Hypoxia sensitivity of a voltage-gated potassium current in porcine intrapulmonary vein smooth muscle cells

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Dospinescu C, Widmer H, Rowe I, Wainwright C, Cruickshank SF. Hypoxia sensitivity of a voltage-gated potassium current in porcine intrapulmonary vein smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 303: L476–L486, 2012. First published July 6, 2012; doi:10.1152/ajplung.00157.2012.—Hypoxia contracts the pulmonary vein, but the underlying cellular effectors remain unclear. Utilizing contractile studies and whole cell patch-clamp electrophysiology, we report for the first time a hypoxia-sensitive K+ current in porcine pulmonary vein smooth muscle cells (PVSMC). Hypoxia induced a transient contractile response that was 56 ± 7% of the control response (80 mM KCi). This contraction required extracellular Ca2+ and was sensitive to Ca2+ channel blockade. Blockade of K+ channels by tetraethylammonium chloride (TEA) or 4-aminopyridine (4-AP) reversibly inhibited the hypoxia-mediated contraction. Single-isolated PVSMC (typically 159.1 ± 2.3 μm long) had mean resting membrane potentials (RMP) of −36 ± 4 mV with a mean membrane capacitance of 108 ± 3.5 pF. Whole cell patch-clamp recordings identified a rapidly activating, partially inactivating K+ current (IKH) that was hypoxia, TEA, and 4-AP sensitive. IKH was insensitive to Penitrem A or glutryl in PVSMC and had a time to peak of 14.4 ± 3.3 ms and recovered in 67 ms following inactivation at +80 mV. Peak window current was −32 mV, suggesting that IKH may contribute to PVSMC RMP. The molecular identity of the potassium channel is not clear. However, RT-PCR, using porcine pulmonary artery and vein samples, identified Kv1.5, Kv2.1, and BK, with all three being more abundant in the PV. Both artery and vein expressed STREX, a highly conserved and hypoxia-sensitive BK channel variant. Taken together, our data support the hypothesis that hypoxic inhibition of IKH would contribute to hypoxic-induced contraction in PVSMC.

Hypoxia and its role in pulmonary vein smooth muscle cells

The vasoactivity of the pulmonary veins (PV) in response to several contractile stimuli has been demonstrated in numerous studies with hypoxia (68), norepinephrine (29), histamine, and 5-HT (54), as well as thromboxane A2 (16, 47), acetylcholine (17), and prostaglandin E2 (28, 62), all inducing vasoconstriction. Furthermore, previous reports indicate that the veins are more reactive, for example, to the thromboxane mimetic U-46619 (3) or hypoxia (68) than the arteries in the pulmonary circulation or contract under conditions that cause arterial relaxation (20). This contractile difference highlights the potential for increased tone in the PV to significantly contribute to an increased total pulmonary vascular resistance (48). Post-capillary venuconstriction will raise upstream microvascular hydrostatic pressures with recent reports suggesting that, in healthy humans, hypoxic pulmonary venuconstriction facilitates capillary recruitment (57). However, despite this potential role, the cellular mechanisms contributing to hypoxia-induced PV contraction remain unclear.

Interest in PV physiology has significantly increased over the last decade not least because of its role as an important source of ectopic atrial fibrillation (7, 24). Pulmonary venous constriction can contribute to edema formation (14) for example as a result of congestive heart failure (5) or as a result of ascent to high altitudes (22, 31). In vascular occlusive diseases, cellular proliferation results in extensive fibrotic tissue deposition within the lumen of the vessel (39) and proliferation of smooth muscle in the medial layer (67). This results in pulmonary venous occlusion and the subsequent development of interstitial edema (44). The clinical manifestations may be very similar to idopathic pulmonary arterial hypertension (PAH) (55) with between 5–10% of histological cases initially considered to be PAH occurring as a result of pulmonary venoocclusive disease (27, 39). Intervention strategies exist; however, the prognosis remains poor, and PAH-specific therapies, such as vasodilators, may exacerbate edema formation (12, 38, 44). Understanding those factors that regulate contractility and proliferation in the PV smooth muscle cell (PVSMC) is essential to identify any PVSMC-specific targets.

In vascular smooth muscle, membrane potential affects [Ca2+]i, regulation, which in turn will affect contractility and the regulation of cell proliferation. A study by Michelakis et al. (37) reported a role for K+ in the regulation of rat PV tone, molecularly identifying several Kv, Kc, and BKca mRNA subunits in both the smooth muscle and cardiomyocyte cell population. In the rodent PV, Kv contribute to contractile tone (37), and, given Kv help maintain smooth muscle resting membrane potential (RMP) (42) and their O2 sensitivity (25), these channels would appear to be potential candidates for the PV response to acute hypoxia. However, the effects of hypoxia on channel activity, such as Kv, in PVSMC are not known, and direct comparisons between pulmonary venous and arterial contractility have previously highlighted functional differences (28, 29, 47, 54, 62, 68).

Thus in this study, we have set out to determine the effects of hypoxia on K+ channel activity in PVSMCs, identifying a hypoxia-sensitive current (IKH) that rapidly activates and only partially inactivates. From its biophysical properties, IKH has a window current that is maximal at membrane potentials similar to RMP, suggesting that hypoxic inhibition of this channel will contribute to hypoxia-induced contractility in the PV.

MATERIALS AND METHODS

Contractile studies. PVs with a mean diameter of 2 ± 0.5 mm (5th order) were isolated from fresh porcine lungs obtained daily from a local abattoir. All experimental procedures were in accordance with UK legislation and were approved by Robert Gordon University...
animal care and use committee. Vein segments were cut into rings 3–5 mm in length and mounted in a four-channel Myobath (World Precision Instruments, Stevenage, UK). Data were acquired using Chart 5 software and sampled at 2 Hz. Each bath was filled with a physiological saline solution of the following composition (in mM): 119 NaCl, 4.7 KCl, 1.18 MgSO4, 25 NaHCO3, 2.52 CaCl2, and 5 glucose. Bath solution was bubbled continuously with a 95% O2 and 5% CO2 combination (normoxia) or a 95% N2, 5% CO2 combination for hypoxic conditions. Bath temperature was maintained at 37 ± 1°C using a thermo-circulator (FH16-D; Grant Instruments, Cambridge, UK).

mRNA expression profiling. Fifth-order intrapulmonary veins and size-matched intrapulmonary arteries from the left middle or cranial lobe were dissected out and cleaned of connective tissue. Small sections (~1 mm²) were subsequently snap frozen in liquid nitrogen. Total RNA was extracted using the High Pure RNA Tissue kit (Roche Applied Science, Indianapolis, IN) following the manufacturer’s guidelines, and concentration was determined using a Nanodrop 1000 spectrophotometer (ThermoScientific, Wilmington, DE). cDNA was synthesized using the Transcriptor High Fidelity cDNA Synthesis kit (Roche Applied Science, Indianapolis, IN) following the manufacturer’s instructions. To allow a degree of quantitative comparison between expression levels across tissues, we standardized the amount of total RNA used in each RT reaction. End-point PCR analysis was carried out using GoTaq DNA polymerase (Promega, Madison, WI) for Kv1.5 (Kcna5), Kv2.1 (Kcnb1), Kv4.3 (Kcnb1), and BK core subunit (Kcnma1). The primer sequences were the following: Kv1.5 forward (5'-AGG ATC TGC AGC CCC TTG GAG T); Kv2.1 forward (5'-CCT TGT CAT CCT CAT CTC CAT CA) and reverse (5'-GTC CTT CCC TAC TGT TTG) and reverse (5'-GTC CTT CCC TAC TGT TTG) and reverse (5'-GTC CTT CCC TAC TGT TTG) and reverse (5'-GTC CTT CCC TAC TGT TTG). Each pair of primers were designed to amplify all known mRNA variants of the gene of interest. Amplicons were obtained by using an initial denaturing step at 94°C for 5 min and then denaturing at 94°C for 30 s, annealing at 56°C for 45 s, and extension for 45 s at 72°C using 30 cycles with a final 7-min extension at 72°C.

Negative controls included RT controls, for which no reverse transcriptase was added, and PCR controls. None of the controls produced any detectable amplicon, ruling out genomic or other contamination. Amplicons were run on a 1.5% agarose gel and visualized using ethidium bromide. All amplicons were sequenced and shown to match the expected genes.

Cell isolation. Single smooth muscle cells were isolated from fifth-order porcine intrapulmonary veins using a modified version of a previously described protocol (13). Venous segments were opened longitudinally and placed in iced HEPES-based dissociation solution (composition in mM: 128 NaCl, 5.4 KCl, 0.95 KH2PO4, 0.35 Na2HPO4, 4.16 NaHCO3, 10 HEPES, 10 n-glucose, and 2.9 sucrose; pH 7.3) supplemented with 1.5 mg/ml papain and 0.75 mg/ml DL-dithiothreitol. Venous segments were then stored on ice for 1–2 h followed by incubation in a water bath at 37°C for 8–10 min. Thereafter, the tissue was washed at least three times using enzyme-free fresh dissociation solution and transferred to dissociation solution containing 1.5 mg/ml collagenase for further 6–8 min at 37°C. After being rinsed in enzyme-free fresh dissociation solution, gentle trituration, using a fire-polished wide-bore Pasteur pipette, yielded numerous elongated smooth muscle cells. Cell viability was determined by exposing isolated cells to a high-K⁺ bath solution (80 mM KCl) and monitoring any contractile response. Subsequently, cells were stored at 4°C and remained viable for at least 5–6 h.

Phase-contrast photomicrographs of sample cells were acquired with a Leica DMi400B inverted microscope (Leica Microsystems CMS, Jena, Germany), analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD), and cell morphometric measurements were calculated using perimeter and surface area under conditions identical to those used in the electrophysiological experiments. A tridimensional estimation of cell surface (S) was calculated by the

Fig. 1. Hypoxia-induced contractions in the pulmonary vein (PV). A: sample trace of hypoxia-induced contractions from an intrapulmonary vein. Trace is representative of 12 similar recordings from 12 animals. B: representative traces of the effect of Ca²⁺ channel inhibitors on the hypoxia-induced contractile response. C: effects of Ca²⁺ on hypoxia-induced contraction. Data represent mean values ± SE. *P < 0.05, **P < 0.01, n = 12.
approximation of cell area to the surface of an ellipsoid. The particular type of ellipsoid used was a prolate spheroid. The long axis was considered equivalent to the cell length (l), and the other two axes were both equal to the width (w).

An estimate of surface area was established by approximation of the surface of an ellipsoid using a formula by Knud Thomsen: 

\[ S = 4\pi \left( a^p b^q + a^t c^p + b^t c^q + c^t a^p \right)^{1/3} \]

where \( p = \log(3) = \ln(3)/\ln(2) \approx 1.17 \), and \( a, b, \) and \( c \) are the semi-axes of the ellipsoid; here \( a = \frac{l}{2}, b = c = \frac{w}{2} \).

**Electrophysiology.** Recordings were made from freshly isolated PVSMC, at room temperature (22–25°C), using the whole cell configuration of the patch-clamp technique using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). Pipettes were fabricated from filaments, thin-walled borosilicate glass capillaries (1.5 mm OD × 1.17 mm ID; Clark Electromedical Instruments, Reading, UK) using a two-stage micropipette vertical puller (PP-830; Narishige, Tokyo, Japan) and heat-polished using a microforge (MF-830; Narishige, Tokyo, Japan) to a final resistance of 3–4 MΩ. Whole cell currents and membrane potential were low pass filtered at 1 kHz, sampled at 2–4 kHz with a DigiData 1200 series interface (National Instruments, Austin, TX) and recorded using the WinWCP V3.7.6 software (John Dempster, Strathclyde University, Glasgow, UK). Leak currents were subtracted offline using standard protocols contained within the software suite, and cell membrane capacitance was calculated from the time constant of the capacitive current decay in response to a depolarizing step from −80 mV. The extracellular (bath) solution contained (in mM): 110 KCl, 2.5 MgCl₂, 1.2 CaCl₂, 10 HEPES, 3.6 ATP (pH 7.2). A calculated liquid junction potential was 4.88 mV was left uncorrected. Access resistance was 3.65 × 10¹⁰ HEPES, 3.6 ATP (pH 7.2). A calculated liquid junction potential.

**Reagents and solutions.** Tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), nifedipine, papain, and collagenase were obtained from Sigma Aldrich (Dorset, UK) and Penitrem A and glyburide from Biomol (Exeter, UK). Drugs were prepared as stock solutions in deionized water or bath solution, with the exception of Penitrem A and glyburide, which were delivered to the recording chamber in DMSO (maximum bath concentration 0.02%).

**RESULTS.**

In contractile studies, 85% of porcine PV rings responded with a rapid and transient increase in tension when exposed to hypoxic conditions, in the absence of any preconstriction (Fig. 1A). This rapidly reverted to baseline levels when bubbling was switched back to the normoxic gas. Of note was the ability of the vessel to respond to repeated hypoxic insults with a consistent contractile response with the average peak hypoxia-induced contraction being 5.3 ± 0.7 g (56 ± 7% of the 80 mM KCl control response; \( n = 12 \)). Removal of extracellular Ca²⁺ resulted in an 82 ± 8% reduction of this hypoxic response. However, the
calcium channel blocker nifedipine (10 μM) reduced the hypoxic contractile response by only 53 ± 5% \((n = 6)\) (Fig. 1, B and C).

With Ca\(^{2+}\) influx significantly contributing to the hypoxia-induced contraction of the PV, we examined the effects of inhibitors that would affect membrane potential, specifically the effects of K\(^{+}\) channel blockers. The K\(_v\) channel inhibitor 4-AP (5 mM) significantly increased baseline tension by 44% \((P < 0.05, \ n = 12,\ \text{Fig. 2B})\), and these effects could be inhibited by the removal of extracellular Ca\(^{2+}\) or by the addition of the Ca\(^{2+}\) blocker nifedipine (Fig. 2B). Preincubation with either 4-AP (5 mM) or TEA (5 mM) significantly reduced the hypoxic response (Fig. 2C) to 24 ± 3 and 3 ± 1%, respectively \((P < 0.05, \ n = 12;\ \text{Fig. 2B})\). However, the BK\(_{Ca}\) channel blocker Penitrem A (500 nM) \((35)\) or the K\(_{ATP}\) channel blocker glyburide (10 μM) did not affect the hypoxia-induced contraction \((P > 0.05, \ n = 12)\), thus suggesting a role for K\(_v\) channel inhibition in the PV hypoxic contractile response.

To clarify the effects of hypoxia on PVSMC K\(^{+}\) channel function, individual PVSMC were isolated from fifth-order intrapulmonary vessels. In Ca\(^{2+}\)-free dissociation solution, freshly isolated PVSMC appeared elongated and fully relaxed. These cells were spindle shaped, without striations and with a single, centrally located nucleus. No cardiomyocytes typical of rodent intrapulmonary veins \((37)\) were observed in the cell population isolated from the porcine PV. Exposure of isolated cells to Ca\(^{2+}\) containing bath solution did not have a significant impact on cell morphology (Fig. 3A). In Ca\(^{2+}\)-containing conditions, the estimated cell length and width were 159.1 ± 2.3 μm and 8.6 ± 0.1 μm, respectively \((n = 258\) cells). Cell surface area ranged between 1,045.3 μm\(^2\) and 5,854.5 μm\(^2\) with a mean of 3,367.4 ± 52.3 μm\(^2\) (Fig. 3B, left), and in Ca\(^{2+}\)-containing bath solution, RMPs, measured under current clamp (0 pA), ranged from −18 mV to −55 mV with a mean of −36 ± 4 mV \((n = 221,\ \text{Fig. 3B, middle})\), with a membrane capacitance of 108 ± 3.5 pF \((n = 145,\ \text{Fig. 3B, right})\).

To provide an insight into the current molecular underpinnings, we investigated and compared the mRNA expression profile of a range of voltage-gated potassium channels and associate subunits in porcine venous and arterial tissues by using the RT-PCR method. Specifically, we chose to investigate channel subunits that have previously been reported to be involved in oxygen sensitivity. Using equal amounts of total RNA extracted from each tissue for retrotranscription provided an indication of the relative abundance of the channel mRNA in end-point PCR analysis. A representative electrophoretic gel displaying the ampiclons is shown in Fig. 3C. We observed that both tissues expressed bands at the expected length for K\(_{v1.5}\), K\(_{v2.1}\), K\(_{v4.3}\), BK, and the subunit K\(_{\beta1.2}\). Closer inspection of the gels revealed that mRNA expression levels of K\(_{v1.5}\), K\(_{v2.1}\), and BK were consistently higher in venous than in arterial tissue \((n = 3)\), whereas expression levels of K\(_{v4.3}\) and the subunit K\(_{\beta1.2}\) appeared equal in both tissues (see also MATERIALS AND METHODS). The α-subunit K\(_{v1.4}\) was not present in end-point PCR analysis and failed to appear with mRNA expression, thus not supporting an involvement of the K\(_{v1.4}\) subunit in porcine PVSMC.

Fig. 3. Passive membrane properties and K\(^{+}\) channel mRNA expression in porcine PV smooth muscle cell (PVSMC). A: freshly isolated myocytes from intrapulmonary veins (phase-contrast microscopy). B: frequency distribution of resting membrane potential (RMP) \((\text{middle, } n = 221)\) and membrane capacitance \((\text{right, } n = 145)\). C: messenger RNA expression profile of selected K\(^{+}\) channels in 5th-order PVs (V) and size-matched pulmonary arteries (PAs) \((A)\) obtained by RT-PCR. Band length: K\(_{v1.5}\): 505 bp; K\(_{v2.1}\): 560 bp; K\(_{v4.3}\) : 462 bp; BK2αβ : 670 bp; BK\(_{\text{STREX}}\) : 845 bp. Contrast was inverted across the gel for the sake of clarity. Controls indicate no DNA contamination (genomic or otherwise). D: STREX variant amino acid sequence in porcine tissue. Gray box outlines the alignment of selected motifs across selected mammalian species showing the conserved hypoxia motif in the porcine STREX sequence. [Adapted from Ref. 36; Copyright 2005 National Academy of Sciences, U.S.A.].

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in either arterial or venous tissue samples (data not shown). However, both venous and arterial tissues expressed the BK variant known as STREX on top of the shorter, ZERO, variant. Insertion of STREX dramatically modifies BK channel properties such as voltage/calcium sensitivity, modulation by enzymes, and sensitivity to drugs in a range of tissues (51). In addition the STREX variant, a 58-amino-acid sequence, is highly conserved across vertebrate species (36); however, expression in porcine tissues has not been reported so far. As may be expected, amino acid alignment in the STREX region showed that the porcine new sequence displays a very high degree of homology with other species, especially the human species (Fig. 3D).

In single PVSMC, the effects of hypoxia on K\textsuperscript{+} currents were recorded using whole cell patch-clamp electrophysiology. Applying incremental depolarizing voltage steps resulted in a family of rapidly activating outward currents with an average current density at +80 mV of 29.2 ± 2.6 pA/pF. The K\textsuperscript{+} channel blockers TEA (5 mM) and Penitrem A (500 nM) inhibited I\textsubscript{out} by 72 ± 6% (P < 0.05, n = 12) and 61 ± 4% (P < 0.05, n = 17) at +80 mV, respectively (n = 17 Fig. 4A). The Penitrem A-insensitive current (I\textsubscript{out}) was reduced by the K\textsubscript{+} channel blocker 4-AP (5 mM, 35 ± 5%, P < 0.05, n = 6), and hypoxia reduced it by 37 ± 5% (P < 0.05, n = 6, Fig. 4B); however, 10 μM glyburide had no significant effect (P > 0.05, n = 5).

The effect of depolarizing steps (−80 mV to +80 mV) during normoxic control, hypoxia, and subsequent normoxic recovery on I\textsubscript{out} were determined. Activation time constants were resolved by fitting the rising phase of the current recorded with a double exponential function (Fig. 5A). Hypoxia increased τ\textsubscript{fast} from 7 ± 1 ms to 17 ± 1 ms and τ\textsubscript{slow} from 27 ± 7 ms to 474 ± 357 ms (P < 0.05, n = 6), suggesting that hypoxia inhibited a rapidly activating component of I\textsubscript{out}. This effect was reversible, with τ\textsubscript{fast} and τ\textsubscript{slow} recovering during normoxic perfusion (8 ± 0.5 and 27 ± 2 ms, respectively, P > 0.05, control vs normoxia, n = 6, see Fig. 5B). Subtraction of the current records obtained before and during hypoxic perfusion revealed the hypoxia-sensitive, difference current (I\textsubscript{KH}), as shown in Fig. 6A. This current activated rapidly, with a peak current density of 3.4 ± 1 pA/pF (n = 6, Fig. 6B) and a time to peak of 14 ± 3 ms at +80 mV. This current then partially inactivated with a steady-state current ~53% of peak current density. In the presence of 5 mM 4-AP, I\textsubscript{KH} was significantly reduced (P < 0.05, n = 6, Fig. 6B). The temporal effects of hypoxia on I\textsubscript{KH} were examined by applying +80-mV depolarizing steps before, during and after hypoxic perfusion, subtracting the hypoxia-insensitive current from all other current traces and plotted as a function of time (Fig. 7A). Peak and steady-state I\textsubscript{KH} were attenuated by 70 ± 5% (P < 0.05, n = 6) ~1 min from the start of the hypoxic flow (Fig. 7B) and were by definition completely inhibited by the end of the hypoxic period.

Steady-state inactivation of I\textsubscript{KH} was examined with 1-s conditioning voltage steps of varying amplitude (−100 to +20 mV) followed by a 500-ms common test pulse (+80 mV). The resulting test currents decreased as the conditioning pulse increased (Fig. 8A). The inactivation curve was obtained by plotting normalized peak test currents ([I(I\textsubscript{max}−I\textsubscript{min}))/I\textsubscript{max}−I\textsubscript{min}] as a function of the conditioning pulse and the data fitted with a Boltzmann function (Fig. 8B). The voltage for half-inac-
HYPOXIA INHIBITS PULMONARY VEIN $K^+$ CHANNELS

A

B

**Fig. 5.** The effect of hypoxia on activation of Penitrem A-insensitive current. A: activation of currents during normoxia, hypoxia, and recovery. Dashed lines represent exponential fits during activation phase of a $K^+$ current recorded from a single cell during normoxia, hypoxia, and recovery. *Inset:* sample trace recorded during a voltage step (−80 to +80 mV) in normoxia and hypoxia. B: bar graph (right) illustrating the effect of normoxia, hypoxia, and recovery on the current activation time constant ($\tau_{ac}$). Bar graph (left) effect of normoxia, hypoxia, and recovery on current density. Both graphs, $*P < 0.05$, $n = 6$.

A movement (V_{0,5}) for the $I_{KH}$ current was derived, giving a value of −58.5 mV and a slope of 17.6 mV.

For the voltage dependence of activation, peak currents elicited by stepping from −80 mV to potentials ranging from −50 mV to +100 mV were converted into conductance using the equation $G = I(V_t - E_K)$, where $V_t$ is the test potential, and $E_K$ is the potassium equilibrium potential. Data values were subsequently normalized and fitted with a Boltzmann function (Fig. 8B, left) giving a $V_{0,5}$ activation of −13 mV and a slope of 14.9 mV. The areas under the inactivation and activation curves overlapped to give a window of current availability between −60 mV to +20 mV, characterized by partial activation and incomplete inactivation of channels, with peak window current availability at −32 mV (Fig. 8B, inset). Time-dependent recovery from inactivation of $I_{KH}$ was investigated using a two-pulse protocol. Conditioning and test potentials were stepped from the holding potential of −80 mV to +80 mV (Fig. 9A). The time interval between conditioning potential and test potential was increased (10–290 ms), and $I_{KH}$ peak test currents were normalized to the corresponding peak conditioning currents. Mean values were plotted as a function of the time interval between the conditioning and test potentials with the rate of recovery, after inactivation, being well described by a single exponential function with a time constant ($\tau_{rec}$) of 67 ms (Fig. 9B).

**DISCUSSION**

To the best of our knowledge, this study provides the first electrophysiological characterization of a hypoxia-sensitive, voltage-gated $K^+$ current in freshly isolated porcine PVSMCs. Using contractile and electrophysiological studies, we have identified a TEA-, 4-AP-sensitive hypoxic response in fifth-order PVSMC.

Hypoxia induced a transient contractile response followed by a more pronounced relaxation, usually to below baseline. These results are in agreement with the contractile responses reported in the PVs of various animal species (59, 68, 69). Hypoxia consistently induced contractile responses in the absence of any pretone, a finding similar to that reported by Uzun and Demeryürek (59). Of particular note was the ability of the PV to respond to repeated hypoxic insult without any obvious loss of contractile response. A previous study examining the effects of chronic hypoxia on contractility in a rodent model identified that the acute HPV response was preserved although agonist-induced responses were blunted (69). This, the authors suggested, indicated that the PVSMC was more responsive to acute hypoxic insult following exposure to chronic hypoxia and highlighted potential differences between the cellular mechanisms initiating the hypoxic response in the PV and pulmonary artery (PA). A recent study from Taylor et al. (57) has outlined the potential of hypoxic contraction in the human PV to facilitate gas exchange, by increasing downstream pulmonary pressure and so facilitating capillary recruitment. Maintaining hypoxia sensitivity in venous smooth muscle, even under conditions of chronic hypoxia, would clearly be advantageous. This study highlights that the porcine PV hypoxic contractile response is robust, reproducible, and is sensitive to conventional $K^+$ channel blockers, strongly suggesting a role for these channels in the hypoxia-induced contractility in PV rings.

Using electrophysiological studies, we identified a hypoxia-sensitive current ($I_{KH}$) in PVSMC, which is a rapidly activating, partially inactivating current with a fast time of recovery from steady-state inactivation. The biophysical features of this current are consistent with previously reported voltage-gated, Ca$^{2+}$-independent $K^+$ currents with rapid rates of activation and steady-state inactivation in pulmonary smooth muscle (32). The rapid rate of activation of $I_{KH}$ was comparable to observations made in rabbit portal vein smooth muscle (time to peak ∼20 ms) (4) but slower than the ultrafast activating...
current found in murine portal vein smooth muscle (time to peak of 4.1 ms) (63). The potential at which $I_{KH}$ half-inactivated was within the range seen in other vascular muscle (−78 to −38 mV), whereas the time of recovery from steady-state inactivation was comparable with data from gastrointestinal smooth muscle (1) but faster than in rat retinal arterioles (118.7 ms) (35), human mesenteric arteries (254 ms) (55), and rabbit PA (> 10 s) (43). In previous reports, the modulation of $K_v$ currents during hypoxia has been demonstrated in other tissues. For example, in rabbit pulmonary neuroepithelial bodies, which function as airway oxygen sensors, an $A$-type $K^+$ channel was hypoxia sensitive (19); in rodent neurons, an $A$-type current was regulated by the cellular redox state (50); and in cultured rat PA cells, a rapidly activating, inactivating $K_v$ current was reversibly inhibited by hypoxia (19). In rodent neurons, an $A$-type $K^+$ current which function as airway oxygen sensors, an $A$-type $K^+$ current has been demonstrated in other tissues.

Michelakis et al. (37) reported a role for 4-AP-sensitive $K^+$ channels in maintaining basal tone in the rodent PV. Our findings support this and suggest that hypoxia-induced contractility in the porcine PV requires functional $K^+$ channels and inhibiting these impairs this response. This is in contrast to the findings of Halla et al. (23), where 4-AP induced relaxation in piglet PV preparations. The order of branch used was smaller, and whether these differences represent functional differences along the venous tree or are developmental differences is not clear. Although changes in PV contractility suggest a role for $K^+$ inhibition in smooth muscle, the role of the endothelium in the PV hypoxic response has not been specifically looked at and cannot be excluded. In the rat PA, $K^+$ channel blockers have been shown to inhibit endothelial functional responses to hypoxia, reducing nitric oxide production (30).

In agreement with previously reported findings by Zhao et al. (68) and Uzun and Demiryürek, (59), the hypoxia-induced contraction required extracellular $Ca^{2+}$. In our study the application of a $Ca^{2+}$ channel blocker did not inhibit the hypoxic response by as much as removing extracellular $Ca^{2+}$. A recent study by Thakali et al. (58) has suggested that, in vein smooth muscle, increasing intracellular $Ca^{2+}$ effectively “silences” the $Ca^{2+}$ channel, reducing the efficacy of $Ca^{2+}$ channel block and so reducing vessel relaxation. Such an effect could account for the results reported here, but equally the lack of effect of $Ca^{2+}$ channel blockade may also reflect a role for $Ca^{2+}$ release from the sarcoplasmatic reticulum (SR). Further work is required to clarify this possibility although previous reports have suggested that the PV relies on the influx of extracellular $Ca^{2+}$ more than PAs during contraction (34) perhaps reflecting potential differences in the $Ca^{2+}$ stores such as the SR. In pulmonary arterial smooth muscle for example, there is approximately twice the volume of SR compared with mesenteric vein smooth muscle (15).

The PVs play an active role in the regulation of pulmonary blood flow. Kadowitz et al. (29) found that ~50% of the total increase in pulmonary vascular resistance, caused by sympathetic nerve stimulation under conditions of steady flow, was due to venoconstriction. Pulmonary venous constriction may result in edema formation during congestive heart failure (5) and is also important in the development of high-altitude pulmonary edema (22, 31). Acute alveolar hypoxia increases PV pressure (18), and exposure to chronic hypoxia is known to produce medial thickening in the intrapulmonary veins in animal models and in healthy human high-altitude residents (41, 61). Indeed, many of the elements associated with the progression of pulmonary hypertension occur in the PVs during disease. For example, in patients suffering from chronic bronchitis and emphysema, lesions consisting of medial hypertrophy, arteriolization, and the development of longitudinal bundles of smooth muscle cells in the intima have been identified in small intrapulmonary veins (53, 60). A case study by Zhang et al. (67) has suggested that medial thickening in the PA is secondary to PV occlusive disease, highlighting a potential role for the PV in the development of pulmonary hypertension.

The morphology of the porcine PVSMC was typically smooth muscle in appearance with sizes comparable to previous reports in rabbit (60–120 μm long, 8) and human-cultured PASMC (132.6 ± 3.5 μm long, 63). However, in this study, the length of porcine PVSMCs were almost twice that of porcine PASMC (77.8 ± 2 μm long, 52). The difference in resting cell length may reflect differences in intracellular $Ca^{2+}$ (40), and therefore a difference between arteries and veins in the handling of $Ca^{2+}$ under basal conditions. In common with human and canine PVs (33), the myocardial sleeve of the porcine PV is restricted to the proximal regions of the extrapulmonary veins.
monary veins, and no cardiomyocytes were identified in cell isolates.

The membrane capacitance of porcine PVSMC was $108/ \mu F$. This value is higher than most reported for myocytes in PAs, e.g., $28/ \mu F$ in freshly isolated rabbit PASMC (8) and $9/ \mu F$ in murine PASMC (29). However, Yuan et al. (64) described PA myocytes with a membrane capacitance of $141/ \mu F$ with cell sizes comparable to those reported in this study.

Smooth muscle contractility and proliferation are affected by levels of $[Ca^{2+}]$, which is regulated, in part, by changes in membrane potential (42). In general, the resting membrane potential of vascular smooth muscle cells is determined by the function of both BKCa channels (49) and Kv channels (11) although recent data question this assumption in PASMC (6). Two major components of the Kv current with kinetically distinct features have been identified (66). These are the delayed rectifier, which is a slowly activating, slowly inactivating current, and the transient A-type current that has rapid activation and inactivation kinetics. A wide range of delayed-rectifier and A-type currents with diverse biophysical and pharmacological properties has been reported. This functional diversity is partly due to the variety of genes encoding the pore-forming $K_v$ α-subunit ($K_{v1-11}$), the capability of $K_v$ α-subunits to form heterotetrameric complexes, and other processes such as splice variants (9). In human PASMC, $K_{v3.4}$, $K_{v4.2}$, and $K_{v4.3}$ have been identified as likely candidates for an A-type current (26), and in the rat PV, several members of the $K_v$ α-subfamily, specifically $K_{v1.1-1.6}$, $K_{v2.1}$, $K_{v3.1}$, and $K_{v4.2}$ are expressed, contributing to $K_v$ regulation of pulmonary venous tone (37). Other important factors that confer further functional and structural diversity are the interactions of $K_v$ α-subunits with modulatory $K_v$ β-subunits ($K_v1-3$) that may affect the gating of the pores of the channel (9). For example, in HEK293 cells, transfection of the auxiliary $K_v1.2$ subunit added hypoxia sensitivity to an A-type $K_{v4.2}$ channel (45).

Using RT-PCR, we have identified the presence of two known hypoxia-sensitive $K_v$ α-subunits, specifically $K_{v1.5}$ and $K_{v2.1}$ (37), as well as the BK ZERO and STREX splice variants. The identification of the STREX splice variant in the porcine pulmonary vasculature is a novel observation, and certainly, in terms of the channels structure/function relationship, hypoxia is known to regulate the gating properties of this channel (36). From our data, there appears to be a differential expression of these subunits mRNA between artery and vein, being more abundant in the PV. Certainly, these channels would contribute to the functional contractile responses recorded. Not all subunits were differentially expressed: mRNA expression for channel subunit $K_{v4.2}$ appeared to be equally

Fig. 7. Time course of $I_{KH}$ current during normoxic/hypoxic perfusion. A, top: representative experiment with peak $I_{KH}$ current values plotted before (a), during (b) and after (c) perfusion with hypoxic bath solution; dashed lines mark zero current level; ●, time points when the sample traces underneath were obtained. Bottom: representative $I_{KH}$ traces. B: mean peak $I_{KH}$ current density under normoxia, hypoxia (after 1 min) and normoxic recovery. *$P < 0.05$, $n = 6$. 

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expressed in artery and vein, but Kv1.4 was absent from both. However, mRNA expression levels may not necessarily reflect protein expression levels.

Hypoxia regulation of Kv/H2/H9252-subunit has been suggested, possibly coupling cellular redox state to channel function (21). Certainly, in bovine tissue, Coppock et al. (10) identified a differential expression of Kv/H2/H9252-subunits between conduit and resistance PAs. To this end, we sought to identify any differences in Kv/H2/H9252-subunit expression, specifically Kv/H2/H1.2, which is known to increase hypoxia sensitivity in Kv (45). Our data suggested no difference in Kv/H2/H9252mRNA content between artery and vein. As with the Kv/H1/H9251-subunits, these findings may not be reflected in actual protein expression, and no conclusions as to their potential modulatory role can be drawn as yet.

In this study, inactivation of I_KH was incomplete, with a steady-state sustained current present in all recordings. The results presented here can best be explained if, alongside the main A-type current that inactivates completely, I_KH contained a secondary component, a slower or noninactivating current, possibly a delayed rectifier that also possesses hypoxia sensitivity. Such currents have been observed in rodent PAs (2, 25, 46) and veins (37).

The A-type channel function is intrinsically voltage dependent; therefore its functional role in vascular smooth muscle relies on the ability of the channel to maintain its activation at physiologically relevant membrane potentials. However, in most vascular tissues, A-type currents do not contribute to RMP due either to their activation thresholds being more positive than RMP (19) or their voltage of complete inactivation negative to RMP (1). In this study, PVSMC steady-state activation and inactivation curves for I_KH revealed a voltage window of current availability that peaked at −32 mV, close to RMP, suggesting that I_KH is likely to be active under our resting conditions. This is consistent with observations made in murine PASMC, where the voltage window was narrower, between −40 and −10 mV, but the peak availability of −31.5 mV was similarly close to the RMP value of −27.9 mV (32). In smooth muscle from retinal arterioles, McGahon et al. (35) have suggested that the A-type current is active at RMP and that its hyperpolarizing effect on the RMP suppresses membrane excitability. A similar role for Kv, in the rat PV is supported by the observation that application of 4-AP results in contraction (37).

A significant body of previous work with isolated vessels supports an active role for the PVs in regulating pulmonary blood flow, including hypoxic pulmonary vasoconstriction.
With this study, we take this further and show that smooth muscle cells from PVs have intrinsic hypoxia sensitivity, and the mechanism involves a K^+ current. To our knowledge, this is the first study to identify a hypoxia-sensitive K^+ current in the PVSMC of any species. Our results indicate that this current possesses rapid activation kinetics and is only partially inactivated, suggestive of a K^+ -current with both A-type and delayed-rectifier channel properties. The voltage dependence of channel availability indicates that I_{KH} is likely to be involved in the maintenance of RMP. Thus our findings would support the hypothesis that inhibition of I_{KH} by hypoxia is physiologically relevant and would contribute to hypoxia-mediated contraction in porcine intrapulmonary veins.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

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REFERENCES

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