = 5). Real-time PCR using cDNA prepared from rat intralobar PA, and using primers recognizing rat cDNA sequence shared by BKα splice variants, disclosed a corresponding 1.8 ± 0.2–fold increase in total BKα transcript in PA of CH compared to N rats (Fig. 4C; n = 6-7). Interestingly, only PA of CH rats showed an increased expression of BKα protein; mesenteric (MA) and femoral (FA) arteries from the same animals failed to show this abnormality in protein lysates pooled from three N or CH rats (Fig. 4D). Western blots also were performed to compare expression of the BKβ1 subunit using the same pooled PA lysate. Immunoreactivity corresponding to anti-BKβ1 was not significantly different between PA of N and CH rats (Fig. 4E; n=6) despite a marked increase in anti-BKα immunosignal (Fig. 4A), suggesting an increased abundance of BKα pore proteins in PA of CH rats compared to accessory BKβ1 proteins.

To resolve the question of whether BK channels in CH rats retain normal properties after the development of PH, we compared unitary conductance, hypoxia-, Ca2+-, and LC- sensitivity
of BK channels between I-O patches from PASMCs of N and CH rats. Briefly, we detected BK channels of apparently similar phenotype in PA of N and CH rats, and the properties of high Ca\textsuperscript{2+}-sensitivity, insensitivity to hypoxia, and activation by LC were indistinguishable (Fig. 5). For example, the unitary conductance of BK channels in PASMCs of CH rats was determined to be 246 ± 4 pS, which was not significantly different than the value of 225 ± 4 pS in N rats (Fig. 5A; n = 6-12, data for N rats plotted from Fig. 1B). Although BK\textalpha subunits were more abundant in PA of CH rats (Figs. 4A, 4B), the hypoxia-insensitive STREX- variant persisted as the predominant isoform, representing 93.7 ± 0.4 % (n = 6) of total BK\textalpha transcripts in PA of CH rats compared to 94.3 ± 0.1 % in N rats (Fig. 5C; n = 6, data for N rats plotted from Fig. 2B). Accordingly, BK channels in I-O patches from PASMCs of CH rats were insensitive to acute hypoxia, and failed to alter NP\textsubscript{o} in response to depletion of bath pO\textsubscript{2} (Fig. 5D; n = 11).

Surprisingly, considering that expression of BK\textalpha proteins increased in PA of CH rats independently of the BK\textbeta\textsubscript{1} protein that confers channel sensitivity to [Ca\textsuperscript{2+}]\textsubscript{i}, Ca\textsuperscript{2+}-activation curves revealed a Ca\textsuperscript{2+} \textit{EC}_{50} value of -5.93 ± .01 \textit{log} mol/L for BK channels in PASMC from CH rats, which was nearly identical to the value of -5.90 ± 0.02 \textit{log} mol/L obtained in PASMCs of N rats (Fig. 5B; n = 5-12 per group, N rat data plotted from Fig. 1C). Similarly, the NP\textsubscript{o} of BK channels in PASMCs of CH rats markedly increased by 26 ± 16-fold in response to 45 μmol/L LC, the BK\textbeta\textsubscript{1} dependent BKCO (Figs. 5E, 5F; n = 9). Collectively, these data imply that BK channels are highly upregulated in PASMCs from a CH rat model of PH, and these channels retain normal properties including sensitivity to BK\textbeta\textsubscript{1}-dependent BKCOs.

\textit{hBK} channels in human PASMCs show higher Ca\textsuperscript{2+}-sensitivity and are activated by BKCO. In a final series of studies designed to ensure the relevance of our findings in rat PASMCs to \textit{hBK} channels in human PASMCs, we performed key experiments using 500-1000
μm diameter PA from 12 human lung samples. Western blots detected the BKα and BKβ1 subunits in protein lysates from human PA (Fig. 6A). The multiple bands in the anti-BKβ1 blot may represent multiple post-translational modifications of the BKβ1 subunit or non-specific immunoreactivity, although these bands were not observed in Western blots of cloned human BKα subunits or rat BKα subunits (Figs. 1A and 4E, respectively). Patch-clamp analysis of hBK channel currents in I-O patches from human PASMCs revealed a standard unitary conductance of 222 ± 5 pS (Fig. 6B; n = 3-4). PCR analysis revealed that the hypoxia-insensitive STREX− variant accounted for 99.5 ± 0.1% of BKα transcript expressed in human PA (Fig. 6D; n=3), implying a homogenous population of STREX− BK channels resistant to hypoxia. This finding concurred with our next observation that BK channel NP_o recorded in I-O patches from freshly isolated human PAMSCs was not significantly altered when patches were exposed to hypoxic bath solutions (Figs. 6E, 6G; n = 5). However, the Ca^{2+}-activation curve for hBK channels exhibited a consistent leftward shift compared to that of BK channels from rat PASMCs, resulting in an EC_{50} value of -6.06 ± 0.01 log mol/L (n = 2-5 per point) compared to BK channels in PASMCs from N rats, which exhibited an EC_{50} value of -5.90 ± 0.02 log mol/L (Fig. 6C, N rat data plotted from Fig. 1C). Finally, hBK channels also exhibited high sensitivity to LC, showing a 141 ± 31 -fold increase in NP_O in response to 45 µmol/l LC (Figs. 6F, 6H; n = 4, 8). Due to the elevated Ca^{2+} -sensitivity of hBK channels, this experiment was performed under conditions designed to reduce basal channel activity in order to accurately quantitate LC-induced increases in NP_o and identify multiple stacked openings. Accordingly, patch potential was held at +40 mV to increase single-channel amplitude, but bath [Ca^{2+}] was reduced 100-fold to 10^{-8} mol/L (instead of 10^{-6} mol/L used for rat PASMCs). Thus, the magnitude of NP_o increase in response to LC cannot be directly compared to that of N or CH rats.
DISCUSSION

The biological and pharmacological properties of BK channels vary between smooth muscle cells of different vascular beds, and BK channels in small PA are understudied. Here, we provide new findings to demonstrate that: i) the BK channel population in rat PASMCs is composed primarily of hypoxia-insensitive STREX⁻ splice variants; ii) BKα subunits in rat PASMCs are functionally coupled to BKβ₁ subunits to form channel complexes that are highly Ca²⁺-sensitive and activated by LC, a BKβ₁-dependent BKCO; iii) PA of CH rats with established PH show an increased abundance of BKα but not BKβ₁ subunits; yet these channels appear phenotypically normal; and iv) BKα subunits in human PASMCs also are STREX⁻ splice variants highly coupled to BKβ₁; these hBK channels show higher Ca²⁺-sensitivity than rat BK channels and are activated by LC. Collectively, these results imply that the properties of BK channels in PASMCs are consistent with those desired for a pharmacological target to increase K⁺ efflux in PASMCs, an ionic flux that is compromised during the development of PH.

The oxygen-sensitivity of BK channels varies between cell types, and serves a variety of important functions. For example, BK channels of carotid body chemoreceptor cells, which are essential for the maintenance of systemic O₂ homeostasis, are highly oxygen–sensitive. A splice insert, STREX, confers intrinsic oxygen-sensitivity to the pore-forming BKα subunit independently of auxiliary channel subunits or cytosolic factors. A recent study detected STREX⁺ BKα transcripts in porcine PA, but their abundance was not defined. Our findings suggest that STREX⁺ BKα variants are rare in rat and human PASMCs, and the vast majority of pulmonary BKα transcripts represent oxygen-insensitive STREX⁻ variants. Accordingly, the NP₀ of BK channels in isolated patches of rat and human PASMCs was unaffected by acute hypoxia,
a property that may confer an important advantage for therapeutic strategies relying on the pharmacological opening of BK channels for the treatment of PH.

Our finding that BKα subunits in rat PASMCs are functionally coupled to BKβ₁ subunits to form highly Ca²⁺-sensitive BK channels contrasts with results of an earlier report.¹⁶ These authors suggested that BKα:BKβ₁ coupling and Ca²⁺-sensitivity is lower in rat PASMCs compared to CASMCs. However, their analysis only compared channel NPₐ at three concentrations of [Ca²⁺]ᵢ without establishing a concentration-response curve to calculate EC₅₀ values; additionally, BKα:BKβ₁ coupling was not evaluated using BKβ₁–selective openers. In contrast, we observed that the high Ca²⁺-sensitivity of BK channels in rat PASMCs is nearly identical to BK channels formed by cloned BKα and BKβ₁ proteins in xenopus oocytes.²³ Notably, the Ca²⁺-sensitivity of BK channels varies between different circulatory beds. It is low in BK channels of hamster and rat skeletal muscle arterioles, rendering the channels inactive under resting conditions.²¹,²² In contrast, BK channels in rat CASMCs exhibit high Ca²⁺-sensitivity, which is linked to higher expression of the BKβ₁ subunit.²¹ Thus, our finding of high Ca²⁺-sensitivity of BK channels in rat PASMCs argues for functionally coupled BKα-BKβ₁ complexes.

We also observed that BK channels in rat PASMCs are activated by LC, a BKβ₁-dependent BKCO. Dopico et al.²⁸ initially reported LC-elicited activation of BK channels in rabbit PASMCs in a panel of preparations. However, although LC is useful as a pharmacological tool to identify the presence of functional BKβ₁ subunits, is not suitable for in vivo use. Off-target effects include the release of intracellular Ca²⁺ and hepatotoxicity.²⁹,³⁰ The Ca²⁺-releasing action of LC may confound vascular reactivity assays designed to evaluate its vasodilator effect.³¹ Tamoxifen, another compound with BKCO properties, initially was thought to activate
BK channels by binding to BK\(\beta_1\) subunits;\(^{32}\) however, later findings suggest a more complex interaction.\(^{33}\) To our knowledge, there are no BK\(\beta_1\)-selective BKCOs suitable for \textit{in vivo} use. However, the availability of other BK\(\beta_1\)-dependent BKCOs including dehydrosoyasaponin-1 and \(\beta\)-estradiol suggests the feasibility of targeting BK\(\beta_1\) subunits to activate vascular BK channels.\(^{34,32}\) This strategy may offer a distinct advantage due to the tissue-specific expression of BK\(\beta_1\) in smooth muscle cells. Accordingly, LC activates BK channels by binding to BK\(\beta_1\) subunits in smooth muscle cells, but does not bind to other BK\(\beta\) subunits (\textit{i.e.}, \(\beta_2\), \(\beta_3\), \(\beta_4\)) that compose BK channels in other tissues, potentially minimizing side effects.\(^{25}\)

Our studies also address the uncertain fate of BK channels in PASMCs during CH-induced PH. Resnik \textit{et al.}\(^5\) reported an increased expression of BK\(\alpha\) subunits in whole-lung protein lysates of Sprague-Dawley rats exposed to hypobaric chronic hypoxia for 3 weeks. However, Bonnet \textit{et al.}\(^9\) subsequently reported reduced BK\(\alpha\) expression in intralobar arteries of Wistar rats exposed to hypobaric hypoxic conditions. Our results clearly indicate that BK\(\alpha\) transcript and protein markedly increase in intralobar arteries of Sprague-Dawley rats exposed to CH for three weeks. The reason for the discrepancy in findings between laboratories is not readily apparent, but may relate to different rats strains, differential effects of normobaric hypoxia (our study) vs hypobaric hypoxia\(^9\), or other unrecognized factors. Notably, in our studies, BK\(\beta_1\) expression in PA of CH rats was normal despite a concomitant increase in BK\(\alpha\) expression, a finding that implies a relative deficit of BK\(\beta_1\) subunits. Thus, we anticipated reduced BK channel sensitivity to \([\text{Ca}^{2+}]_i\) and LC in PASMCs from CH rats. Instead, BK channels properties in PASMCs of N and CH rats were indistinguishable, suggesting that BK\(\beta_1\) subunits may be expressed in excess in PASMC of N rats. Collectively, our data provide initial evidence that PA of CH rats express an over-abundance of apparently normal BK channels that
are sensitive to BKCOs, suggesting they represent credible pharmacological targets to restore K\(^+\) efflux to PASMCs during PH.

Earlier studies of \(h\)BK channels have been limited to cultured human PASMCs,\(^{35}\) whereas our final experiments explored BK channel properties in freshly isolated human PASMCs. Importantly, \(h\)BK channels appear to represent STREX\(^-\) splice variants insensitive to acute hypoxia, implying they would be available for activation during hypoxemia, a feature of some forms of PH. Similar to rat, \(h\)BK channels also appear to be functionally coupled BK\(\alpha\)-BK\(\beta_1\) complexes sensitive to the BKCO, LC. However, \(h\)BK channels exhibit higher Ca\(^{2+}\)-sensitivity than their rat counterparts. This finding raises the possibilities that the \(h\)BK\(\alpha\) isoform may be distinct from rat, or alternatively regulated differently by Ca\(^{2+}\)-sensitizing mechanisms, concepts that deserve further attention.

Our study has several limitations. First, we could not use the whole-cell patch-clamp technique to confirm that the upregulation of BK channels in PA of CH rats was associated with increased BK current density at the whole-cell level, although we attempted these studies. In the whole-cell configuration, we observed overwhelming KV channel current in PASMCs even under conditions that elicit abundant BK channel current in other cells, a previously reported phenomenon.\(^{36}\) For this reason, we focused on single-channel recordings to define BK channel properties without interference from other K\(^+\) channels. Another potential limitation involved the use of human donor tissue lacking detailed patient health histories, which implies that patient age, gender, genetic background and disease processes may have influenced our results. Nevertheless, \(h\)BK channel properties including Ca\(^{2+}\)-sensitivity were consistent between PASMCs from different lung samples.
Human forms of PH show diverse clinical presentations with multiple genetic and environmental factors influencing the disease process. \(^{37}\) Medications for PH have been introduced and refined to optimize patient outcomes, but resistance to vasodilator drugs remains a key challenge, largely related to structural remodeling of the pulmonary vasculature. \(^{38, 39}\) A BKCO, NS1619, was recently reported to ameliorate monocrotaline-induced pulmonary hypertension in rats, \(^{40}\) which is a promising result, but unfortunately NS1619 also exhibits several off-target effects including inhibition of L-type voltage-gated Ca\(^{2+}\) channels, \(^{41}\) making it difficult to credit the BK channel alone for the therapeutic effect. Earlier investigations have demonstrated that sarcolemmal K\(^+\) efflux in PASMCs establishes a negative membrane potential, which inactivates voltage-dependent Ca\(^{2+}\) channels to lower pulmonary vascular tone. \(^{42}\) Sarcolemmal K\(^+\) efflux also inhibits proliferation of PASMCs by inducing apoptotic signaling pathways. \(^3\) Our new findings show that human PASMCs express hypoxia-insensitive hBK channels readily activated by BK\(\beta_1\) subunit-dependent BKCOs, implicating hBK channels as receptive targets for increasing K\(^+\) efflux in human PASMCs. Collectively, our findings combined with earlier discoveries by other laboratories may encourage the development of new BKCOs with favorable solubility, pharmacokinetic and hemodynamic profiles optimized for the treatment of PH.
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FIGURE LEGENDS

Figure 1. Western blot of PA lysate (third lane) from a normoxic rat reveals BKα and BKβ₁ subunits. To confirm the specificity of antibodies, the first and second lanes contain lysates from HEK cells expressing either the BKα or BKβ₁ subunit, respectively (A). BK current in an I-O patch of a PASMC from a normoxic rat during a 800-ms voltage ramp from -80 to +80 mV reveals a unitary conductance of 225 ± 4 pS (n = 5-11 per point) (B). Exposure to a range of Ca²⁺ concentrations reveals similar Ca²⁺-sensitivities between BK channels in I-O patches from PASMCs and CASMCs clamped at +20 mV (n = 4-7 per point) (C). Representative K⁺ current recordings reveal increasing BK channel opening in an I-O patches of rat PASMC (D) or CASMC (E) exposed to elevations of [Ca²⁺]i at a patch potential of +20 mV.

Figure 2. The alternatively spliced STREX sequence located between the S8 and S9 regions of the BKα subunit confers intrinsic oxygen-sensitivity. Inset depicts the assembled BK channel complex (A). The relative abundance of BKα transcript containing the STREX sequence in rat PA was 5.7 ± 0.1% as determined by real-time qPCR (n = 7) (B). The open-state probability (NP₀) of BK channels in I-O patches of PASMCs from normoxic rats failed to respond to hypoxic bath solution (n = 7) (C). A sample recording demonstrates the lack of effect of hypoxic bath solution on BK channel current (patch potential was -40 mV; [Ca²⁺]i was 10⁻⁶ mol/L) (D).

Figure 3. Exposure to lithocholate (LC) fails to increase BK channel opening in I-O patches of HEK293 cells expressing cloned BKα subunits only (A), but LC activates BK channels in HEK293 cells co-expressing cloned BKα and BKβ₁ subunits (B). LC (45 µmol/L) activates BK channels in I-O patches of rat PASMCs exposed to normoxic (C) or hypoxic (D) bath solutions, confirming the presence of a functionally coupled BKβ₁ subunit (n = 9-12; patch potential was...
+20 mV; \([\text{Ca}^{2+}]_i\) was 10\(^{-6}\) mol/L). Bar graph shows average change in NPo of cloned BK\(\alpha\) or BK\(\alpha\)-\(\beta\) channels exposed to LC in I-O patches of HEK293 cells (E). Bar graph depicts average increase in NPo of BK channels in I-O patches of PASMC in response to three concentrations of LC (n = 4-5; patch potential was +20 mV; \([\text{Ca}^{2+}]_i\) was 10\(^{-6.17}\) mol/L) (F). LC activates BK channels in I-O patches of rat PASMCs in the presence of hypoxia (G).

**Figure 4.** Western blot reveals accentuated immunoreactive bands corresponding to the BK\(\alpha\) pore protein in PA lysate from chronic hypoxic (CH) compared to normoxic (N) rats (n = 5 each). The dotted lines on the blot images mark the location of two deleted lanes which were determined to be statistical outliers by the Grubbs’ outlier test (p < 0.05) (A). Bar graph derived from data shown in (A) depicts the average 3.2 ± 0.5 -fold increase in BK\(\alpha\) protein expression in PA lysate of CH compared to N rats (B). Real-time RT-PCR reveals a 1.8 ± 0.2-fold increase in BK\(\alpha\) transcript expression in PA from CH compared to N rats (n = 6-7) (C). Western blots using protein lysate from pulmonary (PA), mesenteric (MA) or femoral (FA) arteries of N and CH rats indicate that only PA respond to chronic hypoxia by increasing BK\(\alpha\) abundance (each lane was loaded with lysate pooled from 3 rats) (D). Western blot suggests a similar expression level of BK\(\beta_1\) subunit protein between PA lysate from CH and N rats, as confirmed by averaged values from 6 experiments using different lysate preparations (E).

**Figure 5.** Summarized data from I-O patch recordings of PASMCs reveal a unitary BK channel conductance of 246 ± 4 pS in PASMCs of CH rats (n = 5-12 per point), which was nearly identical to the conductance of 225 ± 4 pS measured in PASMCs of N rats (A). BK channels in inside-out patches of PASMCs exhibit similar \(\text{Ca}^{2+}\)-sensitivity between CH and N rats (n = 4-7...
per point) \( (B) \). Quantitative real-time PCR reveals that the abundance of STREX-containing BKα transcript remains low \((6.3 \pm 0.4\% )\) in PA of CH rats \( (n = 6) \) \( (C) \). Exposure to acute hypoxia \((3.4 \pm 1.5\% \text{ O}_2\) of BK channels in I-O patches of PASMCs from CH rats does not significantly alter NP\(_0\) \( (n = 11) \) \( (D) \). BK channels in I-O patches of PASMCs from CH rats are activated by LC \((45 \mu\text{mol/L})\) \( (E) \), as confirmed by a bar graph depicting averaged values \( (n = 9) \) \( (F) \).

**Figure 6.** Western blot reveals BKα and BKβ\(_1\) subunits in protein lysates from human PA \( (A) \). Unitary \( h \text{BK} \) channel conductance in human PASMCs was \( 222 \pm 5 \) pS, a value nearly identical to BK channels in PASMCs of N rats \( (B) \). The Ca\(^{2+}\)-sensitivity of \( h \text{BK} \) channels in human PASMCs is higher than BK channels in PASMCs of N rats \( (n = 2-5 \text{ per point}) \). Asterisks indicate a statistical difference between EC\(_{50}\) values using a non-linear fit comparison \( (C) \). Quantitative real-time PCR using human PA reveals that only rare \((0.47 \pm 0.08\% )\) BKα transcripts contain the STREX insert that confers oxygen sensitivity \( (n = 3) \) \( (D) \). Averaged values confirm that acute hypoxia does not significantly affect the NP\(_0\) of \( h \text{BK} \) channels in PASMCs freshly isolated from human lungs \( (n = 4) \) \( (E) \). Summarized data from \( h \text{BK} \) channels in I-O patches of human PASMCs verify that LC \((45 \mu\text{mol/L})\) profoundly increases NP\(_0\) \( (n = 4, 8) \) \( (F) \). Exposure of an I-O patch from a human PASMC to hypoxic bath solution fails to affect \( h \text{BK} \) current (patch potential was \(-40 \text{ mV}; [\text{Ca}^{2+}]_i \) was \( 10^{-6.17} \) mol/L) \( (G) \). Representative recording of \( h \text{BK} \) channel current in an I-O patch from human PASMC reveals that LC \((45 \mu\text{mol/L})\) profoundly activates the channel (patch potential was \(+40 \text{ mV}; [\text{Ca}^{2+}]_i \) was \( 10^{-8} \) mol/L \( (H) \).
Supplemental Figure 1. After exposure to 3 weeks of chronic normobaric normoxia (N) or hypoxia (CH), rats were anesthetized with isoflurane and cardiovascular parameters were measured. Right ventricular systolic pressure (RVSP) was significantly higher in CH compared to N rats (A), a finding that corresponded to an increased right ventricle/ left ventricle+septal ratio (RV/(LV+S)) in the CH animals (B). Systemic mean arterial pressure (MAP) (C) and heart rate (D) were not significantly different between N and CH rats.

Supplemental Figure 2. To rule out the possibility that the loss of soluble cytosolic factors in I-O patches accounted for the failure of hypoxia to affect BK channel activity, we exposed BK channels in cell-attached (C-A) patches of rat PASMCs to hypoxic bath solution. Although the cytosolic milieu is preserved in C-A patches, hypoxia did not significantly affect the open-state probability (NP₀) of BK channels (n=3).
Figure 2

A

B

C

D

Figure 2
Figure 3

A. HEK: α alone
- Control
- + LC (45 μmol/L) 5 pA 2 s

B. HEK: α + β1
- Control
- + LC (45 μmol/L) 5 pA 2 s

C. PASMC
- Control
- Normoxia
- Hypoxia
- Hypoxia + LC (45 μmol/L) 5 pA 2 s

D. PASMC
- Normoxia
- Hypoxia
- Hypoxia + LC (45 μmol/L) 5 pA 2 s

E. HEK
- Fold change in NP0
- α
- α + β1

F. PASMC
- Fold change in NP0
- Control (DMSO)
- 5 (LC, μmol/L)
- 15 (LC, μmol/L)
- 45 (LC, μmol/L)
- Hypoxia
- LC

G. PASMC
- Fold change in NP0
- Hypoxia
- LC

Figure 3
Figure 5
Figure 6

A

B

C

D

E

F

G

H

Figure 6
Supplemental Figure 1

A

RVSP (mm Hg)

N

CH

B

RV/(LV + S)

N

CH

C

Systemic MAP (mm Hg)

N

CH

D

Heart rate (bpm)

N

CH

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