Oxygen Sensitivity of Cloned Voltage-Gated K⁺ Channels Expressed in the Pulmonary Vasculature

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Abstract—Hypoxic pulmonary vasoconstriction is initiated by inhibiting one or more voltage-gated potassium (Kv) channel in the vascular smooth muscle cells (VSMCs) of the small pulmonary resistance vessels. Although progress has been made in identifying which Kv channel proteins are expressed in pulmonary arterial (PA) VSMCs, there are conflicting reports regarding which channels contribute to the native O₂-sensitive K⁺ current. In this study, we examined the effects of hypoxia on the Kv1.2, Kv1.5, Kv2.1, and Kv9.3 α subunits expressed in mouse L cells using the whole-cell patch-clamp technique. Hypoxia (PO₂ ≈ 30 mm Hg) reversibly inhibited Kv1.2 and Kv2.1 currents only at potentials more positive than 30 mV. In contrast, hypoxia did not alter Kv1.5 current. Currents generated by coexpression of Kv2.1 with Kv9.3 α subunits were reversibly inhibited by hypoxia in the voltage range of the resting membrane potential (Em) of PA VSMCs (≈28% at −40 mV). Coexpression of Kv1.2 and Kv1.5 α subunits produced currents that displayed kinetic and pharmacological properties distinct from Kv1.2 and Kv1.5 channels expressed alone. Moreover, hypoxia reversibly inhibited Kv1.2/Kv1.5 current activated at physiologically relevant membrane potentials (≈65% at −40 mV). These results indicate that (1) hypoxia reversibly inhibits Kv1.2 and Kv2.1 but not Kv1.5 homomeric channels, (2) Kv1.2 and 1.5 α subunits can assemble to form an O₂-sensitive heteromeric channel, and (3) only Kv1.2/Kv1.5 and Kv2.1/Kv9.3 heteromeric channels are inhibited by hypoxia in the voltage range of the PA VSMC Em. Thus, these heteromeric channels are strong candidates for the K⁺ channel isoforms initiating hypoxic pulmonary vasoconstriction.

Key Words: Kv channel ■ hypoxia ■ pulmonary artery ■ heteromeric

Hypoxia induces constriction of the small pulmonary resistance arteries, a process known as hypoxic pulmonary vasoconstriction (HPV).¹ This constrictor response is the opposite of that which occurs in resistance vessels of the systemic circulation. In these vessels, hypoxia results in vasodilation.² In the fetus, HPV contributes to high pulmonary arterial resistance by diverting blood flow through the ductus arteriosus.³ In the adult, HPV reduces blood flow through atelectatic or underventilated areas of the lung where ventilation is not adequate for oxygenation.³ In this way, short-term HPV is an essential mechanism that helps match ventilation to perfusion, diverting blood flow away from poorly ventilated regions of the lung to maximize arterial saturation.⁴ However, when hypoxia becomes more generalized, as seen in patients suffering from either long-term obstructive lung diseases or high altitude exposure,⁴,⁵ the subsequent pulmonary vasoconstriction causes an increase in pulmonary arterial pressure that can lead to the development of pulmonary hypertension.

In pulmonary arterial (PA) vascular smooth muscle cells (VSMCs), voltage-gated potassium (Kv) channels play an important role in setting the resting membrane potential (Em ≈ −55 mV) and, consequently, vascular tone.⁶–⁸ It is thought that hypoxia reversibly inhibits Kv channels and, thereby, regulates vasoconstriction.⁷,⁹–¹² This hypothesis is supported by the observation that hypoxia inhibits whole-cell K⁺ currents and causes membrane depolarization in both acutely isolated and cultured PA VSMCs.⁷,⁹,¹¹–¹⁴ Membrane depolarization is followed by an influx of calcium through voltage-gated calcium channels, PA VSMC contraction, and vasoconstriction.

Although electrophysiological and pharmacological studies have attempted to identify which Kv channel proteins contribute to the hypoxic-sensitive Kv current in PA VSMCs,¹¹,¹⁴–¹⁶ the literature remains filled with contradictions. Yuan et al¹⁶ used both reverse transcription–polymerase chain reaction (RT-PCR) amplification and Western blotting to determine which of the cloned cardiovascular channels is expressed in rat PA VSMCs in short-term cultures. This group documented the expression of Kv1.1, Kv1.2, Kv1.4, Kv1.5, Kv2.1, Kv9.3, Kv1.1, Kv1.2, and Kvβ2.1 by PCR and confirmed expression of Kv1.2, Kv1.4,
Kv1.5, and Kv2.1 channel proteins by Western blot analysis. In addition, Wang et al. demonstrated that long-term hypoxia down-regulates Kv1.2 and Kv1.5 mRNA and protein expression in cultured PA VSMCs. However, a potential problem with studies of VSMCs in short-term cultures is that, with time, myocytes show alterations in the expression of outward currents.15 Archer et al. used Western blotting of whole lung and acutely isolated VSMCs to argue in favor of the expression of Kv2.1 and Kv1.5 in rat pulmonary arteries. Furthermore, this group reported that the addition of Kv1.5 and Kv2.1 antibodies to the patch-clamp pipet solution inhibits the whole-cell hypoxic-sensitive Kv current. Similarly, Gelband and Gelband demonstrated that anti-Kv1.5 antibodies inhibit the hypoxia-induced membrane depolarization in rat PA VSMCs. Thus, Kv2.1 and Kv1.5 channel subunits may be important molecular components of the native O2-sensitive K+ current.

More recently, Patel et al. used both defined and degenerate RT-PCR primers to suggest that, of the known Kv channels, only Kv1.2, Kv1.3, and Kv2.1 channels are expressed in rat pulmonary arteries. They failed to detect Kv1.5, even when using defined primers specific for this isoform, and disregarded Kv1.2 and Kv1.3 as important O2-sensitive Kv channels in PA VSMCs. However, this group reported that the addition of Kv1.5 and Kv2.1 antibodies to the patch-clamp pipet solution inhibits the whole-cell hypoxic-sensitive Kv current. Similarly, Gelband and Gelband demonstrated that anti-Kv1.5 antibodies inhibit the hypoxia-induced membrane depolarization in rat PA VSMCs. Thus, Kv2.1 and Kv1.5 channel subunits may be important molecular components of the native O2-sensitive K+ current.

The mouse L-cell line expressing human Kv1.5 was used as described previously. The rKv2.1-containing expression vector was transfected into mouse L cells, and stable cell lines were produced and cultured as previously described. Mouse L cells (40% to 60% confluence in 60-mm dishes) were transiently transfected with either rKv1.2 or rKv9.3 and green fluorescent protein (GFP) to assess transfection efficiency and to identify cells for voltage-clamp analysis, as previously described. The transient transfection procedure used either 1 μg of rKv1.2/pCMV 4 or 0.6 μg of rKv9.3 and 0.4 μg of GFP/pCI; each was mixed with 15 μL of the lipofectamine reagent, which was 0.5 mL of serum-free Dulbecco’s modified Eagle medium incubated at 37°C for ~30 minutes. Cells were then incubated with the lipofectamine mixture for 6 to 8 hours; after this time, the mixture was removed and replaced with standard culture medium for 36 to 48 hours. Cells were removed from the dish using brief trypsinization; they were then briefly centrifuged and resuspended in fresh standard culture medium at room temperature for recording within the next 12 hours.

The mouse L cells were used specifically because they endogenously express the Kvβ2.1 subunit, which is needed for efficient expression of Kv1.2. Therefore, the rKv2.1/rKv1.5 experiments were performed in the presence of the Kvβ2.1 subunit. Comparison of the hypoxia sensitivity of Kv1.2 and Kv1.5 ± this β was attempted by using the β-free HEK293 cells. However, Kv1.2 was difficult to express in these cells, perhaps due to the lack of the Kvβ2.1 subunit.

Heteromeric Formation of Kv1.2/Kv1.5 and Kv2.1/Kv9.3 Channels

Mouse L cells stably expressing rKv2.1 or hKv1.5 were transiently transfected with either 0.6 or 1 μg of rKv9.3 and rKv1.5, respectively (see above for details). These DNA amounts gave saturating effects in terms of heteromeric channel formation. After transfection, cells were incubated for 36 hours; then, the transfected cells were incubated with 2 μmol/L dexamethasone for 18 to 24 hours to induce the expression of Kv1.5 or Kv2.1 channels and, thereby, allow heteromeric channel formation of these α subunits. Kv9.3 is electrically silent when expressed as a homomeric channel, but when expressed with Kv2.1, it modifies the activation and deactivation kinetics of the Kv2.1 channel and shifts the activation curve in the hyperpolarizing direction. However, because confirmation of heteromeric assembly between Kv1.2 and Kv1.5 α subunits was more difficult to ascertain, the following criteria were used as a guide to assess heteromeric channel formation. The current kinetics and activation curves generated from Kv1.2 and Kv1.5 homomeric channels and Kv1.2/Kv1.5 heteromeric channels allowed one level of characterization because the activation midpoints were approximately 22 and −12 mV for the Kv1.2 and
Kv1.5 homomorphic channels, respectively. When these channels were coexpressed, activation curve data best fit by the sum of 2 Boltzmann equations were disregarded based on the assumption that these data reflected the summation of distinct populations of Kv1.2 and Kv1.5 homomeric channels. Only data best fit with a single Boltzmann equation were analyzed. The DTX sensitivity of expressed currents was also used to assess heteromeric channel formation because DTX specifically blocks Kv1.2 homomeric channels (K_0.5 ~ 20 mmol/L), whereas Kv1.5 homomeric channels are virtually insensitive (K_0.5 > 300 mmol/L). Recent studies suggest that the DTX sensitivity of a Kv1.2/Kv1.5 heteromeric channel is dominated by the presence of the Kv1.5 toxin-insensitive subunit. Therefore, in the present study, Kv1.2/Kv1.5 heteromeric channels containing ≥1 Kv1.5 DTX-insensitive subunit should be insensitive to 50 mmol/L DTX. Only those currents that were DTX-insensitive were considered to represent heteromeric Kv1.2/Kv1.5 channel complexes. Currents showing any DTX sensitivity were not analyzed further.

Electrophysiology
Experiments were performed in a small volume (180 μL) bath mounted on the stage of an inverted microscope (Nikon) continuously superfused at a flow rate of ~0.5 to 1.0 mL/min. Recordings were made with an Axopatch 200A amplifier (Axon Instruments) using the whole-cell configuration of the patch-clamp technique. Patch pipettes (1 to 2 meqohms) were pulled from borosilicate glass capillaries. Junction potentials were corrected after placing the tip of the electrode in the bath solution and before gigohmm seal formation, which was achieved via gentle suction. After establishing the whole-cell configuration, capacitive transients elicited by symmetrical 10 mV voltage clamp steps from ~80 mV were recorded to calculate cell capacitance and access resistance. Cells expressing currents ~5 nA were discarded. Currents were sampled at 1 to 5 kHz after anti-alias filtering at 0.5 to 2 kHz. Data acquisition and command potentials were controlled by pClamp software (v6.04, Axon Instruments) and stored for later off-line analysis. All experiments were performed at room temperature (21°C to 23°C).

Solutions
The intracellular pipette solution contained (in mmol/L): KCl 110, HEPES 10, K_BAPTA 5, K_ATP 5, and MgCl_2 1; it was adjusted to pH 7.2 with KOH. The bath solution contained (in mmol/L): NaCl 110, KCl 4, MgCl_2 1, CaCl_2 1.8, HEPES 10, and glucose 1.8; it was adjusted to pH 7.35 with NaOH. The effects of hypoxia were studied by switching between normoxic and hypoxic perfusate reservoirs. Normoxic solutions were equilibrated with 100% O_2, whereas hypoxic solutions were achieved by bubbling with 100% N_2 for at least 20 minutes before cell perfusion and by passing a stream of N_2 over the surface of the bath. These procedures produced PO_2 values of ~140 to 160 mm Hg and 30 to 40 mm Hg, respectively. PO_2 was continuously monitored in the bath chamber using an O_2-sensitive microelecrotode (ISO/OXEL-1, World Precision Instruments). The pH and temperature of these solutions were regularly monitored and maintained at pH 7.35 and 21°C to 23°C to guard against pH- and temperature-induced changes in channel function. DTX-1 was kindly provided by Dr R. Hartshorne (Oregon Health Sciences, Portland, Ore) and was added to the bath solution where indicated.

Pulse Protocols and Analysis
The holding potential was ~80 mV, and the cycle time within each protocol was 10 s to allow full recovery from inactivation between pulses. The standard protocol used to obtain current-voltage relationships and activation curves consisted of 250-ms voltage-clamp pulses applied in 10 mV steps between ~70 and 60 mV, unless indicated otherwise. Steady-state current-voltage relationships were obtained by measuring the current at the end of the voltage-clamp pulse and plotted against test potential. Currents were normalized relative to each cell's control (normoxic) current at 60 mV. Deactivating tail currents were recorded at ~40 mV, and activation curves were obtained from the maximum value of the tail current amplitude immediately after the capacitive transients.

Activation curves were fitted with a Boltzmann equation:

\[ y = \frac{1}{1 + \exp\left(-\frac{E - E_0}{k}\right)} \]

where \(k\) represents the slope factor, \(E\) is the membrane potential, and \(E_0\) represents the voltage at which 50% of the channels are open. The time course of tail currents were fit with a sum of 2 exponentials. The activation kinetics were determined by fitting a single exponential to the latter 50% of activation. The curve-fitting procedure used a nonlinear least squares (Gauss-Newton) algorithm, and goodness of fit was judged by the \(x^2\) criterion.

Statistical Analysis
All data are expressed as the mean ± SEM of n cells. Comparisons between groups were made using a paired Student's t test. Values of \(P < 0.05\) were considered significant.

Immunohistochemistry
The production and use of the Kv1.5 N-terminal antibody and the immunolocalization of Kv1.2 and Kv1.5 in rat pulmonary resistance arteries were performed as previously described. The Kv1.2 antibody was produced against a glutathione transferase (GST) fusion protein containing C-terminal sequence amino acids 436 to 495 using previously described protocols. A second, affinity-purified Kv1.2 antibody directed against a synthetic peptide, CNED-FREELKTNACTLANT, was provided by Dr Jeanne Nerbonne (Washington University, St. Louis, Mo). Immunofluorescence staining of transfected tissue culture cells confirmed that both Kv1.2 antibodies detected Kv1.2 protein (data not shown). Rat pulmonary resistance vessels (third division, 250 to 300 μm in diameter) were placed in cryomolds, embedded in Tissue Tek (Baxter), and slowly frozen at ~80°C. Cryosections measuring 10 μm were collected on gelatin-coated coverslips and then incubated with primary antibody followed by biotin-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories) and CY3-conjugated streptavidin (Jackson Immunoresearch Laboratories), as previously described. Samples were analyzed using a Nikon E800 microscope equipped with standard epifluorescence and a Princeton Instruments CCD camera.

Immunogen block of tissue staining was performed to demonstrate antibody specificity. Staining was performed as described above, except that cryosections were stained with antisera that had been preincubated overnight at 4°C with 40 mmol/L of either glutathione transferase (GSH-T) or the appropriate GSH-T fusion protein construct, as previously described. The 112 N-terminal amino acids of Kv1.5 coupled to GSH-T were used to block Kv1.5 antisera binding, while C-terminal residues 429 to 496 of Kv1.2 coupled to GSH-T blocked Kv1.2 antisera binding.

Results
O_2 Sensitivity of Kv2.1 Stably Expressed in Mouse L Cells
Because previous work implicated the Kv2.1 channel as an important molecular component of the PA VSMC O_2-sensitive K^- current, we first investigated the O_2 sensitivity of this channel as stably expressed in mouse L cells. Representative outward currents recorded from a holding potential of ~80 mV to a test potential of 60 mV, before and after 10 minutes of exposure to the hypoxic solution, are shown in Figure 1A. Hypoxia reversibly inhibited Kv2.1 current; in the example shown, hypoxia significantly (\(P < 0.05\)) inhibited the current recorded at 60 mV by ~23%. The onset of Kv2.1 current inhibition occurred within ~1 minute after exposure to the hypoxic solution; inhibition continued to decrease to a new steady state after 7 minutes of exposure to hypoxia. This decrease in current paralleled the decrease in PO_2 levels. The mean current-voltage (I-V) relationship of Kv2.1 (Figure 1B) shows that Kv2.1 currents activated at potentials more posi-
Figure 1. Effect of hypoxia on Kv2.1 current expressed in mouse L cells. A, Representative currents elicited in response to step depolarization from −80 mV to 60 mV before, during, and after 10 minutes of exposure to hypoxic solution. B, Graph showing mean I-V relationship for Kv2.1 current (n=10). Currents were normalized relative to each cell’s control (normoxic) current at 60 mV. C, Mean steady-state activation curves for Kv2.1 current, calculated from peak tail current amplitudes at −40 mV (n=10).

Figure 2. Functional expression and O2 sensitivity of Kv2.1/Kv9.3 heteromeric channels. A, Normalized outward currents recorded from L cells transfected with either Kv2.1, Kv9.3, or Kv2.1 plus Kv9.3 in response to step depolarization from −80 to 60 mV. B, Representative Kv2.1/Kv9.3 currents recorded at 60 mV before, during, and after 10 minutes of exposure to hypoxic solution. Inset shows representative Kv2.1/Kv9.3 currents recorded at −20 mV before, during, and after 10 minutes of exposure to hypoxic solution. C, Graph showing mean I-V relationship of normalized Kv2.1/Kv9.3 current (n=6). D, Bar graph highlighting effects of hypoxia on Kv2.1/Kv9.3 current at more negative potentials (data obtained from C).

Functional Expression and O2 Sensitivity of Kv2.1/Kv9.3 Expressed in Mouse L Cells

Patel et al.15 reported the cloning of a novel Kv channel (Kv9.3) from a rat pulmonary artery; this channel does not functionally express as a homomeric channel when expressed alone, but it assembles with Kv2.1 to form a functional heteromeric channel. Although this group reported that the heteromeric channel was reversibly inhibited by hypoxia, the hypoxic sensitivity of this channel was not fully characterized, and it was detected only in a subset (56%) of transfected COS-7 cells.15 In the present study, 99% of the cells expressing the Kv2.1 channel exhibited a hypoxic response; therefore, the O2 sensitivity of the Kv2.1/Kv9.3 heteromeric channel was examined using the L-cell expression system.

Representative current records from L cells expressing Kv2.1, Kv9.3, or Kv2.1/Kv9.3 are shown in Figure 2A. Kv9.3-transfected cells failed to express any Kv channel activity (Figure 2A; n=3); however, coexpression of Kv9.3 with Kv2.1 produced several important modifications in the biophysical properties of the Kv2.1 channel. Kv9.3 increased activation kinetics (Kv2.1: τ=11.25±1.67 ms; Kv2.1/Kv9.3: τ=7.27±0.56 ms; Figure 2A) and slowed deactivation kinetics (Kv2.1: τ=12.66±0.39 ms, τ=61.2±1.16 ms; Kv2.1/Kv9.3: τ=20.13±1.08 ms, τ=142.12±6.55 ms; Figure 2A) relative to Kv2.1 alone. In addition, Kv9.3 caused a hyperpolarizing shift in the voltage dependence of activation (Kv2.1: V0.5=11.37±1.35 mV, n=15; hypoxia: V0.5=10.73±1.27 mV, n=10), which suggests that the observed decrease in Kv2.1 current was not due to a shift in the voltage dependence of activation.

The effect of hypoxia on the voltage dependence of activation was also examined; mean data are shown in Figure 1C. Hypoxia had no effect on the voltage dependence of activation (control, V0.5=11.37±1.35 mV, n=15; hypoxia, V0.5=10.73±1.27 mV, n=10), which suggests that the observed decrease in Kv2.1 current was not due to a shift in the voltage dependence of activation.

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Figure 2B shows that hypoxia reversely inhibited Kv2.1/Kv9.3 current at 60 mV. Moreover, hypoxic inhibition of Kv2.1/Kv9.3 current was detected in all cells studied. Data summarized in Figure 2C compare the effect of hypoxia on the mean I-V relationship for Kv2.1/Kv9.3 current. Mean hypoxic inhibition of Kv2.1/Kv9.3 current at 60 mV was 21±0.9% (n=6), which was not significantly different from Kv2.1 alone (compare values at 60 mV; Figure 1B). However, in contrast to Kv2.1 alone, a greater amount of Kv2.1/Kv9.3 current was activated at more physiologically important potentials, and this current was reversibly inhibited by hypoxia. The observation that hypoxia inhibited the Kv2.1/Kv9.3 heteromeric current at negative potentials (see inset in Figure 2B for currents at −20 mV), where homomeric Kv2.1 current does not activate, further supports the existence of a Kv2.1/Kv9.3 heteromeric complex. Note that at −20 mV and 300 ms, the currents have not reached steady state. However,
At 80 mV, hypoxic inhibition of rKv1.2 averaged 19% and also shows that hypoxia reversibly inhibited rKv1.2 current. Kv2.1, and Kv2.1/Kv9.3 (Figures 1 through 3). Figure 4A shows a 250-ms pulse to 60 mV was needed to fully activate Kv1.5, whereas the inhibition was 25% at 50% activation. As illustrated in Figure 2D, hypoxia caused a reduction of ∼48% in current activated at −30 mV (P<0.05). These data suggest, therefore, that the Kv2.1/Kv9.3 heteromeric channel may indeed be an important molecular component of the native O2-sensitive K+ current.

O2 Sensitivity of Kv1.5

Some authors suggested that Kv1.5 was an important O2-sensing Kv channel present in PA VSMCs; therefore, the O2 sensitivity of this channel was investigated next. Figure 3 shows the effect of hypoxia on hKv1.5 currents stably expressed in mouse L cells. In contrast to Kv2.1 and Kv2.1/Kv9.3, hKv1.5 current at 60 mV was not inhibited by hypoxia (Figure 3A). Moreover, the mean I-V relationship for hKv1.5 current under control and hypoxic conditions shows that although Kv1.5 current activated at physiologically relevant potentials, this current was not O2 sensitive at any potential (Figure 3B; n=9). Hypoxia also had no significant effect on the rat Kv1.5 channel expressed in the same L cells (data not shown).

Figure 3. Effect of hypoxia on Kv1.5 current expressed in mouse L cells. A, Representative currents elicited in response to step depolarizations from −80 mV to 60 mV before, during, and after 10 minutes of exposure to hypoxic solution. B, Graph showing mean I-V relationship of normalized Kv1.5 current (n=9).

O2 expression and O2 Sensitivity of rKv1.2 Expressed in Mouse L Cells

The O2 sensitivity of rKv1.2 transiently transfected into L cells was examined next. Representative traces of rKv1.2 current elicited in response to 1-s voltage-clamp pulses to 80 mV are shown in Figure 4A. Under normoxic conditions, these currents were noninactivating, delayed-rectifying, outward K+ currents that activated much more slowly than the Kv channels described above. For example, a 1-s voltage-clamp pulse to 80 mV was required to fully activate Kv1.2; a 250-ms pulse to 60 mV was needed to fully activate Kv1.5, Kv2.1, and Kv2.1/Kv9.3 (Figures 1 through 3). Figure 4A also shows that hypoxia reversibly inhibited rKv1.2 current. At 80 mV, hypoxic inhibition of rKv1.2 averaged 19±0.2% (n=7; Figure 4B); this inhibition was not associated with a shift in the voltage dependence of activation (data not shown). Although Kv1.2 current at depolarized potentials (>40 mV) was significantly inhibited by hypoxia, no Kv1.2 current was detected at more physiologically relevant potentials (ie, −50 to −20 mV), suggesting that this homomeric channel is unlikely to contribute to the PA VSMC O2-sensitive K+ current.

Kv1.2 and Kv1.5 α Subunits Coassemble to Form an O2-Sensitive Heteromeric Channel

To determine whether Kv1.2 and Kv1.5 α subunits can assemble to form an O2-sensitive heteromeric channel, L cells stably expressing Kv1.5 were transiently transfected with Kv1.2 cDNA. Only those cells meeting the criteria described in Materials and Methods were analyzed.

The electrophysiological and pharmacological characteristics of K+ currents generated by coexpression of Kv1.2 and Kv1.5 α subunits in L cells were examined first. As illustrated in Figure 5A, coexpression of Kv1.2 with Kv1.5 produced currents that exhibited faster activation kinetics and partial inactivation compared with Kv1.2 alone. Nonetheless, it was possible that this K+ current reflected the summation of distinct populations of Kv1.2 and Kv1.5 homomeric channels rather than heteromeric channels composed of Kv1.2 and Kv1.5 α subunits. Analysis of the voltage dependence of activation, however, revealed that these data were best fit with a single Boltzmann equation with an activation midpoint of −4.55±1.09 mV and k=16.13±0.65 (n=5), compared with 21.73±0.65 mV and k=11.11 (n=7) for Kv1.2 alone and −12.88±0.68 mV and k=8.57±0.54 (n=8) for Kv1.5, suggesting that this K+ current represented a single population of Kv1 channels.

To provide pharmacological evidence for the heteromeric formation of Kv1.2 and Kv1.5 α subunits, the blocking effects of the K+ channel toxin DTX-I on Kv1.2, Kv1.5, and Kv1.2/Kv1.5 currents were investigated. Figure 5C shows that 50 nmol/L DTX-I markedly inhibited the Kv1.2 current elicited during a voltage-clamp pulse to 80 mV. Mean data summarized in Figure 5D show that this concentration of DTX-I inhibited Kv1.2 current by ∼80% (n=6). In contrast, Kv1.5 current was insensitive to 50 nmol/L DTX-I (n=4;
Figure 5. Functional expression and DTX sensitivity of Kv1.2/Kv1.5 heteromeric channel. A, Normalized outward currents recorded from L cells transfected with either Kv1.2, Kv1.5, or Kv1.2 plus Kv1.5 in response to step depolarization from −80 mV to 80 mV. B, Graph showing mean steady-state activation curves for Kv1.2, Kv1.5, and Kv1.2/Kv1.5 currents, calculated from peak tail current amplitudes at −40 mV. C, Representative Kv1.2 currents elicited in response to step depolarizations from −80 mV to 80 mV in absence and presence of 50 nmol/L DTX-I. D, Graph shows effect of 50 nmol/L DTX-I on mean I-V relationship of normalized Kv1.2 current (n=6). E, Representative Kv1.2/Kv1.5 currents recorded at 80 mV from holding potential of −80 mV in absence and presence of 50 nmol/L DTX-I. F, Graph shows effect of 50 nmol/L DTX-I on mean I-V relationship of normalized Kv1.2/Kv1.5 current (n=5).

The electrophysiological and pharmacological data described thus far suggest that heteromeric assembly of Kv1.2 and Kv1.5 α subunits was occurring (Figure 5). To determine whether this DTX-insensitive K+ current was O2 sensitive, the effects of hypoxia were examined. Figure 6A shows that hypoxia significantly inhibited the Kv1.2/Kv1.5 current recorded at 80 mV; in the example shown, hypoxia reversibly inhibited current by ∼18%. In addition, the inset in Figure 6A shows that hypoxia also inhibited this current at −20 mV. Figures 6B and 6C summarize the mean data confirming that hypoxia significantly inhibited current at potentials more negative than −20 mV provides further evidence supporting a heteromeric assembly of Kv1.2 and Kv1.5 α subunits and suggests that this O2-sensitive heteromeric channel may be an important molecular component of the O2-sensitive K+ current in PA VSMCs.

To control the subunit stoichiometry of the Kv1.2/Kv1.5 heteromeric complex, a tandem construct was created in which Kv1.5 was placed upstream of, and in frame with, Kv1.2. This tandem had similar activation kinetics and voltage-sensitivity of the heteromeric currents shown in Figures 5A and 5B, respectively (data not shown). Figure 6D shows the tandem construct also had the expected O2 sensitivity.

To confirm the expression and examine the cellular distribution of the Kv1.2 and Kv1.5 channel α subunits in vivo, cryosections of rat pulmonary resistance vessels were stained with Kv1.2 and Kv1.5 antibodies, as shown in Figure 7. Figures 7A and 7B illustrate that Kv1.2 staining was localized to the smooth muscle layer. Because the anti-GST-Kv1.2 fusion protein antibody had not been used previously to stain vascular tissue, Kv1.2 immunolocalization was repeated with a second antibody directed against a peptide immunogen. The same staining pattern was observed with this antipeptide, the affinity-purified Kv1.2 antibody (data not shown). Figures 7E and 7F show the localization of the Kv1.5 channel protein; Kv1.5 was also localized to the smooth muscle layer, as previously described by Archer et al.14 The remaining panels of Figure 7 show controls for antibody specificity. Figures 7C and 7D illustrate Kv1.2 antibody binding after preincubation with either GSH-T or GSH-T/Kv1.2 fusion protein, respectively. Although the antibody binding was unaffected by preincubation with GSH-T, it was eliminated with the channel containing the fusion protein. The same results were obtained with the Kv1.5 antibody after preincubation with...
Figure 7. Immunolocalization of Kv1.2 and Kv1.5 α subunits in rat pulmonary resistance arteries. Resistance vessels (250 to 300 μm, at least third division) were stained with either Kv1.2 (A through D) or Kv1.5 (E through H) antibodies. A and E represent phase images of tissue sections stained with anti-Kv1.2 and anti-Kv1.5 antibodies, B and F, respectively. C and G show binding of anti-Kv1.2 and Kv1.5 antibodies, respectively, after antibody preincubation with GSH-T. D and H illustrate block of specific binding after preincubation with GSH-T/Kv1.2 and GSH-T/Kv1.5 fusion proteins, respectively. Exposure conditions for C, D, G, and H were identical. Note that reduced magnification images in C, D, G, and H are of longitudinally sectioned vessels identical to those illustrated in panels A, B, E, and F. Calibration bar = 100 μm in A.
GSH-T and GSH-T/Kv1.5 fusion protein (Figures 7G and 7H). Taken together, these data confirm that both the Kv1.2 and Kv1.5 channels are expressed in PA smooth muscle cells.

**Discussion**

The observation that hypoxia reversibly inhibited Kv2.1 current is consistent with previous reports suggesting that this channel is an important Kv channel contributing to the $O_2$-sensitive $K^+$ current in PA VSMCs. It seems unlikely, however, that the Kv2.1 channel alone could account for the native $O_2$-sensitive $K^+$ current because this channel, when heterologously expressed, does not activate in the voltage range of PA VSMC $E_{m}$. Indeed, Patel et al.\(^3\) reported that Kv2.1 coassembles with Kv9.3 in COS-7 cells and *Xenopus* oocytes to form a functional heteromeric channel that activates near the range of the PA VSMC $E_{m}$. In support of this hypothesis, in the present study, coexpression of Kv2.1 and Kv9.3 channel $\alpha$ subunits in mouse L cells generated currents that activated at physiologically relevant potentials (Figure 2). More importantly, however, hypoxia reversibly inhibited Kv2.1/Kv9.3 current in the voltage range of PA VSMC $E_{m}$ (Figure 2). Although Patel et al.\(^3\) reported that the Kv2.1/Kv9.3 heteromeric channel was inhibited by hypoxia, these effects were only detected in a subset (56%) of transfected COS cells. In contrast, in the present study, hypoxia reversibly inhibited Kv2.1/Kv9.3 current in all cells studied. Perhaps $O_2$ sensing and the machinery required to couple it to channel function are endogenously expressed in mouse L cells. The finding that hypoxia inhibited Kv2.1/Kv9.3 current activated at physiologically relevant membrane potentials suggests that this heteromeric channel may be an important component of the $O_2$-sensitive $K^+$ current in PA VSMCs.

Although a number of studies have reported that Kv1.5 is expressed in PA VSMCs,\(^4,16\) the $O_2$ sensitivity of this channel and its role in PA VSMCs is less clear. Two independent groups used antibodies specific for the Kv1.5 channel to argue in favor of Kv1.5 as an important molecular component of HPV.\(^4,18\) Furthermore, Wang et al.\(^17\) suggested that chronic hypoxia down-regulates Kv1.5 mRNA and protein expression in cultured PA VSMCs. Although these data suggest that Kv1.5 channels may contribute to the $O_2$-sensitive $K^+$ current in PA VSMCs, in the present study, hypoxia failed to inhibit Kv1.5 current expressed in mouse L cells, despite the fact that this current activated in the range of the PA VSMC $E_{m}$ (Figure 3). This was not due to a species difference because hypoxia failed to inhibit both human and rat Kv1.5 current. Thus, the inability of Kv1.5 to respond to hypoxia suggests that Kv1.5 homomeric channels do not contribute to the $O_2$-sensitive $K^+$ current in PA VSMCs.

Although the presence of Kv1.2 channel mRNA and protein in PA VSMCs has been documented,\(^5,15\) the idea of Kv1.2 as an $O_2$-sensitive Kv channel has been disregarded by several investigators on the basis of its CTX and DTX sensitivity.\(^4,15\) Kv1.2 is blocked in the nanomolar range ($K_C<20$ nmol/L),\(^22\) whereas the PA VSMC $O_2$-sensitive $K^+$ current is virtually DTX- and CTX-insensitive ($K_C>300$ nmol/L).\(^8,11,15\) Heterologous expression of Kv1.2 in mouse L cells, however, revealed that hypoxia reversibly inhibited Kv1.2 current, although hypoxic inhibition was detected only at depolarized potentials (Figure 4). Data presented in Figures 3 and 4 suggest that Kv1.2, but not Kv1.5, homomeric channels are reversibly inhibited by hypoxia. However, comparison of the electrophysiological and pharmacological properties of Kv1.2 and Kv1.5 homomeric channels with those of the native current suggests that, of these two channels, Kv1.5 encodes a channel most resembling the $O_2$-sensitive $K^+$ current in PA VSMCs. One possible explanation for the discrepancies between our data and the literature is that Kv1.2 and Kv1.5 $\alpha$ subunits assemble to form an $O_2$-sensitive heteromeric channel. Indeed, it has been demonstrated in vitro that Kv1.2 and Kv1.5 $\alpha$ subunits can assemble to form a functional heteromeric channel.\(^23,28\)

Our electrophysiological and pharmacological data suggest that Kv1.2 and Kv1.5 $\alpha$ subunits can assemble to generate currents with distinct kinetic and pharmacological properties from those displayed by either Kv1.2 or Kv1.5 homomeric channels (Figure 5). It seems unlikely that this current reflected the summation of distinct populations of Kv1.2 and Kv1.5 homomeric channels rather than a heteromeric channel for several reasons. The activation curve data were best fit with a single Boltzmann function, suggesting the presence of a single population of Kv1.2/Kv1.5 heteromeric channels. It should be noted, however, that in 65% of the voltage-clamped cells, activation curve data were best fit with 2 Boltzmann functions, suggesting that summation of distinct populations of Kv1.2 and Kv1.5 homomeric channels was occurring. However, in 35% of the cells, activation curves were best fit with a single Boltzmann function, suggesting that in these cells, heteromeric channel formation between Kv1.2 and Kv1.5 $\alpha$ subunits was most likely. This low percentage of true heteromeric complexes most likely reflects the less efficient transient expression of Kv1.2 relative to stable Kv1.5 expression. The percentage of GFP-positive cells expressing Kv1.2 current, when transfected into normal L cells, was 80%, and the current densities were lower than those observed in the cells stably expressing Kv1.5. Thus, the reason only 35% of the Kv1.5-expressing cells showed complete heteromeric channel formation after transient transfection with Kv1.2 is probably due to the fact that (1) only 80% of the cells express any Kv1.2 current and (2) in those cells expressing Kv1.2, less than half make enough Kv1.2 protein to ensure complete heteromeric formation given the amount of Kv1.5 protein already expressed in the cell line.

These heteromeric currents were also insensitive to 50 nmol/L DTX, consistent with the idea that the presence of the Kv1.5 toxin-insensitive channel subunit dominates the pharmacology of the heteromeric channel.\(^24,28\) More importantly, however, the DTX-sensitive currents were reversibly inhibited by hypoxia at physiologically relevant potentials (from $-40$ to $-20$ mV). The latter observation cannot be explained by the summation of distinct populations of Kv1.2 and Kv1.5 homomeric channels because at these negative potentials (reflecting current flow purely through Kv1.5 channels), hypoxia would not be expected to inhibit current due to the fact that Kv1.5 channels are $O_2$ insensitive. The finding that hypoxia inhibited current at these negative potentials (Figure 6) further argues in favor of heteromeric formation of Kv1.2
and Kv1.5 α subunits. It seems, therefore, that Kv1.2 and Kv1.5 α subunits can assemble to form a functional heteromeric channel and that the presence of two Kv1.2 subunits, as in the tandem, confer O$_2$ sensitivity on this heteromeric channel. The Kv1.2/Kv1.5 heteromeric current is a delayed rectifier with apparent slow inactivation (activation between −40 and −30 mV) and a lack of DTX sensitivity. Thus, it does resemble the O$_2$-sensitive currents recorded from native rat PA VSMCs.

In summary, the present study examined the O$_2$ sensitivity of several cloned Kv channels expressed in mouse L cells in an attempt to identify which of these Kv channels may contribute to the O$_2$-sensitive K$^+$ current in PA VSMCs. Our results suggest that (1) Kv1.2 and Kv2.1, but not Kv1.5, homomeric channels are reversibly inhibited by hypoxia; (2) Kv1.2 and Kv1.5 α subunits assemble to form an O$_2$-sensitive heteromeric channel; and (3) of these channels, only the Kv1.2/Kv1.5 and Kv2.1/Kv9.3 heteromeric channels are reversibly inhibited by hypoxia in the voltage range of the PA VSMC E$_{m0}$. Although beyond the scope of the present study, an obvious question deals with the mechanism linking hypoxic conditions to Kv channel function. Possible mechanisms include channel internalization, channel protein phosphorylation, or direct O$_2$ sensing by channel sulfhydryl groups. Whatever the mechanism, it is channel isoform-specific and influenced by subunit composition. A major limitation of our study is that it deals with cloned channels expressed in heterologous systems and not with native VSMCs. Confirmation of the expression of heteromeric complexes and their regulation by hypoxia in native PA VSMCs is the next step in elucidating the molecular mechanisms responsible for hypoxia-induced pulmonary vasoconstriction.

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References

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