Preferential Expression and Function of Voltage-Gated, O$_2$-Sensitive K$^+$ Channels in Resistance Pulmonary Arteries Explains Regional Heterogeneity in Hypoxic Pulmonary Vasoconstriction

Ionic Diversity in Smooth Muscle Cells

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Abstract—Hypoxic pulmonary vasoconstriction (HPV) is initiated by inhibition of O$_2$-sensitive, voltage-gated (Kv) channels in pulmonary arterial smooth muscle cells (PASMCs). Kv inhibition depolarizes membrane potential (E$_{m}$), thereby activating Ca$^{2+}$ influx via voltage-gated Ca$^{2+}$ channels. HPV is weak in extrapulmonary, conduit pulmonary arteries (PA) and strong in precapillary resistance arteries. We hypothesized that regional heterogeneity in HPV reflects a longitudinal gradient in the function/expression of PASMC O$_2$-sensitive Kv channels. In adult male Sprague Dawley rats, constrictions to hypoxia, the Kv blocker 4-aminopyridine (4-AP), and correolide, a Kv1.x channel inhibitor, were endothelium-independent and greater in resistance versus conduit PAs. Moreover, HPV was dependent on Kv-inhibition, being completely inhibited by pretreatment with 4-AP. Kv1.2, 1.5, Kv2.1, Kv3.1b, Kv4.3, and Kv9.3. mRNA increased as arterial caliber decreased; however, only Kv1.5 protein expression was greater in resistance PAs. Resistance PASMCs had greater K$^+$ current (I$_K$) and a more hyperpolarized E$_{m}$ and were uniquely O$_2$- and correolide-sensitive. The O$_2$-sensitive current (active at $-65$ mV) was resistant to iberiotoxin, with minimal tityustoxin sensitivity. In resistance PASMCs, 4-AP and hypoxia inhibited I$_K$ 57% and 49%, respectively, versus 34% for correolide. Intracellular administration of anti-Kv1.5 antibodies inhibited correolide’s effects. The hypoxia-sensitive, correolide-insensitive I$_K$ (15%) was conducted by Kv2.1. Anti-Kv1.5 and anti-Kv2.1 caused additive depolarization in resistance PASMCs (Kv1.5$>$Kv2.1) and inhibited hypoxic depolarization. Heterologously expressed human PASMC Kv1.5 generated an O$_2$- and correolide-sensitive I$_K$ like that in resistance PASMCs. In conclusion, Kv1.5 and Kv2.1 account for virtually all the O$_2$-sensitive current. HPV occurs in a Kv-enriched resistance zone because resistance PASMCs preferentially express O$_2$-sensitive Kv-channels. (Circ Res. 2004;95:308-318.)

Key Words: immunoelectropharmacology ■ laser capture microdissection ■ voltage-gated channels ■ pulmonary circulation ■ adenoviral gene transfer

The adult pulmonary circulation is a low-resistance circuit designed for gas exchange that is perfused by a thin-walled, afterload-intolerant right ventricle. The pulmonary vasculature consists of large, elastic, extraparenchymal “conduit” arteries and small, muscular intrapulmonary arteries, which control regional distribution of blood flow and largely determine pulmonary vascular resistance (PVR). Hypoxic pulmonary vasoconstriction (HPV) is a widely conserved mechanism for ventilation–perfusion matching.$^1$ With segmental hypoxia (eg, atelectasis), resistance pulmonary arteries (PAs) serving the hypoxic lobes constrict, diverting blood to better-oxygenated segments, thereby optimizing systemic Po$_2$, without increasing PVR.$^{2,3}$ Microangiography$^4$ and micropuncture$^5$ reveal that HPV primarily occurs in resistance PAs (<200 μmol/L diameter, division 4, and distal), with lesser contributions from larger intrapulmonary arteries and pulmonary veins. Conversely, conduit PAs (division 1 to 2) dilate or fail to constrict to hypoxia.$^6$ This anatomical (proximal–distal) and functional (conduit-resistance) distinction extends to the cellular level, with evidence for K$^+$ channel diversity within and between segments.$^7$ Whole-cell potassium currents (I$_K$) in conduit PA smooth muscle cells (PASMC) manifest contributions from both voltage-gated (Kv) and large-conductance, calcium-sensitive (BK$_{Ca}$) channels; however, I$_K$ in resistance PASMCs predominantly reflect Kv current.$^3$ This diversity may reflect the dual origins of the adult pulmonary circulation from vascular beds with different embryological origins that fuse late in development.
Conduit PAs originate from the sixth aortic arch, whereas resistance PAs originate from the mesenchymal lung bud by capillary plexus expansion.\textsuperscript{10,11}

HPV is initiated by inhibition of Kv channels.\textsuperscript{12-16} Constrictor responses to hypoxia and 4-aminopyridine (4-AP), a Kv channel inhibitor, are greatest in resistance PAs. Likewise Kv current density is greater in resistance PASMCs.\textsuperscript{7,17} Candidate O\textsubscript{2}-sensitive Kv channels include Kv1.5,\textsuperscript{18,19} Kv2.1/9.3,\textsuperscript{19,20} Kv3.1b,\textsuperscript{21} and Kv1.2.\textsuperscript{22}The importance of Kv1.5 and Kv2.1, 4-AP-sensitive channels, and redox-sensitive channels to HPV is emphasized by 2 models that manifest selective suppression of HPV, the chronic hypoxic pulmonary hypertension (PHT) model, and the Kv1.5 knockout mouse. In chronic hypoxia, emphasis on 2 models that manifest selective suppression of HPV, the chronic hypoxic pulmonary hypertension (PHT) model, and the Kv1.5 knockout mouse. In chronic hypoxia, impairment of HPV results from loss of Kv1.5 and Kv2.1 expression with concordant suppression of O\textsubscript{2}-sensitive I\textsubscript{K}.\textsuperscript{14,23,24}

Electrophysiology

Whole-cell electrophysiology was performed on enzymatically dispersed PASMCs from conduit or resistance PAs (see online supplement).\textsuperscript{30,31} PASMCs and CHO cells (control or infected with adenovirus for Kv1.5 or Kv2.1)\textsuperscript{23} see online supplement) were studied at 25°C and 35°C using voltage and current clamp configurations. Currents were evoked by 200-ms test pulses, filtered at 1 kHz, and sampled at 2 to 4 kHz. Two-step voltage protocols were used: from −70 to +70 mV in 20-mV steps and from −70 to −10 mV in 5-mV steps (to better-characterize the O\textsubscript{2}-sensitivity of the current controlling E\textsubscript{M} while avoiding Kv inactivation). Current density was calculated by dividing average plateau phase I\textsubscript{K} by the manually measured cell capacitance (pA/pF). To record E\textsubscript{M}, cells were held at their resting E\textsubscript{M} in current-clamp mode.

Heterologous Expression of Kv1.5 and Kv2.1

To assess the sensitivity and specificity of the anti-Kv antibodies for their respective channels, CHO cells, which do not express an endogenous Kv current, were infected with replication-deficient serotype-5 adenovirus encoding genes for green fluorescent protein and either human PASMC-derived Kv1.5 or rat Kv2.1 (see online supplement).\textsuperscript{18,25,31,32}

Immunoelectropharmacology was performed in the whole-cell configuration, as previously described.\textsuperscript{19} In addition, single-channel experiments were performed to identify the O\textsubscript{2}-sensitive channels using the inside-out patch configuration (see online supplement). Preliminary experiments established that 1 μL of Kv1.5 or Kv2.1 antibody in 150 μL of internal pipette solution depolarized PASMCs. The difference in I\textsubscript{K} between the recording 1 to 2 minutes after membrane rupture and a recording at 10 minutes was defined as the “antibody effect.” IgG1 antibodies created from glutathione-S-transferase–fusion proteins were directed against intracellular, carboxyl terminus amino acids (542 to 602 of rat Kv1.5 and 509 to 853 of rat Kv2.1). No “roll-off” of I\textsubscript{K} was observed in controls for up to 30 minutes. Significant changes in the biophysical stability of the seal or appearance of leak current disqualified cells from analysis.

LCM and qRT-PCR

qRT-PCR was performed on LCM samples from flash-frozen lung (see online supplement). Expression was normalized to the SMC-specific reporter, SM22α.

Immunoblotting

PAs were frozen in liquid N\textsubscript{2}, and homogenized in buffer containing an antiprotease cocktail before being placed on a 7.5% gel.\textsuperscript{19,34} Signal intensity was normalized to actin expression (Santa Cruz Biotechnology, Santa Cruz, Calif.).

Drugs and Statistics

Drugs are given in saline and, unless otherwise stated, are from Sigma-Aldrich Inc (St. Louis, Mo). Correolide was a gift from John Obenchain (Merck & Co, Inc, Rahway, NJ). Kir6.1 antibody was a gift from Dr H. Nakayama, Kumamoto, Japan. Antibodies for immunoelectropharmacology were obtained from US Biological (Swampscott, Mass) and Sigma-Aldrich. Values are given as mean±SEM. Intergroup comparisons are made using an ANOVA with post hoc testing using Fisher probable least significant differences test. P<0.05 was considered statistically significant.

Results

HPV Is Strongest in Resistance PAs and Is Endothelium-Independent

Acetylcholine-induced relaxation of PA rings was similar in conduit and resistance PAs (Figure 1A and 1B). In endothelium-intact rings, contraction to low-dose correolide (1 to 10 μmol/L) was similar in magnitude to HPV and was strongest in resistance PAs. Both the absolute constrictor
response to hypoxia (Figure 1C) and the constriction expressed as a percentage of phenylephrine constriction were greatest in resistance PAs (Figure 1E). 4-AP constriction was also strongest in resistance arteries (Figure 1E). Iberiotoxin, a specific BKCa channel inhibitor, did not significantly constrict either segment whereas BaCl, an inhibitor of inward rectifier K+ channels, caused similar constriction in conduit and resistance PAs (data not shown). Endothelial denudation converted acetylcholine relaxation to constriction (Figure 1B) but did not alter the magnitude or onset kinetics of HPV. Hypoxia caused no constriction when superimposed on constriction to 10 mmol/L 4-AP, although phenylephrine constricted these rings normally (Figure 1F), suggesting that the Kv channels mediating HPV are a subset of the resistance PASMC family of 4-AP-sensitive channels.

Increased Expression of Kv mRNA and Kv1.5 Protein in Resistance PAs

qRT-PCR on LCM-derived specimens demonstrates a stepwise, proximal-to-distal increase in expression of Kv channel mRNA, relative to the SMC reporter, SM22α (Figure 2A and 2B). However, only Kv1.5 protein was more abundant in resistance PAs (Figure 2C).

Resistance PASMCs Are Enriched in O2-Sensitive Kv Current

SMCs from resistance and conduit PAs have voltage-gated, rapidly-activating, noninactivating I_K (Figure 3). However, resistance PASMCs have greater normoxic current density, despite smaller cell size, as measured by capacitance (Figure 3B). Subtraction analysis shows that Kv current in resistance PASMCs activates at the resting E_m of ≈-60 mV (whether measured at 25°C or 37°C, Figures 3D and 4B), consistent with the participation of Kv channels in setting the PASMC E_m. 4-AP and hypoxia reversibly inhibit ≈57% and ≈49% of I_K, respectively (at 35°C, +70 mV), in resistance PASMCs (Figure 3C and 3D), whereas correolide only inhibits ≈34% of I_K (Figure 5D). Thus, hypoxia inhibits the same channels as 4-AP, whereas the Kv1.5 inhibitor correolide leaves approximately one third of the O2-sensitive uninhibited, a component that is attributable to Kv2.1. The O2-sensitive current
is not blocked by iberiotoxin but is partially inhibited by tityustoxin (Figure 4C). Hypoxia’s effects onset within 1 minute and are reversible. Conduit PASMCs have virtually no hypoxia-sensitive, correolide-sensitive, or 4-AP-sensitive \( I_K \) (Figure 5).

**Human PASMC Kv1.5 Channels Are Inhibited by Correolide and Hypoxia**

CHO cells lack endogenous Kv current and manifest only an ohmic current (Figure 6A). Cells expressing Kv1.5 were selected based on their GFP fluorescence (Figure 6B). This heterologous expression system was used to study the specificity of immunoelectropharmacological current-dissection technique and to characterize the \( O_2 \)-sensitivity of this candidate \( O_2 \)-sensitive channel. The specificity of the anti-Kv1.5 and anti Kv2.1 antibodies was confirmed by showing that they inhibit their respective clonal Kv channels but do not cross-react. Anti-Kv2.1 does not inhibit Kv1.5 current (Figure 6C) and vice versa (see online data supplement). Moreover, antibodies to an unexpressed \( K^+ \) channel, anti-Kir2.1, had no effect in Kv1.5-CHO cells (Figure 6C). \( I_{K,15} \) in infected CHO cells was similar to \( I_K \) in resistance PASMCs (ie, rapidly activating, noninactivating, and reversibly inhibited by hypoxia, 4-AP, and correolide; Figure 6D and 6E).

**Electrophysiological Effects of Correolide Reflect Inhibition of Kv1.5**

Resistance PASMCs are relatively hyperpolarized compared with conduit PASMCs and, unlike conduit PASMCs, depolarize in response to correolide in a dose-dependent manner (Figure 7A and 7B). In the presence of correolide, hypoxia has reduced ability to depolarize the resistance PASMC, indicating a predominant, but not exclusive, role for Kv1.x family channels in hypoxic depolarization (Figure 7A). Similarly, most (but not all) of the hypoxia-sensitive \( I_K \) is inhibited by anti-Kv1.5 (Figure 7C). To assess the extent to which correolide-induced depolarization was caused by Kv1.5 inhibition, endogenous Kv1.5 was blocked using immunoelectropharmacology. Pretreatment with anti-Kv1.5, but not anti-Kv2.1, eliminated the depolarizing effects of correolide (Figure 7C and 7D).

**Kv1.5 and Kv2.1 Set \( E_M \) and Account for \( O_2 \)-sensitive \( I_K \) in Resistance PASMCs**

Antibodies against Kv1.5 cause more depolarization than anti-Kv2.1 (Figure 7E and 8A), and the combination of
anti-Kv1.5+2.1 caused greater depolarization than either antibody alone. The combination completely blocked hypoxic depolarization of the PASMC (Figure 8A), achieving a greater inhibition of hypoxic depolarization than correolide alone (Figure 7B), indicating a contributory role for the correolide-insensitive channel Kv2.1 (Figure 7A). Single-channel data confirm that the most resistant PASMC patches show activity of only 2 to 3 discrete K⁺ channel conductances (not shown). Anti-Kv1.5 decreases the open probability of a 33-pS channel (Figure 8B).

Discussion

O₂-sensitive K⁺ channels mediate acute responses to changes in P-O₂ in the PASMC,2,3 the carotid body type-1 cell,35 adrenomedullary cells,36 neuroepithelial bodies,37 and the ductus arteriosus SMC.31 Although this role for K⁺ channels is widely conserved, there is significant diversity in the channel types implicated (various Kv channels versus BK Ca). Moreover, the downstream response to channel inhibition (vasoconstriction, neurotransmitter release) varies among species, between tissues, and with maturation.3

This study offers 5 findings that increase our understanding of K⁺ channel diversity in vascular biology. First, resistance PAs constitute an electrically unique zone. HPV occurs here because resistance PASMCs have a virtual monopoly on the functional expression of O₂-sensitive Kv channels (Figures 2 through 4), specifically Kv1.5 and, to a lesser extent, Kv2.1 (Figure 8A). Second, E_M in resistance PASMCs is set by Kv1.5 and Kv2.1 (Figures 7 and 8), with a minor contribution from a tityustoxin-sensitive channel (Kv1.2 and/or Kv1.3) (Figure 4C). Third, it is the SMC, not endothelial, K⁺ channel heterogeneity, that accounts for the divergent response to hypoxia and 4-AP. The current study definitively shows that HPV and 4-AP constriction are endothelium-independent (Figure 1E). Fourth, the specificity of the immunoelectropharmacology technique for dissecting the complex mosaic of ionic currents in vascular SMCs was validated, both in PASMCs and in CHO cells heterologously expressing a single Kv channel type (Figure 6C and online Figure I). Finally, human PA Kv1.5 was found to be intrinsically sensitive to hypoxia, 4-AP, and correolide, similar to the O₂-sensitive I_K in resistance PASMCs (Figure 6).

Hypoxia constricts resistance PAs by an endothelium-independent mechanism, at least for the first hour of hypoxia (Figure 1E and 1F). Although the endothelium modulates HPV,38 the endothelium-independence of the core mechanism has been reported.39,40 Proponents for an obligatory role of the endothelium in HPV acknowledge that the initial phase

![Figure 3. Hypoxia inhibits I_K in resistance, but not conduit, PASMCs. A, Representative traces of the hypoxia-sensitive I_K and PASMC current density. B, Resistance PASMC are smaller than conduit PASMCs, as measured by capacitance. C, Only resistance PASMCs have a large O₂-sensitive I_K component at 25°C. D, Subtraction analysis shows that the hypoxia-inhibited portion of I_K activates at -65 mV and accounts for half of the current of resistance PASMC.](image-url)
of HPV is endothelium-independent. Many of the studies in which endothelium is found to be “essential” have studied HPV only after first “priming” (precontracting) with agents such as prostaglandin F2α. This results in hypoxic responses comprising a transient initial constriction, a relaxation, and then a slowly developing secondary phase. The confounding effects of priming are unknown, but HPV in vivo does not require priming, nor is priming required for robust HPV in healthy, isolated resistance PA rings (Figure 1). HPV increases in strength over 60 minutes to a plateau, without an initial transient contraction–relaxation phase, despite the documented absence of acetylcholine relaxation (Figure 1F), consistent with HPV in humans. Carlson reported, “There is an immediate vasoconstrictor response to hypoxia in the human lung and there is no further potentiation or diminution, of the response during a 60-minute period of hypoxia.”

The current study provides new details of molecular expression profiles in the pulmonary circulation and confirms the view that PASMCs are arrayed in a rich mosaic, in which resistance arterial segments, which control regional blood flow and vascular resistance, have greater levels of expression of Kv channels than do conduit arteries, whose function it is to deliver deoxygenated blood to the entire lung. Hypoxic vasoconstriction is solely found in the pulmonary circulation (hypoxia dilates most systemic arteries) and its localization appears to be unique, largely as a result of the preferential presence of hypoxia-inhibited K currents in resistance PASMCs. Systemic arterial SMCs, such as canine renal and rodent mesenteric SMCs, lack a hypoxia-inhibited IK. In this regard, the conduit PA is essentially a systemic artery.

It has been difficult to isolate the molecular species of the Kv channel(s) responsible for setting EM and conducting the O2-sensitive currents. Single-channel studies have demonstrated that hypoxia rapidly and reversibly inhibits several K currents (channel types), including a 37-pS KDR (now classified as a Kv channel and very similar to the 33-pS conductance we measure for PASMC Kv1.5; see online Figure III). The pharmacology of the O2-sensitive K currents in PASMCs (rapidly activating, slowly-inactivating, 4-AP–sensitive, resistant to charybdotoxin and glyburide) suggests certain candidate O2-sensitive chan-

Figure 4. Hypoxia depolarizes EM at 35°C by inhibiting a Kv current in resistance PASMCs. A, Representative tracing and mean±SEM showing reversible hypoxic depolarization of a PASMC by hypoxia. B, Resistance PASMCs have a large O2-sensitive component to IK. The hypoxia–subtraction current is similar to that recorded at 25°C. C, The hypoxia-inhibited component of IK is evident at −65 mV and is not impaired by iberiotoxin and only slightly reduced by tityustoxin.
nels, including Kv1.2, Kv1.5, Kv2.1, and possibly Kv3.1b. Each of these channels is present in PASMCs and each has its proponents. Likewise, this pharmacology tends to exclude a role for 4-AP–insensitive channels (BK Ca), rapidly inactivating channels (Kv1.4 and Kv4.3), and charybdotoxin-sensitive channels (e.g., homotetrameric Kv1.2 or Kv1.6 channels).

There is direct evidence that HPV is dependent on Kv in that preconstriction with the Kv blocker 4-AP eliminates subsequent HPV, even though the PA constricts vigorously to phenylephrine (Figure 1F). Moreover, the identities of the relevant O2-sensitive Kv α-subunits are confirmed to be Kv1.5 and Kv2.1. The strong parallel between correolide and hypoxic constriction suggests that of the candidate Kv channels, a Kv1.x channel is central to the mechanism of HPV.

Correolide's specificity for Kv1.5 channels (at least in PAs) is supported by the finding that its ability to depolarize PASMCs is blocked by pretreatment with anti-Kv1.5, but not anti-Kv2.1, antibodies (Figure 7E). Theoretically, correolide can inhibit Kv1.2; however, tityustoxin, an inhibitor of Kv1.2/Kv1.3, inhibits only a small portion of the hypoxia-sensitive I_K (Figure 4C). Moreover, after exposure to anti-Kv1.5 plus anti-Kv2.1, little responsiveness to hypoxia remains (Figure 8A). Finally, although mRNA for many Kv channels is more abundant in resistance versus conduit PAs, only Kv1.5 protein is more abundant distally (Figure 2).

Further support for the physiological relevance of Kv1.5 to HPV comes from studies of heterologously expressed Kv1.5. The Kv1.5 channel used in these studies was derived from human PA mRNA, which is important not only because of tissue heterogeneity in formation of heterotetramers and association with β-subunits but also because of the presence of tissue-specific alternatively spliced variants. Overturf et al cloned Kv1.5 from canine colonic SMCs and noted that whereas there was conservation of the channel's transmem-
brane S1 to S6 segments with other Kv1.5 species, homology decreased to 74% to 82% in the NH2 and COOH terminal segments, with unproven, but likely, functional consequences. Thus, Kv1.5 isoforms with discrete pharmacology, O2 sensitivity, and slope conductances (10 pS for colonic SMC versus 32 pS for PASMC) exist, highlighting the need to specify the origins of the Kv1.5 isoform being studied. In CHO cells, human PA–Kv1.5 recapitulates the characteristics of the endogenous O2-sensitive IK in PASMCs (voltage-gated, inhibited by 4-AP, correolide, and hypoxia, Figures 4 and 6).

It is often questioned whether the K+ channels are active at sufficiently negative potentials to set the resting EM of PASMCs. We carefully studied this using a protocol that minimized the risk of inactivating Kv channels by avoiding exposure to potentials above −10 mV (Figure 4C) at physiological temperatures (35°C). There is an outward Kv current at −65 mV, which is inhibited by hypoxia (Figure 4B). Inhibiting this current depolarizes the PASMC. Thus, the consequence of enriched Kv expression in resistance PAs (Figure 2) is a relative hyperpolarization of resistance versus conduit PASMCs (∼ −60 mV versus ∼ −35 mV; Figures 4A and 8A). As would be predicted, inhibition of both Kv1.5 and Kv2.1 depolarizes resistance PASMCs to an EM similar to those in control conduit PASMCs (∼ −35 mV) and eliminates hypoxic depolarization (Figure 8A). The relative lack of effect of either anti-Kv antibody or correolide on conduit PASMC EM suggests that other channels, transporters, and/or pumps set EM in conduit PASMC.

Kv2.1, alone or in combination with Kv9.3, has a pharmacological profile and O2-sensitivity, consistent with a role in HPV.19,20,43 Kv2.1 contributes to setting the resting EM in PASMCs and to HPV19 (Figure 8A). The current study suggests that Kv2.1 accounts for the portion of 4-AP-sensitive current that is correolide-insensitive. Hogg et al also used immunoelectropharmacology to demonstrate that anti-Kv2.1 inhibited normoxic IK (∼67% at +50 mV) and reduced the hypoxia-responsive IK.43 They did not assess the effects of anti-Kv1.5 and concluded that Kv2.1 plays a crucial role in HPV and in setting EM. The quantitative difference in the importance of Kv2.1 may relate to the dose and specificity of the Kv2.1 antibody used. Moreover, they did not rigorously establish the specificity of the anti-Kv2.1 antibodies. Our data

![Figure 6. Human PA Kv1.5 is inhibited by hypoxia, correolide, and 4-AP. A and B, CHO cells have no intrinsic Kv current but, when infected with an adenovirus carrying Kv1.5, develop an IK (the GFP reporter allows selection of green cells for patch clamp studies). C, Specificity of the immunoelectropharmacology was confirmed by demonstrating that only the anti-Kv1.5 antibody inhibited IK in Kv1.5-infected CHO cells. D and E, Representative and mean ± SEM showing that IK in a CHO cell is O2-sensitive and has similar sensitivity to correolide and 4-AP as IK in a resistance PASMC.](image-url)
clearly show that the antibodies directly inhibit their target channels without cross-reactivity (Figures 6C and 8 and online Figure I). Smirnov et al, using conventional patch clamp techniques, found 2 populations of conduit PASMCs: one with 4-AP and tetraethylammonium (TEA) sensitivity and the other with only 4-AP sensitivity. In contrast, resistance PASMCs uniformly demonstrated a 4-AP-sensitive current. Although they performed no molecular studies, the pharmacology suggested the TEA-sensitive Kv current was Kv2.1, and the exclusively 4-AP-sensitive current was Kv1.5, an interpretation consistent with previous publications.

Patch-clamp surveys of PASMC electrical phenotypes have revealed greater functional expression of L-type calcium channels and Kv channels in resistance PASMCs, and more BKCa current in conduit PASMCs. In addition to this longitudinal diversity, phenotypically and electrophysiologically distinct cell populations coexist side-by-side within a single segment, constituting an electrical mosaic. In the pulmonary circulation, SMC diversity is not restricted to the expression of ion channels. Frid et al identified 4 populations of PASMCs in calf conduit PA, based on expression of contractile and cytoskeletal proteins (actin, myosin, calponin, desmin, and metavinculin). These "phenotypes" responded differently to chronic hypoxia, with increased proliferation exclusively in "meta-vinculin-negative" PASMCs. Regional heterogeneity of K+ channel expression has been reported in the heart and brain, where differences in distributions and properties of K+ channels contribute to regional differences in the seizure susceptibility.

In conclusion, K+ channel diversity is a common but underappreciated phenomenon that needs to be considered in the design and interpretation of vascular biology studies. It is the enrichment of resistance PASMCs with the O2-sensitive Kv channels that accounts for the localization of HPV to this zone of the pulmonary circulation. Regional ionic diversity permits resistance PAs to match local perfusion to ventilation without increasing PVR and right ventricular afterload. The molecular basis for this ionic diversity requires further study.

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**Vascular Reactivity:** Vascular reactivity was assessed as previously described\(^1\). The optimal tension was defined as the tension at which the constriction to phenylephrine (1µM) was maximal (range 400-2000 mg). Care was taken to ensure rings were dissected on ice and mounted within 20 minutes of excision. Tension was recorded using a transducer connected to an analog-digital converter (ADInstruments, Mountain View, CA).

**Enzymatic dispersion of PASMCs:** Enzymatic dispersion was performed on freshly isolated PAs each day. The same cocktail was used on conduit and resistance PAs and conformed to prior published protocols\(^1\). Immediately after harvesting from anesthetized, adult male Sprague Dawley rats, PAs were placed in “Ca++-free” Hanks solution for 30 min at 4°C and then digested in a solution containing (mg/ml): papain 1.0, bovine albumin 0.75 and dithiothreitol 0.85 (4°C for 15 min and then 37°C for 10 min). Arteries were washed with Hank’s solution (without EGTA) for 15 minutes and then placed on ice in Hank’s solution supplemented with glucose (1mg/ml). Gentle trituration produced a suspension of single PASMCs.

**Whole cell patch clamp solutions:** Cells were pipetted into a perfusion chamber of an inverted microscope stage and were superfused with a solution containing (mM) NaCl 140; KCl 4.2; CaCl\(_2\) 1.5; KH\(_2\)PO\(_4\) 1.2, MgCl\(_2\) 0.5, HEPES 10; glucose 10 (pH 7.4). Pipettes (resistance 1-3MΩ) were filled with a solution containing (mM): KCl 134, KH\(_2\)PO\(_4\) 1.2, MgCl\(_2\) 1.0, HEPES 5, pH 7.30, Na\(_2\)ATP 5 and EGTA 5. For
immunoelectropharmacology the antibody was added to the intracellular solution. Studies were performed both at 25ºC and 37ºC.

**Single channel patch clamp solutions:** Inside out K+ single channels were recorded with the symmetrical KCL (140mM) condition, as previously described\(^1\). Pipettes (resistance 3-5 MΩ) were filled with a solution containing (mM) KCL 140; CaCl\(_2\) 1.0; KH\(_2\)PO\(_4\) 1.2; MgCl\(_2\) 1.0; HEPES 10; glucose 5.0 (pH 7.4). Bath solution was identical with Pipettes solution except for the bath solution contained 5 mM Na2ATP and pH was 7.2. For immunoelectropharmacology the antibody was added to the extracellular solution.

**LCM and qRT-PCR:** Lung sections were mounted without a coverslip on nuclease-free microscope slides, dehydrated using the HistoGene LCM Frozen Section Staining Kit and then large, intermediate and small resistance PAs were harvested using the PixCell II LCM system (Arcturus, Mountainview CA), as previously described\(^2,3\). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed on LCM specimens, as previously described\(^2,3\).

To perform qRT-PCR, total RNA was extracted from LCM Cap using PicoPure RNA Isolation Kit (Arcturus). qRT-PCR was performed using the relevant primer (500nM), a species-specific TaqMan probe (200nM), and TaqMan One-Step RT-PCR Master Mix (25µl total volume). The assay was performed on an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). 40 cycles of PCR are
performed and mRNA abundance expresses as $2^{\Delta\Delta C_t}$, a ratio of the relative expression of Kv channels normalized to expression of a reporter (e.g. SM22-α) and a calibrator sample. The probes and resulting products have been fully validated and there was no contamination with DNA.

Infection of CHO cells with Adenovirus:

**Adenovirus with human Kv1.5 and a GFP reporter**: $(1.2 \times 10^{10})$. Transient infection of CHO cells with adenovirus was performed as previously described. The infection was carried out with 50 µl of viral stock at an estimated MOI of 300. The virus was infected with serum free media for 6 hours followed by replacement of the whole media including viral particles with media with serum for 18 hours of incubation. The adenovirus carrying rat Kv2.1 had a lower titre $4 \times 10^9$ and the MOI was 1000.

**On-line Figure Legends**:

**Figure 1 online: Quantification of $K^+$ channel expression**: Channel protein expression in conduit vs. resistance PAs was measured using immunoblot technique. PAs were isolated from four rats. The relevant $K^+$ channel band was scanned and the intensity normalized to the reporter actin using Adobe Photoshop. Only Kv1.5 protein is more abundant in resistance PASMCs.

**Figure 2 online: Validation of the specificity of Kv2.1 antibody**
Inhibition of specific PASMC Kv channels was achieved by immunoelectropharmacology. Specificity of the immunoelectropharmacology was confirmed by demonstrating that only the anti-Kv2.1 antibody inhibited \( I_K \) in Kv2.1-infected CHO cells.

**Figure 2 online: Validation of the specificity of Kv2.1 antibody**

Inhibition of specific PASMC Kv channels was achieved by immunoelectropharmacology. Specificity of the immunoelectropharmacology was confirmed by demonstrating that only the anti-Kv2.1 antibody inhibited \( I_K \) in Kv2.1-infected CHO cells.

**Figure 3 online: Anti-Kv1.5 inhibits a 33 pS channel in resistance PASMCs**

Probability histograms corresponding to Inside-out patch clamp data in Figure 8 showing that anti-Kv1.5 inhibits a 32 pS Kv channel (PA-Kv1.5). That patches were excised from resistance PASMCs (representative of \( n=8 \)). Note the inhibition of 2-channel (2.6 pA) and 1-channel (1.3pA) openings with the antibody. Anti-Kv1.5 reduces channel open probability. The slope conductance of PASMC Kv1.5 in symmetrical \( K^+ \) was 32.8 pS.

**References for on-line supplement:**


CHO-Kv2.1

Figure 1: On-line

- Control
- Kv2.1 antibody 1:150
- Kv1.5 antibody 1:150

**p<0.05

n = 5
Figure 2: On-line

* p<0.05 value greater in resistance PA
Figure 3: On-line

A

B

\[ I (\text{pA}) = pS \times V (\text{mV}) \]

\[ pS = 32.8 \pm 2.8 \]

\[ R = 0.98 \]

\[ n = 8 \]