Graded response of $K^+$ current, membrane potential, and $[Ca^{2+}]_i$ to hypoxia in pulmonary arterial smooth muscle

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Olschewski, Andrea, Zhigang Hong, Daniel P. Nelson, and E. Kenneth Weir. Graded response of $K^+$ current, membrane potential, and $[Ca^{2+}]_i$ to hypoxia in pulmonary arterial smooth muscle. Am J Physiol Lung Cell Mol Physiol 283: L1143–L1150, 2002. First published August 9, 2002; 10.1152/ajplung.00104.2002.—Many studies indicate that hypoxic inhibition of some $K^+$ channels in the membrane of the pulmonary arterial smooth muscle cells (PASMCs) plays a part in initiating hypoxic pulmonary vasoconstriction. The sensitivity of the $K^+$ current ($I_k$), resting membrane potential ($E_m$), and intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) of PASMCs to different levels of hypoxia in these cells has not been explored fully. Reducing $P_O2$ levels gradually inhibited steady-state $I_k$ of rat resistance PASMCs and depolarized the cell membrane. The block of $I_k$ by hypoxia was voltage dependent in that low $O_2$ tensions (3 and 0% $O_2$) inhibited $I_k$ more at 0 and −20 mV than at 50 mV. As expected, the hypoxia-sensitive $I_k$ was also 4-aminopyridine sensitive. Fura 2-loaded PASMCs showed a graded increase in $[Ca^{2+}]_i$, as $P_O2$ levels declined. This increase was reduced markedly by nifedipine and removal of extracellular $Ca^{2+}$. We conclude that, as in the carotid body type I cells, PC-12 pheochromocytoma cells, and cortical neurons, increasing severity of hypoxia causes a proportional decrease in $I_k$ and $E_m$ and an increase of $[Ca^{2+}]_i$.

hypoxic pulmonary vasoconstriction; patch clamp; electrophysiology; ion channels; oxygen

The regional distribution of pulmonary blood flow is determined in part by local variations in alveolar $O_2$ tension. The pulmonary arterial vasoconstriction in response to hypoxia reduces the blood flow through poorly ventilated alveoli, matches lung perfusion to ventilation, and prevents systemic hypoxemia (19, 38). The small pulmonary arteries are unique in that they constrict in response to a fall in $O_2$ tension, whereas systemic arteries tend to dilate. The “dose-response” relationship of hypoxia and pulmonary vasoconstriction has been demonstrated in the intact animal (33) and in the isolated perfused lung (32). In each case, the threshold for the onset of hypoxic pulmonary vasoconstriction (HPV) is ~60–70 mmHg. In human lungs, graded hypoxia significantly reduces the perfusion of the lung ventilated with a hypoxic gas mixture (10).

There is a large body of data from other $O_2$-sensitive tissues, such as the carotid body (16–18), PC-12 cell line (45), pulmonary neuroepithelial body (39, 40), and neocortical neurons (12, 13), indicating that $K^+$ channels play an essential role in their responsiveness to changes in $O_2$. The $P_O2$-channel activity dose-response curve is relevant to secretory behavior, neuronal excitability, and the physiology of $O_2$ delivery and transport to the brain (14, 15). $K^+$ current amplitude of the type I cells in the carotid body is inversely proportional to the degree of hypoxia (17). Lowering of $P_O2$ also causes a dose-dependent increase in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$; see Refs. 3–5 and 37). In the PC-12 cell line, the magnitude of hypoxia-induced inhibition of $K^+$ channels shows a graded response to the severity of hypoxia (45). In cortical neurons, $P_O2$ regulates the channel open probability, without changing the single-channel conductance. Exposure to progressively lower $P_O2$ gradually reduces the open probability (12, 13). In contrast, how progressive changes in $P_O2$ modify $K^+$ channel activity, resting membrane potential ($E_m$), and $[Ca^{2+}]_i$ in pulmonary arterial smooth muscle cells is still uncertain. To address these questions, we have examined the direct effect of different $O_2$ tensions on $K^+$ channels, restoring $E_m$ and $[Ca^{2+}]_i$ in freshly dispersed, rat pulmonary arterial smooth muscle cells.

METHODS

The animal study was approved by the Institutional Animal Care and Use Committee of the Minneapolis Veterans Affairs Medical Center and conforms to current National Institutes of Health and American Physiological Society guidelines for the use and care of laboratory animals.

Cell isolation. Rat pulmonary artery smooth muscle cells (PASMC) were freshly dissociated for electrophysiological studies every day. Male Sprague-Dawley rats (body wt 300–400 g) were anesthetized with 50 mg/kg ketamine and 5 mg/kg xylazine, and the heart and lungs were removed en bloc. Resistance pulmonary arteries (4th–6th divisions) were dissected free and placed in $Ca^{2+}$-free Hanks’ solution (see Solutions). The arteries were then transferred to Hanks’ solution containing 0.5 mg/ml papain, 1 mg/ml albumin, and 1 mg/ml dithiothreitol without EGTA and kept at 4°C for 30 min. After this time, the arteries were incubated at 37°C for

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13 min. The arteries were washed thoroughly in enzyme-free Hanks' solution for at least 10 min and then maintained at 4°C. Several digestions were done each day to ensure cell viability. Gentle tituration produced a suspension of single cells, which was then separated into aliquots in a perfusion chamber on the stage of an inverted microscope (Diaphot 200; Nikon) for the studies.

**Experimental protocol.** After a brief period to allow a partial adherence to the bottom of the recording chamber, cells were perfused via gravity with a bath solution (see Solutions) at a rate of 2 ml/min for the recording of K⁺ current (I₂) and resting Eₘ or [Ca²⁺]. After equilibration under normoxic conditions (2 min), PASMCs were exposed to graded levels of hypoxia for 4 min followed by 4 min of reperfusion with normoxic extracellular solution to verify a recovery. All experiments were done using fresh cells isolated from at least three animals and were performed at 30°C. The electrophysiological studies were carried out in low light intensity because of the light sensitivity of amphotericin B.

**Current recording.** Whole cell recordings were performed using the amphotericin-perforated patch-clamp technique (26). Patch pipettes were pulled from glass tubes (PG 150T; Warner Instruments). The pipettes were fire-polished directly before the experiments and had a resistance of 2–3 MΩ when filled with intracellular solution. The patch-clamp amplifiers were Axopatch 200A (Axon Instruments, Foster City, CA) in all voltage- and current-clamp experiments. Offset potentials were nulled directly before formation of a seal. Whole cell capacitance and series resistance were corrected (usually 80%). Only cells with series resistance <10 MΩ were kept. The effective corner frequency of the low-pass filter was 1 kHz. The frequency of digitization was at least two times that of the filter. For Eₘ experiments, cells were held in current clamp at their resting Eₘ (without current injection). Eₘ stability was always determined for at least 1 min before any recording. The data were stored and analyzed with commercially available software (pCLAMP version 8.1; Axon Instruments).

**Pulse protocols and analysis.** Smooth muscle cells from small pulmonary resistance vessels were voltage-clamped at a holding potential of −70 mV. The standard protocol used to obtain current-voltage relationships consisted of 300-ms voltage-clamp pulses applied in 10-mV steps between −70 and +50 mV. Measuring the current at the end of the voltage-clamp pulse and plotting this against the test potential obtained steady-state current-voltage relationships. Currents were normalized relative to each cell's control (normoxic) current at +50 mV.

**Measurement of [Ca²⁺].** [Ca²⁺] was measured by dual-excitation ratiometric imaging, using fura 2 (9). Freshly dispersed cells were transferred to the experimental chamber and incubated in Ca²⁺-free extracellular solution with 0.1 μM fura 2-AM and 0.8 μM pluronic acid for 20 min at room temperature. The plates were then washed with extracellular solution containing 2.0 mM CaCl₂ and incubated at room temperature for a further 20 min. Plates were rewarshed and placed on the stage of an inverted microscope and perfused with a warmed extracellular solution (30°C). This loading method allows low concentrations of fura 2 to be quickly introduced in the cells without the potential effects on cell morphology that may occur from long exposures to high concentrations. Changes in [Ca²⁺] were measured using a cooled charge-coupled device camera (Hamamatsu) with MetaFluor image capture and analysis software (Universal Imaging, West Chester, PA). Measurements were made every 5 s. Background fluorescence was recorded from each dish of cells and subtracted before calculation of the 340- to 380-nm ratio. [Ca²⁺] was calculated according to the method of Grynkiewicz et al. (9). A dissociation constant of 220 nM was calculated from in vitro calibration. Maximal and minimal ratio values were determined at the end of each experiment by first treating the cells with 1 μM ionomycin (maximal ratio) and then chelating all free Ca²⁺ with 10 mM EGTA (minimal ratio). Any cells not responding to ionomycin were disregarded, as were cells showing significant photobleaching. Peak increases in [Ca²⁺] were measured during each intervention, and data are given as averaged peak values.

**Solutions.** The Hanks' solution contained (in mM) 145 NaCl, 4.2 KCl, 1.2 KH₂PO₄, 10 HEPES, 10 glucose, and 0.1 EGTA (pH was adjusted to 7.4 by KOH). The extracellular or bath solution contained (in mM) 115 NaCl, 5.4 KCl, 2.0 CaCl₂, 1 MgCl₂, 10 glucose, 1 NaHPO₄, and 25 NaHCO₃ (pH 7.4 when bubbled with 5% CO₂). The standard intracellular pipette solution contained (in mM) 145 KCl, 1 MgCl₂, 1 K₂ATP, 0.1 EGTA, 10 HEPES 10, and 120 μg/ml amphotericin B (pH was adjusted to 7.2 by KOH). The effect of hypoxia was studied by switching between normoxic and hypoxic perfusate reservoirs. Normoxic solutions were equilibrated with 21% O₂, 5% CO₂, and 74% N₂. Hypoxic solutions were achieved by bubbling with 10% O₂, 5% O₂, 3% O₂, and 0% O₂ (plus 5% CO₂-balance N₂) for at least 20 min before cell perfusion and by blowing N₂ over the surface of the experimental chamber using a modified dish (30). These procedures produced P O₂ values in the experimental chamber of 140–160 mmHg (21% O₂), 60–80 mmHg (10% O₂), 35–44 mmHg (5% O₂), 24–30 mmHg (3% O₂), and 11–17 mmHg (0% O₂), respectively. O₂ levels were measured with a Rapidlab Chiron blood gas analyzer from samples taken directly from the experimental chamber containing the PASMC during perfusion, which allows an exact measurement of P O₂. By the use of a small recording chamber (400 μl), high perfusion rate (2–3 ml/min), and short dead space, bath exchange could be achieved in <30 s. P O₂ was 36–42 mmHg, and pH was 7.37–7.42 under these conditions.

Fura 2-AM and pluronic acid were obtained from Molecular Probes (Eugene, OR). All other compounds were purchased from Sigma Chemical (St. Louis, MO). All drugs were dissolved in Hanks' solution, with the exception of nifedipine, which was dissolved in ethanol as a stock solution. pH of solutions containing drugs was tested and corrected to eliminate potential pH-induced effects. Stock solutions in ethanol were diluted at least 1:10,000 in the bath solution. At this concentration, the vehicle alone had no effect on the baseline levels of Ca²⁺.

**Statistical analysis.** Numerical values are given as means ± SE of n cells. Intergroup differences were assessed by a factorial ANOVA with post hoc analysis with Fisher's least significant difference test. P values <0.05 were considered significant. In Figs. 1–8, the SE is indicated when it exceeds the symbol size.

**RESULTS**

Whole cell outward Iₖ in PASMCs under hypoxia. When the cells were superfused with a bath solution bubbled with different concentrations of O₂ (10%, 5%, 3%, 0%), whole cell outward Iₖ in PASMC from resistance vessels was recorded after 4 min of exposure to the low O₂ level (Fig. 1). Under these conditions, hypoxia rapidly inhibited Iₖ. This decrease in Iₖ was parallel to the decrease in P O₂ levels. The averaged current-voltage relationships for Iₖ under normoxic and
Fig. 1. Effect of hypoxia on whole cell outward $K^+$ current of pulmonary artery smooth muscle cells (PASMCs). Representative 300-ms traces demonstrate $K^+$ currents from PASMCs under normoxic conditions (left and right) and after a 4-min exposure to low $O_2$ tension (middle). Currents were evoked from a holding potential of −70 to +50 mV in incremental depolarizing 10-mV steps with 5 cells in each group.

Fig. 2. Averaged whole cell current-voltage ($I$-$V$) plots of steady-state outward $K^+$ currents recorded from PASMCs. Currents were recorded during control and after a 4-min exposure to low $O_2$ tension (measured at 290–300 ms) and normalized to maximum current under normoxia [measured current divided by maximum current ($I/I_0$)] at +50 mV. Values are means ± SE.
Fig. 3. Effect of 4-aminopyridine (4-AP) and low O₂ tension on outward K⁺ current from PASMCs. A: representative K⁺ current traces were recorded during control and after exposure to 5 mM 4-AP or 5 mM 4-AP with 3% O₂ by the voltage step from −70 to 0 mV. B: averaged whole cell I-V relationship of outward K⁺ currents normalized to maximum current under normoxia (I/Iₒ) and after exposure to 5 mM 4-AP or 5 mM 4-AP with 3% O₂. Values are means ± SE.

Voltage dependence of the inhibition of steady-state outward current by hypoxia. The effect of hypoxia on Iₖ at several potentials was also examined. Currents were normalized relative to each cell’s control (normoxic) current at +50, 0, and −20 mV and plotted against the O₂ level in the bath solution (Fig. 5). Low O₂ tension at or below 70 mmHg inhibited the whole cell K⁺ current. It was remarkable that hypoxic inhibition of Iₖ was voltage-dependent, being greater at more negative Eₘ.

Effect of hypoxia on Eₘ in PASMCs. In rat PASMCs, the average resting Eₘ value, measured by the current-clamp technique, was −47.6 ± 2.0 mV (Fig. 6). Superfusion with bath solution containing 10% O₂ had no effect on Eₘ (−47.2 ± 1.7 mV, n = 5). Decreasing the O₂ concentration of the bath solution caused further depolarization. Bath solution bubbled with 5% O₂ depolarized to −42.0 ± 4.9 mV (n = 5), 3% O₂ to −35.8 ± 3.9 mV, and 0% O₂ to −22.6 ± 6.4 mV. The depolarizing effect of 5 and 3% O₂ on resting Eₘ was reversible. The superfusion of the cell chamber with bath solution bubbled with 0% O₂ depolarized Eₘ irreversibly during the recording period of 4 min.

Effects of hypoxia on [Ca²⁺]i in PASMCs. Average resting [Ca²⁺]i in rat PASMC under normoxic conditions was 91.3 ± 1.8 nM (n = 214). When the cells were superfused with the bath solution bubbled with different levels of O₂, hypoxia caused an increase in [Ca²⁺]i in a dose-dependent manner (Fig. 7). Extracellular solution bubbled with 5 and 0% O₂ increased [Ca²⁺]i by 184.5 ± 23.3 nM (n = 17) and by 512.1 ± 50.5 nM (n =

hypolectic conditions are shown in Fig. 2. The block of Iₖ under different hypoxic conditions was reversible to 90–97% (at +50 mV) after a 4-min recovery time, except after exposure to 0% O₂ in the bath solution (77% recovery at +50 mV).

Effect of the K⁺ channel inhibitor 4-aminopyridine. To characterize the O₂-sensitive outward Iₖ, their sensitivity to the classic K⁺ channel blocker 4-aminopyridine (4-AP) was examined. Representative Iₖ traces that were recorded during control and after exposure to 3% O₂ or 5 mM 4-AP under hypoxic conditions (3% O₂) are shown in Fig. 3A. Extracellular application of 5 mM 4-AP inhibited Iₖ to 17.6 ± 4.6% at 0 mV (n = 5), and no further decrease was induced by the additional application of bath solution containing 5 mM 4-AP and bubbled with 3% O₂ (14.6 ± 4.0 at 0 mV; n = 5), suggesting that hypoxia may be acting on 4-AP-sensitive channels.

Effect of the Ca²⁺-activated K⁺ channel inhibitor iberiotoxin. To investigate the composition of the hypoxia-insensitive Iₖ in PASMC, their sensitivity to the Ca²⁺-activated K⁺ channel (Kᵥca) blocker iberiotoxin (ITX) was examined. Representative Iₖ traces that were recorded during control and after exposure to 0% O₂, or 100 nM ITX under hypoxic conditions (0% O₂), are shown in Fig. 4A. When cells were dialyzed with extracellular solution bubbled with 0% O₂, the current was inhibited to 38.6 ± 4.7% at +50 mV (n = 5; Fig. 4B). Application of ITX under hypoxia caused an additional inhibition of Iₖ to 11.6 ± 5.1% at +50 mV (n = 3).

Fig. 4. Effect of low O₂ tension and iberiotoxin (ITX) on outward K⁺ current from PASMCs. A: representative outward K⁺ current traces were recorded during control and after exposure to 0% O₂ or 100 nM ITX under hypoxic conditions (0% O₂) by a voltage step from −70 to +50 mV. B: averaged whole cell I-V relationship of outward K⁺ currents normalized to maximum current under normoxia (I/Iₒ) and after exposure to 0% O₂ or 100 nM ITX in hypoxic conditions with 0% O₂. Values are means ± SE.
24), respectively. To determine whether Ca$^{2+}$ influx from extracellular sources was required for the increase of [Ca$^{2+}$]$_i$, PASMCs were perfused with Ca$^{2+}$-free extracellular solution (10 mM EGTA) during the 4 min of hypoxia. Removal of extracellular Ca$^{2+}$ markedly reduced the response of [Ca$^{2+}$]$_i$ to severe hypoxia, but there was still an increase in [Ca$^{2+}$]$_i$, of 174.1 ± 38.8 nM (P < 0.001; n = 17), suggesting a role of intracellular stores in the increase of [Ca$^{2+}$]$_i$, during hypoxia (Fig. 7). In another set of experiments, the hypoxic challenge was applied to PASMCs after treatment with 10 μM nifedipine (2 min), an L-type Ca$^{2+}$ channel antagonist, or 30 μM La$^{3+}$ (2 min), which is a nonselective blocker of Ca$^{2+}$ entry. The addition of nifedipine or La$^{3+}$ had no effect on resting [Ca$^{2+}$]$_i$ (n = 28 and n = 26). The hypoxia-induced increase in [Ca$^{2+}$]$_i$ was reduced markedly by La$^{3+}$ (change in [Ca$^{2+}$]$_i$ = 95.1 ± 17.9 nM; n = 17; P < 0.001) and by nifedipine (change in [Ca$^{2+}$]$_i$ = 144.6 ± 33.4 nM; n = 17; P < 0.001; Fig. 7), suggesting that part of the increase is caused by Ca$^{2+}$ influx via voltage-dependent Ca$^{2+}$ channels.

Effects of KCl and 4-AP on [Ca$^{2+}$]$_i$ in PASMCs. KCl (50 mM) caused an increase in [Ca$^{2+}$]$_i$, of 743.7 ± 92.5 nM (n = 11; Fig. 8). Pretreatment of PASMCs with 10 μM nifedipine or 30 μM La$^{3+}$ for 2 min before exposure to 50 mM KCl was begun almost completely abolished the KCl-induced increase in [Ca$^{2+}$]$_i$ (change in [Ca$^{2+}$]$_i$ = 69.7 ± 28.3 nM; n = 18; P < 0.001 for nifedipine and change in [Ca$^{2+}$]$_i$ = 56.8 ± 19.6 nM; n = 11; P < 0.001 for La$^{3+}$). The application of 5 mM 4-AP increased [Ca$^{2+}$]$_i$ by 285.1 ± 41.9 nM (n = 21; Fig. 8). Nifedipine or La$^{3+}$ significantly inhibited the 4-AP-induced increase in [Ca$^{2+}$]$_i$ (change in [Ca$^{2+}$]$_i$ = 60.6 ± 22.5 nM; n = 10; P < 0.001 for nifedipine and change in [Ca$^{2+}$]$_i$ = 47.2 ± 14.8 nM; n = 9; P < 0.001 for La$^{3+}$).

These results suggest that the 4-AP-induced increase in [Ca$^{2+}$]$_i$ is largely the result of Ca$^{2+}$ influx through voltage-dependent Ca$^{2+}$ channels, which are opened by the membrane depolarization resulting from decreased voltage-dependent K$^+$ channel activity.

**DISCUSSION**

Our observations demonstrate a graded response of $I_k$, $E_m$, and [Ca$^{2+}$]$_i$ to increasing hypoxia. The decrease in current started between 80 and 40 mmHg $P$O$_2$ and was significant at 30 mmHg. The finding that electrophysiological and [Ca$^{2+}$]$_i$ changes occur at these $O_2$ tensions would be compatible with the levels of hypoxia reported to cause vasoconstriction in the isolated lung and intact animals. The hypoxia-induced inhibition of 4-AP-dependent voltage-gated K$^+$ (Kv) channels in rat PASMCs causes membrane depolarization and an increase in [Ca$^{2+}$]$_i$, as previously reported (2, 43). More importantly, the effect of hypoxia in reducing $I_k$ was maximal at more negative $E_m$, close to the resting $E_m$, reinforcing the conclusion that it may be physiologically relevant.

HPV is a physiological response whereby desaturated mixed venous blood is diverted away from hypoxic alveoli, thus optimizing the matching of perfusion and ventilation and preventing arterial hypoxemia. It seems likely that the smooth muscle cells are both sensors of hypoxia and effectors of vasoconstriction (31, 38). Several different mechanisms have been proposed to explain HPV. The first mechanism involves hypoxic inhibition of one or more K$^+$ channels that set resting $E_m$ in the vascular smooth muscle cells of the small pulmonary resistance vessels, leading to cell depolarization, opening of voltage-gated Ca$^{2+}$ channels, and myocyte contraction (1, 21, 25, 38, 42). The second proposal is that hypoxia induces release of Ca$^{2+}$ from intracellular stores (28). This Ca$^{2+}$ could contribute to contraction, either directly or through blockade of K$^+$ channels (8, 24, 35). Finally, sensitization of actin/myosin might

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**Fig. 5.** Voltage dependence of the blockade of steady-state outward K$^+$ currents recorded from PASMCs at low O$_2$ tension. The currents, elicited by a test potential of +50 mV (●), 0 mV (■), or −20 mV (▲; holding potential −70 mV), were averaged from all of the cells tested and normalized to the maximal amplitude of each of the current records (n = 5). Connecting line was drawn by eye. Values are means ± SE. *P < 0.05 for difference between −20 and 0 and +50 mV; #P < 0.05 for difference between 0 and +50 mV.

**Fig. 6.** Effects of hypoxia on resting membrane potential ($E_m$) measured with current-clamp ($I = 0$) in PASMCs. $E_m$ was measured after a 4-min exposure to 10% (n = 7), 5% (n = 7), 3% (n = 5), and 0% (n = 7) O$_2$. Values are means ± SE. *P < 0.05 for difference from control; #P < 0.001 for difference from control.
lead to more contraction at any given cytosolic Ca\(^{2+}\) level (44). HPV could in fact involve participation of two, or even all three, of these mechanisms.

It has been demonstrated that hypoxic vasoconstriction of pulmonary arterial smooth muscle cells is in part mediated by the inhibition of voltage-dependent K\(^+\) channels, leading to cell depolarization, Ca\(^{2+}\) influx, and myocyte contraction (1, 21, 25, 38, 42). The PO\(_2\)-dependent K\(^+\) channel activity dose-response curve is relevant to the physiology of HPV. To determine how a gradual reduction in PO\(_2\) from normoxia to severe hypoxia inhibits K\(^+\) channels, we measured the voltage-current relationship in PASMCs from resistance arteries using the amphotericin-perforated patch-clamp technique. Hypoxia decreased the steady-state \(I_k\) and the decrease in current paralleled the decrease in PO\(_2\) levels. The hypoxia-inhibitory response was nearly completely reversed upon return to normoxia. These results are consistent with those of Zhu et al. (45), who showed that, in PC-12 cells, exposure to progressively lower PO\(_2\) gradually reduced the O\(_2\)-sensitive \(I_k\). Jiang and Haddad (12, 13) also showed, in excised patches from cortical neurons, a dose-dependent inhibition of open probability of single K\(^+\) channels by graded hypoxia, without alteration of single channel conductance.

In the present study, we observed that the blockade of \(I_k\) by hypoxia is voltage dependent. This is important in that it demonstrates that the hypoxic inhibition of \(I_k\) is more marked at physiologically relevant \(E_m\). This observation might be explained in two ways. The first hypothesis involves the presence of different Kv channels, with different sensitivities to hypoxia, in the PASMC. It would suggest that the hypoxia-sensitive Kv channels are open during normoxia at more negative \(E_m\) than the non-hypoxia-sensitive Kv channels. After the \(I_k\) was reduced by 5 mM 4-AP, no further decrease was caused by the additional application of hypoxia, suggesting that hypoxia may be acting on 4-AP-sensitive voltage-gated channels. The pharmacological properties of this K\(^+\) channel are thus similar to those described previously in rat PASMCs (1). Rabbit PASMCs are also known to express O\(_2\)-sensitive K\(^+\) channels that are susceptible to block by 4-AP (7, 21). The potential candidate Kv channel \(\alpha\)-subunits that could form O\(_2\)-sensitive channels in PASMCs are Kv1.2 (11, 36), Kv1.5 (2, 36), Kv2.1 (2, 11, 23), Kv3.1 (22), and Kv9.3 (11, 23). The second explanation of the voltage

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**Fig. 7.** Hypoxia increased intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) measured in rat PASMCs. Superfusion of PASMCs with a bath solution bubbled with 5% (\(n = 17\)) and 0% (\(n = 24\)) O\(_2\) significantly increased [Ca\(^{2+}\)]\(_i\). Preincubation with 30 \(\mu\)M La\(^{3+}\) (\(n = 17\)) or 10 \(\mu\)M nifedipine (2 min; \(n = 17\)) or omitting extracellular Ca\(^{2+}\) significantly decreased the hypoxia-induced increase in [Ca\(^{2+}\)]\(_i\). Values are means ± SE. *\(P < 0.05\) for difference from control; ***\(P < 0.001\) for difference from control.

**Fig. 8.** Effects of 50 mM KCl and 5 mM 4-AP on [Ca\(^{2+}\)]\(_i\) in PASMCs. Bath application of 50 mM KCl (\(n = 11\)) or 5 mM 4-AP (\(n = 21\)) increased [Ca\(^{2+}\)]\(_i\). Pretreatment with 30 \(\mu\)M La\(^{3+}\) or 10 \(\mu\)M nifedipine (2 min) abolished the KCl- or 4-AP-induced increase in [Ca\(^{2+}\)]\(_i\). Values are means ± SE. ***\(P < 0.001\) for difference from control.
dependence of hypoxic inhibition of $I_k$ relates to the fact that $K_{Ca}$ channels, which are not $O_2$ sensitive in the PASMCs of the adult rat, are only activated at more positive test potentials, as shown in Fig. 4. Consequently, hypoxia might inhibit less of the total $I_k$ at more positive $E_m$.

Membrane depolarization and hyperpolarization through inhibition and activation of $K^+$ channels are important mechanisms regulating smooth muscle cell contraction and relaxation. Under current-clamp conditions, lowering $P_{O_2}$ from normoxia to different hypoxic levels resulted in progressive depolarization of resting $E_m$. This observation is consistent with similar observations made in canine PASMCs (25) and cultured rat PASMCs (42). Although superfusion with bath solution containing 5 or 3% $O_2$ depolarized $E_m$ of PASMCs reversibly, severe hypoxia caused depolarization that was not fully reversible in the few minutes permitted in our protocol.

In most proposed mechanisms of HPV, an increase of $[Ca^{2+}]_i$ is necessary to elicit constriction of the vessels. In excitable cells, $[Ca^{2+}]_i$ is increased by $Ca^{2+}$ influx through $Ca^{2+}$-permeable channels and/or by $Ca^{2+}$ mobilization from intracellular $Ca^{2+}$ stores (e.g., endoplasmic/sarcoplasmic reticulum). We have shown that $[Ca^{2+}]_i$ begins to rise significantly in response to hypoxic challenge when $P_{O_2}$ is below 35–44 mmHg (5% $O_2$) and is graded with the severity of hypoxia. This observation confirms work in rat carotid body type I cells (3–5, 37) and in porcine PASMCs (29).

The proposal that the hypoxic $[Ca^{2+}]_i$ response results mostly from $Ca^{2+}$ influx is further supported by the observation that it is considerably attenuated by 30 $\mu$M $La^{3+}$ or 10 $\mu$M nifedipine. More specifically, the data with nifedipine imply the role of voltage-dependent L-type $Ca^{2+}$ channels, which supports the hypothesis that membrane depolarization triggers the rise of $[Ca^{2+}]_i$. Our results are consistent with previous reports demonstrating that voltage-dependent $Ca^{2+}$-channel blockers such as verapamil or SKF-525 significantly reduce HPV in isolated perfused lungs (20) and that nifedipine markedly inhibits the hypoxia-induced $Ca^{2+}$ rise in $[Ca^{2+}]_i$ in type I cells (4) and pulmonary rings (27). Omitting $Ca^{2+}$ markedly reduced the hypoxia-induced rise of $[Ca^{2+}]_i$ in our experiments. Similar observations have been reported in type I cells of the carotid body. The hypoxia-induced increase in $[Ca^{2+}]_i$ is entirely inhibited in the absence of extracellular $Ca^{2+}$ in these cells (4, 34). However, although the increase in $[Ca^{2+}]_i$ is smaller in the absence of extracellular $Ca^{2+}$ in PASMCs (8, 28), it is not absent, suggesting that both intracellular and extracellular $Ca^{2+}$ are important. If the principal effect of hypoxia is the inhibition of particular voltage-dependent $K^+$ channels, then 4-AP and high extracellular $K^+$ would be expected to stimulate entry of $Ca^{2+}$ through voltage-gated $Ca^{2+}$ channels. In our study, 4-AP and KCl did in fact increase $[Ca^{2+}]_i$ primarily through entry of $Ca^{2+}$ through voltage-gated $Ca^{2+}$ channels. These results confirm previous findings in rat (41) and canine (6) PASMCs and provide additional insight that $K_v$ are important regulators of $[Ca^{2+}]_i$ in these cells.

Although the response of PASMCs to hypoxia almost certainly involves both influx of extracellular $Ca^{2+}$ and release of $Ca^{2+}$ from internal stores, this study focuses on the former. It provides evidence of a graded response of $I_k$, $E_m$, and $Ca^{2+}$ to hypoxia, as seen in other $O_2$-sensitive tissues.

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REFERENCES


