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Tetramerization domain mutations in KCNA5 Identified in pulmonary arterial hypertension patients affect channel kinetics and cause abnormal subcellular localization

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Publication Date
2009

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Tetramerization Domain Mutations in KCNA5 Identified in Pulmonary Arterial Hypertension Patients Affect Channel Kinetics and Cause Abnormal Subcellular Localization

A Dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Elyssa D. Burg

Committee in charge:

Jason X-J Yuan, Chair
Kim Barrett
Joan Heller Brown
Judd Landsberg
Frank Powell
Geert Schmid-Schönbein

2009
The dissertation of Elyssa D. Burg is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2009
Epigraph

I have endeavored to state the higher and more abstract arguments by which the study of physical science may be shown to be indispensable to the complete training of the human mind, but I do not wish it to be supposed that because I may be devoted to more or less abstract and unpractical pursuits I am insensible to the weight which ought to be attached to that which has been said to be the English conception of Paradise – namely, ‘getting on.’ Now the value of a knowledge of physical science as a means of getting on, is indubitable.

*Thomas H. Huxley*
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1.2.1 Calcium as a Trigger for Contraction in PASMC

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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AVD</td>
<td>apoptotic volume decrease</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>calcium ion</td>
</tr>
<tr>
<td>[Ca$^{2+}$]_cyt</td>
<td>cytosolic Ca$^{2+}$ concentration</td>
</tr>
<tr>
<td>CH</td>
<td>chronic hypoxia</td>
</tr>
<tr>
<td>CO</td>
<td>cardiac output</td>
</tr>
<tr>
<td>Co-IP</td>
<td>co-immunoprecipitation</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CTEPH</td>
<td>chronic thromboembolic pulmonary hypertension</td>
</tr>
<tr>
<td>D</td>
<td>aspartate</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2'-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>E</td>
<td>glutamate</td>
</tr>
<tr>
<td>E211D</td>
<td>K$_V$1.5 harboring glutamate to aspartate mutation at amino acid position 211</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>E$_K$</td>
<td>potassium equilibrium potential</td>
</tr>
<tr>
<td>E$_m$</td>
<td>resting membrane potential</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>G</td>
<td>glycine</td>
</tr>
<tr>
<td>G182R</td>
<td>K$_V$1.5 harboring glycine to arginine mutation at amino acid position 182</td>
</tr>
</tbody>
</table>
G182R/E211D  $K_{V1.5}$ harboring both the G182R and E211D mutations

HA  hemagglutinin

hPASMC  human pulmonary artery smooth muscle cell

$I_{K(V)}$  whole cell voltage gated potassium current

I-V  current-voltage relationship

IPAH  idiopathic pulmonary arterial hypertension

$K^+$  potassium ion

$[K^+]_i$  intracellular $K^+$ concentration

$[K^+]_e$  extracellular $K^+$ concentration

$KCNA5$  gene encoding the voltage gated potassium channel, shaker-related subfamily, member 5

$K_V$ channel  voltage gated potassium channel

$K_{V1.5}$  voltage gated potassium channel, shaker-related subfamily, member 5 protein

$Na^+$  sodium ion

$Na^+/K^+$-ATPase  sodium potassium ATPase

P  pore region (of a $K_V$ channel)

PA  pulmonary artery

PAEC  pulmonary artery endothelial cell

PAH  pulmonary arterial hypertension

PAP  pulmonary arterial pressure

PASMC  pulmonary artery smooth muscle cell

PH  pulmonary hypertension
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PTE</td>
<td>pulmonary thromboendarterectomy</td>
</tr>
<tr>
<td>PVR</td>
<td>pulmonary vascular resistance</td>
</tr>
<tr>
<td>R</td>
<td>arginine</td>
</tr>
<tr>
<td>RV</td>
<td>right ventricle</td>
</tr>
<tr>
<td>RVH</td>
<td>right ventricular hypertrophy</td>
</tr>
<tr>
<td>S1-S6</td>
<td>transmembrane domains (of a Kv channel)</td>
</tr>
<tr>
<td>SPH</td>
<td>secondary pulmonary hypertension</td>
</tr>
<tr>
<td>T1 domain</td>
<td>N-terminal tetramerization domain of a Kv channel</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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Acknowledgments

Since my eighth grade science fair project (on recombinant DNA, a term I couldn’t even pronounce at the time it was assigned me), I wanted to be a biologist. Getting to this point would not have been possible without the help of educators, friends, colleagues and fellow students.

A large part of my project relied on standard molecular biology techniques. For my mastery, however incomplete, of these methods I would like to extend my deepest thanks to members, both past and present, of the Boss-Pilz lab, including Tong Zhang and Stephanie Turner. I also offer a very appreciative recognition to Darren Casteel. Thanks also to Darren for his aid in making the mutant constructs and for teaching me much about experimental design. Throughout my project, I had numerous collaborators, without whom certain aspects of this work would, quite literally, not have been possible. Therefore, I would also like to thank Brinda Rana and Beatriz Lozano-Ruiz for their help in genotyping patient samples, as well as Igor Tsigelny for the modeling of wildtype and mutant Kv1.5.

The original identification of the two mutations, the characterization of which is the main focus of this dissertation, was initially published in: Remillard CV, Tigno DD, Platoshyin O, Burg ED, Brevnova EE, Conger D, Nicholson A, Rana BK, Channick RN, Rubin LJ, O'Connor D T, and Yuan JX. Function of Kv1.5 channels and genetic variations of KCNA5 in patients with idiopathic pulmonary arterial hypertension. Am J Physiol Cell Physiol 292: C1837-1853, 2007. Furthermore, the figure of Kv1.5 which illustrates the location and side chains of the G182, E211, 182R
and 211D residues within Kv1.5, the 500-ms pre-pulse inactivation curve data, and the whole cell \( I_{K(V)} \) data from HEK-293 cells transfected with WT-Kv1.5 or the mutant constructs (which appear in Chapter 4) were submitted for publication in the following paper: Elyssa D. Burg, Oleksandr Platsoshyn, Jason X.-J. Yuan, and Igor F. Tsigelny. Tetramerization domain mutations in KCNA5 affect channel inactivation. *Intl J Cell Biol*, 2009. Lastly, the genotyping data which appears in Table 2 (Chapter 4) of this dissertation has been co-authored with Brinda K. Rana and Beatriz Lozano-Ruiz.

A very memorable, enjoyable and educational part of my graduate career was spent in the HHMI Med-into-Grad Program with UCSD’s Pulmonary and Critical Care Division. For making this program possible, I would like to thank Patricia Finn and my advisor Jason Yuan. For unceasingly guiding me and showing me all that was ‘cool’ in the hospital, for putting up with my endless stream of ‘what’s that?’, and for really taking the time to teach me about the PTE service in depth, I would also like to thank William Auger, Philippe Montgrain and Maureen Cavanagh. I’d also like to extend my heartfelt appreciation to Judd Landsberg who, in the months preceding my entry into the program, met with me often to prepare me for the imminent onslaught of clinical information. Judd was also invaluable in helping me prepare for my clinically oriented BMS minor examination and has proven to be an enormously helpful committee member. Thank you.

Everyone who passed through Jason Yuan’s lab during my tenure there has also contributed to my success as a researcher. I would like to extend a special appreciation to Carmelle Remillard who originally taught me how to patch and,
through subsequent years, endured my questions, frustrations and occasional moments of unadulterated joy when I got a good seal. I would also like to thank Oleksandr Platoshyn, who I am certain can patch blindfolded with the lights off and both hands tied behind his back, for his guidance and help with the electrophysiology experiments herein presented. In addition, Steve Keller was instrumental in teaching me many molecular biology techniques. To Amy Firth, Euna Ko, Joe Mauban, Aiko Ogawa, Weijuan Yao (Dr. Yao), Ying Yu and Shen Zhang, I also extend my heartfelt thanks for teaching, helping and keeping me sane in the lab.

I would also like to thank the heretofore unmentioned members of my committee, both past and present: Kim Barrett, Wayne Giles, Joan Heller Brown, Frank Powell, and Geert Schmid-Schönbein. You offered valuable insight and suggestions throughout this whole process and got me thinking of the significance of my project from angles I had not previously explored.

My list of acknowledgments would not be complete without a heartfelt ‘thank you’ to my advisor, Jason Yuan. At the end of my first year of graduate school with the choice of a lab looming large, I approached Jason for discussion on this matter. Armed with all the naivete of someone who has never actually patch clamped anything, I asked whether I could join his lab and focus on single cell recordings. Jason has never ceased being an excellent mentor. Through all the stages of my project, Jason offered invaluable insight, both theoretical and practical, and sharpened my scientific thinking. His emphasis on integrating clinical relevance in both research and education
is exemplary of what the biomedical field strives to accomplish and certainly added a much appreciated dimension to my graduate education.

I am also indebted to my friends –Meghan, Micah, Stephanie and Katie –who had the unassigned duty of keeping me sane throughout graduate school even if they didn’t realize it. I also thank Kelly and James who daily reminded me of the intellectual world outside of science. Finally, I want to thank my father and my mother, each of whom has been a source of unwavering support in all my endeavors.
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**Burg ED**, Platoshyn O, Rana BK, Lozano-Ruiz B, Yuan JX-J. Tetramerization domain mutations in \( KCNA5 \) identified in pulmonary arterial hypertension patients affect channel kinetics and cause abnormal subcellular localization. **American**

Burg ED. Tetramerization Domain Mutations in KCNA5 Identified in Pulmonary Arterial Hypertension Patients Affect Channel Kinetics and Cause Abnormal Trafficking Patterns. NIH National Graduate Student Research Festival, Bethesda, Maryland, 2008. Poster Presentation.


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Abstract of the Dissertation

Tetramerization Domain Mutations in KCNA5 Identified in Pulmonary Arterial Hypertension Patients Affect Channel Kinetics and Cause Abnormal Subcellular Localization

by

Elyssa D. Burg

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2009

Professor Jason X-J Yuan, Chair

Voltage-gated K⁺ (Kᵥ) channels regulate resting membrane potential, apoptosis and proliferation in pulmonary artery smooth muscle cells (PASMC). The T1 domain of Kᵥα subunits is necessary for channel assembly, subcellular localization and association with regulatory Kᵥβ subunits. As Kᵥ channel dysfunction, including decreased Kᵥ1.5 channel transcript, has been linked to idiopathic pulmonary arterial hypertension (IPAH), a genetically heterogeneous disease, the KCNA5 gene (which encodes Kᵥ1.5) of IPAH patients was screened to identify IPAH-related mutations.
Two non-synonymous polymorphisms were found in \textit{KCNA5} in IPAH patients. These encode a glycine to arginine mutation at position 182 (G182R) and a glutamate to aspartate mutation at position 211 (E211D), and localize to highly conserved residues within the T1 domain. To study the properties of the mutations, HEK-293 and COS-1 cells were transiently transfected with wildtype \textit{K}_\textit{V}1.5 (WT-\textit{K}_\textit{V}1.5), G182R, E211D or G182R/E211D channels. Whole-cell patch clamp recordings revealed that while the mutant subunits form functional channels, the G182R channel inactivates faster than the WT-\textit{K}_\textit{V}1.5 channel at potentials greater than -20 mV, while both the E211D and G182R/E211D channels inactivate faster than WT-\textit{K}_\textit{V}1.5 at potentials less than -20 mV. Furthermore, both activation and channel closure are slowed in mutant channels.

\textit{K}_\textit{V}1.5 channels undergo complex glycosylation prior to cell surface expression. Immunoblot studies in HEK-293 cells suggest that a greater proportion of the G182R and G182R/E211D channels are present in their immature form than WT-\textit{K}_\textit{V}1.5 channel. Furthermore, protein expression of the mutants is decreased in HEK-293 and COS-1 (G182R < 20% of WT-\textit{K}_\textit{V}1.5). Immunocytochemical analysis of HEK-293, COS-1 and PASMC confirm that while WT-\textit{K}_\textit{V}1.5 is robustly expressed on the cell surface, G182R, E211D and G182R/E211D are retained intracellularly. While mutant channels maintain their ability to interact with \textit{K}_\textit{V}\textit{\beta} subunits, overexpression of \textit{K}_\textit{V}\textit{\beta}1.3 does not rescue G182R expression in COS-1 cells.

Overall, the mutant channels inactivate more quickly and are localized to the plasma membrane less efficiently than the wildtype channel. These data suggest a role
for the G182 and E211 residues in Kv1.5 channel kinetics and subcellular localization and suggest that, in a small subset of IPAH patients, these mutations may contribute to Kv1.5 channel dysfunction.
Chapter 1. Introduction

Two nonsynonymous mutations were identified in the KCNA5 gene, which encodes the voltage-gated potassium (Kv) channel Kv1.5, of idiopathic pulmonary arterial hypertension (IPAH) patients. IPAH is a devastating disease characterized by elevated pulmonary arterial pressure (PAP) and is thought to be genetically heterogeneous. Kv channel dysfunction in pulmonary artery smooth muscle cells (PASMC) is thought to play a role in IPAH pathogenesis. The two mutations localized to the N-terminus tetramerization (T1) domain of the Kv1.5 channel. The T1 domain is instrumental in mediating channel assembly and interaction with regulatory Kvβ subunits. In addition, the T1 domain affects the pore of the channel. Therefore, the focus of this project was to characterize the effects of the mutations on both the electrophysiological properties and the subcellular localization of Kv1.5 with an overall goal of elucidating the channel structure-function relationship as well as the possible pathogenic role of these mutations in IPAH.

1.1 Cellular Resting Membrane Potential and Ion Equilibria

Maintenance of proper ionic equilibria is instrumental for the proper function of all cells. From near immediate effects, such as action potential propagation or cellular contraction, to later responses such as changes in gene transcription or a commitment to apoptosis, to housekeeping functions including volume regulation, changes in local and global ion concentrations play a role in diverse cellular functions. Ion channels pass ions at near-diffusion-limited rates down their electrochemical
gradient by providing an aqueous pore through which ions passively flow. Physiologically relevant ions include sodium (Na\(^+\)), calcium (Ca\(^{2+}\)), chloride (Cl\(^-\)), magnesium (Mg\(^{2+}\)) and potassium (K\(^+\)) for which there are thought to exist more channel-encoding genes than for any other channel. Dysfunctional ion regulation at the channel level has been linked to various human diseases, including pulmonary hypertension (313, 321), episodic ataxia (165) and long QT syndrome (151, 330).

At the core of ion handling, the electrogenic Na\(^+\)/K\(^+\)-ATPase pump extrudes 3 Na\(^+\) for every 2 K\(^+\) ions it brings in, thus generating a high intracellular K\(^+\) concentration ([K\(^+\)]\(_i\)) of ~140 mM and a negative charge differential across the membrane. Conversely, extracellular K\(^+\) ([K\(^+\)]\(_e\)) is low (~4mM). Together, the chemical (or concentration) and electric gradients acting on a K\(^+\) ion comprise its electrochemical gradient. The potential at which these two forces are balanced such that K\(^+\) will be at equilibrium across the membrane is its equilibrium potential E\(_K\). At rest, K\(^+\) channels are the most permeable of all ion channels, making K\(^+\) conductance higher than that of any other ion at rest. Therefore, the cell’s resting membrane potential (E\(_m\)) is most closely approximated by E\(_K\). The Nernst equation is used to calculate the equilibrium (or reversal) potential of K\(^+\) (in volts, V):

\[
E_K = (RT/F) \times \ln([K^+]_e/[K^+]_i)
\]

where R is the universal gas constant (8.314 J/(K mol)), T is the temperature (in Kelvin, 310 K for physiological conditions), and F is the Faraday constant (9.65 x 10\(^4\) C/mol). Given external and internal K\(^+\) concentrations of 4 mM and 150 mM, respectively, E\(_K\) = -97 mV. However, resting E\(_m\) of most cells is generally less
negative than this, lying between -40 and -60 mV, as $E_m$ is actually a composite of equilibrium potentials of various ions, including not only $K^+$, but also $Na^+$ and $Cl^-$. Resting $E_m$ in vascular smooth muscle cells (VSMC) for example, is between -70 and -40 mV (198), which is closer to $E_K$ than to $E_{Cl}$ (-25 mV), $E_{Na}$ (+60 mV) or $E_{Ca}$ (+130 mV). The Goldman-Hodgkin-Katz equation for membrane potential takes into account multiple ions and their permeabilities:

$$E_m = \left(\frac{RT}{F}\right) \times \ln\left\{\frac{(P_{Na}[Na^+]_o+P_{K}[K^+]_o+P_{Cl}[Cl^-]_o)/(P_{Na}[Na^+]_i+P_{K}[K^+]_i+P_{Cl}[Cl^-]_i)}{(P_{Na}[Na^+]_o+P_{K}[K^+]_o+P_{Cl}[Cl^-]_o)}\right\}$$

where $P_x$ is the relative permeability of ion $X$, and the other terms are as above.

1.1.1 Role of $K^+$ Channels in the Regulation of $E_m$ in PASMC

Potassium channels are ubiquitously found in both excitable and nonexcitable cells, including neurons, cardiac myocytes and VSMC where they regulate resting membrane potential (198), repolarization after action potentials (96), apoptosis (24, 28, 42), proliferation (199), cell volume (28, 288) and vascular tone (215, 234). Members of all four $K^+$ channel families, $K_V$ (215, 221), calcium dependent ($K_{Ca}$) (215), inward rectifier ($K_{IR}$) (40, 272) and two-pore domain ($K_{2P}$) (89), have been found in PASMC where they are involved in various aspects of cell function. As $K^+$ permeability is relatively high under resting conditions, the activity of $K^+$ channels, notably voltage gated $K^+$ ($K_V$) channels, plays a large role in setting resting $E_m$ in PASMC (7, 8, 198, 265, 315, 322). Other $K^+$ currents as well as $Ca^{2+}$ and $Cl^-$ currents also play a role in setting $E_m$ (140, 207).
The amplitude of whole cell $K_V$ currents ($I_{K(V)}$) depends directly on the number of available functional channels at the plasma membrane ($N$), the total current through a single channel ($i_{K(V)}$) in amperes, and the steady state open probability of a $K_V$ channel ($P_{open}$) according to the following equation:

$$I_{K(V)} = N \times i_{K(V)} \times P_{open}.$$  

Single channel current depends on both the voltage potential ($\Delta V$) in volts and resistance ($R$) in Ohms across the membrane and is determined by Ohm’s Law:

$$i_{K(V)} = \Delta V/R = \Delta V \times g_K = (E_m-E_K) \times g_K$$

where the $K^+$ conductance ($g_K$) is the inverse of the resistance measured in siemens, and the driving force on $K^+$ ions is the difference between $E_m$ and $E_K$. Thus, if there are fewer functional $K_V$ channels at the membrane due to a decrease in transcription, translation, channel assembly or translocation to the cell membrane, or if either the conductance or open probability decreases, resting $I_{K(V)}$ decreases, leading to depolarization.

1.2 Effects of Depolarization in PASMC

Excitable and contractile cells, such as neurons and PASMC, have voltage dependent calcium channels (VDCC). Diminished $I_{K(V)}$ causes membrane depolarization which triggers VDCC opening, thus promoting $Ca^{2+}$ influx and raising cytosolic $Ca^{2+}$ levels ($[Ca^{2+}]_{cyt}$) (315) (Figure 1). $K_V$ channel blockers can depolarize $E_m$ sufficiently to allow for $[Ca^{2+}]_{cyt}$ increase. For example, application of 4-AP, a $K_V$ channel blocker, to primary culture rat PASMC depolarized $E_m$ and increased $[Ca^{2+}]_{cyt}$.
whereas sodium nitroprusside (which generates NO) opened \( K_V \) channels and hyperpolarized the membrane, thus preventing the 4-AP induced rise in \([Ca^{2+}]_{cyt}\) (320). In PASMC, increased \([Ca^{2+}]_{cyt}\) triggers proliferation and contraction (174, 220). Conversely, membrane hyperpolarization occurs when more \( K^+ \) channels open than were so at rest, making it more difficult to open VDCC and thus less likely that the cell will contract (Figure 1). Overexpression of \( K_V1.5 \), but not \( K_V1.4 \), hyperpolarizes mouse fibroblasts (63), indicating that \( K_V1.5 \) specifically may be important in regulating \( E_m \).

![Figure 1: Role of \( K_V \) Channels in the Regulation of \( E_m \) in PASMC.](image)

It has been suggested that a 3-mV depolarization would lead to a two-fold increase in \( Ca^{2+} \) influx (197, 198). As VDCC open at potentials more positive than -30 mV, depolarizations over a small voltage range near \( E_m \) (around -40 mV for PASMC) opens L-type calcium channels and can lead to a sustained elevation of \([Ca^{2+}]_{cyt}\) (68, 197, 315). Because of its rapid and ubiquitous role in cell signaling, resting \([Ca^{2+}]_{cyt}\) is
kept low, ~100 nM, by various mechanisms including calcium extrusion, sequestration into the endoplasmic or sarcoplasmic reticulum and intracellular buffering. Therefore, most increases in cellular calcium, even local events, trigger a cellular response.

1.2.1 Calcium as a Trigger for Contraction in PASMC

In human PASMC (hPASMC), $E_{m}$, through its control of VDCC activity (specifically L-type channels), and $K_V$ channels through their regulation of $E_{m}$, are large determinants of contractility (197, 215, 251). PASMC, as the contractile cell type in resistance pulmonary arteries (PA), play a large role in setting PA tone.

An increase in $[Ca^{2+}]_{cyt}$ is necessary for contraction in PASMC (16, 45, 174, 197). Both $Ca^{2+}$ influx through plasma membrane $Ca^{2+}$ channels and $Ca^{2+}$ release from intracellular stores (e.g., the sarcoplasmic reticulum) contribute to a rise in $[Ca^{2+}]_{cyt}$. In rat pulmonary arterial rings, removal of extracellular $Ca^{2+}$ prevents pharmacologically induced contraction (174), indicating that $Ca^{2+}$ influx through plasmalemmal channels is necessary for contraction. When $[Ca^{2+}]_{cyt}$ increases, it binds to calmodulin which then activates myosin light chain kinase (MLCK). Activated MLCK phosphorylates the regulatory light chain of myosin, allowing for the activation of myosin ATPase. The ensuing hydrolysis of ATP provides the energy source needed for the cross-bridging cycles between myosin and actin filaments. These cross-bridging interactions constitute cellular contraction (268), and in the case of concerted contraction of VSMC, vasoconstriction. Chronic PASMC contraction is thought to be partly
responsible for the elevated pulmonary arterial pressures observed in some instances of IPAH.

1.2.2 Calcium as a Trigger for Proliferation in PASMC

In addition to causing contraction, increased \([Ca^{2+}]_{\text{cyt}}\) is also a major stimulus for cellular proliferation. Increases in \([Ca^{2+}]_{\text{cyt}}\) and nuclear calcium trigger Ca\(^{2+}\)-dependent gene transcription in vascular smooth muscle cells (91, 92, 190). Both nuclear and cytosolic calcium pools promote proliferation by activating calcium dependent kinases (e.g., CaMK), immediate early genes and other transcription factors (e.g., c-fos, NFAT, CREB) which are necessary for cell growth (9, 15, 260). Ca\(^{2+}\) can also affect gene expression through its interaction with PKC and calmodulin or by activation of cell cycle proteins (cyclins and cyclin dependent kinases). In addition to stimulating quiescent cells to enter the cell cycle (\(G_0\) to \(G_1\) transition), Ca\(^{2+}\) is also required for progression through the \(G_1\) to S and \(G_2\) to mitosis checkpoints, as well as mitosis itself (15, 39, 116, 180).

In PASMC specifically, both increased \([Ca^{2+}]_{\text{cyt}}\) and intracellularly stored Ca\(^{2+}\) are thought to play a role in proliferation (80). In the presence of serum and growth factors, calcium depletion inhibits growth of PASMC (80). Furthermore, proliferating hPASMC have decreased \(I_{K(V)}\), depolarized \(E_m\) and increased resting \([Ca^{2+}]_{\text{cyt}}\) compared to growth arrested PASMC (220), supporting the hypothesis that decreased \(I_{K(V)}\) depolarizes the PASMC membrane, leading to an increase in \([Ca^{2+}]_{\text{cyt}}\) and resultant proliferation.
1.3 Classification of K\(^+\) Channels

Although all K\(^+\) channels are capable of exquisite selectivity for K\(^+\) ions, they are gated by different stimuli and have different structures. K\(^+\) channels are classified into 4 subgroups based on their structure and physiological activators and inhibitors: voltage gated (K\(_V\)), calcium activated (K\(_{Ca}\)), inward rectifier (K\(_{IR}\)), and two pore domain channels (K\(_{2P}\)). K\(^+\) channels are the most diverse group of ion channels, encoded for by at least 70 mammalian genes and expressed as more than 100 principal pore forming α subunits (41, 90).

1.3.1 K\(_V\) Channels

K\(_V\) channels are found in a wide variety of cell types including vascular smooth muscle cells, neurons, lymphocytes, and cardiac cells where they play a role in cardiac and neuronal repolarization, apoptosis, volume regulation and proliferation. The Drosophila voltage gated Shaker channel, the first K\(^+\) channel cloned, was isolated on the basis of flies’ shaking leg response to ether anesthesia (210). The mammalian Shaker-related channels comprise the K\(_V\)1 subfamily. K\(_V\)1 channels activate rapidly at potentials close to the resting membrane potential and are sensitive to pharmacological block by 4-AP. Three Drosophila related K\(_V\) channel genes, Shab, Shaw and Shal, which correlate with mammalian K\(_V\)2, K\(_V\)3 and K\(_V\)4 subfamilies, respectively, were identified by homology screening with Shaker cDNA probes, and human homologues of all 4 subfamilies have been found (31, 216).
Pore-forming \(K_v (K_v\alpha)\) subunits have six membrane spanning domains and tetramerize to form functional channels (Figure 2A). \(K_v\) channels can associate with cytoplasmic modulatory \(K_v (K_v\beta)\) subunits. Altogether, \(K_v\alpha\) subunits are represented by some 40 mammalian genes and are grouped into subfamilies \(K_v1-12\), of which members of \(K_v5\) (327), \(K_v6\) (228), \(K_v8\) (103, 249) and \(K_v9\) (213, 214, 250) are electrically silent modulatory pore forming subunits. For example, no current was observed in frog oocytes injected with \(K_v6.1\) cRNA; however, when \(K_v6.1\) was coexpressed with \(K_v2.1\), the observed current exhibited decreased rates of inactivation and TEA sensitivity and a leftward shift in half maximal activation compared to currents from \(K_v2.1\) by itself (228). Alternatively, \(K_v8.1\), when coexpressed with members of the \(K_v2\) or \(K_v3\) subfamilies, abolished all functional expression of these outwardly rectifying potassium currents (103).

Human PASMC express a diversity of \(K_v\) channels including \(K_v1.1-1.7, K_v2.1, K_v3.1, K_v3.3-3.4, K_v4.1-4.2\), e.g. (221), although \(K_v1.5\) is one of the major channels. Channels composed of combinations of these subunits underlie the variety of voltage sensitive potassium currents present in hPASMC. The structure and regulation of \(K_v\) channels will be taken up in greater detail in section 1.4.
1.3.2 $K_{Ca}$ Channels

Calcium activated potassium ($K_{Ca}$) channels, which are subclassified by their relative conductances into large ($BK$, $BK_{Ca}$, hSlo, or MaxiK channels; 200-260 pS), intermediate (IK, 100-200 pS), and small (SK, 3-15 pS) conductance subfamilies, have also been found in smooth muscle cells where they contribute to control of myogenic tone and $E_m$ (25, 196, 200, 215, 283). HPASMC express $K_{Ca}1.1$, $K_{Ca}2.2$, $K_{Ca}2.3$, $K_{Ca}3.1$ and the modulatory $K_{Ca}\beta$ subunits $K_{Ca}\beta1-4$ (221). The genetic identity of BK channels, which are encoded for by the Slo locus, like $K_V$ channels, was initially discovered in *Drosophila* (9), although calcium sensitive $K^+$ currents were first observed at the single channel level a decade earlier in rat myotubes (208). Cloning of the Slo gene from mouse revealed that multiple mammalian BK channels are encoded for by the single gene (30).
BK channels have three sites, all of which lie within the C-terminal tail, that influence channel sensitivity to calcium and other divalent cations such as magnesium (254, 296). Different sensitivities of the sites to calcium concentrations confer a wide range of sensitivity to the channel (296). In contrast to BK channels, which are activated by voltage and intracellular calcium directly, both IK and SK channels are voltage insensitive and activated by \([\text{Ca}^{2+}]_{\text{cyt}}\) through calmodulin, which is constitutively bound to the C-terminus of the channel (253, 290). SK and IK pore forming subunits, like those of the K\(_V\) subfamily, have 6 transmembrane domains and 1 pore region (Figure 2Bb). BK channels, however, have an additional transmembrane domain (T0) through which they associate with regulatory K\(_{\text{Ca}\beta}\) subunits (Figure 2Ba)(277). Interestingly K\(_{\text{Ca}\beta}1\), which alters the kinetics and increases calcium sensitivity of BK channels, can also interact with the calcium activated chloride channel CLCA1 to alters current kinetics and calcium sensitivity (84).

In addition to modulation by K\(_{\text{Ca}\beta}\) subunits, like other K\(^+\) channels, BK channels can be regulated by phosphorylation. For example, phosphorylation of the human BK channel by the proto-oncogene c-Src inhibits these currents (6). The Drosophila counterpart (dSlo) can likewise be phosphorylated by Src tyrosine kinase and can additionally interact with the catalytic domain of PKA through its C-terminal domain (289).
1.3.3 $K_{IR}$ and $K_{ATP}$ Channels

Inwardly rectifying $K^+$ ($K_{IR}$) and ATP-sensitive $K^+$ ($K_{ATP}$) channels have been found in various cell types including arterial smooth muscle cells and may play a role in controlling resting membrane potential in PASMC (40, 96, 229). $K_{IR}$ and $K_{ATP}$ channels belong to the same structural subfamily of $K^+$ channels ($K_{IR}$, Figure 2C) (129, 270) which is divided into 7 subfamilies ($K_{IR}$1-7). The pore forming subunits, which have two transmembrane domains and one pore, assemble as tetramers to form functional channels (97, 120, 123, 129, 303). Inward rectification is accomplished by block of outward currents by high affinity bound intracellular magnesium and polyamines including spermine and putrescine (65, 155). Regions in both the C-terminus and in the second transmembrane domain contribute to these associations (158, 294, 302).

$K_{ATP}$ channels are composed of $K_{IR}6$ subunits and sulphonylurea receptors (SUR; Figure 2C, left) (105) which are members of the ATP-binding-cassette protein family and have 13 transmembrane domains. As their name suggests, $K_{ATP}$ channels are sensitive to ATP, being inhibited by ATP binding (to the $K_{IR}$ subunit) when cellular phosphorylation potential is high and activated by magnesium-bound nucleotides (binding to the SUR) when metabolic activity falls (201). Thus is cellular metabolic state linked to electrical activity. However, sensitivity of these channels to ATP has been shown to depend on cell specific factors such as phospholipids (14).

$K_{IR}$ channels have been found in a variety of cell types in the pulmonary vasculature, including PASMC (40). An inwardly rectifying conductance was also
found in a subset of freshly isolated rat PA endothelial cells (PAEC); these cells were distinct from the majority of cells which displayed strong delayed rectifier conductances (98). In pulmonary veins (PV), specifically in PV cardiac myocytes, $K_{IR}$ channels may play a role in regulating vascular tone (182).

1.3.4 $K_{2P}$ Channels

$\text{K}^+\text{ leak}$ (or background) current ($I_{\text{K(leak)}}$) maintains $E_m$ at potentials below the threshold for action potential firing in excitable cells. It is widely believed that these currents are carried by $K_{2P}$ channels, so named for their subunit structure. $K_{2P}$ channels are unique among $K^+$ channels in that they assemble as dimers. Each pore forming subunit has 4 transmembrane domains and 2 pore regions (Figure 2D). $K_{2P}$ channels are generally considered to contribute to background $K^+$ conductance, as they are voltage insensitive, lack both activation and inactivation kinetics, and are open at voltages around resting membrane potential (54). Although most $K_{2P}$ channels are voltage insensitive, at least one member, $K_{2P}2.1$ (TREK-1), can be rendered reversibly voltage sensitive by phosphorylation (21). $K_{2P}$ channels are sensitive to changes in pH, being inhibited by extracellular acidosis, and volatile anesthetics such as halothane (144). Like the other three subfamilies of $K^+$ channels, $K_{2P}$ channels, specifically $K_{2P}3.1$ (TASK-1), have been found in PASMC, where they are thought to contribute to setting the resting membrane potential (89, 205).
1.3 Role of \( K_V \) Channels in PASMC

In addition to influencing \( E_m \), \( K_V \) channels have been shown to play a role in hypoxic pulmonary vasoconstriction (8, 265, 316), apoptosis and volume regulation (26, 28, 126) and proliferation (220) in PASMC.

1.3.1 Volume Regulation

Cell volume is an actively regulated process that depends on both water and ion movement across the cell membrane(135). Water moves across animal cell membranes through aquaporins which are expressed in various mammalian tissues and cells, including PASMC (237, 240, 284). Passive water movement creates hydrostatic pressure gradients that are not well tolerated by animal cells, however, necessitating the movements of ions to maintain osmotic balance. Regulatory volume decrease (RVD) returns a cell to its original volume after swelling induced by hypotonic challenge. RVD occurs with the extrusion of \( K^+ \) and \( Cl^- \) ions accompanied by the passive exit of water to maintain the osmotic balance across the cell membrane (27, 237). \( K_{Ca} \), \( K_{IR} \), \( K_{2P} \) and \( K_V \) channels including \( K_V 1.5 \) are known to be involved in regulation of cell volume (63, 136). RVD is mechanistically similar to one of the earliest stages of apoptosis, apoptotic volume decrease (AVD).

1.3.2 Introduction to Apoptosis

Within a cell, regulation of volume maintains cellular homeostasis, but within a multicellular organism, a balance between apoptosis and proliferation is required to
ensure tissue homeostasis. Apoptosis, or programmed cell death, occurs during development to separate fingers and toes, for example, and after severe DNA damage. Apoptosis is distinct from necrosis which, resulting from acute cellular injury or ATP depletion, results in cellular swelling and uncontrolled release of enzymes and signaling molecules from lysosomes. Necrosis is often detrimental to surrounding tissue as it can trigger inflammatory responses and the death of otherwise normal cells, whereas apoptosis triggers phagocytosis by immune system macrophages and other cells of the monocyte lineage. Morphologically, early apoptosis is characterized by membrane blebbing, cell shrinkage and a loss of the normal asymmetry of the cell membrane, in which phosphatidylserine (PS), normally located only on the inner leaflet, is exposed on the outer leaflet. Later, caspases, the effector molecules of apoptosis, induce nuclear fragmentation, chromosome condensation and protein cleavage.

Mitochondria, surrounded by an inner and outer membrane, lie at the heart of apoptotic signaling. Mitochondrial function and permeability is so linked to ion fluxes that a mitochondrial K\(^+\) cycle has been well described (75). The volume of mitochondria, like that of animal cells, is largely regulated by K\(^+\) flow (75). Both K\(_{\text{Ca}}\) and mitochondrial K\(_{\text{ATP}}\) channels pharmacologically distinct from their plasmalemmal counterparts have been found on the inner mitochondrial membrane (106, 263, 301). Mitochondrial K\(_{\text{ATP}}\) channels are thought to play a role in ischemic preconditioning (4, 191) and mitochondrial reactive oxygen species (ROS) production (117).
Altered mitochondrial membrane permeability lies at the heart of apoptosis. There are two main apoptotic pathways: the extrinsic pathway and the intrinsic, or mitochondrial, pathway. While the former depends on activation of death receptors at the plasma membrane and the latter for an intracellular signal for apoptotic induction, the pathways overlap in that they both involve altered mitochondrial membrane permeability that results in release of proapoptotic signaling molecules, including cytochrome c, Smac/Diablo, AIF and Omi/HtrA2 from the mitochondrial matrix (83, 128, 237).

1.3.2.1 Apoptotic Volume Decrease

Although mitochondria play a key role in apoptosis, K$^+$ and K$^+$ channels are also involved in two main aspects of apoptosis: apoptotic volume decrease (AVD) and caspase activity (28, 310). Cell shrinkage is one of the first morphological characteristics of apoptosis. AVD is an early prerequisite for cellular commitment to apoptose in many cells and, like RVD, is characterized by activation of both K$^+$ and Cl$^-$ channels (24, 162, 311). RVD and AVD are mechanistically similar. The efflux of both K$^+$ and Cl$^-$ results in an imbalance in cellular osmolarity, for which the cell compensates by extruding water, ultimately resulting in cell shrinkage. In many cell types, including lymphocytes, neurons and vascular smooth muscle cells, the increased K$^+$ efflux is required for cell shrinkage and apoptosis; conversely, preventing K$^+$ efflux attenuates both AVD and apoptosis (20, 24, 48, 125, 126, 162, 224, 297, 311). That AVD is required for apoptosis is illustrated by the observations that in lymphocytes
challenged with apoptotic inducers, DNA fragmentation is observed only in cells that underwent AVD and had low intracellular K$^+$ levels, while it was not observed in cells that maintained volume and [K$^+$]$_{cyt}$ levels (24, 47, 102, 185). Although AVD is a generalized characteristic of apoptosis, it may be differentially regulated depending on the mechanism of apoptotic induction. For example, initiator caspase-8 (but not caspase-9) activation is needed for death receptor Fas-induced AVD, whereas caspase-9 (but not caspase-8) activity is needed for intrinsic pathway AVD (285).

$K_V$ channels have been implicated in AVD. Staurosporine (ST) is a potent apoptotic inducer that, through depolarization of the mitochondrial membrane potential, induces AVD and cytochrome c release. In PASMC, ST also increases $I_{K(V)}$ (125). Rat PASMC transfected with $K_V$1.5 demonstrate increased $I_{K(V)}$, accelerated ST-induced cell shrinkage and higher levels of both basal and ST-induced apoptosis compared to control PASMC (26). Furthermore, inhibition of $I_{K(V)}$, either by $K_V$ channel block with 4-AP or by high extracellular K$^+$ (40mM), decreases ST-mediated apoptosis in hPASMC by diminishing the ST-induced increase in $I_{K(V)}$ despite the persistence of mitochondrial membrane depolarization (125). These data point to the importance of an initial ST-induced volume decrease through $K_V$ channels that is required for apoptosis in PASMC.

In addition to $K_V$ channels, $K_{Ca}$ channels are also involved in AVD. An increase in [Ca$^{2+}$]$_{cyt}$ can trigger AVD by activating $K_{Ca}$ channels, thus leading to cell shrinkage (137). Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), like ST, triggers apoptosis through dissipation of the mitochondrial membrane potential.
FCCP-induced apoptosis, however, induces increased $I_{K(Ca)}$ rather than $I_{K(V)}$ in PASMC, and blockage of $K_{Ca}$ channels attenuates FCCP-induced apoptosis (126). Certain apoptotic inducers, however, may effect AVD by an increase in outward $K^+$ currents through both channel types. Nitric oxide (NO), for example, which also induces apoptosis in PASMC, increases both $K_V$ and $K_{Ca}$ currents. Block of either outward driving force on $K^+$ or pharmacological block of either channel type reduced $K^+$ currents and inhibited apoptosis (127). Cytosolic application of cytochrome c in PASMC increases outward potassium currents; this effect was not dependent on caspase activity and occurred before nuclear condensation (224), indicating that cytochrome c may have its proapoptotic effect in part by initiating early AVD. Altogether these data point to the importance of early $K^+$ efflux through both $K_V$ and $K_{Ca}$ channels in PASMC apoptosis.

The importance of an early outward $K^+$ flux in apoptosis in PASMC is also illustrated by the effect of antiapoptotic proteins on $I_{K(V)}$. Bcl-2 is an antiapoptotic protein which inhibits cytochrome c release and proapoptotic Bcl-2 family members such as Bax and Bak. The apoptosis repressor with caspase recruitment domain protein (ARC) is also antiapoptotic, preserving mitochondrial integrity and thus preventing cytochrome c release. Overexpression of Bcl-2 in PASMC or ARC in cardiomyocytes decreases the current density of $I_{K(V)}$, prevents staurosporine-mediated increases in $I_{K(V)}$ and inhibits ST-induced apoptosis (56, 57). Furthermore, Bcl-2 overexpression downregulates $K_V$ mRNA expression, including that of $K_V1.5$ (56).
These results suggest that an inhibition of $K_V$ channel function may be one of the mechanisms through which antiapoptotic proteins exert their effects.

1.3.2.2 Role of $K^+$ in Caspase Activation

AVD is not the only aspect of apoptosis in which $K^+$ regulation figures prominently. Caspases are the effector molecules of apoptosis. They are proteases that cleave and/or inactivate cellular proteins, including cytoskeletal elements, the nuclear lamina and inhibitors of apoptosis, thus achieving the final stages of apoptosis. Once caspases are active, the cell is committed to apoptosis. After an initial apoptotic trigger and early AVD, caspase activation ultimately leads to nuclear and DNA degradation as well as disruption of the cell membrane. Although the initial $K^+$ efflux of apoptosis is caspase independent (23, 224), $[K^+]_i$ plays a role in modulating caspase activity, as physiological $[K^+]_i$ concentrations inhibit endogenous nuclease, caspase and protease activity as well as nuclease-induced chromatin fragmentation (102). The protective effect of $[K^+]_i$ may be due to its ability to inhibit the formation and activation of the apoptosome, the caspase-activating complex formed by the oligomerization of Apaf-1 (32, 273). In pharmacologically induced (ST, e.g.) apoptosis in lymphocytes and PASMC, $K^+$ channel blockade suppresses the activation of effector caspases and endonucleases, while loss of $K^+$ is associated with caspase and endonuclease activation (24, 48, 102, 162). While caspases are the final effector molecules of apoptosis, their activation by decreased $[K^+]_i$ is not specific to the context of apoptosis.
During cellular membrane repair after challenge with bacterial pore forming toxins, a decrease in $[K^+]_i$ was found to promote the activation of caspase-1 (88).

1.3.3 $K^+$ Channels in Proliferation

While $K_V$ channels play an indirect role in regulating proliferation through their control on $E_m$ and resultant effect on VDCC (cf. section 1.1.1), $K^+$ channel regulation of $E_m$ in nonexcitable cells which do not have VDCC has also been implicated in proliferation. Inhibition of $K^+$ channels leads to membrane depolarization that inhibits proliferation by decreasing the driving force on calcium entry into the cytosol. $K_V$ channel block in activated T lymphocytes inhibits both activation-induced proliferation and lymphokine production by preventing a rise in $[Ca^{2+}]_{cyt}$ (70, 150). Additionally, $K^+$ channel blockers, in addition to inhibiting $K^+$ currents, inhibit DNA synthesis in stimulated lymphocytes (50), although a direct dependence on $K^+$ efflux for DNA synthesis was not established.

$K^+$ channel activity has also been directly implicated in cellular proliferation. Cells may depend on hyperpolarization mediated by an increase in outward $K^+$ current to proliferate in response mitogenic agents. In human myeloblastic leukemia cells, epidermal growth factor (EGF), which activates the MAPK/ERK proliferative pathway, activates $K^+$ channels; however, blockade of $K^+$ channels prevents EGF-induced activation of ERK, and this effect was independent of extracellular calcium (298). Membrane hyperpolarization may be a general requirement for cellular
proliferation even in cells with VDCC such as VSMC. In native contractile VSMC, EGF, a potent mitogen, induces hyperpolarization through an increase in BK current (108). Iberiotoxin, which blocks BKCa channels, prevents the EGF-induced increase in proliferating cell nuclear antigen (PCNA) (108), indicating that BK channel activity may be necessary for EGF-stimulated proliferation. Thus, K\textsuperscript{+} channel activity may be one of the upstream components of the MAPK mitogenic response. Overall, the interaction between plasmalemmal K\textsuperscript{+} channels and VDCC must be taken into account when considering the effect of K\textsuperscript{+} channel activity on proliferation.

1.3.4 Imbalances in Apoptosis and Proliferation in PASMC in IPAH

Imbalances in proliferation and apoptosis are hallmarks of many diseased states, including neurodegenerative disorders, cancers and IPAH. Major pathological findings in the pulmonary vasculature of IPAH patients include abnormal PASMC proliferation, medial hypertrophy and pulmonary vascular remodeling (164, 230). PASMC from IPAH patients are resistant to apoptotic stimuli and have decreased $I_{K(V)}$ compared to PASMC from secondary pulmonary hypertension (SPH) patients (313, 326) (cf. sections 2.3 and 2.4). Thus, the balance toward PASMC proliferation and decreased apoptosis leads to medial hypertrophy and arterial lumen obliteration. The resistance to apoptosis, as well as chronic cellular contraction, and decreased $I_{K(V)}$ in PASMC from IPAH patients support the hypothesis that dysfunctional K\textsubscript{V} channels in IPAH contribute to pathogenic PASMC hyperplasia.
1.3.5 Effect of Hypoxia on $K^+$ Channel Expression and Function

Physiological response to low oxygen partial pressures, i.e. hypoxia, distinguish small pulmonary arteries from those of the systemic circulation. While the systemic vasculature dilates to compensate for lower oxygen delivery to tissue, pulmonary arterioles, but not conduit pulmonary arteries, constrict to match ventilation to regional blood flow. This is known as hypoxic pulmonary vasoconstriction (HPV). Acute hypoxia contracts PASMC, constricts pulmonary arteries and increases pulmonary arterial pressure in animals and humans (161, 192, 227, 318). People who are chronically exposed to hypoxia (e.g. from living at altitude or from respiratory diseases such as chronic obstructive pulmonary disease (COPD) or sleep apnea) may develop pulmonary arterial hypertension (PAH), as chronic hypoxia causes pulmonary vascular remodeling after the initial HPV response (238).

PASMC in resistance arterioles have been directly implicated in HPV (269). Hypoxia is thought to inhibit the activity of $K^+$ channels, including $K_v$1.5 (8), in PASMC, thus decreasing $I_{K(V)}$. In fact, sensitivity of hPASMC to hypoxia depends on the expression levels of $K_v$1.5 (223). In response to chronic hypoxia (CH), protein and mRNA levels of $K_v$1.5, as well as $K_v$1.1, $K_v$1.2 and $K_v$2.1, decrease (222, 287); $K_v$9.3 has also been implicated in HPV (104, 213). Decreased $I_{K(V)}$ causes depolarization and entry of calcium through voltage dependent L-type channels, thus causing cellular contraction (227, 265, 316). CH causes $E_m$ depolarization and an increase in $[Ca^{2+}]_{cys}$ in PASMC but not in mesenteric arterial smooth muscle cells.
(MASMC) (222), indicating that hypoxia selectively inhibits $K_V$ channel function in a physiologically relevant mechanism.

$K^+$ channels may not themselves be oxygen sensors. Cytochrome P450 requires NADPH as an oxygen donor cofactor to catalyze monooxygenase reactions. Inhibition of this enzyme inhibits steady state $I_{K(V)}$ in PASMC (319), although other cell specific oxygen sensing mechanisms are thought to exist (3). Similarly, in type I carotid body cells, hypoxia inhibits $K^+$ currents, and inhibition of cytochrome P450 inhibits $I_{K(V)}$ as well as $I_{K(Ca)}$ (94). These data suggest that cytochrome P450 may form one link between oxygen sensing and $K^+$ channel function.

$K_V\beta$ subunits, which associate with and modulate $K_V$ channels, are members of the oxidoreductase family of enzymes (85). Thus, $K^+$ channel oxygen sensing may be rather direct. Other evidence also indicates a role for $K_V\beta$ subunits in oxygen sensing. As mentioned, conduit PA do not constrict in response to hypoxia. As $K_V\beta$ subunit expression increases from the second to fourth branch off the main intralobal PA (43), the presence of $K_V\beta$ subunits may be required for HPV. However, the mRNA expression levels of various $K_V\beta$ subunits do not differ between PASMC and MASMC (219), suggesting that while the presence of $K_V\beta$ subunits may be required to confer HPV onto a cell, it is not sufficient. Whatever the exact mechanism of oxygen sensing, the hemodynamic effects and molecular mechanisms of HPV closely mimic PAH; therefore CH has been used as a model to study PAH (232) (cf. section 2.6).
1.4 Structure of K<sub>V</sub> Channels

Each K<sub>V</sub> subunit is encoded by a separate gene. The K<sub>V</sub>1.5 subunit, for example, is coded by the KCNA5 gene at chromosome 12p13 clustered with KCNA1 and KCNA6. It was first cloned from human ventricle (271). KCNA5, an intronless gene that lacks splice sites, codes for a ~67 kDa protein of 612 residues. Functional considerations of all K<sub>V</sub> channels include the passage of ions at near diffusion rates ($10^8$ ion/sec) (186) while open, K<sup>+</sup> selectivity and the ability to sense and respond to voltage changes across the membrane. K<sub>V</sub> channels can exist in three main conformations: open, inactivated and closed (Figure 3). The channel is open when both the selectivity filter and all inactivation gates are open (Figure 3A), inactivated when inactivation gates are closed (Figure 3B) and closed when the pore is closed and inactivation gates open (Figure 3C). K<sub>V</sub> channels are generally thought to pass through the inactivated state before entering the closed state from which they can open again and current is unable to pass in both the closed and inactivated states. The time constant of activation ($\tau_{act}$) is an indication of how quickly the channel opens after membrane depolarization, while the time constant of deactivation ($\tau_{deact}$) measures the rate of channel closure after a voltage stimulus is removed. Lastly, the inactivation time constant ($\tau_{inact}$) indicates the rate of channel inactivation, regardless of the inactivation mechanism (cf. section 1.4.3).
Functional $K_V$ channels are composed of four pore forming $K_V\alpha$ subunits that either homo- or hetero- tetramerize in vivo, although certain $K_V\alpha$ subunits can associate only with subunits of the same subfamily (167, 225, 259, 286). Each $K_V\alpha$ subunit has 6 transmembrane domains (S1-S6), cytosolic N- and C- termini, and voltage sensing residues in S4 (152, 153, 211) (Figure 2A). Each of these functional considerations is governed, for the most part, by an independent domain within the $K_V$ channel.

1.4.1 Structure of the Pore and Selectivity Filter of $K_V$ Channels

$K_V$ channels achieve $K^+$ selectivity through a highly selective aqueous pore lined by the four pore domains (P) of the tetramerized subunits. This domain sits between S5 and S6, dipping partway into the membrane without crossing into the intracellular environment (93, 305, 308) (Figure 2A). In the assembled channel, the four S5-P-S6 regions are surrounded radially by the S4 voltage sensing segments (114, 152). The selectivity filter is located within the pore toward the extracellular side; its

**Figure 3: Schematic Representation of Three States of a $K_V$ Channel.** Purple boxes represent transmembrane domains; red circles, inactivation domains; straight black lines, selectivity filter. A) Depicts channel in open state when both the pore and inactivation domains assume the conducting conformation. B) The channel is depicted in its inactivated state in which the pore gate may be open but the closed inactivation gate prevents conductance C) Depicts channel in closed state where the pore is closed and the inactivation gates open. Although this diagram represents a $K_V$ channel with fast N-type inactivation, slowly inactivating $K_V$ channels adapt the
sequence is TVGYG. Continuous with this filter but proximal to the intracellular side is an inner cavity which accommodates hydrated K$^+$ ions (53). Further proximal to the intracellular side lies a cross bundle of α helices that forms the channel gate close to the interface between the membrane and intracellular environment (53). Although this overall structure of the pore is specific to Kv channels, the filter itself is conserved among K$^+$ channels (157).

The K$^+$ filter accomplishes its selectivity through a series of four coordinated oxygen atoms on peptide backbone carbonyl groups as well as the side chain hydroxyl groups of the threonine residues of the filter sequence. These oxygen atoms stabilize dehydrated K$^+$ ions by substituting for the waters of hydration (329), and the structure is held rigid so that smaller atoms (Na$^+$, e.g.) are unable to pass through the filter (329). In fact, the pore favors dehydrated K$^+$ to dehydrated Na$^+$ ions at a ratio of 1000:1. While the length across the membrane is ~30Å, the length of the selectivity filter is only 12Å (53, 113, 152), thus effectively compressing the membrane’s electric field to this length for K$^+$.

Crystallographic studies of the bacterial KcsA K$^+$ channel provided the first insights into how the conduction gate regulates selectivity. In addition to the selectivity conferred by the precise coordination of a K$^+$ ion among oxygen atoms in the filter, the filter adapts a nonconductive state in the absence of K$^+$ and the presence of Na$^+$ (282, 323). When the conduction gate is open, the selectivity filter is exposed to the high intracellular K$^+$; conversely, when the gate is closed, the filter is exposed to low extracellular K$^+$ levels and changes its conformation to the nonconductive
conformation (186, 329). In its conductive conformation, the filter contains two K$^+$ ions separated by one water molecule (186, 282). It is the binding of dehydrated K$^+$ ions to the selectivity filter which induces a conformational change from the nonconductive to conductive states, thus allowing ions to pass (53, 282, 329).

1.4.2 Structure of the Voltage Sensor of K$V$ Channels

K$^+$ channels can be categorized generally as ligand or voltage gated. The function and identity of the domains involved in open-gating vary and depend on the stimulus to which the channel responds. For example, a bacterial K$^+$ channel that opens in response to intracellular Ca$^{2+}$ has an intracellular gating ring at the cytoplasmic side of the membrane that uses the free energy of Ca$^{2+}$ binding to perform mechanical work to open the pore (112). K$V$ channels, on the other hand, sense changes in $E_m$ through domains that cross the membrane. These voltage sensors, upon detection of a positive electric field, sensed by positive residues on the S4 segment, perform positive work to open the pore, which is more stable in its closed than open state (248, 307). Early studies established that charged residues on the S4 linkers contribute to the gating charge associated with activation, thus indicating that part of the S4 domain moves in response to channel activation (148). Repeated leucine residues around the pore region may also play a role in the voltage sensitivity of activation and inactivation in K$^+$ channels (172).

While the movement of the S4 segment underlies the coupling between voltage sensing and channel opening, the exact mechanism is unknown. The inner bundle $\alpha$
helices (as mentioned in the previous section) form the gate (or inner pore). The S4-S5 linker, located cytoplasmically (Figure 2A, Figure 4A), is thought to translate the motion of the S4 sensor to the inner pore thus closing or opening the ion permeation pathway (153). Crystal structures indicate that the voltage sensors, in a helix-turn-helix structure composed of part of S3 and all of S4, act as paddles, transferring their charge from the inner leaflet to the outer leaflet of the membrane upon membrane depolarization (111, 114, 115, 153). However, studies of gating currents and of luminescence energy transfer between protein domains within the channel reveal that the S4 segment may rotate within the membrane with a small 1-2.5Å transmembrane displacement (17, 34, 226). Whatever the precise mechanism of voltage sensing, the voltage sensing motifs are modular, as they can be transferred between channels (5). More recent crystallization studies of the voltage sensor paddle from eukaryotic Kv2.1 transferred onto Kv1.2 support the idea that the voltage sensor charges do indeed translocate across the membrane (154).

1.4.3 Structure of the Inactivation Domains of Kv Channels

Many voltage gated channels, including Kv channels, inactivate after opening when maintained at depolarized potentials, thus allowing for cell repolarization. Certain Kv channels, Kv1.4 and Kv4.2 for example, have fast inactivating currents known as A-type currents which are characterized by N-type (fast) inactivation, while other channels such as the delayed rectifiers Kv1.5 and Kv2.1 have slowly inactivating or noninactivating currents. The difference is thought to lie mainly in the N-terminus:
the presence there of an inactivation peptide, tethered to the cytoplasmic face of the channel, confers rapid inactivation by obstructing the pore and is commonly referred to as the ball and chain model (101). Thus the presence of one inactivation peptide is sufficient to render N-type inactivation onto a channel. The inactivation peptide lies at the extreme N-terminus of the protein and is connected to the rest of the N-terminal domain by a flexible linker. Modulatory Kvβ subunits that confer rapid inactivation have a structurally and mechanistically similar inactivation peptide.

Inactivation domains enter the pore as an extended peptide, for which the receptor site lies within the central cavity (328). Inactivation peptides, like Kv voltage sensors and pore domains, are modular. They confer rapid inactivation on channels whose endogenous inactivation peptide has been deleted or which do not have endogenous N-type inactivation (77, 324). Furthermore, transplanting the N-terminus from a rapidly inactivating channel onto a slowly inactivating outwardly rectifying channel converts the recipient channel into an inward rectifier (35), consistent with the hypothesis that the newly conferred rapid inactivation reduces conduction at depolarized potentials, rendering these channels inwardly rectifying (266).

Classically, slow C-type inactivation referred to any inactivation that remained after N-terminal deletion. C-type inactivation was first described in the rat Kv1.3 channel simply as slow inactivation with a mechanism distinct from that of A-type currents (29). Kv1.5 also has slow inactivation which remains incomplete after 5-10 seconds of depolarizing stimulus. As ion channels became better understood, it became apparent that the designation of C-type as all slow inactivation was too simple,
as there exist more than one type of slow inactivation. However, the mechanisms and
domains involved in slow inactivation are still less well understood than those of N-
type inactivation. Classic C-type inactivation involves a collapse of the pore’s
selectivity filter and a resultant block of the ion conduction pathway (209, 306) and is
thought to depend on the interaction of all 4 channel subunits (204). While early
studies on C-type inactivation in *Drosophila* Shaker channels suggested that the N-
terminus was not involved in slow inactivation (233), the recently discovered U-type
inactivation, as seen in Kv1.5 homotetramers, is thought to involve the N-terminus
(130). The N-terminus of Kv channels, in addition to domains for N- and U- type
inactivation, can also contain a dominant-negative N-type inactivation-prevention
(NIP) domain (245) which prevents N-type inactivation mediated by peptides from
either Kvβ or Kvα subunits in Kv1.6 (245).

Although the N-terminus may be the major factor in determining slow or fast
inactivation, other regions of voltage gated K+ channels are involved in inactivation.
Mutational analysis of a conserved glutamate residue in the extracellular domain
between S5 and P indicates that the extracellular end of S5 may form hydrogen bonds
with the P-S6 loop to open a slow inactivation gate (139). Furthermore, in addition to
the initial rearrangement of S4 upon voltage sensing, a second stage of S4 movement,
which is accompanied by rearrangements of the entire pore domain, occurs during
inactivation (74). Thus, in addition to the N-terminus, the S4-S5-P-S6 segment also
plays a role in Kv channel inactivation.
1.4.4 Structure and Function of the T1 Domain in \( K_V \) Channels

1.4.4.1 Role of the T1 Domain in Tetramerization

Like many aspects of \( K_V \) channel function, subunit tetramerization is largely governed by a single specific domain. The cytosolic N-terminal tetramerization (T1) domain is involved in subunit association (147, 258, 259, 299) as well as \( K_V\alpha-K_V\beta \) associations (cf. section 1.5.1, Figure 4). Like the pore region, the T1 domain, composed of 120-130 amino acids, is highly conserved. Within the \( K_V \) 1 subfamily, T1 exhibits 76-90% amino acid identity, suggesting that most residues are essential for proper function. Single point mutations disrupt both \( K_V\alpha-K_V\alpha \) and \( K_V\alpha-K_V\beta \) interactions (18, 257). Most highly conserved residues are within the hydrophobic core of the folded domain indicating a shared structural framework. Polar interfaces which face outward from this core dictate \( K_V\alpha-K_V\alpha \) associations (124).

Figure 4: Schematic Diagram of \( K_V \) Channel Assembly and the T1 Domain. A) \( K_V \) subunits have six transmembrane domains (S-1 through S-6) and cytosolic N- and C-termini. The red region in the shaded box represents the T1 domain through which both \( K_V \) subunit interactions and \( K_V\alpha-K_V\beta \) interactions occur. B) Schematic view perpendicular to pore axis. Red oval indicates T1-T1 interaction between alpha subunits (others left out for clarity). Three \( K_V\beta \) subunits are shown in green. P, pore region.
Although T1 domains are conserved across Kv subfamilies, Kvα subunit interactions are generally limited to associations within a single subfamily; this specificity is conferred by the T1 domain (141, 259, 299). Specificity may be due in part to the presence of an intermolecular Zn$^{2+}$ binding motif that is present in members of Kv2, Kv3 and Kv4 and their homologous subfamilies, but not in Kv1 channels (18, 109). Specific amino acid interactions also play a role in specifying Kvα-Kvα assembly (195). The T1 domains of an assembled channel, like the individual Kvα subunits, interact in a rotationally symmetric tetramer (124), and isolated T1 domains self tetramerize (1, 217), indicating that this is the most stable conformation. While the role of T1 in tetramerization is widely recognized, the S1 domain may also be involved (11).

1.4.4.2 Structure of the T1 Domain

Interestingly, the T1 domain begins to acquire its tertiary structure even as the rest of the Kv protein is still within ER-bound ribosomes (121, 122). In fact, T1 may start acquiring its secondary structure as early as when it is in the ribosomal tunnel (122). Given this data, it is not surprising that Kv1 channels tetramerize cotranslationally in the ER (49, 156). In the assembled membrane-spanning channel, the T1 domain is 15-20Å from the cytoplasmic opening of the pore and is attached to the first transmembrane domain through a flexible T1-S1 linker (152). It is through the spaces created by these linkers that K$^+$ ions are thought to gain access to the pore (46,
119). Given its proximity to the pore, it is not surprising that alterations in T1 structure, in addition to affecting subunit association, can also affect the function of the pore.

1.4.4.3 The T1 Domain is Involved in $K_V$ Channel Gating

Mutations in the membrane-facing surface of the T1 domain introduce T1 conformational changes and shift of the channel’s activation curve as well as a slowing or speeding of channel closing rate (46), suggesting that the structure of T1 is coupled to channel gating. Interestingly, the conformational change induced by these mutations is paralleled upon binding of a $K_V\beta$ subunit (38), indicating that the electrophysiological effects of $K_V\beta$ subunits on $K_V$ channels may be due in part to T1 conformational change. Additionally, a different mutation in the T1 polar interface causes little overall structural change but affects gating by stabilizing the closed conformation of the $K_V1.2$ channel (184). These data indicate that the T1 structure plays a role in channel gating. There is also evidence that interaction among the cytoplasmic N-terminus, S4-S5 linker and C-terminus modulate the activation time course of $K_V$ channels in a voltage dependent manner (252).

1.4.4.4 The N-terminus of $K_V$ Channels Interacts with Modulatory Proteins that Affect Channel Activity

In addition to its direct effects on the pore, T1 interacts with other proteins that modulate channel activity. The classic example of such modulation is by $K_V\beta$ subunits (cf. section 1.5.1); however other such modulatory proteins have also been discovered.
The N-terminus of hK\textsubscript{V}1.5 contains two copies of the proline rich SH3-domain binding motif with which Src tyrosine kinase can directly associate (99). Phosphorylation of hK\textsubscript{V}1.5 by Src suppresses channel current (99). PDZ domains are another widespread protein-protein interaction motif. K\textsubscript{V}1.5 has both a canonical C-terminal PDZ-binding domain and a unique N-terminal PDZ-binding site in its T1 domain (59). Binding of PSD95 to each of these domains differentially affects the magnitude of whole cell K\textsubscript{V} currents in transfected HEK-293 cells (59). SAP97, another PDZ-containing protein, depends on the presence of K\textsubscript{V}1.5’s N-terminus, but not its C-terminal, PDZ domain, to increase currents through the channel, an effect that may be mediated by protein kinase C (58, 171). Overall, these data suggest that the N-terminus of K\textsubscript{V}\alpha subunits has a role in channel kinetics beyond its widely recognized role in tetramerization and K\textsubscript{V}\alpha-K\textsubscript{V}\beta interaction.

1.4.4.5 The Role of the T1 Domain in Channel Subcellular Localization

Interactions between K\textsubscript{V} Channels and modulatory proteins, in addition to affecting K\textsuperscript{+} current, also influence subcellular localization. While other regions of K\textsubscript{V} channels, including the outer pore region of K\textsubscript{V}1.1 and K\textsubscript{V}1.4 (331) and the C-terminal phosphorylation site of K\textsubscript{V}1.2 (304), influence cell surface expression, the T1 domain is widely considered one of the dominant determinants in subcellular localization. The clustering that differentially distributes ion channels and receptors in neuronal soma, dendrites, nodes of Ranvier and postsynaptic density regions, for example, depends on protein-protein interactions between PDZ-containing proteins such as PSD95 or
ankyrin G and ion channels. A specific cytoplasmic loop of the voltage gated Na\(^+\) channel Na\(_V\)1, through its association with ankyrin G, is necessary and sufficient for targeting Na\(_V\) channels to the axonal initial segment (76). K\(_V\)1.5, in addition to its N- and C-terminal PDZ domains which interact with SAP97 and PSD95 to influence current amplitude, can associate with α-actinin-2 through its N-terminal region, thus linking it to the actin cytoskeleton (170). This interaction may have an important role in clustering K\(_V\)1.5 channels to relevant cellular domains in polarized cells.

Due to the divergent biophysical properties of individual subunits, tetramer composition plays a large part in determining the electrophysiological properties of the assembled channel; however, tetramer composition also affects channel localization within the cell. For example, K\(_V\)1.1 homotetramers localize to the endoplasmic reticulum, while homotetrameric K\(_V\)1.4 channels are trafficked to the cell surface (167, 331). Interestingly, K\(_V\)1.1 and K\(_V\)1.4 coexpression results in increased K\(_V\)1.1 heterotetramer surface expression, an effect that is dose-dependent on K\(_V\)1.4 (167). K\(_V\)1.1 subunits have a dominant ER retention signal that prevents homotetrameric K\(_V\)1.1 channels or any heteromeric channel with more than one K\(_V\)1.1 subunit from being expressed on the cell surface (281). Additionally, interference with normal channel assembly by a dominant negative truncated K\(_V\)1 polypeptide results in the ER-retention of channel complexes and their subsequent degradation (69). Thus, the T1 domain plays an important role in determining channel subcellular localization and electrophysiological properties by mediating interactions both among pore forming subunits and between channels and modulatory proteins.
1.5 Kvβ Subunits Increase Current Diversity of Kv Channels

Kvβ subunits are 35-42 kDa cytoplasmic proteins and were first isolated in the late 1980s by their ability to bind dendrotoxin (212, 235), a snake venom known to block K+ channels. The first recognized function of Kvβ subunits was their ability to confer rapid inactivation on slow- or non- inactivating delayed rectifier Kv channels. However in the years following their initial discovery and characterization, other functions of Kvβ subunits were elucidated. Kvβ subunits can modify other electrophysiological properties of Kv channels including conductance and open gating properties, thus diversifying currents from a given pool of Kvα subunits. Kvβ subunits also modify the subcellular localisation and expression levels of Kvα subunits (67, 239, 243) and provide a link between oxygen sensing and Kv channel function. There are presently three known Kvβ subfamilies, with the largest, Kvβ1, composed of three members. Kvβ1 subunits are splice variants of a single 250 kb gene (KCNA1B) that is divided into 14 exons (60, 143).

1.5.1 Kvβ Subunits Induce Fast Inactivation on Delayed Rectifier Kv Channels which Associate with Kvβ Subunits Through T1 Domains in a Subfamily Specific Manner

In addition to its role in Kvα-Kvα association, the T1 domain is also responsible for Kvα-Kvβ interactions (Figure 4) (257, 312), although the C-terminal of Kv2 subfamily members may also play a role in Kvα-Kvβ association (67). Kvβ subunits are cytoplasmic proteins (255) that associate with T1 through their core (312). Kvβ subunits can associate with the Kvα tetramer in a 1:1 ratio giving an overall
stoichiometry of \( \text{K}_\alpha:4\text{K}_\beta \); however, there can be 0-4 \( \text{K}_\beta \) subunits in the assembled channel complex, depending on their relative concentrations (85, 86, 300). Moreover, there may be more than one type of \( \text{K}_\beta \) subunit in a single \( \text{K}_\gamma \) channel complex (242). The octameric complex has a four-fold axis of symmetry, with T1 facing the channel pore and the \( \text{K}_\beta \) subunits facing outward toward the cytoplasm (86, 152).

Like \( \text{K}_\alpha:4\text{K}_\beta \) associations, those between \( \text{K}_\alpha \) and \( \text{K}_\beta \) subunits is subfamily specific. For example, \( \text{K}_\alpha \beta \text{K}_\beta \) and \( \text{K}_\beta \) subunits associate with \( \text{K}_\gamma \) subfamily members (or their homologs) but not those of the \( \text{K}_\gamma \)2, \( \text{K}_\gamma \)3 or \( \text{K}_\gamma \)4 (or their \textit{Drosophila} homologs) subfamilies (194, 242, 243, 257, 312). \( \text{K}_\gamma \beta \) subfamily members confer rapid inactivation on \( \text{K}_\gamma \)1.5, a slowly inactivating delayed rectifier (257). Like other \( \text{K}_\gamma \alpha:4\text{K}_\beta \) interactions, this was found to be subfamily specific as it was not able to bind to either \( \text{K}_\gamma \)3.4 or \( \text{K}_\gamma \)4.2 (257). Even within a given \( \text{K}_\gamma \alpha \) subfamily, however, certain \( \text{K}_\beta \) subunits may have subunit specific effects, as \( \text{K}_\beta \)1.2 speeds the rate of inactivation of \( \text{K}_\gamma \)1.4 (which itself is a fast inactivating channel) and confers fast inactivation on \( \text{K}_\gamma \)1.5 but has no effect on either \( \text{K}_\gamma \)1.1 or \( \text{K}_\gamma \)1.2 (163, 187). Individual domains within \( \text{K}_\beta \) subunits may have \( \text{K}_\gamma \alpha \) isoform-specific effects. For example, the C-terminus of \( \text{K}_\beta \)1.2 increases the number of functional \( \text{K}_\gamma \)1.2 channels, whereas it reduces \( \text{K}_\gamma \)1.5 currents (2).

One of the earliest recognized effects of \( \text{K}_\beta \) subunits on \( \text{K}_\gamma \) channels was the introduction of a fast inactivating component on \( I_{\text{K}(\gamma)} \) on slowly- or non-inactivating channels (239). This is accomplished through an N-terminal inactivation peptide that acts on the channel pore in a manner similar to \( \text{K}_\alpha \) N-terminal inactivation peptides
(cf. section 1.4.3) (95). Furthermore, inactivation peptides from K\textsubscript{\(\alpha\)}\textsubscript{1.1} and K\textsubscript{\(\alpha\)}\textsubscript{1.4} compete with each other (143), and K\textsubscript{\(\beta\)}\textsubscript{3.1} is capable of inducing inactivation in N-terminal-deleted K\textsubscript{\(\alpha\)}\textsubscript{1.4} (95), indicating that K\textsubscript{\(\alpha\)}\textsubscript{\(\alpha\)} and K\textsubscript{\(\beta\)} inactivation peptides share a similar mechanism.

1.5.2 K\textsubscript{\(\beta\)} Subunits Link K\textsubscript{\(\alpha\)} Channel Function to Signaling Events and Cellular Redox State

While K\textsubscript{\(\beta\)} subunits affect electrophysiological properties of K\textsubscript{\(\alpha\)} channels they also modulate other aspects of channel function. K\textsubscript{\(\beta\)} subunits can affect sensitivity to modulation by PKA and PKC phosphorylation, as well as sensitivity to cellular redox state and pharmacological channel blockers such as bupivacaine and quinidine (12, 13, 82, 169). For example, K\textsubscript{\(\beta\)} subunits associate with PKC\(\zeta\) through PKC-zeta-interacting-proteins (ZIP) (81). Furthermore, K\textsubscript{\(\beta\)} subunits are themselves aldo-keto reductases that have a bound NADPH cofactor (85, 173, 291). Both cofactor binding and binding pocket structure play a role in the ability of K\textsubscript{\(\beta\)} subunits to localize K\textsubscript{\(\alpha\)} complexes to the cell surface (33) and in their ability to inactivate K\textsubscript{\(\alpha\)} currents (12, 291), linking cellular redox potential to K\textsubscript{\(\alpha\)} channel localization and function.

1.5.3 K\textsubscript{\(\beta\)} Subunits Act as Chaperones to K\textsubscript{\(\alpha\)} Channels

K\textsubscript{\(\beta\)} subunits also act as chaperones, as they affect channel subcellular localization and expression levels (67, 167, 241). Like K\textsubscript{\(\alpha\)}\textsubscript{-K\(\alpha\)} associations, K\textsubscript{\(\alpha\)}\textsubscript{-K\textsubscript{\(\beta\)}} association through T1 occurs in the ER (193). This association early in the
biosynthetic pathway has been shown to enhance the stability of Kvα subunits and to increase their expression at the plasma membrane (67, 261, 276). For example, Kvβ2 promotes Kv1.2 N-linked glycosylation, increases the stability of Kv1.2 protein and increases efficiency of Kv1.2 cell surface expression in transfected mammalian cells (261). Even in cells with little endogenous Kv expression, Kvβ subunit coexpression with Kv1.2 promotes cell surface expression of the Kv channel (33). Kv1.1/Kv1.2 and Kv1.1/Kv1.4 heteromeric channels are expressed in both the ER and on the cell surface, but Kvβ subunits promote the surface expression of each heteromer (167).

The ability of a Kvβ subunit to affect electrophysiological properties and its ability to affect Kvα protein stability may depend on different domains, as the N-terminus of Kvβ1.2 inactivates and slows deactivation of Kv1.2 currents, whereas the C-terminus of Kvβ1.2 increases the number of functional channels (2). In addition to Kvβ subunits, regions within the pore, transmembrane domains and C-terminus of Kv subunits are involved in controlling surface expression and intersubunit assembly (145, 168, 278).

Additionally, calnexin, a classic ER chaperone, promotes cell surface expression of Kv channels in neurons, but this effect was epistatic with that of Kvβ subunits, suggesting the two chaperones share a common pathway (166).

Thus, while other associations and domains within Kvα subunits influence channel localization and electrophysiological properties, the T1 domain, through its role in facilitating Kvα-Kvα and Kvα-Kvβ associations, interacting with clustering proteins and interacting with the pore itself, is a key determinant of Kv channel localization and function.
1.6 $K_V$1.5 is a Classic Delayed Rectifier Subject to Modulation by $K_V\beta$ Subunits

$K_V$1.5 was first cloned from human ventricle and its electrophysiological properties marked it as a delayed rectifier with very slow partial inactivation (incomplete after 5 sec at +60 mV, e.g.) (267, 271). $K_V$1.5 is essential for generating normal currents in ventricular myocytes (64, 146) and also plays a prominent role in VSMC. The $K_V$1.5 pore forming subunit, encoded for by the human $KCNA5$ locus, has been found in a variety of vascular cells, including human PASMC and rabbit portal vein myocytes (221, 275, 322). It is also present in freshly isolated rat PAEC (98). Overexpression of $K_V$1.5 causes hyperpolarization and enhances apoptosis in hPASMC (26).

Like currents from other $K_V$ channels, $K_V$1.5 currents can be modified by the presence of $K_V\beta$ subunits. In $K_V$1.5, a 90 amino acid region that overlaps with the T1 domain was found to be sufficient for interactions with $K_V\beta1$, while a 9 residue region therein contained was necessary for $K_V\beta$ subunit-mediated inactivation (257). All three members of the $K_V\beta1$ subfamily and $K_V\beta3.1$ induce hyperpolarizing shifts in the activation curve, slow deactivation and induce fast inactivation on $K_V$1.5 (13, 60, 61, 142, 280). $K_V\beta2.1$ affects $K_V$1.5 function in much the same way (279). The addition of the first 87 residues of $K_V\beta1.3$, which includes its inactivation peptide, onto the N-terminus of $K_V$1.5 confers fast inactivation similar to that induced by coexpression of $K_V\beta1.3$ and $K_V$1.5, supporting the hypothesis that the inactivation peptide of $K_V\beta$ subunits, like those of $K_V\alpha$ subunits, act as independent domains (280). Not all inactivations conferred by $K_V\beta1$ subunits on $K_V$1.5 are identical, however. N-terminal
sequence differences between Kvβ1.1 and Kvβ1.2 are thought to be responsible for the divergent characteristics of the fast transients induced by these subunits on Kv1.5 currents (143, 257). In addition to conferring inactivation onto Kv1.5 channels, Kvβ1.3 reduces Kv1.5 sensitivity to block by bupivacaine and quinidine, possibly through reducing the affinity of the drugs for their receptor sites on the channel (82).

Effects of Kvβ subunits on Kv1.5 activity may also depend on other cell specific factors. For example, when Kvβ3.1 is coexpressed with Kv1.5 in mammalian cells, it induces rapid inactivation, whereas in Xenopus oocytes, it does not (95, 142). However, Kvβ1.1 is able to confer rapid inactivation on Kv1.5 in both mammalian cells and oocytes (143, 239, 245). Pongs and coworkers examined this difference by studying the effects of chimeric Kvβ subunits composed of various Kvβ3.1 and Kvβ1.1 domains and proposed that the ability of Kvβ3.1 to inactivate Kv1.5 currents depends on its oxidoreductase enzymatic activity which does not function in oocytes (12). Thus, Kv1.5, like other Kv channels, is subject to modulation by Kvβ subunits which interact with the channel through its T1 domain.

1.6.1 The Effect of Kvβ Subunits can be Modulated by Phosphorylation

The effect of Kvβ subunits on Kv1.5 currents can be modulated by serine/threonine and tyrosine kinases. PKC activation has minimal effect on Kv1.5 current when Kv1.5 is expressed alone, but when it is coexpressed with Kvβ1.2, PKC dramatically reduces Kv1.5 currents (295). In mammalian HEK-293 cells, the Kvβ1 (Kvβ1.3 and Kvβ1.2)-induced fast inactivation and shift in the activation curve depend
on phosphorylation by PKC, whereas modulation by Kvβ2.1 did not (132). Both Kvβ1.3 and Kvβ1.2 have PKA consensus sites in their C terminus, while Kvβ1.3 has two additional sites in its N-terminus. Unlike PKC, PKA, through phosphorylation of the Kvβ subunit, reduces Kvβ1.3-induced fast inactivation (131); this effect was not observed for Kvβ1.2 induced inactivation of Kv1.5, however.
Chapter 2. Pulmonary Arterial Hypertension

During my tenure at UCSD, I had the privilege of participating in the HHMI Med-into-Grad Program with the Pulmonary and Critical Care Division at UCSD’s Thornton and Hillcrest Hospitals as well as the VA San Diego Hospital. This chapter is included in this dissertation to illustrate the clinical relevance of the dissertation project.

2.1 The Pulmonary Circulation

The pulmonary circulation is a high flow, low resistance system that relies on arterial distension and recruitment to keep pressures relatively stable and adapt to changing cardiac output. Normal mean pulmonary arterial pressure (PAP) is ~12-14 mmHg. Pulmonary hypertension is defined as PAP >25 mmHg at rest or >30 mmHg on exercise, but can exceed 90 mmHg in some cases (236, 246). In PAH patients, recruitment and distension cannot compensate for increased arterial pressures so the right heart adapts by hypertrophying to overcome the increased pressures of the pulmonary arterial tree. Eventually, however, right ventricular hypertrophy (RVH) becomes pathogenic and leads to right heart failure. As such, PAH is also accompanied by elevated right heart pressures and decreased cardiac output.

PA tone, largely determined by the contractile activity of PASMC in resistance arterioles, is the primary determinant of PAP. PAP is the product of pulmonary vascular resistance (PVR) and cardiac output (CO):

\[ \text{PAP} = \text{PVR} \times \text{CO}. \]
PVR is inversely proportional to the fourth power of the radius, $r$, of the arterial lumen according to Poiseuille’s equation which describes viscous fluid flow through a tube of radius $r$:

$$PVR = \frac{8L \eta}{\pi r^4},$$

where $L$ is the total length of the blood vessels and $\eta$ is the coefficient of blood viscosity. As radius is raised to the fourth power, both PVR and PAP are exceptionally sensitive to changes in arterial lumen diameter.

2.2 What is Pulmonary Arterial Hypertension?

$K_v$ channel dysfunction, including $K_v1.5$, in PASMC has been linked to pulmonary arterial hypertension (PAH), a rare but devastating disease which, if left untreated, leads to right heart failure and death. PAH affects people of all ages, including newborns. 2-5 people per million per year are diagnosed with idiopathic pulmonary arterial hypertension (IPAH) (71, 176). Patients present with a range of nonspecific symptoms, including exertional dyspnea, chest pain, fatigue, syncope, cyanosis, and peripheral edema. The generality of these symptoms and the rarity of the disease make diagnosis a difficult task, and the mean time from onset of symptoms to diagnosis is two years (247), although up until recently it was as much as five years.

While PAH is commonly the result of an underlying disorder or a known risk factor, such as left heart failure, connective tissue disease, chronic obstructive pulmonary disease (COPD), amphetamine use or HIV infection, and can thus be classified as secondary pulmonary hypertension (SPH), the cause of idiopathic
pulmonary arterial hypertension (IPAH) is unknown, although it is likely the result of multiple insults, including both genetic and environmental factors (244, 264, 314). Familial PAH (fPAH) is defined as IPAH in two or more affected blood relatives, but its exact genetic cause is unknown.

The intimal and medial hypertrophy, PASMC proliferation, remodeling, muscularization, concentric laminar fibrosis and sustained vasoconstriction that accompany PAH decrease vascular compliance and obliterate the arterial lumen, thus increasing both PVR and PAP. An increase in PVR increases the stroke work of the right ventricle and thus exposes the RV to pressure overload. Initially, adaptive hypertrophy of the RV overcomes the pressure overload but in time progresses to contractile dysfunction and decompensated right heart failure. Major pathological findings of IPAH include medial hypertrophy due to PASMC proliferation, muscularization of the arterial walls due to PASMC invasion of the intimal layer, endothelial cell proliferation, and concentric laminar fibrosis (164, 247). Pathological findings of pulmonary arterial hypertension are present in muscular pulmonary arteries (<500µm diameter) to the resistance pulmonary arterioles (<100µm in diameter). Thus, PAH is often referred to as ‘small vessel disease’ to distinguish it from obstructions in the main pulmonary artery that can cause PAH such as in chronic thromboembolic pulmonary hypertension (CTEPH).
2.3 Dysfunctional PASMC Contribute to PAH Pathogenesis

A balance between apoptosis and proliferation is needed for the normal function of organs. The main function of mature VSMC is contraction, and as such, in a fully developed PA the proliferation rate of VSMC is very low. However in response to combinations of local environmental stimuli VSMC may proliferate abnormally. In PASMC from IPAH patients, increased proliferation and decreased apoptosis are favored (87, 231). Indeed, the pulmonary vascular remodeling observed in PAH is due in part to increased activity of an elastase which induces expression of growth factors, pro-proliferative signals such as tenascin, and fibronectin which signal PASMC migration and proliferation (230). Treatment with an elastase inhibitor is able to completely reverse established PAH in a rat model of the disease in part by inducing VSMC apoptosis (44). Additionally, abnormal PASMC depolarization, which has been observed in PASMC from IPAH patients, contributes not only to chronic vasoconstriction and increased proliferation (by increasing [Ca^{2+}]_{cyt}), but also may inhibit PASMC response to vasorelaxant factors, as PASMC depolarization attenuates acetylcholine induced endothelium-dependent vasodilation in intact rat pulmonary arteries (256). Given PASMC dysfunction in IPAH pathology and the central role $K_V$ channels play in PASMC contraction, proliferation, apoptosis and migration, it is not surprising that $K_V$ channel dysfunction, including $K_V1.5$, has also been linked to PAH.
2.4 Dysfunctional Kv Channel Function and Expression in IPAH

A number of observations have suggested that the cellular abnormalities of IPAH may be due in part to an imbalance in ion equilibria and Kv channel dysfunction. PASMC from IPAH patients have higher resting [Ca\(^{2+}\)]\(_{\text{cyt}}\) and a more depolarized E\(_{\text{m}}\) than PASMC from either non-PAH controls or SPH patients (313, 321). This may be due to a decrease in Kv channel function which has also been observed in PASMC from IPAH patients. Both higher resting [Ca\(^{2+}\)]\(_{\text{cyt}}\) and depolarized E\(_{\text{m}}\) are characteristic of proliferating PASMC compared to growth arrested PASMC (220), indicating that PASMC in IPAH patients may have an intrinsic and abnormal tendency to proliferate in the vasculature. In addition to promoting proliferation, increased [Ca\(^{2+}\)]\(_{\text{cyt}}\) in PASMC is a major stimulus for both cellular contraction and migration.

Indeed, Kv channel function in PASMC from IPAH patients is inhibited compared to PASMC from SPH patients. PASMC from IPAH patients have decreased Kv1.1, Kv1.2, Kv1.3, Kv1.4 and Kv1.5 mRNA transcript and decreased whole cell \(I_{K(V)}\) amplitude and density compared to control PASMC (313, 321). Kv channel dysfunction in PAH may also stem from unidentified circulating mitogenic factors, as normal hPASMC grown in serum from SPH patients proliferate more and have both decreased \(I_{K(V)}\) and depolarized E\(_{\text{m}}\) compared to hPASMC grown in serum from normotensive subjects (149). These results are consistent with the hypothesis that downregulated Kv channel expression and activity in IPAH-PASMC depolarize the cell, thus leading to calcium entry through VDCC. In addition to downregulated Kv channel expression and function, enhanced Na\(^+\)/Ca\(^{2+}\) exchanger member 1 (NCX1)
protein and enhanced \( \text{Ca}^{2+} \) entry via operation in the reverse mode in PASMC from IPAH patients may provide additional mechanisms that contribute to elevated \([\text{Ca}^{2+}]_{\text{cyt}}\) observed in PASMC from IPAH patients (325). Increased \([\text{Ca}^{2+}]_{\text{cyt}}\) in PASMC could lead to the sustained pulmonary vasoconstriction and medial hypertrophy observed in IPAH. In addition to increasing \([\text{Ca}^{2+}]_{\text{cyt}}\) and thus increasing proliferation, dysfunctional \( \text{K}_V \) channels may inhibit apoptosis by decreasing the potential for AVD. The decreased \( I_{K(V)} \) observed in IPAH cells is unlikely to be merely an effect of PAH, since PASMC from SPH patients who had comparable PAP and PVR do not have \( \text{K}_V \) channel alterations compared to normal PASMC (313, 321).

2.5 IPAH is Caused by Multiple Genetic and Environmental Factors

Although there is no single genetic cause of IPAH, heterogeneous mutations in the bone morphogenetic protein (BMP) receptor type II (\( \text{BMPR2} \)) gene (which encodes the BMPR-II protein) have been linked to both familial and sporadic PAH (51, 52, 134, 274). BMPR-II is a cell surface receptor of the TGF-\( \beta \) superfamily of signaling molecules that is needed for recognition of BMP ligands. BMPR-Ia is another receptor molecule involved in this pathway. BMP ligand binding triggers receptor oligomerization, stimulating transcription factors of the Smad family which activate genes involved in cell growth arrest and induction of apoptosis through Bcl-2 downregulation (107, 203, 326).

BMP signaling in PASMC induces apoptosis (133). In hPASMC, BMPs promote apoptosis by activating caspases, releasing cytochrome c, and downregulating
antiapoptotic Bcl-2 (133). BMPR-II mutations found in IPAH patients diminish the antiproliferative and proapoptotic effects of BMP signaling (159, 160, 202). Mutations in the BMPR-II receptor identified in PAH patients renders normal PASMC resistant to BMP-induced apoptosis (133), indicating that a loss of pro-apoptotic BMP signaling, which would result in an overall increase in PASMC in the pulmonary vasculature and thus remodeling and arterial lumen obstruction, lies at the core of many cases of PAH. In PASMC, BMP-2 (a ligand for the BMP receptor) treatment increases whole cell $I_{K(V)}$ and $K_V1.5$ mRNA and protein (62, 309). (As has been noted, increased $I_{K(V)}$ in PASMC is associated with apoptosis (26, 126)). However, PASMC from IPAH patients are more resistant to apoptosis induced by BMP than cells from normotensive control or SPH patients (326). Similarly, while BMP ligand (BMP-2, -4 and -7) inhibits serum-stimulated DNA synthesis and proliferation in PASMC from normal subject or SPH patients, BMPs fail to inhibit the same in PASMC from IPAH patients (189). These data suggest an overall resistance to apoptosis in PASMC from IPAH patients and that PASMC from IPAH patients react abnormally to endogenous proliferation and antiproliferation signals in the vasculature.

Although $BMPR2$ mutations are the single most prevalent genetic factor associated with IPAH known at this time, they do not account for all instances of the disease: ~70% of familial PAH cases have $BMPR2$ mutations; the prevalence of the mutations is 10-40% in sporadic IPAH and the penetrance is incomplete, as only ~20-30% of BMP-RII mutant carriers develop the disease (10, 188, 264). Indeed, a large study of ten IPAH families identified another locus for PAH (244), and there is
emerging evidence that a polymorphism in the serotonin transporter is linked to PAH susceptibility (55). Environmental factors, such as amphetamine use and HIV infection, have been correlated to PAH as well. Even in the background of known PAH risk factors such as fenfluramine use, mutations such as one found in the serotonin 5-HT (2B) receptor may predispose individuals to PAH (19). Thus, there are likely various genetic modifiers that influence disease pathogenesis.

2.6 Animal Models of PAH

While differences in $K_V$ channel activity and expression between PASMC from IPAH patients and from non-PAH subjects have been observed in man, animal models also support a role for $K_V$ channel dysfunction in PAH. Exposure to chronic hypoxia (CH), treatment with monocrotaline (MCT, an endothelial toxin), and/or introduction of $BMPR2$ mutations have been used to induce PAH in laboratory animals. Additionally, fawn hooded rats are spontaneously pulmonary hypertensive.

Although $BMPR2$ mutations have been linked to IPAH, the exact mechanism by which they mutations contribute to IPAH are unknown; however, mice harboring smooth muscle-specific inducible BMPR-II dominant negative mutations found in humans have cardiac characteristics typical of IPAH, including increased PAP, increased right to left ventricle + septum weight ratios, and PA muscularization (292). Interestingly, despite global smooth muscle cell expression of the dominant negative BMPR-II, mice developed symptoms of PAH without abnormalities in systemic blood pressure (293). Mice with a conditional heterozygous or homozygous knockout of
BMPR2 in pulmonary endothelial cells are similarly predisposed to develop PAH (100). On a molecular level, the mice with the global smooth muscle cell BMPR-II mutation have decreased K_V channel transcript and K_V1.5 protein in whole lung tissue (292, 309), echoing the reduced K_V1.5 transcript found in human PASMC from IPAH patients (321). Treatment of these mice with nifedipine, an L-type Ca^{2+} channel blocker reduces right systolic pressures (309). These studies demonstrate that loss of normal BMP signaling in smooth muscle cells is enough to produce a PAH phenotype. However, a combination of genetic and environmental factors are thought to be necessary for disease onset in humans harboring BMPR2 mutations (10). Thus, K_V channel dysfunction may underlie PAH pathogenesis in the background of BMPR2 mutations.

Chronic hypoxia and monocrotaline (MCT) administration have each been used to induce PAH in rats. CH and MCT-treated rats develop hemodynamic abnormalities that resemble those seen in PAH, including an increase in PVR, right ventricular (RV) hypertrophy, and pulmonary arterial remodeling (179, 181). In PASMC, hypoxia decreases I_{K(V)} and K_V gene expression (66, 222, 287, 316). In piglets, CH, by reduces K_V1.2 protein and depolarizes PASMC (66).

The role of apoptosis, or lack thereof, in PAH has also been studied in animal models of PAH. In VSMC, survivin is an important inhibitor of apoptosis. In a small PAH patient sample, survivin was found to be expressed in the PA of six patients with PAH, but not in the PA of three patients without PAH; furthermore, it is expressed in rats with MCT-induced PAH but not in rats without PAH (178). Administration of a
dominant negative form of survivin in MCT-treated rats reverses PAH and lengthens survival, leading to hemodynamic improvements including decreased PVR, RV hypertrophy and PA medial hypertrophy due to mitochondria-dependent apoptosis of PASMC and a decrease in proliferation (178). These changes are accompanied by an increase in $K_V$ current in PASMC (178). Additionally, dichloroacetate (DCA) can both reverse and prevent the hemodynamic abnormalities of PAH induced by either CH or MCT, including increased PVR, RV hypertrophy and PA remodeling (179, 181). On a molecular level, both DCA treatment in CH- and MCT-induced PAH and survivin gene therapy in MCT-induced PAH act through mechanisms that involve a restoration of expression and function of $K_V$ channels. DCA rescues both the decrease in potassium currents and $K_V2.1$ channel expression induced by CH and the decrease in $I_K$ and protein and mRNA expression of $K_V1.5$ induced by MCT (179, 181). On a cellular level, DCA leads to PASMC apoptosis and decreased proliferation in the PA medial layer (179). Dominant negative survivin gene therapy also increases $I_{Kr(V)}$ and causes PASMC apoptosis (178). MCT-induced PAH is also completely reversed (as measured by PAP and arterial structure) in rats treated with an elastase inhibitor; this effect was due to myocyte apoptosis and loss of the extracellular matrix which had supported abnormal PASMC growth (44).

Lastly, fawn hooded rats (FHR) have been used to study the molecular mechanisms of PAH and the effects of DCA treatment. FHR are spontaneously pulmonary hypertensive at 40 weeks of age, demonstrating increased PAP and right ventricular thickness compared to consomic controls (22). On the molecular level,
FHR-PAH is characterized by normoxic activation of hypoxia-inducible-factor-1-alpha (HIF-1α) which inhibited expression of Kv1.5 (22). Oral DCA administered to FHR improves PAH and increases survival, while DCA treatment in cultured PASMC from FHR eliminates HIF-1α activation and restores Kv1.5 protein and RNA expression (22).

Together, these promising studies suggest that future therapeutics may act to increase Kv channel expression and/or activity, as this may result in increased apoptosis of abnormally proliferating PASMC in IPAH.

2.7 Treatment of IPAH

Prior to drug developments of the last ten years, lung transplantation and calcium channel blockers were the only treatments for IPAH. Nifedipine and diltiazem provided an early therapeutic approach but helped only a small percent of IPAH patients (<10-30%) (37, 246). The observation that three signaling pathways – prostacyclin, endothelin, and nitric oxide – are involved in PAH pathophysiology led to the development of three additional classes of drugs. Endothelin-1 is both an endogenous smooth muscle mitogen and vasoconstrictor and is upregulated in PA endothelial cells of PAH patients (79). Thus, endothelin receptor antagonists such as bosentan have proven effective at treating PAH by improving hemodynamics, exercise capacity and survival (36, 72, 175). Additionally, production of NO and prostacyclin (PGI₂), both potent vasodilators and inhibitors of SMC proliferation, are decreased in PAH patients. Prostacyclin analogues (73, 206) and phosphodiesterase-5 inhibitors
such as sildenafil (118, 206), which prolong the effects of NO, have also been found effective at treating IPAH. These drugs have both vasodilator and antiproliferative effects and have a variety of administrative routes, including inhalation, oral, and intravenous infusion by permanent indwelling catheter.

Drugs are commonly used in combinations determined on a per patient basis. For example, combination sildenafil and prostanoid treatment has been found to improve exercise capacity and pulmonary hemodynamics in patients with severe PAH (78), and inhaled iloprost, a prostacyclin analogue, when added to bosentan monotherapy regimens improves patients’ exercise capacity and hemodynamics (177). However, some patients do not respond to any pharmacological treatment or deteriorate after an initial beneficial period. These patients remain on the lung transplant list. Thus, an early screen of people at risk for developing PAH (HIV, known BMPR2 mutation, and/or relatives with IPAH, e.g.) would provide a basis for early and preventative treatment. As the various genetic factors that contribute to IPAH are elucidated, such a screen would include increasingly many genes that are involved in IPAH. Such a screen would reduce the time to diagnosis and lead to earlier medical intervention.

2.8 Chronic Thromboembolic Pulmonary Hypertension

Chronic thromboembolic pulmonary hypertension (CTEPH) is a type of PAH, the initial cause of which is well defined. Initially, a thrombus from the peripheral venous circulation lodges in the PA where it fails to spontaneously resolve. The
immediate rise in PAP caused by the blockage, however, is followed by a decline as ventricular hypertrophy compensates for the increased pressure load. Over time, however, cardiac output continues to decrease until pulmonary hypertension is fully developed. For many patients, CTEPH can be cured surgically with pulmonary thromboendarterectomy (PTE) (110) in which clot material is removed from the PA. Interestingly, surgically removed clot material is often a cast of the pulmonary arterial tree, indicating that the initial clot burden continued to grow after becoming lodged in the PA. Furthermore, clots are often recanalized, an additional indication of growth and abnormal cellular proliferation in CTEPH.

Certain patients who undergo PTE have poor post-operative outcomes (~15% of PTE patients), characterized by persistent pulmonary hypertension (often quoted as PVR > 500 dynes × s/cm⁵). Post-operative mortality in these patients is much higher (~20-30%) than in patients who have a post-operative PVR <500 dynes × s/cm⁵ (3-7%). Patients with high post-operative PVR are suspected to have underlying distal vessel PAH (i.e., IPAH or other forms of PAH in addition to CTEPH) which is intractable to surgery. These patients would benefit tremendously from pre-operative treatment for underlying distal vessel disease before undergoing PTE. A genetic screen provides a noninvasive and inexpensive way to accomplish the goal of identifying people at high risk for PAH to determine who, among CTEPH patients, should be treated for distal vessel PAH before undergoing PTE.
2.9 Goals of the Dissertation Research as Related to PAH

$K_V$ channels play an important role in controlling PASMC contraction, proliferation, and apoptosis and have been implicated in IPAH pathogenesis. Because of the progressive and life-threatening nature of IPAH and CTEPH, the import of an early diagnosis, allowing for earlier treatment, cannot be underscored. $BMPR2$ mutations are not the only genetic contributor to IPAH, and as the cellular mechanisms and abnormalities of PAH become better understood, other genetic risk factors will be found. The role of $K_V$ channels in PAH pathogenesis is widely supported by observations in both IPAH patient tissue samples and animal models of PAH, and it is with the wider goal of contributing to an understanding of disease etiology and genetic risk factors that this research on $K_V1.5$ channel mutations found in a small population of IPAH patients has been undertaken. Specifically, two mutations that were identified in the $KCNA5$ gene of IPAH patients and which localize to the T1 domain are characterized in terms of their effects on electrophysiological properties and subcellular localization of the $K_V1.5$ channel.
Chapter 3. Materials and Methods

3.1 Cells and Culture

All cell culture materials were purchased from Gibco/Invitrogen unless otherwise noted. Normal human pulmonary artery smooth muscle cells (hPASMC) were purchased from Lonza and cultured in smooth muscle growth media (SMGM, Lonza), which consisted of smooth muscle basal media (SMBM) supplemented with insulin (5 µg/ml), human fibroblast growth factor-B (2 ng/ml), gentamicin sulfate and amphotericin-B (0.1%), fetal bovine serum (FBS, 5%), and epidermal growth factor (0.5 ng/ml). PASMC from IPAH patients and SPH patients were maintained in identical fashion. HEK-293 (ATCC) and COS-1 (ATCC) cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with 4.5g/L D-glucose and 10 mg/L sodium pyruvate, supplemented with FBS (10%), L-Glutamine (0.01%), and penicillin/streptomycin (1%).

All cells were incubated in a humidified environment at 37°C and 5% CO₂. Media was changed 24 hours after initial seeding and every 48 hours subsequently. When cells reached 80-90% confluency, cells were gently washed with phosphate buffered saline (PBS; for COS-1 and HEK-293 cells) or hepes buffered saline solution (HBSS; for PASMC), incubated with 3mL of trypsin/EDTA solution until detachment (3-5 min), and then incubated with an equal amount of trypsin neutralizing solution. For PASMC, a 0.25% trypsin/EDTA solution (Lonza) was used, whereas COS-1 and HEK-293 cells were trypsinized with a 0.05% solution. The cell suspension was then
transferred to a sterile 15-ml round bottom tube, centrifuged at room temperature for 7 minutes at 1500 rpm, and then resuspended in appropriate growth media and seeded. PASMC between passages 3 and 8 and HEK-293 and COS-1 cells between passages 2 and 12 were used for experiments.

3.2 Constructs

3.2.1 Wildtype \( K_{\text{V}1.5} \) (WT-\( K_{\text{V}1.5} \)) and Mutant \( K_{\text{V}1.5} \) (E211D, G182R, E211D/G182R) Constructs

The KCNA5-pCMS-EGFP bicistronic vector (Figure 5) expresses both \( K_{\text{V}1.5} \) and EGFP proteins (26). Because EGFP is expressed separately from the \( K_{\text{V}1.5} \) protein, it serves only as a marker of cell transfection without interfering in the folding or function of \( K_{\text{V}1.5} \). To make the mutant construct, a gel purified EcoRI—NotI fragment of the KCNA5-pCMS-EGFP construct containing the coding region of the human KCNA5 gene (bps -18 to +1903, NM_002234), was subcloned into pBluescript SK (Stratagene). The resulting KCNA5-pBSSK vector was sequenced to verify identity and integrity of the insert. Mutagenesis was performed with Stratagene’s commercially available QuikChange II (XL) Site-Directed Mutagenesis Kits as follows. KCNA5-pBSSK dsDNA template was incubated with each or both of the mutant primer pairs (sequences below), dNTP, reaction buffer, and water. \( PfuUltra \) HF DNA polymerase was added and the reactions were subject to the following cycling parameters: 95°C for 30 seconds, followed by 20 cycles of 95°C for 30
seconds, 55°C for 1 minute, and 65°C for 7 minutes. Methylated parental DNA was digested with DpnI and used to transform DH5α E. coli or XL10-Gold ultracompetent cells. There is a naturally occurring ClaI restriction site at position 1190 of the KCNA5 coding sequence, which is downstream of the mutagenesis sites. The EcoRI-ClaI fragment of the mutated KCNA5-pBSSK (containing the mutation(s) and the ClaI-NotI fragment of the original KCNA5-pBSSK were then subcloned back into the pCMS-EGFP vector (Clontech) (Figure 5). DNA purified from transformed colonies was sent for sequencing to verify the presence of the mutation and the integrity of the insert.

The following primer pairs were used for site directed mutagenesis (lowercase letters indicate a nucleic acid mutation; nucleic acid mutations that result in amino acid substitutions are in bold. Silent mutations were inserted to create NaeI and AgeI diagnostic restriction sites, which are underlined):

G182R:
5'-CTACTACTACCAGTCGGGGGCCGCTGCGGAGGCCGGTC-3'
5'-GACCGGCCTCCGCAGcCGGC CCCgGGACTGTAGTAG-3'

E211D:
5'-GACGAGGCCATGGA cCGTTCCGCGAGGATGAG-3'
5'-CTCATCCTCGCGGAcCGgTCCATGGCCTCGTC-3’
3.2.2 K_β^{1.3}-HA Construct

The K_β^{1.3} which was tagged with hemagglutinin (K_β^{1.3}-HA) construct was kindly provided by Dr. Katherine T. Murray and is described in detail elsewhere (295).

3.3 Transfection

Two to three days after seeding, cells were transiently transfected at 60-80% confluency with either Fugene 6 (Roche) for staining preparations (better adherence of cells to glass coverslides with Fugene 6) or with Lipofectamine 2000 (Invitrogen) for all other experiments. Transfection reagent (in μl) and DNA (in μg) was added directly to cells in growth media in the following ratios: 4:1 for Fugene 6 and 10:4 for
Lipofectamine 2000. In both instances, Opti-MEM Reduced Serum Media (Gibco) was used to dilute DNA and transfection reagents. Specifically, DNA and Lipofectamine 2000 were separately diluted in Opti-MEM, incubated at room temperature for 5 minutes before being combined, incubated at room temperature for 30 minutes and then added to cells. Fugene 6 was similarly diluted in Opti-MEM for 5 minutes before DNA was added directly to these reactions. These complexes incubated at room temperature for 30-45 minutes before being added to cells. Lipofectamine 2000 complexes were removed from cells 4 hours after incubation in a humidified environment at 37°C and 5% CO₂. Cells were washed gently in growth media and the incubated in fresh growth media until used for experimentation 24-72 hours after transfection. The short (4 hour) incubation time with Lipofectamine 2000 complexes was chosen to optimize the balance between the integrity of cell membranes for patch clamping and transfection rate. Under these conditions, transfection rate was between 50 and 80%. Fugene 6 complexes remained on cells until they were prepared for staining 24-48 hours after transfection; these conditions produced a low transfection rate (~10-30%), but little toxicity was observed.

3.4 Electrophysiology

3.4.1 Solutions for Electrophysiology

Cells were seeded onto glass coverslips, transfected 24-72 hours later with Lipofectamine 2000 and used for electrophysiological recordings 24-48 hours after
transfection. External solution was perfused through the recording chamber at \( \sim 2 \) ml/min. All chemicals were from Sigma. External solution for recording whole-cell \( I_{K(v)} \) contained (in mM): 141 NaCl, 4.7 KCl, 3 MgCl\(_2\), 1 EGTA, 10 glucose, and 10 hepes and was adjusted to pH 7.4 with NaOH. Internal (pipette) solution consisted of the following (in mM): 135 KCl, 4 MgCl\(_2\), 10 Heps, 10 EGTA, and 5 Na\(_2\)ATP and was adjusted to pH 7.2 with KOH. 4-AP (5 mM) was dissolved in external solution. All solutions were sterile filtered before use.

3.4.2 Electrophysiological Recordings

25-mm glass coverslips plated with cells were placed into the recording chamber of a Nikon Eclipse TE300 inverted microscope and superfused with external solution by gravity perfusion. All experiments were performed at room temperature (22-24°C) on a vibration isolation table (TMC) and recorded with an Axopatch-1D amplifier using a Digidata 1322A interface (Axon Instruments). Borosilicate glass tubes (Sutter Instruments) were pulled on a Model-97 electrode puller (Sutter Instruments) and fire polished on a MF-83 microforge (Narishige Scientific Instruments) to resistances of 2-4 M\( \Omega \). Pipettes were filled with appropriate solution through a syringe and attached to a microelectrode that was mounted onto a CV-4 preamplifier headstage (Axon Instruments). Silver wire was soaked in bleach to create silver chloride microelectrodes. Pipette manipulation was accomplished with a Sutter MP-225 motorized micromanipulator. Single channel records where recorded in the cell-attached mode (Figure 6) after achieving a 2-5 G\( \Omega \) seal with the membrane.
Whole cell recordings were made after additional suction was applied to the seal to break into the cell (Figure 6).

**Figure 6: Illustration of Cell-Attached and Whole-Cell Patch Clamp Configurations.** Once a gigaohm seal is achieved between the patch pipette and the cell, single channel recordings are made in the cell-attached mode. If additional suction is applied to the seal, whole-cell access is gained.

### 3.4.3 Pulse Protocols

Administration of voltage protocols and data acquisition was achieved with pClamp 8.2 (Axon Instruments) on a Dell Optiplex GX110 running Windows 2000. Prior to recording, the cell’s capacitance, resting membrane potential ($E_m$), and holding current (the current that is injected into the cell to hold it at the command potential) were noted. All holding potentials were -70 mV and all voltage steps were in 20-mV increments. Pulse protocols for recording I-V relationships stepped from -80 to +80 mV for 300 ms. Inactivation protocols consisted of a stepped prepulse between -90 and +60 mV, followed by a 5-ms return to the holding potential, followed by a 150-ms test-pulse to +60 mV. Channel availability was calculated as the ratio of the
amplitude of the current during the test-pulse to the amplitude of the current during the pre-pulse. Tail currents were recorded from a standard double pulse protocol in which the pre-pulse stepped from -80 to +60 mV for 300-ms. This was followed by a test-pulse to -40 mV. Single channel recordings were made at 110, 90 or 70 mV.

3.5 Reverse Transcriptase PCR

Total RNA was isolated from HEK-293, COS-1 and COS-7 cells, and RT-PCR using a standard TRIZOL (Invitrogen) and chloroform extraction. Briefly, cells were washed twice in PBS and incubated in TRIZOL at room temperature for 10 minutes. Cell lysate was transferred to a microcentrifuge tube and chloroform added in a 1 (cell lysate):0.2 (chloroform) ratio. Tubes were shaken by hand and allowed to sit at room temperature for 3 minutes. Samples were then centrifuged at 12,000 rpm at 4°C for 15 minutes and the aqueous phase was transferred to a new tube which was subject to the same centrifugation protocol. To the aqueous phase isopropyl alcohol was added in a 1:1 ratio and vortexed vigorously for 15 sec. Samples were incubated at room temperature for 10 minutes and then centrifuged at 12,000 rpm for 10 minutes at 4°C. Supernatant was discarded and to the samples was added 75% EtOH (in DEPC-treated water). After vortexing, samples were centrifuged at 7,000 rpm for 5 minutes at 4°C from which the supernatant was removed. RNA was allowed to air dry at room temperature and was dissolved in DEPC-treated water. After quantification with a spectrophotometer, RNA was either stored at -70°C or used to make cDNA.
cDNA was prepared from equal amounts of total cell RNA with the Superscript First-Strand cDNA Synthesis kit (Invitrogen) according to the manufacturer’s instructions. cDNA was subject to PCR using the Platinum PCR SuperMix High Fidelity reagent (Invitrogen). The PCR protocol was as follows: 3 min at 94°C followed by 32 cycles of (30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C), followed by 10 min at 72 °C and then held at 4°C. The sequences of sense and antisense primers (Table 1) were designed from the coding regions of human Kv1.5 and various human Kvβ subunits. Primer fidelity and specificity were examined with NCBI BLAST program. As a control for integrity of total RNA, primers specific for GAPDH were also used (the first GAPDH primer was used in the examination of Kvβ mRNA while the second was used in the study of Kv1.5 mRNA. PCR products were run on a 1.5% agarose gel which contained 0.008% Gel Star Nucleic Acid Gel Stain (Cambrex). Net intensity values of the PCR product bands, measured using a Kodak Electrophoresis Documentation System (Eastman Kodak, Rochester, NY), were normalized to GAPDH; the ratios are expressed as arbitrary units for quantitative comparisons.
Table 1: Oligonucleotide Sequences of the Primers Used for RT-PCR. *Accession numbers in GenBank for the sequences used in designing the primers.

<table>
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<th>Location (nt.)</th>
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<td>5'-GGCAAAGAGCACCTTGGGAGA-3'</td>
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3.6 Immunoblot and Co-immunoprecipitation

Cell lysis was performed 24 hours after transfection in 580 µl lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl, pH = 7.4) supplemented with 7x Complete Mini protease inhibitor cocktail (Roche) and 100 µg/ml PMSF. Lysates were kept on ice for 10 minutes and then centrifuged at 4°C at 14,000 rpm for 15 minutes. The insoluble fraction was discarded and Laemmli Sample Buffer (BioRad) with 10% β-mercaptoethanol was added to the soluble fraction in a 2:1 ratio. Samples were vortexed briefly and boiled at 100°C for 5 minutes. Protein samples were separated on 4-20% Tris-Glycine Gels (Invitrogen) for 90 minutes at constant voltage (100 V). Running buffer, which was stored at room temperature as a 10x solution and diluted for use, consisted of (mM): 248 Tris Base, 1918 glycine, and 35 sodium dodecyl sulfate (SDS). Protein samples were then transferred to nitrocellulose membranes (Whatman) for 110 minutes at a constant current (A=250 mA) using the Mini Protein 3 System (BioRad) at 4°C. Transfer
buffer consisted of (in mM): 100 Tris Base, 190 glycine, and 20% methanol. After transfer, membranes were incubated in blocking buffer (5% nonfat dry milk powder, 5 mL 10x TBS-T) for 1 hour and subsequently incubated overnight with primary antibody in PBS-T/Albumin (PBS-T/A) at 4°C. PBS-T/Albumin consisted of 0.1% Tween 20 and 1% bovine serum albumin (BSA) in PBS. After primary antibody incubation, membranes were washed twice in TBS-T (37 mM NaCl, 15.4 mM Trizma HCl, 4.6 mM Tris Base and 0.001% Tween 20) and incubated with appropriate secondary antibody dissolved in PBS-T (0.1% Tween 20) at room temperature for one hour. After washing twice more in TBS-T, membranes were exposed to SuperSignal Chemiluminescent substrate for HRP (Pierce) for 5 minutes and developed on BioMax Light Film (Kodak). Films were scanned and the intensities of the bands analyzed using ImageJ software (NIH). The following antibodies were used: rabbit anti-hK\textsubscript{V}1.5 at 1:600 dilution (Alomone Labs), mouse anti-GAPDH at 1:500 (Chemicon), goat anti-rabbit IgG-HRP at 1:2000 (Santa Cruz) and goat anti-mouse-IgG-HRP at 1:1000 (Jackson).

For co-immunoprecipitation, 5% of the total cell lysate was run as a loading control and the rest was incubated with 1:160 anti-K\textsubscript{V}1.5 antibody (Alomone) for 45 minutes on ice. Protein A/G beads (Pierce) were then added and the mixture gently rocked for 1 hour at 4°C. The unbound fraction was washed 4x in ice cold lysis buffer. Subsequent preparation of the protein fraction for loading onto the gel follows the procedure outlined for immunoblots. Membranes were probed for HA-tag (mouse anti-HA, Santa Cruz). For the MG-132 studies, cells were either treated with 10 µM
MG-132 (Calbiochem) dissolved in DMSO (Sigma) or an equal volume (1:1000) or DMSO for 24 hours before cell lysis.

3.7 Immunostaining

To minimize toxicity of cells on glass coverslips used for immunostaining, Fugene 6 (Roche) was used to transfect cells. 24 hours after transfection of hPASMC or 48 hours after transfection of HEK-293 and COS-1 cells, cells were washed on ice three times with PBS. After fixing cells in 4% paraformaldehyde/PBS for 15 minutes at room temperature, coverslips were incubated in blocking solution (2% BSA, 2% FBS, 0.1% Triton X-100 in PBS) for 1 hour. Coverslips were then exposed to rabbit anti-KV1.5 antibody (Alomone, 1:1000 in blocking solution) for 2 hours at room temperature. After washing cells with PBS, they were incubated with bovine anti-rabbit rhodamine-conjugated IgG antibody (Santa Cruz) for 45 minutes. Cells were also stained with DAPI (Invitrogen) to label nuclei. After washing again in PBS, coverslips were mounted onto glass slides using Fluoromount-G (Electron Microscopy Sciences) and visualized under appropriate fluorescent filters on an Olympus IX70 microscope. The SoftWoRx suite was used for deconvolution and image processing.

3.8 Thymidine Incorporation

24 hours after transfection in 6 well dishes, cells were serum starved in DMEM without FBS for 24 hours. Media was then changed to DMEM with 10% FBS to induce proliferation and 3H-thymidine (1 μCi/ml final concentration) was added. Cells
were allowed to incubate for 6 hours. They were then washed with cold PBS once and with cold 7.5% trichloroacetic acid (TCA) twice. Remaining cellular material was lysed with 0.5M NaOH at 37°C for 30 minutes. Samples were then transferred into individual scintillation vials to which 5 ml Ecolume scintillation fluid (ICN) was added. $^3$H counts were read on a Beckman LS3801 liquid scintillation counter for 1 minute each.

3.9 Protein Modeling

The structural modeling of $\text{K}_\text{V}1.5$ was based on previous x-ray crystallographic data of the $\text{K}_\text{V}1.2$ channel (protein database ID:2a79, 1qdw, 1qdv, and 1dsx), with which $\text{K}_\text{V}1.5$ is 77% identical, and structural homology comparisons with short peptide sequences for the divergent sequences of $\text{K}_\text{V}1.5$. We created the model of $\text{K}_\text{V}1.5$ using the protein modeling software InsightII (Accelrys, San Diego).

3.10 Protein Sequence Alignment

The alignments of the T1 domains of various $\text{K}_\text{V}1$, $\text{K}_\text{V}2$, $\text{K}_\text{V}3$, and $\text{K}_\text{V}4$ subfamily members was performed using the ClustalW software (138).

3.11 Statistical Analysis

The composite data are expressed as means ± standard error (SEM). Statistical analysis was performed using unpaired Student's $t$ test or one-way analysis of variance (ANOVA) as indicated. Differences were considered to be significant when $P<0.05$. 
Chapter 4. Results: Tetramerization Domain Mutations in Kv1.5 Alter Channel Kinetics

4.1 Whole Cell $I_{K(V)}$ and Channel Kinetics in PASMC from IPAH Patients are Altered Compared to Secondary Pulmonary Hypertension Patients

As has already been established (cf. sections 2.3 and 2.4), the dysfunction of Kv channels, including reduced current amplitude and protein expression of Kv1.5, has been implicated in IPAH. Kv channel function in PASMC from IPAH patients were further examined by comparing whole cell Kv currents ($I_{K(V)}$) and channel kinetics in these cells to control PASMC from secondary pulmonary hypertensive (SPH) subjects.

I-V recordings were generated using a standard pulse protocol which stepped from -60 to +80 mV in 20-mV increments (holding potential = -70 mV). Both $I_{K(V)}$ amplitude and current density in PASMC from IPAH patients were found to be significantly lower when compared to control PASMC from SPH patients (Figure 7A). For example, at +80 mV, $I_{K(V)}$ in control cells was $1621.918 \pm 154.92$ pA, while that in IPAH cells was $279.78 \pm 42.04$ pA (SPH n=29, IPAH n= 22; p<0.001) (Figure 7Ac, Figure 7Ca). The averaged $I_{K(V)}$ recording (Figure 7B) and the summarized data of $I_{K(V)}$ amplitude (Figure 7Ca) at +80 mV for PASMC from both IPAH and SPH patients also indicate that $I_{K(V)}$ is significantly decreased in PASMC from IPAH patients compared to PASMC from SPH patients.
As kinetic time constants indicate intrinsic properties of channels, \( \tau_{\text{act}} \), \( \tau_{\text{inact}} \) and \( \tau_{\text{deact}} \) of \( I_{K(V)} \) in PASMC from both IPAH and SPH patients were measured by fitting various components of \( I_{K(V)} \) traces (activation, inactivation, and closure upon voltage stimulus withdrawal) at +80 mV depolarization with a single exponential curve (Figure 7Cb-d). All three time constants, \( \tau_{\text{act}} \), \( \tau_{\text{inact}} \) and \( \tau_{\text{deact}} \) were significantly lower in PASMC from IPAH patients compared to PASMC from SPH patients (\( \tau_{\text{act}} \): 3.57 ± 0.28 ms in SPH compared to 1.03 ± 0.08 ms in IPAH, \( P < 0.001 \); \( \tau_{\text{inact}} \): 176.80 ± 13.45 ms in SPH compared to 38.17 ± 4.42 ms in IPAH, \( P < 0.001 \); and \( \tau_{\text{deact}} \): 2.00 ± 0.13 ms in SPH compared to 1.49 ± 0.12 ms in IPAH, \( P < 0.01 \) (Figure 7C). These data suggest intrinsic gating abnormalities in \( K_V \) channels expressed in PASMC from IPAH patients compared to PASMC from SPH patients and led to the further investigation of the role of \( K_V \) channels in IPAH.
Figure 7: Kv Currents in PASMC from IPAH Patients have Lower Amplitude and Altered Kinetics Compared to PASMC from SPH Patients. PASMC were isolated from IPAH patients or from SPH control patients and subject to standard electrophysiological protocols to determine $I_{K(V)}$ amplitude and kinetic time constants. A) Representative recording from PASMC from SPH (a) and IPAH (b) patients. The pulse protocol is shown (inset). The I-V data is summarized in (c) and the current density in (d) ($n=29$ SPH control; $n=22$ IPAH). B) The averaged amplitude of $I_{K(V)}$ measured at +80 mV (a) as well as the current normalized to its maximum value (b). C) Boxplots show the average current amplitude (a), and time constants of activation (b), inactivation (c) and deactivation (d) for $I_{K(V)}$ recorded in PASMC from SPH patients (control, gray fills) and IPAH patients (black fills). Data are presented as mean ± SEM; $n=68$ control; $n=61$ IPAH. SPH control and IPAH data were compared with student’s t-test: **P<0.01; ***P<0.001.
4.2 Two Nonsynonymous Mutations Found in the KCNA5 Gene of IPAH Patients Localize to the Highly Conserved N-terminal Tetramerization Domain of Kv1.5

Two non-synonymous mutations in the KCNA5 gene (which encodes the voltage gated potassium channel Kv1.5) were identified in IPAH but not normotensive (Non-IPAH) patients (Figure 8). These mutations, which encode for a glycine (G) to arginine (R) mutation at amino acid 182 (ggg→agg; G182R) and a glutamate (E) to aspartate (D) mutation at residue 211 (gag→gac; E211D) (Figure 8), localize to the N-terminal tetramerization domain (T1) of Kv1.5 (Figure 4). The g/a mutation which encodes G182R in Kv1.5 was found in two of 413 IPAH patients and zero of 184 NPH (Non-IPAH) patients, while the g/c mutation which encodes the E211D mutation in Kv1.5 was found in 11 of 414 IPAH patients and zero of 184 NPH patients (Table 2). In all instances, IPAH patients who had either mutation were heterozygous for the allele, making the allele frequency for the G182R minor variant 0.0024 and 0.013 for the E211D minor variant (Table 2).

Both the G182R and E211D mutations occur at highly conserved residues within the T1 domain. The glycine at position 182 is 100% identical in the T1 domains of all 17 human Kv1, Kv2, Kv3 and Kv4 subunit examined (Kv1.1-7, Kv1.10, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4, Kv4.1, Kv4.2, Kv4.3) (Figure 9A) while the glutamate residue at position 211 is ~60% identical over these subunits (Figure 9B).
Figure 8: Identification of Two Mutations Found in the KCNA5 Gene of IPAH Patients. Chromatograms of the regions containing G182R (A) and E211D (B) (boxed in red) and their corresponding amino acid sequences (black boxes on right) are shown. Sequences from patient with pulmonary hypertension secondary to thromboembolic disease (Non-IPAH) are shown as controls. G182R was found in 2 of 413 IPAH patients, while E211D was found in 11 of 414 IPAH. Neither mutation was found in 184 Non-IPAH controls.
Table 2: Frequency of \(K_V\) Channel Mutations in IPAH Patients. Occurrence of the G182R mutation in KCNA3 and KCNA5, E211D in KCNA5 and V220M in KCNA10 is shown for IPAH and NPH patient populations and for alleles and allele frequency. Total number for each group is indicated in parenthesis.

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<th>E211D (KCNA5)</th>
<th>V220M (KCNA10)</th>
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<td>2 (413)</td>
<td>11 (414)</td>
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<td>0 (184)</td>
<td>0 (184)</td>
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<tr>
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<td>2 (826)</td>
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<tr>
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Figure 9: Alignment of T1 Region Encompassing the G182 and E211D Residues from Various Human \(K_V\) Channels. The residues which correspond to G182 (A) or E211 (B) are shown in bold print. The sequences are from the NCBI database with the indicated accession numbers: KCNA1: NM_000217; KCNA2: NM_004974; KCNA3: NM_002232; KCNA4: NM_002233; KCNA5: NM_002234; KCNA6: NM_002235; KCNA7: NM_031886; KCNA10: NM_005549; KCNB1, NM_004975; KCNB2: NM_004770; KCNC1: NM_004976; KCNC2: NM_139136; KCNC3: NM_004977; KCNC4: NM_004978; KCND1, NM_004979; KCND2: NM_012281; KCND3: NM_004980.
Because of the high conservation in the T1 domain of Kv channels and the role Kv channels play in IPAH, a screen to determine whether there were other N-terminal mutations in the Kv channels of IPAH patients was performed. The N-termini of Kv1.1 (encoded by the KCNA1 gene), Kv1.2 (KCNA2), Kv1.3 (KCNA3), Kv1.4 (KCNA4), Kv1.5 (KCNA5), Kv1.6 (KCNA6) and Kv1.10 (KCNA10) in 234 IPAH patients and 184 NPH controls were examined for nonsynonymous mutations. One IPAH patient was heterozygous for the g/a mutation (G182R) in KCNA3 (Kv1.3 channel) (Table 2). In addition to these G182R and E211D mutations, a mutation that encodes a valine to methionine amino acid substitution at position 220 (V220M) of Kv1.10 was found in 30 IPAH patients (all were heterozygous for the minor variant) and 23 NPH controls (20 were heterozygous and 3 homozygous for the minor variant) (Table 2). Although the allele frequency for the mutation which encodes V220M was higher than that of any other mutation in IPAH patients (0.0641), it was higher in the NPH control population (0.071), indicating that this mutation is not IPAH-specific. The valine at position 220 is in the first transmembrane domain (S1) of Kv1.10 and is not well conserved.

4.3 Protein Modeling Reveals the Orientation of the G182R and E211D Residues

The two mutations in Kv1.5, G182R and E211D, result in amino acid side chain substitutions in highly conserved residues of the T1 domain. The G182R mutation introduces a long positively charged amino acid side chain (3 carbon atoms and a guanidinium group [CH₅N₃]⁺, the side chain of arginine) where before existed...
only a hydrogen atom (the side chain of glycine), while the E211D mutation shortens
the acidic side chain from two carbon atoms to one. It was of initial interest to
determine the orientation these mutations would adapt in the assembled protein.
However, a complete model of Kv1.5 was lacking, and most information of Kv
structure is based on bacterial channels as well as Kv1.2. Based on previous X-ray
crystallographic data of Kv1.2 (PDB IDs #2a79, 1qdw, 1qdv and 1dsx), which has
77% amino acid identity to Kv1.5, and structural homology comparisons with short
peptide sequences for the divergent sequences of Kv1.5, the protein modeling software
InsightII (Accelrys, San Diego) was used to model Kv1.5 and to determine the relative
orientations of the mutant side chains (Figure 10). The model of the wildtype Kv1.5
protein indicates that both the G182 and E211 residues are close to the ion permeation
pathway. Specifically, G182 is located on an outward facing loop between two alpha
helices within the cytoplasmic core of T1 (Figure 10A, B). The acidic side chain of
E211 faces outward from the region just upstream of the T1-S1 linker very close to the
intracellular opening of the pore (Figure 10A, B). The positively charged guanidinium
group introduced by G182R protrudes beyond the surface of the wildtype T1 interface;
furthermore, the E211D mutation buries the negatively charged carboxyl group within
the surface of the T1 domain (compare Figure 10B to Figure 10C). As polar interfaces
in the T1 domain mediate Kv channel assembly and interaction with modulatory
proteins, these mutations could be expected to affect channel function.
Figure 10: The G182R and E211D Mutations in Kv1.5 Alter the Orientations of Conserved Amino Acids. A) Ribbon diagram of wildtype Kv1.5 with side chains of the glycine at position 182 (GLY182) and the glutamate at position 211 (GLU211) indicated (left) with close up of the region shown on the right. Four differently colored subunits are shown, but the residues are indicated only on the orange subunit for clarity. The top of the channel is extracellular while the bottom is cytoplasmically located. B) Space filling diagram of wildtype Kv1.5 with the side chains of glycine (GLY182) and glutamate (GLU211) indicated (left). Colored subunits correspond to (A). Close up of region with GLY182 and GLU211 (right). C) Space filling diagram of mutant Kv1.5 with the side chains of the arginine at position 182 (ARG182) and the aspartate at position 211 (ASP211) indicated (left). Close up of region with ARG182 and ASP211 (right).
4.4 G182R, E211D and G182R/E211D Mutant Subunits Form Functional Kv1.5 Channels

The G182R and E211D residues, which lie in close proximity to both the pore opening and the T1 polar interfaces that mediate Kvα-Kvα and Kvα-Kvβ interactions, were expected to affect the functional properties of the channel, specifically of the T1 domain. Therefore, a functional characterization of the G182R and E211D mutant channels, as well as the doubly mutant G182R/E211D channel, including electrophysiological properties (channel kinetics and pharmacological inhibition), channel association with Kvβ subunits and channel subcellular localization, was undertaken. While Kv dysfunction in PASMC has been linked to IPAH, PASMC express high levels of endogenous Kv1.5. Therefore, an expression system which would allow the study of the effects of the G182R and E211D mutations in isolation was needed. The majority of experiments were conducted in (human) HEK-293 and (mammalian) COS-1 cells, neither of which express high levels of endogenous Kv1.5 (Figure 11).

![Figure 11: COS-1 and HEK-293 Cells Express Little Endogenous Kv1.5. COS-1, HEK-293 and human PASMC were transiently transfected with either empty vector (EGFP) or wildtype Kv1.5 (WT-Kv1.5) as indicated at bottom. Whole cell lysate was probed on immunoblot for Kv1.5 (67 kDa, top) and GAPDH (36 kDa, bottom) as a loading control.](image)

Due to the high conservation of the residues at positions 182 and 211 (Figure 9) and the essential role of the T1 domain in channel formation, there was a likelihood
that the mutant proteins would not form functional $K_v1.5$ channels. Therefore, HEK-293 cells were transiently transfected with water (mock), wildtype $K_v1.5$-EGFP (WT-$K_v1.5$), $K_v1.5$(G182R)-EGFP (G182R), $K_v1.5$(E211D)-EGFP (E211D), or $K_v1.5$(G182R/E211D)-EGFP (G182R/E211D) to measure whole cell current-voltage (I-V) relationships (Figure 12). As the constructs were bicistronic vectors that expressed EGFP from a promoter separate from that of $K_v1.5$, green fluorescence (EGFP) was used as an indication of transfection which was not present in cells transfected with water (mock) (Figure 12A).

Representative traces of currents elicited by a standard voltage step protocol from -80 to +60 mV (from a holding potential of -70 mV) in HEK-293 cells transiently transfected with the indicated vector are shown (Figure 12Bb) along with summarized respective I-V curves (Figure 12Cb). There was no significant difference in $I_{K(V)}$ amplitude among HEK-293 cells transfected with WT-$K_v1.5$, G182R, E211D or G182R/E211D channels (Figure 12Bb, Figure 12Cb). For example, at +60 mV, steady state currents for cells transfected with WT-$K_v1.5$ were $14080.74 \pm 835.989$ pA ($n = 15$), while cells transfected with G182R, E211D or G182R/E211D had steady state current amplitudes of $13950.6 \pm 541.825$ ($n = 15$), $12607.8 \pm 785.798$ ($n = 14$) and $12902.5 \pm 990.739$ ($n = 16$) pA, respectively (Figure 12Cc). Additionally, $I_{K(V)}$ from HEK-293 cells transfected with WT-$K_v1.5$ or G182R, E211D or G182R/E211D protein demonstrated similar activation thresholds (at -30 mV) (Figure 12Cb). HEK-293 cells transfected with water (mock) had a peak $I_{K(V)}$ of $525.53 \pm 125.60$ pA at +60 mV (Figure 12Ba, Figure 12Ca), compared to ~12-15 nA for cells transfected with
**Kv1.5.** The low current amplitude in mock transfected cells (~4% of $I_{K(V)}$) in cells transfected with WT-Kv1.5, G182R, E211D or G182R/E211D) supports the fact that currents recorded in HEK-293 cells transfected with WT-Kv1.5, G182R, E211D or G182R/E211D arise mostly from the transfected channel. Cell capacitance was recorded from each transfected cell to control for cell size and followed a normal distribution (Figure 12E).

Differences in channel kinetics were observed in the I-V recordings between WT-Kv1.5 and mutant Kv1.5 channels. While WT-Kv1.5 currents displayed gradual inactivation, indicated by a slowly decaying current for the duration of the +60 mV pulse, the G182R, E211D and G182R/E211D channels were found to inactivate more rapidly (Figure 12Da). This is demonstrated most clearly by the inset showing the top 5% of the traces during the +60 mV pulse (Figure 12Da). Upon closer examination of the activation of whole cell currents, it was noted that WT-Kv1.5 channels activate more quickly at +60 mV than G182R, E211D or G182R/E211D channels (Figure 12Db). Normalized current traces were fit to single exponential equations of the form $I_{K(V)} = (1-e^{-bx})$, where $b$ is the time constant of activation ($1/\tau_{act}$) and $x$ the time in ms] to calculate $\tau_{act}$. WT-Kv1.5 currents had the fastest activation ($\tau_{act} = 1.43 \pm 0.07$ ms), while G182R (1.72 ± 0.05 ms), E211D (1.75 ± 0.05 ms) and G182R/E211D (1.90 ± 0.04 ms) had slightly slower activations. Thus, while Kv1.5 subunits with the G182R and/or E211D mutations form functional channels that are readily detectable in HEK-293 cells by standard electrophysiological techniques, differences between WT-Kv1.5 and mutant channel kinetics led to the further examination of channel kinetics.
Figure 12: $K_{v}1.5$ Channels with G182R, E211D and G182R/E211D Mutations are Functional.

HEK-293 cells were transiently transfected with water (mock), empty vector (EGFP), WT-$K_{v}1.5$, G182R, E211D or G182R/E211D. A) Transfected cells were identified by green fluorescence and used for patch clamp experiments. B) Representative recordings of $I_{K(V)}$ from HEK-293 cells transfected with water (a) or the indicated vectors (b). The pulse protocol stepped from -80 to +60 mV in 20-mV increments from a holding potential of -70mV. C) Summarized I-V curves from HEK-293 cells transfected with water (a) or the indicated vectors (b). Summarized $I_{K(V)}$ amplitudes at +60 mV are also shown (c). D) Averaged normalized $I_{K(V)}$ traces over the initial 300 ms of the voltage pulse (a). Inset details the traces. The averaged normalized $I_{K(V)}$ traces over the initial 10 ms details the activation kinetics with $\tau_{act}$ values summarized on right (b). E) Histogram showing cell capacitances for all cells used in patch clamping experiments. All data are presented as mean ± SEM. EGFP, n=3; WT-$K_{v}1.5$, n=15; G182R, n=15; E211D, n=14; G182R/E211D, n=16. Data were compared with ANOVA and Tukey’s post-hoc test; no significant differences were found.
4.5 G182R, E211D and G182R/E211D Mutant Kv1.5 Channels Inactivate Faster than WT-Kv1.5 Channels

Although certain properties of $I_{K(V)}$, including current amplitude and activation threshold, were found to be similar between wildtype channels and G182R, E211D or G182R/E211D mutant channels, channel kinetics provide another mechanism through which to examine differences in channel structure-function. Therefore, the inactivation and deactivation kinetics of $I_{K(V)}$ in HEK-293 cells transiently transfected with WT-Kv1.5 or mutant Kv1.5 were examined. The inactivation protocol (Figure 13A, inset) consisted of a relatively long pre-pulse (500 ms) to different voltage steps intended to inactivate all Kv1.5 channels. This was followed by a short (5-ms) step to the holding potential (-70 mV) followed by a test pulse to a potential sufficiently depolarized (+60 mV) to open Kv1.5 channels. As pre-pulses at different voltages will inactivate proportional numbers of channels, the current observed during the test pulse (Figure 13Ab) is an indication of the relative availability of the channels following inactivation.

The traces of $I_{K(V)}$ during the inactivation protocol are revealing. The WT-Kv1.5 currents do not significantly inactivate after a 500-ms prepulse, whereas G182R $I_{K(V)}$ demonstrates significant inactivation. For example, the average WT-Kv1.5 current within the first 20 ms after depolarization to +60 mV is 14938 pA, while it is 15204 pA during the last 20 ms before return to the resting potential. Alternatively, the average G182R current for the first 20 ms after depolarization to +60 mV is 12962 pA, while it decreases to 10616 pA toward the end of the pulse, indicating significant
inactivation which was still incomplete after 500 ms. These observations in inactivation kinetics led to the further examination of differences in channel kinetics between WT-K$_V$1.5 and mutant channels.

Plotting the ratio of the amplitude of steady state $I_{K(V)}$ during the test pulse ($I_{T2}$) to the amplitude of the steady state current during the pre-pulse ($I_{T1}$) against the pre-pulse potential gives the steady state inactivation curve (Figure 13B, Figure 13C). Compared to WT-K$_V$1.5, the G182R mutant channel demonstrates a distinct leftward shift in the curve at potentials more positive than -20 mV (Figure 13B), indicating that a greater proportion of G182R channels are inactivated for a given test potential greater than -20 mV. Although the G182R/E211D channel contains the G182R mutation, this channel demonstrated similar inactivation kinetics to the wildtype channel at potentials more positive than -20 mV (Figure 13C). However, the E211D and G182R/E211D channels demonstrated a leftward shift in inactivation curves compared to WT-K$_V$1.5 at potentials more negative than -20 mV (Figure 13C). To determine half maximal inactivation potentials ($V_{1/2}$), steady state inactivation curves were fit to a Boltzmann equation of the form $I_{T2}/I_{T1}$ (%) = $\frac{1}{1 + \exp\left(\frac{(V_{1/2} - V)}{k}\right)}$ where V represents the pre-pulse potential and k the slope factor which represents the steepness of voltage dependence of inactivation. $I_{K(V)}$ in HEK-293 cells transfected with WT-K$_V$1.5 was found to be half inactivated at 10.03 mV (Figure 13B). The leftward shift in the G182R curve is apparent by the $V_{1/2G182R}$ = -6.88 mV (Figure 13C, red lines). Cells transfected with E211D or G182R/E211D had $V_{1/2}$ values of 4.00 mV and 25.35 mV, respectively. These data suggest that compared to WT-K$_V$1.5 the
G182R mutation causes faster inactivation at potentials positive to -20mV and that both the E211D and G182R/E211D cause faster inactivation at potentials negative to -20 mV. These data are consistent with the faster inactivation observed in the I-V traces of HEK-293 cells transfected with G182R, E211D or G182R/E211D compared to WT-K_v1.5 (Figure 12Da).
Figure 13: Measurement of WT-Kv1.5, G182R, E211D and G182R/E211D Inactivation Curves. A) A standard two-pulse inactivation protocol (inset) was used to determine channel availability after 500-ms inactivating pre-pulse in HEK-293 cells transiently transfected with WT-Kv1.5, G182R, E211D or G182R/E211D. Representative recordings from cells transfected with the indicated vector are shown (a). Detail view of representative current recorded during test pulse from HEK-293 cells transfected with WT-Kv1.5 is shown for clarity (b). B, C) Inactivation curves are plotted as current amplitude during test pulse ($I_{T2}$) over the amplitude during pre-pulse ($I_{T1}$) against the pre-pulse potential (left). A Boltzmann equation of the form $I_{T2}/I_{T1} (%) = [1/(1 + \exp {(V_{1/2} - V)/k})$] was used to fit the inactivation curves (right). $V_{1/2}$ (pink lines in B) from cells transfected with WT-Kv1.5, G182R, E211D or G182R/E211D channels were calculated from the fitted curves. WT-Kv1.5, blue; G182R, green; E211D, red; G182R/E211D, black. All data are presented as mean ± SEM. WT-Kv1.5, n=8; G182R, n=4; E211D, n=7; G182R/E211D, n=6.
4.6 The G182R Mutation Decelerates Channel Closure

In addition to inactivation, $K_v$ channels can exist in a deactivated, or closed, state and thus can also be characterized by deactivation kinetics. To this end, tail currents, which are indicative of the rate of channel closure after removal of a depolarizing voltage stimulus, were examined in HEK-293 cells transiently transfected with WT-$K_v$1.5, G182R, E211D or G182R/E211D channels. A standard tail current protocol was used in which pre-pulses between -80 and +60 mV were followed by a test pulse to -40 mV (Figure 14A, inset), at which there is still a driving force on $K^+$ ions. A representative current recording from HEK-293 cells transfected with WT-$K_v$1.5 is shown with the tail currents magnified for clarity (Figure 14A). Tail current I-V relationships are similar among cells transfected with WT-$K_v$1.5, G182R, E211D or G182R/E211D (Figure 14B), indicating that the WT-$K_v$1.5 channel and the mutant channels have similar conductances and half activation potentials. Moreover, the voltage threshold of activation of all four channels is -30 mV, consistent with the data from the whole cell I-V curves (Figure 12C).

To determine the time constants of channel closure ($\tau_{\text{tail}}$) (Figure 14D), tail current decay curves (Figure 14C) were fitted to a single exponential Boltzmann function. The G182R mutation was found to significantly decelerate channel closure, as $\tau_{\text{tail}}$ in cells transfected with G182R and G182R/E211D channels were higher than $\tau_{\text{tail}}$ in cells transfected with WT-$K_v$1.5 (24.46 ± 0.94 ms, n=12 for G182R, p=0.01; and 20.81 ± 0.73 ms, n=10 for G182R/E211D, compared to 17.57 ± 0.85 ms, n=11 for WT-$K_v$1.5) (Figure 14D). However, the difference between WT-$K_v$1.5 and
G182R/E211D was not statistically significant. Furthermore, the E211D channel showed a trend toward slower closure (\( \tau_{\text{tail}} = 22.18 \pm 2.14 \), n=14), although it did not differ significantly from the wildtype channel (Figure 14D). Thus, the G182R mutation slows channel closure in both G182R and G182R/E211D mutant channels.
Figure 14: Measurement of WT-Kv1.5, G182R, E211D and G182R/E211D Tail Currents in HEK-293 Cells. 

A) A standard pulse protocol (inset) was used to elicit tail currents in HEK-293 cells transiently transfected with WT-Kv1.5, G182R, E211D or G182R/E211D. A representative recording from cells transfected with WT-Kv1.5 is shown with the tail currents indicated by the black arrow and shown magnified in the right panel. B) I-V relationships are plotted as raw amplitude (left) and normalized to 100% within each group (right) as a function of the pre-pulse voltage. C) Averaged raw tail current traces (left) and averaged tail current traces normalized within each group (right) are shown. D) Tail current traces were fitted to a Boltzmann equation with a single exponential to generate tail current decay time constants ($\tau$). WT-Kv1.5 (blue fills and bars) n=11; G182R (green fills and bars) n=12; E211D (red fills and bars) n=14; G182R/E211D (black fills and bars) n=10. Data are presented as mean ± SEM. Data were compared with ANOVA and Tukey’s post-hoc test; ** P<0.01 compared to WT-Kv1.5.
4.7 Channel Block by 4-AP is similar between WT-KV1.5 Channels and G182R, E211D and G182R/E211D Mutant Channels

Differences in channel structure function are often revealed during pharmacological block. Therefore, the inhibition of $I_{K(V)}$ by 4-aminopyridine (4-AP), a $K_V$ channel blocker, in HEK-293 cells transiently transfected with WT-KV1.5, G182R, E211D or G182R/E211D channels was examined during a standard I-V pulse protocol (Figure 15A, inset at bottom). $I_{K(V)}$ was recorded in transiently transfected HEK-293 cells before (Control), during (4-AP) and after (Wash) administration of 5 mM 4-AP in the bathing solution (Figure 15A). 4-AP was found to significantly inhibit $I_{K(V)}$ in HEK-293 cells transiently transfected with the WT-KV1.5 channel at all potentials tested at which the channel was activated (Figure 15A, WT-KV1.5 curve). For example, at +60 mV, $I_{K(V)}$ in these cells was 14080.74 ± 835.99 pA. Upon administration of 4-AP, there was 67% inhibition, with an $I_{K(V)}$ amplitude of 4601.54 ± 288.00 pA; however, upon washout of 4-AP, $I_{K(V)}$ recovered to 95% its original value at 13354.62 ± 844.91 pA (Figure 15A, top) Thus, 5 mM 4-AP significantly and reversibly inhibits $I_{K(V)}$ in HEK-293 cells transiently transfected with WT-KV1.5.

In cells transiently transfected with G182R, E211D or G182R/E211D channels, $I_{K(V)}$ shows inhibition and recovery similar to those of the wildtype channel. At +60 mV, $I_{K(V)}$ in cells transfected with the G182R channel, control $I_{K(V)}$ amplitude was 14131.72 ± 779.68 pA, while it decreased to 3354.58 ± 487.45 pA (24% of control $I_{K(V)}$) during exposure to 4-AP, and recovered to 12707.73 ± 795.11 pA (90% of control $I_{K(V)}$) after washout (Figure 15A, second panel). In cells transfected with the
E211D channel, control $I_{K(V)}$ was 13439.93 ± 997.52 pA, decreasing to 3738.16 ± 455.65 pA during 4-AP administration (28% of control $I_{K(V)}$), and recovering to 11825.70 ± 1056.26 pA (88% of control $I_{K(V)}$) (Figure 15A, third panel). Similarly, in cells transfected with the G182R/E211D channel, control $I_{K(V)}$ was 15900.83 ± 1063.23 pA, while 4-AP reduced $I_{K(V)}$ to 37% (5821.07 ± 824.26 pA), and $I_{K(V)}$ nearly fully recovered after washout (15221.38 ± 979.64 pA, 96% of control $I_{K(V)}$) (Figure 15A, bottom panel). Thus, at +60 mV 4-AP blocks ~70% $I_{K(V)}$ in cells transfected with WT-Kv1.5, G182R, E211D or G182R/E211D channels, and $I_{K(V)}$ recovers to ~90% its original value after washout. To examine possible differences in recovery among the channels, we plotted the % inhibition by 4-AP over the various potentials tested during the pulse protocol. No significant differences were observed regarding 4-AP inhibition between HEK-293 cells transfected with WT-Kv1.5 and the mutant G182R, E211D or G182R/E211D channels.
Figure 15: 4-AP Inhibition of WT-KV1.5, G182R, E211D and G182R/E211D Currents in HEK-293 Cells. A) A standard I-V pulse protocol (inset at bottom) was delivered to HEK-293 cells transiently transfected with the indicated vector. Currents were first recorded in standard physiological salt solution (control, left), then with the addition to the bathing solution of 5mM-AP (4-AP, middle), and finally after 5-10 min washout of 4-AP in physiological salt solution (wash, right). I-V curves are shown on the far right. White circles, control; black circles, 4-AP administration; white triangles, washout. B) Summarized data is shown as the percent inhibition of $I_{K(V)}$ during 4-AP exposure. WT-KV1.5, n=15; G182R, n=8; E211D, n=8; G182R/E211D, n=8. Data are presented as mean ± SEM. Data were compared with ANOVA using Tukey’s post-hoc test; no significant differences were found.
4.8 G182R Channels Have Increased Single Channel Conductance Compared to WT-K<sub>V</sub>1.5

While whole cell \( I_{K(V)} \) measurements represent the summation of currents through all activated channels of a given cell, single channel recordings provide invaluable insight as to individual channel properties. Single channel current (\( i_{K(V)} \)) of WT-K<sub>V</sub>1.5, G182R, E211D and G182R/E211D was recorded in the single channel patch clamp configuration in which an unbroken seal is formed between the patch pipette and a patch of membrane (Figure 6).

Representative traces of single channel currents recorded at the indicated potentials in HEK-293 cells transfected with WT-K<sub>V</sub>1.5, G182R, E211D or G182R/E211D channels are shown (Figure 16A). Dashed gray lines represent the closed state of the channel, while outward current through open channels is recorded as a deflection upward from the closed state. The WT-K<sub>V</sub>1.5 recording reveals two channels in the membrane patch, indicated by the two levels of channel opening (Figure 16A, black bars to right of 90-mV recording). Because \( i_{K(V)} \) during two channel openings is a simple multiple of the amplitude of a single channel opening, the WT-K<sub>V</sub>1.5 channel has a single conducting (open) state. Single channel current amplitude was recorded at +70 mV, +90 mV, and +110 mV; \( i_{K(V)} \) at these potentials were 0.2735 pA, 0.4960 pA and 0.6800 respectively (Figure 16B, left).

Single channel recordings were also made from cells transfected with G182R, E211D or G182R/E211D channels. A representative recording from cells transfected with the G182R channel also reveals two channels (Figure 16A, G182R). However, in
contrast to the WT-K,V1.5 channel, G182R has at least two conducting states, as each major opening level (solid black bars to right of 90-mV recording) has a corresponding subconducting level (dashed lines) (Figure 16A, G182R). At +70 mV, +90 mV and +110 mV, G182R $i_{K(V)}$ is 0.4070 pA, 0.7560 pA, and 1.1090 pA, respectively (Figure 16B). The recording from cells transfected with E211D indicates one open configuration (Figure 16A) and single channel amplitudes of 0.4435 pA, 0.5620 pA and 0.7940 pA at +70 mV, +90 mV and +110 mV, respectively (Figure 16B, E211D). Lastly, the G182R/E211D channel, like G182R, has two conducting states which are best seen on the recording at +70 mV (Figure 16A, black arrows). In HEK-293 cells transfected with the G182R/E211D channel, $i_{K(V)} = 0.3575$ pA at +70 mV, 0.6390 pA at +90 mV and 0.8810 pA at +110 mV (Figure 16B at right).

The slope of the I-V curve gives the single channel conductance (g) (Figure 16C, left). Single channel conductance for WT-K,V1.5 was $14.84 \pm 0.80$ pS. While all three mutant channels, G182R, E211D and G182R/E211D had higher single channel conductances (G182R, 21.586 ± 1.56 pS; E211D, 18.98 ± 2.69 pS; and G182R/E211D, 18.33 ± 1.57 pS), only that of the G182R channel was found to differ significantly (P<0.05); however, the trend toward higher conductance in E211D and G182R/E211D is apparent (Figure 16C). In addition to $i_{K(V)}$, the product of single channel open probability and the number of available channels (NP$_{open}$) determines the whole cell current $I_{K(V)}$ through a given population of K,V channels. This product was determined for HEK-293 cells transfected with WT-K,V1.5, G182R, E211D or G182R/E211D channels (Figure 16C, right). The K,V1.5 channel had an NP$_{open}$ of 0.529 ± 0.18. The
NP\textsubscript{open} for the G182R, E211D and G182R/E211D channels were higher than that of the wildtype channel (0.75 ± 0.12, 0.59 ± 0.12, and 0.54 ± 0.20 respectively), but these differences were not found to be statistically significant (Figure 16C, right).

**Figure 16: Single Channel Recordings of WT-KV1.5, G182R, E211D and G182R/E211D Currents in HEK-293 Cells.** A) Single channel currents were recorded from cells transfected with the indicated vectors at +110 mV, +90 mV and +70 mV. Dashed gray lines indicate the closed state. Solid bars to the right of the recordings indicate open levels of one and two channels, while dashed black lines to the right of the recordings indicates subconductance levels. In the G182R/E211D recordings (right), the sublevels are indicated in the +70 mV recording with black arrows. B) Representative single channel I-V relationships are shown for WT-KV1.5 (left panel), G182R (second panel), E211D (third panel) and G182R/E211D (right). C) Slope conductances, as determined from the slope of the regression line (y=mx+b, where m is the slope), were calculated for each of the channels and are shown in summarized form on the left. The product of the number of channels (N) and the open probability (P\textsubscript{open}) was calculated for each of the channels and is shown summarized on the right. All channel types, n=3. Data are presented as mean ± SEM. Data were compared with ANOVA using Tukey's post-hoc test, *P<0.05.
4.9 Conclusions

4.9.1 Summary of Electrophysiology Results

Two mutations, G182R and E211D, in the T1 domain of Kv1.5 were found in a small population of IPAH patients (Figure 8). Given the reduced $I_{K(V)}$ and expression of Kv channels in PASMC from IPAH patients compared to non-PH and SPH controls (Figure 7, sections 2.3 and 2.4), these mutations were studied to determine whether they might contribute to disease phenotype. Furthermore, as both mutations occur at highly conserved residues within the T1 domain (Figure 9), which is itself highly conserved among Kv channels, the examination of these mutations was expected to elucidate the channel’s structure-function relationship.

Both of the singly mutant channels as well as the doubly mutant channel, G182R/E211D, were able to form functional channels in transiently transfected HEK-293 cells (Figure 12C). This was evident by their similar $I_{K(V)}$ amplitudes (readily distinguished from cells transfected with water) and voltage thresholds of activation compared to WT-Kv1.5 (Figure 12C). However, kinetic differences were observed in the I-V traces of the mutant channels compared to those of WT-Kv1.5, namely the time it took to reach maximal $I_{K(V)}$, especially at higher potentials (Figure 12Db), and the presence of an inactivating component (Figure 12Da). In terms of activation time constants, WT-Kv1.5 currents had the fastest activation ($\tau_{\text{act}} = 1.43 \pm 0.07$ ms), while G182R (1.72 ± 0.05 ms), E211D (1.75 ± 0.05 ms) and G182R/E211D (1.90 ± 0.04 ms)
had slightly slower activations, although these differences were not statistically significant (ANOVA, P>0.05).

Given these observations from the I-V traces, the kinetics of $I_{K(V)}$ were further examined. $I_{K(V)}$ in cells transfected with G182R demonstrated a leftward shift in the steady state inactivation curve at potentials greater than -20 mV compared to WT-$K_{V}1.5$ (Figure 13B). $V_{1/2}$ for the WT-$K_{V}1.5$ channel was 10.03 mV, while $V_{1/2}$ for G182R was -6.88 mV. The E211D and G182R/E211D channels also demonstrated leftward shifts in their inactivation curves but at potentials less than -20 mV compared to WT-$K_{V}1.5$ (Figure 13C), and had $V_{1/2}$ of 4.00 mV and 25.35 mV, respectively. Deactivation was also examined by measuring the $\tau_{tail}$ as an indication of the rate of channel closure after stimulus removal. G182R by itself was found to significantly decelerate the rate of channel closure compared to WT-$K_{V}1.5$ ($\tau_{tail} = 24.46 \pm 0.94$ ms for G182R and 17.57 ± 0.85 ms for WT-$K_{V}1.5$, P=0.01), while the G182R/E211D channel also had a higher $\tau_{tail}$ compared to WT-$K_{V}1.5$ (20.81 ± 0.73 ms), although this difference was not statistically significant (Figure 14D). The $\tau_{tail}$ of E211D did not differ from WT-$K_{V}1.5$ (22.18 ± 2.14 ms) (Figure 14D).

The effects of pharmacological block by 4-AP, a general $K_{V}$ channel inhibitor, on $I_{K(V)}$ in cells transfected with WT-$K_{V}1.5$, G182R, E211D or G182R/E211D were also examined. 4-AP reversibly decreased current amplitude in cells transfected with WT-$K_{V}1.5$; neither the reversible inhibition nor the percent inhibition differed between WT-$K_{V}1.5$ and any of the mutant channels (Figure 15).
Lastly, single channel recordings were made from cells transfected with WT-
$K_{\text{V}1.5}$, G182R, E211D or G182R/E211D at +70, +90 and +110 mV. It was noticed that both the G182R and G182R/E211D channels, in addition to adapting fully open states, also were able to briefly adapt subconducting states (Figure 16A). Furthermore, single channel conductance of the WT-$K_{\text{V}1.5}$ channel was $14.84 \pm 0.80$ pS (Figure 16C). Both E211D ($18.98 \pm 2.69$ pS) and G182R/E211D ($18.33 \pm 1.57$ pS) had slightly elevated conductances, although these were not found to be statistically significant (Figure 16C). However, the conductance of the G182R channel ($21.586 \pm 1.56$ pS) was significantly increased compared to WT-$K_{\text{V}1.5}$ (Figure 16C).

4.9.2 Discussion

The overall electrophysiological characterization of two mutations found in the T1 domain of $K_{\text{V}1.5}$ in idiopathic pulmonary arterial hypertension patients suggests that the T1 domain is involved in channel kinetics. The initial modeling of mutant $K_{\text{V}1.5}$ suggested that G182R introduced a positively charged group which extends beyond the normal polar interface of the T1 domain. Furthermore, the E211D mutation effectively shortens the length of a negatively charged side chain, burying the group within the core of the T1 domain. As interactions among $K_{\text{V}}$ subunits rely on polar interactions at the T1 interface, it was unexpected that the G182R and E211D mutant proteins would be able to form functional channels. However, whole cell $I_{\text{K(V)}}$ recordings in HEK-293 cells transiently transfected with the WT-$K_{\text{V}1.5}$ or mutant $K_{\text{V}1.5}$ indicated that the mutant channels are able to form functional channels.
However, given the experimental system used, namely transient transfection, it is possible that the overexpression of Kv1.5 channel subunits itself favored channel formation. To this end, it may be helpful in the future to create a stably transfected cell line which would allow the study of channel formation in a more physiological background. However, the observed changes in mutant channel kinetics indicates that even in the assembled channel, the mutations affect function.

The effects of the mutations on channel kinetics, including activation, inactivation and deactivation suggest that the T1 domain plays a role in these aspects of channel function. Channel opening (activation) relies on the positively charged residues of S4 moving in response to a depolarization in the transmembrane electric potential. The activation of all three mutant channels (G182R, E211D and G182R/E211D) was slower than the WT-Kv1.5 channel. A direct effect of G182R on S4 seems unlikely, given its distance from the pore. However, it does lie close to the ion permeation pathway through the center of the T1 domain. The positive charge introduced by G182R may repel K$^+$ ions to such an extent that they are less likely to enter the pore. The pore, however, favors the open conformation in the presence of high (physiological) levels of K$^+$ (186, 282, 323, 329). It is possible, then that the G182R slows channel activation by decreasing the presence of K$^+$ ions at the cytoplasmic opening of the pore and making channel opening more difficult. The E211D residue, which lies along the ion permeation pathway through the side of the T1 domain, may have a similar net effect. The negative residue of this side chain in its wildtype form may be involved in attracting K$^+$ ions to the pore (152). With the loss of
the protruding negatively charged group from the T1 surface, K⁺ ions may be less likely to enter the ion permeation pathway. While the G182R residue lies relatively far from the cytoplasmic pore opening, and therefore relatively far from S4, E211D, given its proximity to the T1-S1 linker, may be expected to interact directly with the arginines on S4. However, even if the glutamate interacted with these arginines, the loss of this interaction by the shortening of the acidic side chain would be expected to speed channel opening, not slow it. Therefore, it is unlikely that either G182R or E211D have their effects through an effect on S4.

Other T1 domain mutations have also been found to affect channel gating. Interestingly, a valine to arginine mutation in Aplysia Kv1.1 (46), which lies on the same linker between two alpha helices in the T1 domain as does G182R, also causes a decrease in channel activation rates. This mutation did not alter either overall T1 conformation or the stability of T1 interactions, leading the authors to postulate that the mutation had its effects through local action of the side chain (46). It is possible that the G182R and E211D mutations alter T1 conformation in such a way as to globally alter channel gating, but such sophisticated modeling was beyond the scope of this dissertation. The G182R residue lies on the polar interface of T1 which is important for subunit interactions. In Kv1.2, the homologous residue lies on an interface of thirty side chains, 21 of which are polar, that makes contacts across the interface (184). A glutamine amino acid 2 residues upstream of the homologous G182 residue in Kv1.2 was completely intolerant to alanine mutation and failed to form functional channels (184). Based on this evidence as well as mutational analysis of
other T1 domain residues, the authors concluded that structural changes in the buried polar interfaces of the T1 core play a role in conformational changes associated with channel opening (184). Therefore, it is possible that both the G182R and E211D, by introducing local changes in T1 structure, affect channel kinetics.

Slow inactivation in Kv1.5 is incompletely understood but may depend on a collapse of the channel pore and/or the N-terminus (130, 209, 306). Faster inactivation was observed in the G182R mutant channel at potentials greater than -20 mV and in both the E211D and G182R/E211D channels at potentials less than -20 mV, as demonstrated by the leftward shifts in their inactivation curves compared to that of the WT-Kv1.5 channel. If both the G182R and E211D mutations destabilize the open conformation of the pore by decreasing local K+ ion concentrations, then a collapse of the channel pore may also explain the observed faster inactivation conferred by the G182R and E211D mutations. Kv1.5 activates at around -30 mV. Interestingly, this is the boundary between the faster inactivation in the G182R channel and that observed in the E211D and G182R/E211D channels. It is possible, then, that the open conformation of the G182R channel is destabilized by the pre-pulse potentials greater than -20 mV which open the channel, thus leading to faster inactivation during the test-pulse. Alternatively, the G182R mutation may stabilize the closed or inactivated state of the channel after pre-pulses greater than -30 mV; thus fewer channels are available to open during the test pulse compared to WT-Kv1.5 channels.

The E211D and G182R/E211D channels, in contrast to the G182R channels, inactivate faster than the wildtype channel at potentials less than – 20 mV. The E211D
mutation may also destabilize the open conformation of the channel (during the test-pulse), but the fact that the G182R/E211D channel mimics the E211D channel (and does not have an inactivation curve that is a combination of G182R and E211D), indicates that the effects of the mutations on channel function are more sophisticated. It is possible that the negative charge at position 211 has a more prominent role in inactivation than the introduction of the positive charge conferred by G182R. Thus, while the negative charge of the wildtype E211 residue may stabilize T1 interactions in the open channel conformation, the lack of it in the 211D mutation may destabilize the channel to a greater extent. The picture is further complicated by the suggestion that T1 is not a maximally stable complex and that while the tetramer assembles, it undergoes conformational changes easily for proper function (184).

Regardless of the exact mechanism of inactivation, at potentials greater than the resting membrane potential (between -40 and -70 mV), Kv channels open to repolarize the membrane. In neurons and cardiomyocytes, this is manifested as the duration of the action potential. In PASMC, the rate of repolarization would influence the duration voltage dependent calcium channels (VDCC) remained open after a depolarization. The G182R channels, therefore, would decrease the duration of VDCC opening, thus limiting the influx of Ca$^{2+}$ compared to the wildtype channel. However, at potentials less than -20 mV, the main function of Kv channels is their contribution to maintaining the resting membrane potential. Compared to WT-Kv1.5, both E211D and G182R/E211D channels would depolarize E_m by increasing the rate of inactivation.
Lastly, G182R was found to decrease the rate of channel closure and increase single channel conductance compared to WT-Kv1.5. While the mechanisms leading to these effects are unknown, both would lead to relatively more hyperpolarization (in terms of duration due to the decreased rate of channel closure and in terms of amplitude due to the increased conductance) associated with channel openings compared to WT-Kv1.5 channels.

4.10 Acknowledgments for Chapter 4

The original identification of the two mutations, the characterization of which is the main focus of this dissertation, was initially published in: Remillard CV, Tigno DD, Platoshyn O, Burg ED, Brevnova EE, Conger D, Nicholson A, Rana BK, Channick RN, Rubin LJ, O'Connor D T, and Yuan JX. Function of Kv1.5 channels and genetic variations of KCNA5 in patients with idiopathic pulmonary arterial hypertension. *Am J Physiol Cell Physiol* 292: C1837-1853, 2007. Furthermore, the figure of Kv1.5 which illustrates the location and side chains of the G182, E211, 182R and 211D residues within Kv1.5, the 500-ms pre-pulse inactivation curve data, and the whole cell $I_{K(V)}$ data from HEK-293 cells transfected with WT-Kv1.5 or the mutant constructs (which appear in Chapter 4) were submitted for publication in the following paper: Elyssa D. Burg, Oleksandr Platoshyn, Jason X.-J. Yuan, and Igor F. Tsigelny. Tetramerization domain mutations in KCNA5 affect channel inactivation. *Intl J Cell Biol*, 2009. Lastly, the genotyping data which appears in Table 2 (Chapter 4) of this dissertation has been co-authored with Brinda K. Rana and Beatriz Lozano-Ruiz.
Chapter 5. Results: Tetramerization Domain Mutations in $K_{v}1.5$ Alter Channel Subcellular Localization

The G182R and E211D mutations are located in the highly conserved T1 domain which has been implicated in channel assembly and localization to the plasma membrane (cf. sections 1.4.4.1 and 1.4.4.5). $K_{v}B$ subunits, which interact with $K_{v}$ channels through their T1 domains, act as chaperones to $K_{v}$ channels, increasing protein expression at the plasma membrane and stabilizing $K_{v}$ channel protein (cf. section 1.5.3). As mutations in the T1 domain are expected to affect both interactions with $K_{v}B$ subunits and channel tetramerization, protein expression levels and subcellular localization patterns of WT-$K_{v}1.5$, G182R, E211D and G182R/E211D channels were also examined.

5.1 The G182R Mutation Causes Incomplete Processing of the Protein in HEK-293 Cells

$K_{v}1.5$ is a surface membrane protein which has a glycosylation site on its extracellular loop between S1 and S2. Oligosaccharides are modified in the biosynthetic pathway from the ER to the Golgi apparatus. Thus, $K_{v}1.5$ can appear as a doublet on immunoblot with the upper band representing the mature sialylated form (present in the Golgi and on the cell surface), while the lower band represents the immature high mannose N-linked oligosaccharide form (present in the ER).

Whole cell protein lysates from HEK-293 cells transfected with WT-$K_{v}1.5$, G182R, E211D or G182R/E211D channels were separated by SDS-PAGE and
transferred to nitrocellulose membranes which were probed with anti-K\textsubscript{V}1.5 antibody. Representative blots from both early (passage < 7) and late (between 8 and 12) passage cells are revealing (Figure 17A, left and right, respectively). While double bands are apparent in the earlier passage cells, they become less noticeable with increased passage when the majority of protein is present in its mature form (Figure 17A). Transfected WT-K\textsubscript{V}1.5 protein was present as a doublet in early passage cells, though most protein was present in the upper band; however, all three mutant channels, which were also detected as doublets, had prominent lower bands in early passage cells (Figure 17A, left). To quantify these differences, the ratio of upper band to lower band was calculated for both early and late passage cells (Figure 17B, left) and for early passage cells only (Figure 17B, right). For the combination of early + late passage cells, WT-K\textsubscript{V}1.5 was found to have an upper/lower band ratio of 4.11 ± 0.80 (Figure 17B, left). The ratio in early + late passage cells for the WT-K\textsubscript{V}1.5 channel did not differ significantly from those of G182R (3.21 ± 0.78), E211D (3.29 ± 0.60) or G182R/E211D (3.18 ± 0.68) (Figure 17B, left).

However, as expected, the ratios from the early passage cells taken along demonstrated some differences between channel types. The ratio of upper/lower band in early passage cells transfected with WT-K\textsubscript{V}1.5 was 2.44 ± 0.41, while the G182R/E211D channels had significantly lower values (1.14 ± 0.21, n=7, P<0.05) (Figure 17B, right). While the G182R ratio (1.23 ± 0.22, n=7) was similar to that of G182R/E211D, this difference was not found to be statistically significant (Figure 17B, right). Although the ratio for the E211D channel (1.70 ± 0.43, n=7) was also lower
than that of WT-K\textsubscript{V}1.5, this difference was also not found to be statistically significant (Figure 17B, right). These data suggest that while most WT-K\textsubscript{V}1.5 protein is processed into its mature form, the G182R mutation (and to a lesser extent the E211D mutation) causes more of the protein to be present in its immature form.

The immunoblots against K\textsubscript{V}1.5, in addition to showing differences in protein processing between the WT-K\textsubscript{V}1.5 and mutant channels containing G182R, also revealed a difference in overall protein expression levels for channels containing E211D (Figure 17A, Figure 17C). This difference was observed in early passage cells and persisted through late passage. The expression level of WT-K\textsubscript{V}1.5 (normalized to GAPDH) was assigned a value of one, and the expression levels of G182R, E211D and G182R/E211D (normalized to GAPDH) expressed as a % compared to WT-K\textsubscript{V}1.5. The total protein expression level (both bands) of G182R/E211D (0.72 ± 0.10 a.u., n=13) was found to exhibit a trend toward lower protein levels compared to WT-K\textsubscript{V}1.5, although this difference was not found to be statistically significant (Figure 17C, upper panel). Neither the G182R (0.97 ± 0.11 a.u., n=13) nor the E211D (0.90 ± 0.12 a.u., n=13) total protein expression levels differed from WT-K\textsubscript{V}1.5 (Figure 17C, both bands). In cells transfected with water (Mock), endogenous K\textsubscript{V}1.5 levels were low (0.31 ± 0.05 a.u., n=13), as expected (Figure 17A, Figure 17C). The decrease in K\textsubscript{V}1.5 protein levels observed in cells transfected with G182R/E211D could be attributable to a decrease in levels of the upper band (0.62 ± 0.09 a.u., n=13, P<0.001) (Figure 17C, bottom panel). Additionally, the level of E211D protein in the upper band relative to WT-K\textsubscript{V}1.5 exhibited a trend toward lower values (0.76 ± 0.06 a.u.,
n=13), although this difference did not reach statistical significance (Figure 17C, lower panel). G182R upper band expression did not differ significantly from WT-

K\textsubscript{\textit{v}}1.5 (0.84 ± 0.09 a.u.) (Figure 17C, lower panel). These results suggest that the E211D mutation, especially in the presence of the G182R mutation, reduces the amount of protein expression in HEK-293 cells, an effect which is attributable to a decrease in mature protein levels. However, these results must be interpreted with caution as immunoblots of transfected cell protein lysates are not strictly quantitative.
Figure 17: K\textsubscript{v}1.5 and Mutant Channel Expression Patterns in HEK-293 Cells. HEK-293 cells were transfected with water (mock), WT-K\textsubscript{v}1.5, G182R, E211D or G182R/E211D and subject to standard immunoblot procedures. A) Representative immunoblots from early (left) and late (right) passage cells transfected with the indicated construct. Blots were probed for K\textsubscript{v}1.5 (67 kDa). B) Summarized data of upper to lower band ratios are shown for both early and late passage cells (left, n=13) and separately for early passage cells (right, n=7). C) WT-K\textsubscript{v}1.5 protein levels were normalized to protein level = 1. Summarized data of protein levels of K\textsubscript{v}1.5 in cells transfected with water (mock), G182R, E211D or G182R/E211D are presented as ratio to WT-K\textsubscript{v}1.5 levels. The data for total protein level (both bands) is shown at the top (n=13) and for only the upper band at the bottom (n=13). Data are presented as mean ± SEM. Data were compared with ANOVA using Tukey’s post-hoc analysis; *P<0.05, ***P<0.001 compared to WT-K\textsubscript{v}1.5.
5.2 Subcellular Localization of WT-\(K_{\text{V}1.5}\) and Mutant \(K_{\text{V}1.5}\) in HEK-293 Cells

To examine the effects of the G182R and E211D mutations on \(K_{\text{V}1.5}\) subcellular localization, transfected HEK-293 cells were subject to standard immunocytochemical staining. As the vector used to transfect HEK-293 cells expressed EGFP separately from the \(K_{\text{V}1.5}\) channel, green fluorescence was used as a marker of transfection of individual cells. Rhodamine-conjugated secondary antibody labeled \(K_{\text{V}1.5}\) red and DAPI was used as a nuclear stain. HEK-293 cells transfected with WT-\(K_{\text{V}1.5}\) demonstrate two distinct patterns of staining (Figure 18). Cell surface staining is visible as positive staining around the perimeter of the cell (Figure 18, white arrows). Additionally, distinct perinuclear packets of staining were also visible (Figure 18, white arrowheads). This data is consistent with immunoblot data suggesting that transfected WT-\(K_{\text{V}1.5}\) protein is present both in its mature form (at the cell surface) and in its immature form (in the ER). HEK-293 cells transfected with G182R demonstrated predominantly perinuclear staining (Figure 18, second column). Similar to HEK-293 cells transfected with WT-\(K_{\text{V}1.5}\), cells transfected with either E211D or G182R/E211D demonstrated both perinuclear (arrowheads) and cell surface staining patterns (arrows), although perinuclear populations appear more prevalent than the surface staining (Figure 18). HEK-293 cells transfected with water (mock) confirm anti-\(K_{\text{V}1.5}\) antibody specificity, and HEK-293 cells transfected with WT-\(K_{\text{V}1.5}\) and incubated without anti-\(K_{\text{V}1.5}\) antibody confirm secondary antibody specificity (Figure 18, two columns at right). These data are consistent with the
hypothesis that the G182R mutation, and to a lesser extent the E211D mutation, causes incomplete processing of the Kv1.5 protein which leads to its intracellular retention.

Figure 18: WT-Kv1.5 and Mutant Channel Subcellular Localization in HEK-293 Cells.
HEK-293 cells were transfected with WT-Kv1.5 (left and far right), G182R (second column), E211D (third column), G182R/E211D (fourth column) or water (Mock, fifth column), fixed, permeabilized and stained with anti-Kv1.5 antibody. Mock column demonstrates anti-Kv1.5 antibody specificity. Cells in the ‘WT-Kv1.5 –Kv1.5 antibody’ column were incubated in blocking buffer without anti-Kv1.5 antibody to demonstrate secondary antibody specificity. In cells transfected with WT-Kv1.5, G182R, E211D or G182R/E211D, green fluorescence indicates transfection (top row). Secondary antibody used was anti-rabbit-IgG conjugated to TRITC (red, second row). All cells were stained with DAPI (blue, third row) to indicate nuclei. Overlaid images (EGFP/TRITC/DAPI) are shown at bottom (Merge). White arrows point to cell surface Kv1.5 staining; white arrowheads to perinuclear clusters of Kv1.5. All fields shown at 60x magnification.
5.3 The G182R Mutation in Kv1.5 Causes Reduced Channel Protein Expression in COS-1 Cells

In order to determine whether the effects of the G182R and E211D mutations on channel processing and subcellular localization were cell-type specific, similar experiments were conducted in COS-1 cells transiently transfected with WT-Kv1.5, G182R, E211D or G182R/E211D (Figure 19A). Transfection efficiency in COS-1 cells, like that in HEK-293 cells, was 60-80%. COS-1 cells, like HEK-293 cells, express little or no endogenous Kv1.5 (Figure 11, Figure 19B). However, COS-1 cells transiently transfected with WT-Kv1.5, E211D or G182R/E211D expressed Kv1.5 protein robustly, as evident by immunoblot with anti-Kv1.5 antibody (Figure 19B). All blots were reprobed with anti-GAPDH antibody, and all Kv1.5 levels expressed in arbitrary units (a.u.) normalized to GAPDH levels. Expression levels among COS-1 cells transfected with WT-Kv1.5, E211D or G182R/E211D were similar (1.02 ± 0.02 a.u. for WT-Kv1.5, n=4; compared to 0.98 ± 0.90 a.u. for E211D, n=4; and 0.96 ± 0.06 a.u. for G182R/E211D, n=4) (Figure 19B). Surprisingly, expression of G182R protein in COS-1 cells was significantly reduced compared to WT-Kv1.5 levels and only slightly higher than in mock transfected cells (0.17 ± 0.06 a.u. for G182R, n=4; compared to 0.04 ± 0.2 a.u. for mock, n=4; and 1.02 ± 0.02 a.u. for WT-Kv1.5) (Figure 19B). To confirm procedural consistency and the integrity of the G182R plasmid, HEK-293 cells were transfected and subject to immunoblot in tandem with COS-1 cells. Protein expression in HEK-293 cells confirmed that G182R protein has expression levels similar to transfected WT-Kv1.5 in HEK-293 cells (1.14 a.u. for
WT-Kv1.5 compared to 1.21 a.u. for G182R in HEK-293 cells, n=1) (Figure 19C, but cf. Figure 17). Thus, greatly diminished protein expression of transfected G182R in COS-1 cells is cell specific.

Figure 19: WT-Kv1.5 and Mutant Channel Protein Expression in COS-1 Cells. COS-1 cells were transiently transfected with water (Mock), WT-Kv1.5, G182R, E211D or G182R/E211D constructs and subject to standard immunoblot procedure. A) Representative fields of cells are shown at 40x magnification. B) Representative immunoblot from COS-1 cells transfected with water (mock) or the indicated vector. Blots were probed with anti-Kv1.5 antibody (top) and reprobed for GAPDH (36 kDa) which served as a loading control (bottom). Summarized data are expressed in arbitrary units (a.u.) as the band intensity of Kv1.5 divided by the intensity of GAPDH (n=4). Data are presented as mean ± SEM and were compared with ANOVA using Tukey’s post-hoc analysis; ***P<0.001 compared to WT-Kv1.5. C) HEK-293 cells were transfected (simultaneously with COS-1 cells) with the indicated vectors and subject to immunoblot analysis to detect Kv1.5 (top) and GAPDH (bottom). Bar graph showing Kv1.5 protein levels relative to GAPDH levels is shown at bottom (n=1).
5.4 Subcellular Localization of WT-K\(V_{1.5}\) and Mutant K\(V_{1.5}\) in COS-1 Cells

To study the effects of the G182R and E211D mutations on the subcellular localization of K\(V_{1.5}\) protein in COS-1 cells, COS-1 cells transfected with WT-K\(V_{1.5}\), G182R, E211D or G182R/E211D protein were subject to standard immunocytochemistry. Cells transfected with WT-K\(V_{1.5}\) show diffuse staining patterns with distinct staining around the cell perimeter, indicative of the presence of cell surface K\(V_{1.5}\), although there are also some concentrated areas around the nucleus (Figure 20, WT-K\(V_{1.5}\)). Cells transfected with the G182R mutant demonstrated either cell surface and perinuclear K\(V_{1.5}\) (Figure 20, second column) or had little to no positive K\(V_{1.5}\) staining despite transfection (Figure 20, third column). COS-1 cells transfected with either E211D or G182R/E211D consistently had both cell surface and perinuclear K\(V_{1.5}\) (Figure 20, E211D, G182R/E211D). Mock-transfected COS-1 cells demonstrated anti-K\(V_{1.5}\) antibody specificity, while COS-1 cells transfected with WT-K\(V_{1.5}\) but not exposed to anti-K\(V_{1.5}\) antibody indicate the specificity of the secondary antibody (Figure 20, right).
Figure 20: WT-K\textsubscript{V}1.5 and Mutant Channel Subcellular Localization in COS-1 Cells. COS-1 cells were transfected with WT-K\textsubscript{V}1.5 (left and far right), G182R (second and third columns), E211D (fourth column), G182R/E211D (fifth column) or water (Mock, sixth column), fixed, permeabilized and incubated with anti-K\textsubscript{V}1.5 antibody. Mock column demonstrates anti-K\textsubscript{V}1.5 antibody specificity. Cells in the ‘WT-K\textsubscript{V}1.5 –K\textsubscript{V}1.5 antibody’ column were incubated without anti-K\textsubscript{V}1.5 antibody to verify secondary antibody specificity. In cells transfected with WT-K\textsubscript{V}1.5, G182R, E211D or G182R/E211D, green fluorescence indicates transfection (top row). Secondary antibody was rhodamine-conjugated anti-rabbit-IgG (red, second row). All cells were stained with DAPI to indicate nuclei (blue, third row). Overlaid images (EGFP/TRITC/DAPI) are shown at bottom. White arrows point to cell surface K\textsubscript{V}1.5 staining; white arrowheads point to perinuclear clusters of K\textsubscript{V}1.5. All fields shown at 60x magnification.

5.5 Subcellular Localization of WT-K\textsubscript{V}1.5 and Mutant K\textsubscript{V}1.5 in Human PASMC

Although human PASMC express endogenous K\textsubscript{V}1.5 (Figure 11), it was expected that transfected K\textsubscript{V}1.5 would tetramerize with endogenous protein, thus forming a population of channels composed of at least one mutant subunit. As the subcellular localization of K\textsubscript{V} channels may be determined by a single subunit acting in a dominant manner, it was possible that transfected G182R, E211D or G182R/E211D protein would affect the localization of this subset of K\textsubscript{V}1.5 channels.
in hPASMC. Therefore, hPASMC were transfected with WT-KV1.5, G182R, E211D or G182R/E211D and subject to standard immunocytochemistry to detect subcellular localization of Kv1.5.

PASMC transfected with WT-KV1.5 have a dispersed pattern of Kv1.5 staining, with Kv1.5 located throughout the cell as well as on the perimeter, indicative of cell surface localization (Figure 21, left). When PASMC transfected with G182R were examined, however, nearly all Kv1.5 was found in packets around the nucleus (Figure 21, G182R), indicative of intracellular retention. Although this was not surprising given the relative abundance of the immature protein form in HEK-293 cells compared to WT-KV1.5 (Figure 17), of G182R observed in HEK-293 cells, the E211D and G182R/E211D mutant channels were observed in similar perinuclear packets in PASMC (Figure 21). Untransfected PASMC demonstrated weak positive Kv1.5 staining that was found both on the cell surface and perinuclear packets (Figure 21, mock).
Figure 21: WT-Kv1.5 and Mutant Channel Subcellular Localization in PASMC. hPASMC were transfected with WT-Kv1.5 (left), G182R (second column), E211D (third column), G182R/E211D (fourth column) or water (Mock, fifth column), fixed, permeabilized, and incubated with anti-Kv1.5 antibody. In cells transfected with WT-Kv1.5, G182R, E211D or G182R/E211D, green fluorescence indicates transfection (top row). Secondary antibody used was rhodamine-conjugated anti-rabbit-IgG and indicates the presence of Kv1.5 (red, second row). All cells were stained with DAPI to indicate nuclei (blue). Overlaid images (EGFP/TRITC/DAPI) are shown at bottom. White arrows point to cell surface Kv1.5 staining; white arrowheads point to perinuclear clusters of Kv1.5. All fields shown at 60x magnification.
5.6 Proteasome Inhibition Increases Expression of Transfected \( K_\text{V}1.5 \) Channels.

Given the decreased protein expression of the G182R channel in COS-1 cells, experiments to determine whether the effect was at the RNA or protein level were undertaken. Although reverse-transcriptase-PCR is not sensitive to slight differences in mRNA levels, especially in transfected cells, it was possible that a significant decrease in mRNA transcribed from the G182R plasmid would be detectable. Whole cell RNA from COS-1 cells transfected with water (mock), WT-\( K_\text{V}1.5 \), G182R, E211D or G182R/E211D was isolated using a standard Trizol and chloroform extraction. RNA was reverse transcribed and the resulting cDNA subject to PCR with primers for either \( K_\text{V}1.5 \) or GAPDH. Despite a lack of high levels of endogenous \( K_\text{V}1.5 \) protein, mock transfected COS-1 cells had readily detectable levels of \( K_\text{V}1.5 \) mRNA (0.81 ± 0.2 a.u., n=2) (Figure 22A), probably due to methodological constraints. It was therefore difficult to determine how much mRNA detected in transfected cells is the result of transfection. Nevertheless, there were no detectable differences in cDNA levels between COS-1 cells transfected with WT-\( K_\text{V}1.5 \) (1.20 ± 0.04 a.u.) and G182R (1.06 ± 0.08 a.u.), E211D (1.12 ± 0.09 a.u.) or G182R/E211D (1.19 ± 0.06 a.u.) (Figure 22). Thus, the decreased expression of G182R protein in COS-1 cells is not due to a complete lack of mRNA.
Figure 22: Expression of Endogenous and Transfected $\text{K}_v 1.5$ mRNA in COS-1 Cells. COS-1 cells were transiently transfected with water (mock), WT-$\text{K}_v 1.5$, G182R, E211D or G182R/E211D plasmids. A) RNA was isolated and reverse transcribed into cDNA which was subject to a standard PCR protocol with $\text{K}_v 1.5$ (top) or GAPDH (bottom, shown as loading control) primers. B) Summarized data are expressed as arbitrary units (a.u.) of $\text{K}_v 1.5$ normalized to GAPDH, n=2. Data are presented as mean ± SEM and were compared with ANOVA using Tukey’s post-hoc analysis; *P<0.05 compared to WT-$\text{K}_v 1.5$.

It was also possible that the G182R mutation caused protein misfolding in the ER which targeted it for proteasomal degradation. MG-132 is a potent cell permeable proteasome inhibitor. COS-1 cells transfected with water (mock), WT-$\text{K}_v 1.5$, G182R, E211D or G182R/E211D were treated with MG-132 (10 µM) or equal volume vehicle (DMSO, 0.1%) and subject to standard immunoblot procedures to detect $\text{K}_v 1.5$ or GAPDH (Figure 23A). Treatment with MG-132 resulted in a general increase in $\text{K}_v 1.5$ protein levels in both transfected cells and mock transfected cells. In mock transfected cells, protein levels increased from 0.011 ± 0.003 a.u. to 0.035 ± 0.017 a.u. (n=5) with MG-132 treatment, although this difference was not statistically significant (Figure 23B). Transfected WT-$\text{K}_v 1.5$ also demonstrated an increase in protein level
(1.438 ± 0.059 a.u. with DMSO compared to 2.103 ± 0.303 a.u. with MG-132, P<0.05, n=5), representing a 146% increase (Figure 23B). The E211D (1.418 ± 0.144 a.u. with DMSO and 2.3609 ± 0.514 a.u. with MG-132, n=5) and G182R/E211D (1.159 ± 0.177 a.u. with DMSO compared to 1.979 ± 0.285 a.u. with MG-132, n=5, P<0.05) protein levels were similarly increased with MG-132 treatment to 166% and 170% of control levels respectively, although the increase in G182R/E211D was statistically significant while the increase in E211D was not (Figure 23B). However, treatment with MG-132 rescued expression of the G182R protein to a greater extent than any of the other transfected proteins including WT-KV1.5 (0.138 ± 0.046 a.u. with DMSO compared to 0.622 ± 0.092 a.u. with MG-132, n=5, P<0.005), increasing to 451% of control levels (Figure 23B). However, the absolute differences between DMSO and MG-132 groups among cells transfected with WT-KV1.5, G182R, E211D or G182R/E211D were not found to differ. These data suggest that although G182R protein expression can be increased by proteasome inhibition, this effect is not specific to the G182R protein.
Figure 23: Proteasome Inhibition Increases Expression of Transfected Kv1.5 Protein. COS-1 cells were transfected with water (mock), WT-Kv1.5, G182R, E211D or G182R/E211D. 24 hours after transfection cells were treated with vehicle (DMSO, 0.1%) or the proteasome inhibitor MG-132 (10 µM) for 24 hours before lysis. A) Whole cell lysates were subject to standard immunoblot procedure to detect Kv1.5 (67 kDa, top) or GAPDH (36 kDa, bottom). B) Summarized data are presented as Kv1.5 normalized to GAPDH. Dark fills represent DMSO treated cells (control) and light fills MG-132 treated cells. Data are presented as % increase on the right. Data are presented as mean ± SEM, n=5. Data were compared with ANOVA using Tukey’s post-hoc analysis. Left: *P<0.05, **P<0.005 compared to control (+vehicle); right: *P<0.05 compared to WT-Kv1.5.

5.7 Overexpression of Kvβ Subunits in COS-1 Cells Does Not Rescue G182R Protein Expression

Kv channels associate with cytoplasmic Kvβ subunits which modify expression levels of Kv channels. It was therefore possible that the dramatic decrease in G182R protein level in COS-1 cells compared to HEK-293 cells was due to differential Kvβ interactions. To examine the expression of Kvβ subunits in HEK-293 and COS-7 cells, RNA was reverse transcribed and subject to PCR to detect levels of
$K_\text{V} \beta 1$, $K_\text{V} \beta 2$ and $K_\text{V} \beta 3$. Both HEK-293 and COS-7 cells expressed $K_\text{V} \beta 1$, $K_\text{V} \beta 2$ and $K_\text{V} \beta 3$; however, expression levels between the cell types varied greatly (Figure 24). HEK-293 cells expressed higher levels of both $K_\text{V} \beta 1$ and $K_\text{V} \beta 2$ than COS-7 cells, although only the difference in $K_\text{V} \beta 2$ was found to be statistically significant (for $K_\text{V} \beta 1$: $0.12 \pm 0.03$ a.u. in HEK-293 cells compared to $0.03 \pm 0.03$ a.u. in COS-7 cells; for $K_\text{V} \beta 2$: $0.42 \pm 0.03$ a.u. in HEK-293 cells compared to $0.02 \pm 0.01$ a.u. in COS-7 cells, $P<0.001$; $n=3$) (Figure 24). Conversely, HEK-293 cells expressed significantly lower levels of $K_\text{V} \beta 3$ than COS-7 cells ($0.17 \pm 0.07$ a.u. compared to $0.71 \pm 0.05$ a.u., $P<0.01$) (Figure 24).
Figure 24: mRNA Expression Levels of K\text{v}\beta Subunits Differ Between COS-7 and HEK-293 Cells. Whole cell RNA from HEK-293 and COS-7 cells was reverse transcribed into cDNA which was subject to standard PCR with primers for human K\text{v}\beta1, K\text{v}\beta2 or K\text{v}\beta3. M, 100-bp DNA ladder. RT-PCR amplified products of GAPDH are shown as loading controls. A) Representative gels are shown. B) Summarized data are presented as K\text{v}\beta band intensity normalized to GAPDH levels (in arbitrary units). Data are presented as mean ± SEM, n=3. Data were compared using t-test analysis between HEK-293 and COS-7 cells; **P<0.01, ***P<0.001 compared to HEK-293 cells.

Given the lower expression of K\text{v}\beta1 and K\text{v}\beta2 subunits in COS-1 cells compared to HEK-293 cells, it was possible that decreased G182R expression in COS-1 cells could be rescued by overexpression of K\text{v}\beta subunits, as K\text{v}\beta subunits are known to have a chaperone function on K\text{v} channel proteins. K\text{v}\beta1 subunits (K\text{v}\beta1.2 and K\text{v}\beta1.3) were therefore coexpressed with WT-K\text{v}1.5 or G182R in COS-1 cells to determine whether G182R expression could be rescued by K\text{v}\beta subunits. In COS-1 cells transfected with only WT-K\text{v}1.5 or G182R, G182R protein levels were ~8% of
WT-Kv1.5 (Figure 25). In COS-1 cells cotransfected with either WT-Kv1.5 or G182R and Kvβ1.2, a similar pattern was observed, with G182R protein levels 6.38 ± 2.54% of WT-Kv1.5 (Figure 25). Similarly in COS-1 cells cotransfected with either WT-Kv1.5 or G182R and Kvβ1.3, G182R was expressed at 4.32 ± 3.26% of WT-Kv1.5 levels (Figure 25). Thus, decreased levels of G182R protein in COS-1 cells cannot be rescued by overexpression of Kvβ subunits.

**Figure 25: Kvβ Subunits Do Not Rescue G182R Expression in COS-1 Cells.** A) COS-1 cells were transfected with WT-Kv1.5 or G182R alone (lanes 1 and 2) or with Kvβ1.2 (lanes 3 and 4) or Kvβ1.3 (lanes 5 and 6). Protein lysate from these cells was subject to standard immunoblot to detect Kv1.5 (top, 67 kDa) or GAPDH (36 kDa), shown as a loading control (bottom). B) WT-Kv1.5 and G182R protein levels were normalized to GAPDH and are summarized in arbitrary units. Black bars, cells transfected with WT-Kv1.5; light fills, cells transfected with G182R. NS = no significant difference (ANOVA with Tukey post-hoc test, P>0.05) compared to G182R –Kvβ. Data are presented as mean ± SEM, n=3.
5.8 G182R, E211D and G182R/E211D Channels Can Associate with K\(\text{V\(\beta\)1.3}

It was possible that the failure of K\(\text{V\(\beta\)}\) subunits to rescue the expression of G182R protein in COS-1 cells was due to an inability of the mutant protein to form complexes with the K\(\text{V\(\beta\)}\) subunits as these interactions rely on the tetramerization domain in which the mutations are found. A functional interaction between WT-K\(\text{V\(\beta\)}1.5\) and K\(\text{V\(\beta\)}1.3\) was confirmed by \(I_{\text{K(V)}}\) recordings of cells transfected with WT-K\(\text{V\(\beta\)}1.5\) alone or in the presence of hemagglutinin-tagged K\(\text{V\(\beta\)}1.3\) (K\(\text{V\(\beta\)}1.3\)-HA). As expected, \(I_{\text{K(V)}}\) from cells transfected with WT-K\(\text{V\(\beta\)}1.5\) alone were very slowly inactivating, whereas in the presence of K\(\text{V\(\beta\)}1.3\), a distinct inactivation is observable (Figure 26A).

Interaction of WT-K\(\text{V\(\beta\)}1.5\), G182R, E211D and G182R/E211D with K\(\text{V\(\beta\)}1.3\) was examined by co-immunoprecipitation in HEK-293 cells which were transfected with both WT-K\(\text{V\(\beta\)}1.5\), G182R, E211D or G182R/E211D and K\(\text{V\(\beta\)}1.3\)-HA. Protein fractions immunoprecipitated with anti-K\(\text{V\(\beta\)}1.5\) antibody were immunoblotted for the HA-tag (Figure 26B, top blot). Whole cell lysate was also immunoblotted for the HA-tag and served as a loading control (Figure 26B, bottom blot). WT-K\(\text{V\(\beta\)}1.5\) interacts strongly with K\(\text{V\(\beta\)}1.3\)-HA (Figure 26B). Although the G182R and E211D mutations were expected to diminish the ability of the K\(\text{V\(\beta\)}1.5\) channel to interact with K\(\text{V\(\beta\)}1.3\), there was no difference among the ability of G182R, E211D or G182R/E211D to bind with K\(\text{V\(\beta\)}1.3\) (0.97 ± 0.21 for WT-K\(\text{V\(\beta\)}1.5\) compared to 1.63 ± 0.60, 1.14 ± 0.29 and 1.57 ± 0.29 for G182R, E211D and G182R/E211D channels, respectively; \(n=3\)) (Figure 26B, lower panel). Thus, the failure of K\(\text{V\(\beta\)}\) subunit overexpression to rescue G182R expression in COS-1 cells is not due to a lack of interaction between G182R protein
and the $K_\nu\beta_1$ subunit. Additionally, neither the G182R nor the E211D mutation significantly alters $K_\nu\beta_1$ binding.

**Figure 26: G182R, E211D and G182R/E211D Mutant Channels Bind with $K_\nu\beta_1.3$ Subunits.**

A) HEK-293 cells were transfected with WT-$K_\nu.1.5$ alone or in the presence of $KV\beta.1.3$-HA. Representative $I_{K(V)}$ recordings are shown (pulse protocol, inset). B) HEK-293 cells were transfected with WT-$K_\nu.1.5$ or $K_\nu\beta.1.3$-HA alone or cotransfected with WT-$K_\nu.1.5$, G182R, E211D or G182R/E211D and $K_\nu\beta.1.3$-HA. Whole cell protein lysate was immunoprecipitated with anti-$K_\nu.1.5$ antibody and immunoblotted for HA tag (IP: $K_\nu.1.5$, WB: HA; top). Whole cell lysate was also immunoblotted for HA tag (Input: Lysate, WB: HA; bottom gel) as a loading control. Inset shows immunoprecipitated fraction from HEK-293 cells transfected with WT-$K_\nu.1.5$ (1), WT-$K_\nu.1.5$+$K_\nu\beta.1.3$ (2) or $K_\nu\beta.1.3$ (3) and immunoblotted against $K_\nu.1.5$ as control. Summarized data are presented as the ratio of the immunoprecipitated band (IP) to the input band (IP/input). Data are presented as mean ± SEM, n=3.
5.9 COS-1 Cells Transfected with G182R Mutant K\textsubscript{V}1.5 Demonstrate a Trend Toward Increased Proliferation

Increased K\textsuperscript{+} efflux through an increased number of K\textsubscript{V} channels in transfected COS-1 cells has opposing effects on cell viability, as it would increase the likelihood of apoptosis (by increasing AVD) while at the same time hyperpolarizing the cell, thus increasing the driving force on Ca\textsuperscript{2+} entry into the cytosol and increasing proliferation. To determine whether the trafficking and electrophysiological effects of the mutations in K\textsubscript{V}1.5 affected cell proliferation, COS-1 cells were subject to standard proliferation assays.

Initially, COS-1 cells were plated in regular growth media, transfected with water (mock), WT-K\textsubscript{V}1.5, G182R, E211D or G182R/E211D and incubated with radiolabeled thymidine (3[H] thymidine) for 6 hours (after 24 hours serum starvation) to measure DNA synthesis. Cells that were mock-transfected cells showed robust DNA synthesis (in counts per minute, CPM, 15858.2 ± 2552.4 CPM, n=3) (Figure 27A). Cells transfected with WT-K\textsubscript{V}1.5, however, showed less DNA synthesis (10762.6 ± 523.7 CPM, n=3), although this difference was not statistically significant (Figure 27A). Proliferation in COS-1 cells transfected with G182R, E211D or G182R/E211D did not differ from cells transfected with WT-K\textsubscript{V}1.5 (9292.7 ± 678.9 CPM, 9615.3 ± 1053.1 CPM, and 10158.6 ± 509.7 CPM, respectively) (Figure 27A).

Because transiently transfected cells do not pass transfected DNA to daughter cells, it was initially thought that untransfected cells dominated the cell population by the end of the 54 hour time course of the 3[H] assay. This could account for the
similar 3[H] thymidine counts between COS-1 cells transfected with WT-Kv1.5 and cells transfected with G182R, E211D or G182R/E211D. Thus a cell counting assay in which transfected cells were counted in a hemocytometer was performed 24 hours after transfection (Figure 27B). However, no difference was observed among EGFP-transfected cells, cells transfected with WT-Kv1.5, and cells transfected with G182R, E211D or G182R/E211D (WT-Kv1.5: 1.19 ± 0.06 (×10^3) cells; G182R: 1.28 ± 0.10 (×10^3) cells; E211D: 1.14 ± 0.09 (×10^3) cells; G182R/E211D 1.11 ± 0.10 (×10^3) cells; n=10) (Figure 27B).

In a time course over 96 hours (n=1), however, it was found that transfection media affected cell proliferation from the start (Figure 27C, compare EGFP to ‘no treatment group’). However, at 96 hours, there was a clear increase in proliferation as cell number started to increase (Figure 27C, left). Thus, the similar CPM 54 hours after transfection (Figure 27A) and the similar cell counts 24 hours after transfection (Figure 27B) among WT-Kv1.5, G182R, E211D and G182R/E211D may be due to an initial decline in proliferation due to transfection. As the media was unchanged during the 96 hour time course, it is not surprising that cell count in the untreated group, which was higher than any of the transfected groups even at 24 hours, started to decrease at 96 hours (Figure 27C). Furthermore, a clear difference was detected between cells transfected with WT-Kv1.5 (3.8×10^4 cells) and cells transfected with E211D (7.8×10^4 cells) or G182R/E211D (8.3×10^4 cells) and a modest difference observed between cells transfected with WT-Kv1.5 and G182R (5.1×10^4 cells) (Figure 27C, left). Therefore, cells were subject to counting 96 hours after transfection (Figure
While no significant difference was observed between WT-Kv1.5 (0.85 ± .014 \((\times 10^3)\) cells) and G182R (1.18 ± 0.04 \((\times 10^3)\) cells), E211D (1.20 ± 0.24 \((\times 10^3)\) cells) or G182R/E211D (1.13 ± 0.29 \((\times 10^3)\) cells) (Figure 27D), there was a clear trend toward a higher cell count for cells transfected with G182R compared to WT-Kv1.5 (\(P=0.07, n=4\)). However, the differences between WT-Kv1.5 and E211D and between WT-Kv1.5 and G182R/E211D were less distinct. Given its reduced expression in COS-1 cells, it is surprising that the G182R cell count differs from that of EGFP, although again, this trend was not statistically significant.
Figure 27: G182R Channels Confer a Trend Toward Increased Proliferation in COS-1 Cells. COS-1 cells were transiently transfected with water (mock), EGFP (empty vector), WT-KV1.5, G182R, E211D or G182R/E211D or maintained in regular growth media (no treatment). A) Transfected COS-1 cells were incubated with 3[H] thymidine for six hours. Lysate was counted on a liquid scintillation counter to quantify DNA synthesis (counts per minute, CPM). Data are presented as mean ± SEM, n=3. B-D) Adherent transfected COS-1 cells were trypsinized and counted on a hemocytometer (B) 24 hours after transfection (normalized to EGFP, data presented as mean ± SEM, n=10), (C) 24-96 hours after transfection (n=1) or (D) 96 hours after transfection (normalized to mock, data presented as mean ± SEM, n=4).
5.10 Conclusions

5.10.1 Summary of Results Regarding Subcellular Localization and Expression of Mutant $K_v1.5$

In addition to channel function, $K_v1.5$ channels, like all proteins, are subject to regulation at the subcellular localization level. $K_v1.5$ is a surface protein and has a glycosylation site on its extracellular loop between S1 and S2. The oligosaccharide is subject to processing during the biosynthetic route to the cell surface. In HEK-293 cells, a human cell line, transfected WT-$K_v1.5$ is present mostly in its mature glycosylated form, whereas both the G182R and G182R/E211D channels were present in relatively greater amounts in their immature forms in early passage cells (Figure 17A, B). Additionally, G182R/E211D demonstrated slightly decreased protein levels which could be attributed to a decrease in the upper band (Figure 17C); however, this result must be interpreted with caution as quantification of protein levels from transfected cells with immunoblot procedures is not strictly quantitative. Immunostaining against $K_v1.5$ in HEK-293 cells demonstrated that while cells transfected with WT-$K_v1.5$ had both perinuclear and surface staining, the G182R protein localized mainly to perinuclear packets with little present on the cell surface (Figure 18). E211D and G182R/E211D staining were seen both on the cell surface and in perinuclear packets (Figure 18).

Transfected COS-1 cells were subject to similar studies to determine whether the effects of the mutations were cell-type specific. Unexpectedly, G182R protein was
present in significantly reduced amounts on immunoblot (~17% of WT-Kv1.5 levels, Figure 19). Immunostaining of transfected COS-1 cells revealed that most WT-Kv1.5 protein was present on the cell surface (Figure 20). Cells transfected with G182R were characterized by two staining patterns: either little positive Kv1.5 staining or localization to both the cell surface and perinuclear packets (Figure 20). Both the E211D and G182R/E211D channels consistently localized to both the surface and perinuclear packets (Figure 20). In transfected hPASMC, WT-Kv1.5 was observed in a diffuse pattern throughout the cell, while G182R, E211D and G182R/E211D were present in distinct clusters around the nucleus (Figure 21).

The low levels of G182R protein in COS-1 cells was further examined. mRNA levels of WT-Kv1.5, G182R, E211D and G182R/E211D were found to be comparable (Figure 22). Furthermore, treatment with the general proteasome inhibitor MG-132 resulted in a general increase in transfected protein levels that was not different among WT-Kv1.5, G182R, E211D and G182R/E211D proteins (Figure 23). In light of differences in Kvβ expression between HEK-293 and COS-1 cells (Figure 24), rescue of G182R protein levels was attempted by overexpression of Kvβ1.2 or Kvβ1.3 (Figure 25); however, neither Kvβ1.2 nor Kvβ1.3 were able to rescue G182R levels in COS-1 cells (Figure 25). Furthermore, this was not due to an inability of G182R to interact with the Kvβ subunits (Figure 26), and both E211D and G182R/E211D channels also retained their ability to interact with Kvβ subunits (Figure 26).
Lastly, the effect of the \( K_{\text{V}}1.5 \) mutations on cell proliferation was examined in COS-1 cells. Cells transfected with G182R demonstrated a trend toward increased proliferation compared to WT-\( K_{\text{V}}1.5 \) (Figure 27).

5.10.2 Discussion

Before \( K_{\text{V}}1.5 \) channels get to the cell surface, they undergo processing and regulation throughout the biosynthetic pathway. Many \( K_{\text{V}}1 \) channels, including \( K_{\text{V}}1.1 \) (and the \textit{Drosophila} Shaker channel), \( K_{\text{V}}1.2 \) and \( K_{\text{V}}1.4 \) are glycosylated on the extracellular loop between S1 and S2 (49, 258, 262). Early core partial glycosylation (of the \textit{Drosophila} Shaker channel) in the ER is followed by processing of the sugar residues into their mature form in the Golgi (193). On immunoblot of \( K_{\text{V}}1 \) subunits, a lower electrophoretic mobility (lighter) suggests an ER pool of immature protein, whereas a higher mobility (heavier) are indicative of sialylated (mature) complexes present in the Golgi and at the plasma membrane (167). Therefore, in HEK-293 cells, immunoblot results suggest that WT-\( K_{\text{V}}1.5 \) is present mainly in its mature form whereas G182R and G182R/E211D are present in a relatively higher proportion in their immature form. Immunostaining of transfected HEK-293 cells support the hypothesis that WT-\( K_{\text{V}}1.5 \) localizes to both the cell membrane and an intracellular population while a higher proportion of G182R is present inside the cell.

COS-1 cells were used for similar experiments to determine whether these effects could be reproduced in another cell type. COS-1 cells transfected with WT-\( K_{\text{V}}1.5 \) display a prominent band. Surprisingly, expression of G182R protein was
significantly reduced compared to wildtype expression levels. Staining data indicate that the wildtype protein is present mostly on the cell surface whereas cells transfected with G182R either fail to stain positive for Kv1.5 or demonstrate a combined pattern of surface and perinuclear staining. Both E211D and G182R/E211D channels also demonstrated this combined pattern. Additionally, in transfected hPASMC, the clusters of G182R, E211D and G182R/E211D adjacent to the nucleus are evident whereas they are absent from hPASMC transfected with WT-Kv1.5. Although proteasome inhibition partially rescued G182R expression, it resulted in similar increases in WT-Kv1.5, E211D and G182R/E211D protein; therefore, the G182R channel does not seem to be selectively targeted for proteasomal degradation.

As transient transfection necessarily results in the overexpression of Kv1.5, the effects of mutations on cellular function may not be dramatic if enough functional protein is expressed. However, in PASMC, where Kv1.5 is one of the dominant Kv channel subtypes expressed, a reduction in Kv1.5 levels or a reduction of expression at the cell surface may very well be detrimental to the cell, resulting in decreased $I_{K(V)}$ and relatively depolarized cells. This would lead to higher $[Ca^{2+}]_{cyt}$ and therefore increased proliferation and contraction.

Overexpression of Kvβ subunits was unable to rescue protein levels of G182R; therefore there may be another unknown chaperone protein which associates with Kv1.5 (but not in the context of the G182R mutation by itself) in COS-1 cells. Alternatively, there may be a Kv1.5-associated protein which negatively regulates its expression (and binds only to the G182R channel) and is present in COS-1 cells but
not in HEK-293 cells. In either case, it is surprising that while G182R protein levels were markedly reduced in COS-1 cells, this effect was not observed for the G182R/E211D protein, as though the E211D mutation rescued the G182R phenotype. This may be the result of simple steric hindrance. For example, if a chaperone protein relies on a positively charged interface to interact with the negative side chain of E211 for WT-K\textsubscript{V1.5} channel expression, this interaction may be preserved in E211D and G182R/E211D channels (Figure 28). However, the presence of the positive side chain of the G182R group in the context of E211 may prevent this interaction by repellent positive charges (Figure 28). In the doubly mutant channel, however, the negatively charged group, because of the shorter side chain of aspartate compared to glutamate, may be sufficiently distant from the positive guanidinium group to allow the interaction between K\textsubscript{V1.5} and the hypothetical chaperone protein (Figure 28). Of course, there may be a variety of regulatory and/or adaptor proteins which interact with these specific residues of the T1 domain of K\textsubscript{V1.5}. The differences in expression patterns of G182R, E211D and G182R/E211D between HEK-293 and COS-1 cell types may be due to differential regulatory proteins (other than K\textsubscript{V}\textbeta1.3) expressed in these cells. For example, even between two mammalian cell lines (mouse L cells and HEK-293 cells), dramatic differences in current properties from transfected K\textsubscript{V1.5} were found to exist (279). Similarly, the effects of mutant K\textsubscript{V1.5} variants were observed in CHO cells but not in HEK-293 cells (218), further suggesting the sensitivity of channel function to specific cell backgrounds.
The trend toward increased proliferation (96 hours after transfection) in the COS-1 cells transfected with G182R is surprising. In nonexcitable cells which do not express VDCC, such as COS-1 cells, a decrease in $I_{K1.5}$ is expected to diminish $[Ca^{2+}]_{cyt}$, and therefore proliferation, by decreasing the driving force on $Ca^{2+}$ influx into the cytosol (70, 150). Even independent of $Ca^{2+}$, proliferation is generally associated with increases in $K_V$ channel activity (298). The cause for the trend toward increased proliferation in COS-1 cells transfected with G182R therefore remains unknown. However, in PASMC which express VDCC, decreased $I_{K1.5}$ (which would result from decreased $K_V1.5$ expression or specifically expression at the plasma membrane) leads to depolarization, higher $[Ca^{2+}]_{cyt}$ and increased proliferation. In these cells which express some other $K_V$ proteins, G182R protein may even act in a dominant negative fashion, associating with other $K_V$ subunits in the ER and retaining
them there and/or decreasing their surface expression and/or decreasing their protein levels altogether. That certain Kv1 subunits are capable of acting in such a dominant negative manner is not without precedent (69, 167, 281).

5.11 Acknowledgments for Chapter 5

Chapter 6. General Conclusions

6.1 Future Directions

As previously mentioned, transient transfection introduces certain experimental limitations. For example, although whole cell $I_{K(V)}$ among cells transfected with WT-$K\beta1.5$, G182R, E211D and G182R/E211D were similar, the presence of overexpressed $K\beta1.5$ may have a “saturating” effect in terms of the number of channels expressed at the plasma membrane. The fact that G182R channels have a slightly increased single channel conductance compared to WT-$K\beta1.5$ suggest that there may in fact exist a difference in $I_{K(V)}$ amplitude in cells which express physiological levels of protein. Overexpression may also result in $K\beta1.5$ channel assembly among mutant subunits which might not so readily occur with less protein. Similarly, small differences in the affinity of mutant $K\beta1.5$ to associate with $K\beta$ subunits may be undetected given the overexpression of both $K\beta1.5$ and $K\beta1.3$ in the co-immunoprecipitation studies. Observations on protein expression levels and cellular functions, such as cell proliferation, are also limited for similar concerns. Furthermore, because the mutations were found in IPAH patients and may reasonably be expected to contribute to PASMC abnormalities associated with IPAH, it would be of tremendous interest to study these mutations in PASMC. However, transient transfection produces highly variable transfection rates in cultured hPASMC. Therefore, stably transfected PASMC lines in which the endogenous $KCNA5$ had been replaced with either the G182R or E211D versions of the gene would provide a better
cell culture system in which to study the effects of the mutations. However, from the perspective of elucidating the effects of the mutations on channel structure-function relationship, HEK-293 and COS-1 cells are ideal as they express little endogenous $K_V$ protein.

Even stably transfected cultured cells may not be the best system in which to study the relevance of the $K_V1.5$ mutations to IPAH, as culturing arterial SMC is known to specifically alter ionic currents compared to freshly isolated cells (317). Furthermore, contractile uterine artery VSMC are associated with $K_V1$ channel expression whereas the main $K_V$ current in cultured (proliferating) uterine artery VSMC was carried by $K_V3.4$ (183). The $K_{Ca}$ current of VSMC may also change as cells transition from a contractile to proliferative phenotype (200). Thus, the function and expression of $K_V$ channels, in addition to expression of regulatory proteins which interact with $K_V$ channels, may change with cell culture. Thus, a transgenic mouse which expressed mutant $K_V1.5$ in PASMC would allow for both fresh cell isolation (to study the effects of the mutations on cellular function) as well as a whole animal system to study the effects of the mutations on the pulmonary vasculature and on hemodynamics. Additionally, PASMC from patients who had the G182R and/or E211D mutations could be isolated and studied (albeit in culture). This would allow for the determination of the effects of the mutations in the physiological environment in which they may cause PASMC abnormalities observed in IPAH patients. Therefore, if a condition specific to these particular IPAH patients’ PASMC caused mutant $K_V1.5$ to result in abnormal proliferation or decreased $I_{K(V)}$ for example, that cell specific
factor could be isolated. However, the difficulty in obtaining PASMC from IPAH patient lungs, especially given the reduced frequency of lung transplants for IPAH patients, makes this approach difficult.

Additionally, although the immunostaining patterns of WT-\(K_v 1.5\) and the mutant channels clearly differ in COS-1 and HEK-293 cells, the precise subcellular location of the perinuclear packets observed in cells transfected with mutant protein remains uncertain. Although the immunostaining experiments were visualized on a deconvolution microscope and images were taken as z-stacks through the cell, to determine precisely the location of the \(K_v 1.5\) clusters, or to verify that the perimeter staining of \(K_v 1.5\) in fact represents cell surface staining, costaining with either a surface marker or an ER or Golgi marker (such as Brefeldin A) would further clarify the precise staining patterns of \(K_v 1.5\).

6.2 General Discussion

\(K_v\) channel dysfunction in PASMC is thought to play a role in the etiology of pulmonary arterial hypertension. \(K_v 1.5\) is one of the major \(K_v\) subunits expressed in hPASMC (221). As such, \(K_v 1.5\) channel dysfunction may underlie certain cases of IPAH. The characterization of two mutations found in the tetramerization domain of \(K_v 1.5\) in idiopathic pulmonary arterial hypertension patients was the main focus of this dissertation. While the T1 domain had previously been implicated in channel gating, subcellular localization and protein expression of the channels, neither the G182 nor the E211 residues had been specifically studied. Results confirm a role of
the T1 domain, and specifically of these residues, in both of these functions. While the effects on channel activation and deactivation were subtle, disease phenotypes generally are so. Rarely is a disease so clearly the cause of one specific genetic mutation. IPAH is no different, and it is widely believed to be the result of multiple factors, including both genetic and environmental insults. The slower activation and faster inactivation observed in the mutant channels may contribute to the increased $[\text{Ca}^{2+}]_{\text{cyt}}$ observed in PASMC from IPAH patients in a small subset of patients.

While the altered kinetics of mutant $K_{V}1.5$ may play a role in PASMC (dys)function, the channels first must get to the plasma membrane for this to be relevant. The observed patterns of mutant $K_{V}1.5$ subcellular localization and protein processing suggest that the channels, especially the G182R channel, may not be efficiently localize to the plasma membrane. This effect may be even more pronounced in pulmonary artery smooth muscle cells of patients in which $K_{V}1.5$ is not artificially overexpressed. The $E_{\text{m}}$ depolarization observed in PASMC from IPAH patients compared to control PASMC, if it is due to decreased $K_{V}1.5$ channel expression at the plasma membrane, may be explained by the G182R mutation in a small subset of IPAH patients. As with the CFTR channel in cystic fibrosis, the G182R channel may function well enough to maintain normal cell function if it could get to the cell surface. However, the real problem of the mutation may lie in getting it to the cell surface.

Lastly, as a better understanding of the genetics of IPAH becomes available, $KCNA5$ may be one of a variety of genes examined to assess risk for developing IPAH.
One can imagine a microarray of known IPAH risk factors, of which \textit{KCNA5} is one, as a standard genetic test. This would have important implications for screening CTEPH patients for a pre-treatment course of drugs before undergoing PTE surgery, for example. Additionally, such a screen could help identify which main pathway(s) (BMP signaling, prostacyclin or PDE-V for example) was deficient in a specific IPAH patient, paving the way for a pharmacogenomic approach in the treatment of IPAH.


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