Proteolytic Cleavage of the Kv1.5 Channel in the S1-S2 Linker Does Not Affect Channel Function

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Abstract

Atrial fibrillation (AF), the most prevalent human cardiac arrhythmia, is characterized by rapid and disorderly electrical activity in the atria of the heart. Kv1.5 channel mediated ultra-rapidly activating delayed rectifier potassium current (I_{Kur}) is critical for timely and adequate atrial repolarization. Since cardiac I_{Kur} is specific to the atria, biomedical research surrounding Kv1.5 poses significant promise for developing clinical strategies to treat AF. In fact, loss-of-function mutations in KCNA5 (encoding Kv1.5) have been identified in patients with AF. Importantly, common pathologies, such as selective atrial ischemia, are capable of stimulating the onset of AF. A well-documented consequence of ischemia is a substantial increase in proteolytic enzyme activities. In this regard, it has been reported that cell-surface Kv1.5 channels are sensitive to cleavage by extracellular proteases, such as proteinase K (PK). In this study, we further examined the effects of extracellular proteases on the function and expression profile of Kv1.5 channels stably expressed in HEK 293 cells. Our results demonstrate that PK cleaves membrane-bound mature (75-kDa) Kv1.5 channels at a single locus in the external S1-S2 linker, yielding 42-kDa N-terminal and 33-kDa C-terminal fragments. Contrary to our expectations, whole-cell voltage clamp analysis showed that PK treatment did not affect Kv1.5 current (I_{Kv1.5}). Examination of plasma membrane proteins isolated via biotinylation indicated that the N- and C-terminal Kv1.5 fragments were both present and stable on the cell-surface. Co-immunoprecipitation (co-IP) studies following PK cleavage suggest that the two Kv1.5 fragments do not associate. Moreover, the PK-generated N- and C-terminal fragments degraded at different rates. These findings indicate that the C-terminal fragment of Kv1.5 (S2-S6,
pore-containing) may be sufficient for current conduction. Our data raises the possibility that cleavage of cell-surface ion channels, assessed by Western blot analysis, does not necessarily result in a loss of channel activities. This novel insight into the Kv1.5 structure-function relationship may be indicative of an inherent protective mechanism for Kv1.5 channel function.
Co-Authorship

Jun Guo created the HEK stable cell lines and periodically assisted with cell culture. Jun Guo and Wentao Li assisted in some patch-clamp experiments. Tonghua Yang assisted in some immunofluorescence microscopy experiments. Dr. Shetuan Zhang assisted in designing the experiments.
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<tr>
<td>AF</td>
<td>Atrial fibrillation</td>
</tr>
<tr>
<td>AV</td>
<td>Atrioventricular</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>Ctrl</td>
<td>Control</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>hERG</td>
<td>Human ether-a-go-go-related gene</td>
</tr>
<tr>
<td>hERG-HEK</td>
<td>HEK 293 cells stably expressing hERG</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>I_{hERG}</td>
<td>hERG current</td>
</tr>
<tr>
<td>I_{Kr}</td>
<td>Rapidly activating delayed rectifier potassium current</td>
</tr>
<tr>
<td>I_{Ks}</td>
<td>Slowly activating delayed rectifier potassium current</td>
</tr>
<tr>
<td>I_{Kur}</td>
<td>Ultra rapidly activating delayed rectifier potassium current</td>
</tr>
<tr>
<td>I_{Kv1.5}</td>
<td>Kv1.5 current</td>
</tr>
<tr>
<td>I_{to}</td>
<td>Transient outward potassium current</td>
</tr>
<tr>
<td>K^+</td>
<td>Potassium</td>
</tr>
<tr>
<td>Kv1.5-HEK</td>
<td>HEK 293 cells stably expressing Kv1.5</td>
</tr>
<tr>
<td>MAGUK</td>
<td>Membrane-associated guanylate kinase</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
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MMPs  Matrix metalloproteinases
Nedd4-2  Neural precursor cell expressed developmentally down-regulated protein 4 sub-type 2
PASMCs  Pulmonary arterial smooth muscle cells
PBS  Phosphate-buffered saline
PK  Proteinase K
PMSF  Phenylmethylsulfonyl fluoride
PVDF  Polyvinylidene difluoride
ROS  Reactive oxygen species
SA  Sinoatrial
SAP97  Synapse associated protein 97
SDS-PAGE  Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S.E.M.  Standard error of the mean
SR  Sinus rhythm
T1  Tetramerization domain
TBST  Tris-buffered saline and 0.1% Tween 20
WB  Western blot
WT  Wild type
Chapter 1: Introduction and Literature Review

Atrial Fibrillation

Atrial fibrillation (AF) is the most common human arrhythmia and is characterized by disorganized rampant electrical activity in localized portions of the atrial wall. During AF, normal electrical rhythm generated by the sinoatrial (SA) node is replaced by swift and irregular impulses that cause a quiver in the atrial myocytes instead of coordinated contractions (Bootman et al., 2006). In fibrillation episodes, atrial cells fire 400-600 times per minute (SA node at rest ~60-80 pulses/min); however, these impulses do not all reach the ventricles due to the limited-impulse carrying capacity of the atrioventricular (AV) node (Nattel, 2002). Accordingly, electrocardiogram (ECG) tracings of AF episodes are characterized by a lack of pronounced P waves (denoting atrial depolarization) and irregular intervals between QRS complexes (corresponding to ventricular depolarization) (Figure 1) (Wakili et al., 2011). The importance of studying this increasingly prevalent heart rhythm disorder was highlighted following the connection of AF to significant patient discomfort, morbidity, and a doubling of mortality (Benjamin et al., 1998).

i) Epidemiology

AF is quickly becoming a global issue; in the United States the number of affected individuals is predicted to increase from 2.3 million to 5.6 million by 2050 (Kannel & Benjamin, 2008). The vast majority of AF occurs in the elderly population, as approximately 9% of individuals over the age of 65 develop sustained AF (Gramley et al., 2010). In fact, the prevalence of this condition increases with age, from 0.5% of people aged 50-60 to nearly 10% of individuals aged 80-90 (Kannel & Benjamin, 2008).
Figure 1. Surface ECG recordings illustrating normal (sinus) cardiac rhythm and atrial fibrillation. **A.** Diagram of the typical electrical conduction system in the human heart. Impulses travel from the SA node to the atria and the AV node before reaching the ventricles. The cardiac events involved in normal/rhythmic heart contraction correspond to the surface ECG recording shown below. **B.** Prototypical atrial fibrillation with disorganized atrial activity as well as subsequent irregular ventricle activation, and the corresponding ECG tracing. Note the lack of regular P waves (atrial depolarization) and abnormal/inconsistent intervals between QRS complexes (ventricular depolarization). Image modified from Wakili et al. (Wakili et al., 2011).
Hence, AF represents a considerable burden to our society’s economy and health care system.

ii) **Prognosis**

Acute AF itself is not life-threatening because ventricular filling is still accomplished, albeit less efficiently, by venous return and blood pressure (Bootman *et al.*, 2006). However, sustained AF promotes irregular and rapid ventricular contractions that cause atrial chamber dilation, which promotes structural or functional atrial remodeling, thereby further favouring AF (Iwasaki *et al.*, 2011). The ventricular dysfunction that accompanies AF is responsible for a decrease in cardiac stroke volume by 20-30% (Alpert *et al.*, 1988). Thus, it is not surprising that AF and the subsequent electrical remodeling are directly linked with many life-threatening conditions. For instance, the decreased cardiac output in AF can lead to congestive heart failure as well as increasing the risk of stroke five-fold due to blood stasis in the atria and clot formation (Hart & Halperin, 2001).

iii) **Mechanisms and Classification**

It has been well documented that many cardiovascular diseases predispose individuals to AF (Allessie *et al.*, 2001; Kozlowski *et al.*, 2010; Nattel, 2002). Over 70% of patients with AF have pre-existing structural heart diseases such as congestive heart failure, cardiac ischemia, cardiomyopathy, and hypertensive heart disease (Kozlowski *et al.*, 2010). This stipulates that earlier recognition and treatment of underlying conditions as well as preventative lifestyle choices may counter AF development. The mechanisms by which disease precipitate AF are not fully understood, but are thought to be associated with atrial dilation and a shortening of the effective refractory period (Nattel,
On the other hand, AF does occur in individuals without recognizable medical conditions; this less documented subset of the arrhythmia is known as lone or idiopathic AF (Allessie et al., 2001). Additionally, a temporal classification system based on the length of fibrillation events is now being employed to further refine chronic AF categorization. AF that spontaneously terminates within one week is termed paroxysmal, and arrhythmic episodes that require electrical or pharmacological cardioversion are known as persistent (Levy, 2000). Permanent AF is the most severe form as it cannot be corrected with our current medical means or the necessary operation is inadvisable (Levy, 2000). In most cases, AF is believed to progress from paroxysmal to permanent as a result of tissue and ion channel remodeling within the atria (de Vos et al., 2010; Nattel et al., 2008).

While rapid progress has been made in understanding the pathogenesis of AF, it has become clear that AF is a complex multifaceted disorder with various potential underlying mechanisms. Paroxysmal AF often originates from spontaneous action potential producing foci/drivers in the cardiac muscle surrounding pulmonary veins (Haissaguerre et al., 1998; Cheung, 1981). Local ectopic firing of the pulmonary vein musculature can lead to single-circuit current re-entry or even multiple-circuit re-entry through atrial remodeling, which can maintain AF (Hobbs et al., 1999). Although less common, ectopic firing that triggers AF can also originate from other sources, such as the ligament of Marshall (a small venous remnant in the left atrium) (Doshi et al., 1999) and the superior vena cava (Tsai et al., 2000).

Importantly, AF can also occur as a result of genetic mutation (Brugada et al., 1997). The familial basis of AF is now receiving more attention and several mutations...
are being associated with AF. This aspect of AF-related research is of particular interest for understanding idiopathic AF. Summation of various studies (as outlined in the Kv1.5 and Atrial Fibrillation section) over recent years has shown that gain- or loss-of-function mutations/defects in relevant ion channel genes can directly cause or increase individual susceptibility to AF. Accordingly, there are presently two conceptual models for ion channel determinants of AF pathology: First, shortening the atrial action potential effective refractory period can permit electrical wavelet re-entry (Nattel, 2002). Second, prolongation of the atrial action potential duration increases the likelihood of AF via more frequent early after-depolarizations (Yang et al., 2009).

iv) Management Strategies

Current medical interventions for AF generally focus on attempting to restore normal sinus rhythm. Ablation techniques, including electrical catheter ablation have the best long-term record, but these methods are costly, invasive, and difficult to implement due to the quantity of AF patients (Ravens & Christ, 2010). Specifically, ablative methods target AF-generating local or ectopic foci that produce harmful electrical signals. Not surprisingly, pharmacological strategies are preferred among patients as opposed to intrusive surgical methods. Unfortunately, most available antiarrhythmic drugs lack atrial specificity and can have paradoxical life-threatening effects on ventricular electrophysiology (Iwasaki et al., 2011; Nattel, 2002). Ionic currents that are exclusive to the atria are promising because they can be targeted with less risk of dangerous ventricular side effects. The value of such drugs remains uncertain since the majority are still in development. Ion channel targeting pharmacology for treating AF is discussed further in the Kv1.5 and Atrial Fibrillation section. It is clear, however, that an
enhanced understanding of AF mechanisms and molecular determinants in the atria could lead to more promising approaches with less inherent risk.

**Atrial Fibrillation, Ischemia, and Proteases**

Ischemia is the restriction of blood flow to bodily tissues. It has been reported that selective atrial ischemia promotes the onset of AF (Sinno *et al.*, 2003). A major consequence of ischemia is insufficient oxygen delivery (among other issues such as a lack of metabolic nutrients), which is needed for oxidative phosphorylation. An insufficient oxygen supply can cause myocardial dysfunction in part due to cardiac remodeling and fibrogenesis, which predispose affected individuals to detriments like heart failure and arrhythmias (including AF) (Gramley *et al.*, 2010). An important association that these studies have uncovered is that cardiac ischemia and hypoxia are able to precipitate AF, yet the scientific explanations for these connections remain incomplete.

On a molecular level, the hypoxia-inducible factor (HIF) has been implicated in the development of AF (Thijssen *et al.*, 2002). HIF is an oxygen-sensing transcription factor that helps cells adapt and survive in reduced oxygen environments. Structurally, HIF-1 is a heterodimer composed of HIF-1α and HIF-1β. Unlike HIF-1α, HIF-1β is constitutively expressed and is not responsive to changes in oxygen (Harris, 2002). When sufficient oxygen is present, HIF-1 is hydroxylated so that it can interact with the von Hippel-Lindau E3 ubiquitin ligase, commencing the destruction sequence of HIF-1α that terminates with proteasomal destruction (Srinivas *et al.*, 1999). During hypoxia, HIF-1 remains active due to dampened prolyl hydroxylation, enabling binding interactions with the hypoxia response elements of target genes, which induces...
expression of those genes (Harris, 2002). Evidence exists suggesting that reactive oxygen species (ROS) are produced by the mitochondria in excess during hypoxia (Guzy et al., 2005; Guzy & Schumacker, 2006). ROS are believed to activate various transcription factors, including HIF-1α, thereby modifying the transcriptional rates of HIF-1α target genes (Chandel et al., 2000; Guzy & Schumacker, 2006).

The known battery of genes that are regulated by HIF-1α is constantly expanding. HIF-1α has been associated with the upregulation of proteases, such as the extracellular matrix metalloproteinases (MMPs) (Choi et al., 2011) and the membrane anchored serine protease matriptase-2 (Maurer et al., 2012). From the perspective of the cell, increasing protease expression during ischemia or hypoxia may be necessary for angiogenesis, which would increase the vascular delivery of nutrients and gases (Semenza, 2002). In accordance with the idea that proteases may cause AF (especially during ischemia), calcium-dependent calpains have been shown to be activated in both AF (Brundel et al., 2002) and hypoxia (Zhang et al., 1998). In addition, our lab has recently found that calpains in the extracellular milieu are capable of degrading human ether-a-go-go-related gene (hERG) K⁺ channels (Lamothe et al., unpublished). When considering all the evidence linking ischemia/hypoxia, proteases, and AF, it is conceivable that ischemia/hypoxia-mediated enhanced proteolytic activities may be cleaving ion channels and producing cardiac arrhythmias.

The Kv1.5 Channel

Kv1.5 channels (also known as HK2 channels) are voltage-sensitive members of the Shaker super-family of potassium channels (Kv1), which were first identified in Drosophila melanogaster (Papazian et al., 1987). These proteins are expressed in
many organs/locations such as the heart, pulmonary arterial smooth muscle cells (PASMCs), the brain, skeletal muscle, pancreatic β-cells, and have crucial functions in cell cycle regulation (Ravens & Wettwer, 2011). In PASMCs, Kv1.5 is important for oxygen sensitive regulation of arterial tone since its downregulation ultimately causes depolarization-induced calcium-mediated vasoconstriction (Archer et al., 2000). In the heart, Kv1.5 channels conduct the ultra-rapidly activating delayed rectifier potassium current (I_{Kur}), which is crucial for atrial action potential repolarization (Fedida et al., 1993; Wang et al., 1993). It is largely accepted that cardiac Kv1.5/I_{Kur} is solely functionally expressed in human atria, not the ventricles, making Kv1.5 an attractive target among groups aiming to develop new AF treatments and prevention strategies (Wang et al., 1993). The underlying concept is that treating arrhythmias by blocking or enhancing an atrial specific current negates the risk of causing potentially lethal ventricular arrhythmias. However, small amounts of Kv1.5 protein and mRNA have been detected in the ventricles of the heart (Mays et al., 1995). Specifically, Kv1.5 channels are concentrated in the atria at the intercalated disks and T-tubules, while immunohistochemical studies have also suggested protein expression at the myocyte cell-surface (Mays et al., 1995).

i) \textbf{I_{Kur} and Other Action Potential Repolarizing Currents}

Cardiac action potentials mediate the electrical impulses occurring within cardiomyocytes. These action potentials are a direct consequence of various ionic currents with unique properties and permeabilities. Cardiac repolarization, which terminates action potentials, is a result of channel-mediated K^+ ion efflux. In the ventricles of the heart, the rapidly activating (I_{Kr}) and slowly activating (I_{Ks}) delayed
rectifier $\text{K}^+$ currents drive repolarization (Li et al., 1996b). An important component of atrial repolarization comes from $I_{\text{Kur}}$. In disease conditions, currents can be increased or decreased, giving rise to a variety of undesired phenotypes. An overview of the ionic currents in ventricular and atrial myocyte action potentials, and the classic changes associated with AF is shown in Figure 2.

ii) Structural Properties

The generic structure of $\text{K}^+$ channels was first described in *Streptomyces lividans* via x-ray crystallography (Doyle et al., 1998). Since then, the resolved crystal structure of Kv1.2 (Long et al., 2005a; Long et al., 2005b) and computer simulations of the Kv1.5 domain sequences have led to the creation of homologous models for describing the three-dimensional structure of Kv1.5 channels (Ander et al., 2008; Yang et al., 2008). Functional Kv1.5 channels are composed of four identical $\alpha$-subunits that assemble to form a tetramer (Figure 3). In humans, these pore-forming $\alpha$-subunits are encoded by the intron-less *KCNA5* gene, which is located on chromosome 12. As shown in Figure 3, each $\alpha$-subunit contains six membrane-spanning domains (S1-S6) (Doyle et al., 1998) composed of 613 amino acids. Like many potassium channels, the N- and C-terminal ends of the $\alpha$-subunits are located intracellularly (Jiang et al., 2003; Long et al., 2005a; Tamargo et al., 2009).

The S1-S4 segment of Kv1.5 comprises the voltage-sensor. More precisely, S4 regulates voltage-dependent activation through the movement of positive amino acid residues (4 Arg residues) in response to changes in membrane potential (Ravens & Wettwer, 2011). Upon membrane depolarization, the positive S4 transmembrane domain is pushed outward due to electrostatic repulsion (Mannuzzu et al., 1996). This
Figure 2. Action potentials in human cardiac myocytes and the major contributing ionic currents. Currents comprising the action potential subtypes are positioned by time and voltage where they contribute. **Left**, ventricular myocyte action potential and key currents. Major outward (repolarizing) currents are $I_{Kr}$ and $I_{Ks}$. **Middle**, atrial action potential in sinus rhythm (SR, normal rhythm) and crucial currents involved. $I_{Kur}$ is important for early-phase repolarization. The currents shown in red are not found in the ventricles. **Right**, atrial action potential during atrial fibrillation (AF). The arrows in blue denote documented changes in current amplitudes in AF patients relative to control patients. The black arrows indicate possible directions of current change due to remodeling of atrial tissue in AF. The red arrows point to changes in resting membrane potential relative to ventricular myocytes. Image modified from: Ravens & Christ (Ravens & Christ, 2010).
Figure 3. Structure of the Kv1.5 ion channel. Scheme illustrating the structure of a single Kv1.5 α-subunit containing six α-helical transmembrane domains (S1-S6) as well as cytoplasmic NH$_2$ and COOH terminal domains (left). The S4 domain is the primary component of the voltage sensor (S1-S4). N-linked oligosaccharides attached to the external S1-S2 linker are also shown. The pore region (P), which selects and permeates K$^+$ ions, is formed by S5, S6, the pore helix/pore loop between S5 and S6, and the selectivity filter within the S5-S6 linker. Functional Kv1.5 channels are formed by assembly of four α-subunits into a tetramer (right). The tetramerization domain (T1) mediates the formation of whole Kv1.5 tetrameric channels. Various Kvβ-subunits can bind each α-subunit (generally at the N-terminus) to modulate channel function and expression. Image modified from: Burg et al. (Burg et al., 2010).
movement of S4 is in some way coupled to the opening of the activation gate within the intracellular S4-S5 linker, enabling the production of current (Ravens & Wettwer, 2011). Although controversial views exist in the literature, some maintain that a paddle motif within the internal S3-S4 linker pushes on the activation gate (S4-S5 linker) to keep it closed until the pressure is alleviated by elevation of the S4 voltage sensor (Long et al., 2005a).

The S5 and S6 transmembrane domains constitute the pore region, which selects and conducts K⁺ ions through the Kv1.5 channel. The S5-S6 linkers of Kv1.5 channel α-subunits curve back into the plasma membrane forming a hydrophobic pore lining (Jiang et al., 2003; Long et al., 2005a; Tamargo et al., 2009). The intracellular most aspect of the aqueous pore is arranged in an “inverted teepee” cavity that contains the K⁺ selectivity filter at the wide outer end (Doyle et al., 1998). The selectivity filter itself utilizes carbonyl groups within a TVGYG amino acid sequence to specifically bind and pass only K⁺ ions (Treptow & Tarek, 2006).

iii) **Iₖur Features, Gating, and Kinetics**

Kv1.5 current (I_{Kv1.5}) has been measured in the atrial myocytes of various species, including humans (Fedida et al., 1993), rats (Boyle & Nerbonne, 1992), and canines (Fedida et al., 2003). Kv1.5 channels were first successfully cloned from a human heart and incorporated into a human cell line more than two decades ago (Fedida et al., 1993). Heterologous systems expressing Kv1.5 have since shown that I_{Kv1.5} closely resembles Iₖur recorded in human atrial myocytes (Nerbonne & Kass, 2005). Indeed, it has been confirmed that Kv1.5 protein is responsible for Iₖur by selectively knocking out this K⁺ current with antisense oligonucleotides directed against
Kv1.5 mRNA (Feng et al., 1997). Due to the fact that Kv1.5 is a member of the voltage-gated K⁺ channel super-family, it is homologous to many other K⁺ channels. Thus, isolating $I_{Kur}$ from other atrial myocyte contaminating currents with similar ranges of voltage-dependence, such as the cardiac transient outward potassium current ($I_{to}$), can be challenging. However, voltage clamp protocols can be adjusted to take advantage of differences in gating kinetics to help discriminate between separate K⁺ currents. In addition, Kv1.5 pharmacological blockade with compounds such as 4-aminopyridine can be used with current subtraction methods to isolate $I_{Kv1.5}$ (Yamane et al., 1995).

Relative to the other delayed rectifier K⁺ currents, upon depolarization, human atrial $I_{Kur}$ activates rapidly with outward rectification (Fedida et al., 1993). Subsequently occurring inactivation of Kv1.5 channels during atrial myocyte action potentials has been shown to be slow and incomplete (Fedida et al., 1993). This inactivation proceeds through a C-type mechanism, whereby the 4 α-subunits collectively change their conformation to constrict the outer mouth of the channel pore (Figure 4A) (Fedida et al., 1999). The S6 transmembrane domain is thought to play an important role in mediating C-type inactivation in Shaker channels (Hoshi et al., 1991). C-type inactivation takes place predominantly when the channel is in the open state and is suspected to be voltage independent (Lopez-Barneo et al., 1993).

C-type inactivation can be decreased by augmenting the external K⁺ concentration, so that the excess K⁺ can bind the conducting pore and limit channel constriction via a foot-in-the-door mechanism (Lopez-Barneo et al., 1993). Recovery of Kv1.5 channels from the inactivated state is slow but can yield a small tail current (Feng et al., 1998b). Moreover, due to the absence of an intracellular amino terminal domain
with an N-type inactivation ball to plug the pore region, Kv1.5 channels do not exhibit N-type (ball and chain) inactivation that is exemplified in Figure 4B (Snyders et al., 1993; Tamkun et al., 1991).

In vivo, $I_{Kur}$ is encoded by four identical Kv1.5 $\alpha$-subunits and four accessory $\beta$-subunits that form $\alpha 4\beta 4$ complexes (Tamargo et al., 2009). The binding of ancillary Kv$\beta$-subunits to $K^+$ channel $\alpha$-subunits in the endoplasmic reticulum (Nagaya & Papazian, 1997) modulates channel trafficking, integration into the membrane, and gating kinetics (Ravens & Wettwer, 2011; Uebele et al., 1998). Crystal structures of Kv$\alpha$-Kv$\beta$ complexes indicate that the $\beta$-subunits assemble with Kv channels near T1 or the N-terminal domain (Gulbis et al., 2000; Sewing et al., 1996). The most prevalent species of Kv$\beta$-subunits in the heart are Kv$\beta$1.2, Kv$\beta$1.3, and Kv$\beta$2.1 (England et al., 1995). In the human atrium, Kv$\beta$1.2 has been shown to assemble with Kv1.5 $\alpha$-subunits, causing a hyperpolarizing shift in the voltage dependence of activation and inactivation, while also slowing the rate of $I_{Kur}$ deactivation (Martens et al., 1999). As shown in Figure 4C, the N-termini of Kv$\beta$1.2 and Kv$\beta$1.3 form an inactivation peptide that is capable of conferring incomplete N-type-like inactivation to Kv1.5 by plugging the inner cavity of the channel pore (Wang et al., 1996). Thus, variations in recorded $I_{Kur}$ in different heterologous cell lines and $I_{Kur}$ in native tissues can in large be attributed to expression or absence of specific Kv$\beta$-subunits.

iv) Synthesis, Glycosylation, Trafficking, and Degradation

In conformity with the biosynthetic pathway of many membrane proteins, DNA encoding Kv1.5 channels is first transcribed into mRNA in the nucleus prior to ribosomal
Figure 4. Schematic representation of Kv1 channel gating mechanisms. A. C-type inactivation, which is typical of Kv1.5 channels, occurs through conformational changes in each α-subunit that constrict the pore region. B. N-type (ball and chain) inactivation, whereby N-terminal “ball” structures from each α-subunit bind a homologous receptor in the pore cavity to trigger channel inactivation. C. Illustration of how N-type inactivation can be conferred to pore-forming channels, like Kv1.5, through interaction with Kvβ-subunits. The conserved C-terminal regions of Kvβ-subunits bind to the T1 domain in the N-terminus of each α-subunit. The more variable N-terminal inactivation “ball” peptides of the Kvβ-subunits can enter and plug the pore cavity through a space between T1 and the transmembrane domains (S1-S6). Voltage changes across the membrane (time in some cases) that mediate transitions between the closed, open, and inactivated states are depicted. Image modified from Ravens & Wettwer (Ravens & Wettwer, 2011).
translation at the endoplasmic reticulum (Dobrev et al., 2012). The incipient protein is post-translationally modified en route to the plasma membrane, including core and complex-glycosylation in the endoplasmic reticulum and Golgi apparatus, respectfully (Dobrev et al., 2012; Schumacher & Martens, 2010). The glycan structure is attached to an asparagine residue located on the external S1-S2 linker (Figure 3) and has been found to cause a hyperpolarizing shift in the voltage dependence of activation (leftward shift the $V_{1/2}$, or activation midpoint) (Schwetz et al., 2010). A study by Gong et al. concluded that glycosylation is not required for surface expression of functional hERG channels, but it does increase the stability of membrane localized channels (Gong et al., 2002). Preliminary data from our laboratory suggest that N-glycosylation is also involved in stabilizing cell-surface Kv1.5 as well as protecting susceptible sites in hERG channels from proteolytic cleavage.

The delicate balance between anterograde and retrograde protein trafficking dictates the plasma membrane expression of channels. These pathways involve many quality-control steps, chaperone molecules, and channel modifications, several of which still remain unknown. Forward trafficking of newly synthesized Kv1.5 channels to the cell-surface appears to rely on microtubule-mediated kinesin transport (Zadeh et al., 2009). Kv1.5 α-subunits associate with cytoskeleton-binding membrane-associated guanylate kinase (MAGUK) proteins, such as synapse associated protein 97 (SAP97), and α-actinin-2. The interaction with SAP97 facilitates membrane anchoring (Abi-Char et al., 2008; Eldstrom et al., 2003), while α-actinin-2 is suggested to play a role in early endosome formation (Maruoka et al., 2000). The function and longevity of membrane localized Kv1.5 channels is governed by an array of post-translational modifications.
Notably, many potential phosphorylation sites are present in Kv1.5 and Kvβ-subunits. In the atria, β-adrenergic receptor and PKA activation increases \( I_{kur} \), while PKC modulation via α-adrenergic receptor stimulation decreases \( I_{kur} \) amplitude (Li et al., 1996a).

Membrane-bound channels must eventually be endocytosed for degradation or recycling back to the cell surface. The mechanisms of Kv1.5 channel internalization and the subsequent fate of those channels remain to be fully elucidated. Specifically, whether or not Kv1.5 internalizes in a caveolin- or clathrin-dependent manner (or both) is unclear. Kv1.5 internalization may be clathrin-dependent since Kv1.5 has been localized to Rab5 GTPase (a monomeric peripheral membrane G protein) positive endosomes (Zadeh et al., 2008), which is well known to be crucial for clathrin-mediated endocytosis (Bucci et al., 1992). On the other hand, Kv1.5 has been shown to associate with caveolin in cell-surface lipid rafts (Martens et al., 2001), which is known to facilitate Rab5-independent internalization (Neel et al., 2005). However, it has been demonstrated that the dynein motor plays a role in Kv1.5 internalization to early endosomes (Choi et al., 2005). Furthermore, Rab GTPases have been found to regulate the recycling and trafficking of internalized channels, including Kv1.5. For example, Rab4 and Rab11 have been implicated in the recycling of internalized Kv1.5 to the plasma membrane (McEwen et al., 2007). Another sub-population of internalized Kv1.5 channels are sorted into Rab7 containing late endosomes for lysosomal or proteasomal degradation (Zadeh et al., 2008).

Potassium channel endocytosis can also be initiated by the covalent attachment of ubiquitin, an 8 kDa protein that tags substrates for proteasomal and lysosomal degradation (Pickart, 2000). Proteins can be ubiquitinated via a joint effort of three
enzymes: an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme (ubiquitin carrier), and an E3 ubiquitin ligase (Lecker et al., 2006). Neural precursor cell expressed developmentally down-regulated protein 4 sub-type 2 (Nedd4-2) is an E3 ubiquitin ligase that is thought to ubiquitinate Kv1.5 channels, signaling their degradation (generally via the 26S proteasome) (Mia et al., 2012). However, data from our lab indicates that Nedd4-2 overexpression selectively decreases hERG current (I_{hERG}) but not I_{Kv1.5}, suggesting that Kv1.5 is not a substrate of Nedd4-2 (Guo et al., 2012). Indeed, Kv1.5 lacks the PPXY (PY) motif that is typically bound by Nedd4-2 WW domains (Staub et al., 2000). Overall, our understanding of the Kv1.5 modulatory processes as well as the complete anterograde and retrograde trafficking pathways is still relatively limited.

v) **Kv1.5 and Atrial Fibrillation**

Certain mutations in ion channel genes have been directly associated with AF. In relation to Kv1.5, channelopathy due to a KCNA5 loss-of-function mutation results in AF type 7 (Olson et al., 2006; Yang et al., 2009). These mutants fail to generate I_{Kur}, which is vital for atrial repolarization. A lack of functional Kv1.5 protein and hence deficient repolarization causes action potential prolongation and early after-depolarizations, a plausible substrate for AF development. Conversely, gain-of-function mutants in genes that encode repolarizing currents, such as I_{Ks} and I_{K1}, can underlie AF pathology by shortening the action potential duration, resulting in more frequent firing (Chen et al., 2003; Xia et al., 2005; Yang et al., 2004). This evidence supports the rationale that familial AF can be addressed by agents that prolong the effective refractory period. However, as described previously, ion channel targeting pharmacology for AF treatment
poses serious risks of ventricular side effects. Moreover, the complexity of AF and the many possible etiologies highlight the value of personalized medicine with uniquely tailored strategies.

The finding that $I_{Kur}$ exists solely in the atria offers the possibility that selective Kv1.5 channel blockers could be developed to correct abnormal heart rhythms without detriment to the ventricles (Wang et al., 1993). Kv1.5 blocking agents are expected to prolong the action potential duration and refractory period to decrease the likelihood of non-rhythmic firing. The majority of compounds that inhibit $I_{Kur}$ bind to the pore cavity while Kv1.5 is in the open state (Decher et al., 2004). Several $I_{Kv1.5}$ blockers have been developed, but their efficacy, channel specificity, and safety profiles have yet to be completely perfected or analyzed (Ford & Milnes, 2008; Tamargo et al., 2009). However, the usefulness of pure $I_{Kur}$ block to revert AF to SR has yet to be demonstrated (Ehrlich & Nattel, 2009), given that most of the developed compounds also impact other channels as well (Ravens, 2010).

It is paradoxical that loss-of-function mutations in the Kv1.5 encoding gene can cause AF by increasing the action potential duration, providing a substrate for early after depolarizations, yet blockade of this channel may be useful to treat AF. Importantly, it has been demonstrated that AF is often associated with a shortening of the atrial action potential (Wettwer et al., 2004), hence blockade of $I_{Kv1.5}$ would correct this anomaly. However, there is some conflict about the potential clinical effectiveness of $I_{Kur}$ targeting drugs. Recently accumulated data concerning AF-mediated remodeling of $I_{Kur}$ is controversial, with evidence for decreased $I_{Kur}$ (Brandt et al., 2000; Van Wagoner et al., 1997) or no significant change (Bosch et al., 1999; Grammer et al., 2000; Workman et
Furthermore, $I_{Kur}$ amplitude is reduced during periods of increased activation (Feng et al., 1998a), such as AF, which may decrease $I_{Kur}$ blocking effectiveness. On the more optimistic end, $I_{Kur}$ block slightly prolongs action potential duration and the effective refractory period in AF-remodeled atrial action potentials (Christ et al., 2008; Wettwer et al., 2004). This implies the possibility that $I_{Kur}$ contributes more to repolarization in AF than in healthy patients.

There is still a need for the development of safe and atrial-selective compounds that can effectively treat AF. Newer therapeutic strategies that focus on the regulation of surface channel density are beginning to gain momentum and they may have advantages over the existing conduction-modulating drugs (Schumacher et al., 2009).

vi) **Kv1.5 and Proteases**

The effects of proteolytic cleavage on the function of Kv1.5 channels are not well understood. In fact, this may represent an avenue of Kv1.5 channel research that has greatly been overlooked, especially since distinct endogenous trypsin-like proteases have been reported to cleave epithelial sodium channels to either enhance or decrease sodium current (Donaldson et al., 2002). Cell-surface Kv1.5 channels have been shown to be sensitive to digestion by extracellularly applied proteinase K (PK) (Choi et al., 2005). PK is a wide spectrum serine protease that is secreted from the fungus *Tritirachium album* Limber and remains stable over a wide range of environmental conditions (Ebeling et al., 1974). Since PK is a membrane impermeable endopeptidase, it selectively cleaves exposed substrate ectodomains when applied externally (Zhou et al., 1998). Its primary applications include the digestion of protein contaminants and nucleases from nucleic acid preparations (Gross-Bellard et al., 1973; Kasche et al.,
1981), and as a tool to assay the proportion of proteins/ion channels that are membrane localized (Choi et al., 2005; Eldstrom et al., 2010; Manganas & Trimmer, 2000; Rajamani et al., 2006).

**Hypothesis and Study Objectives**

As stated in the above introductory material, the Kv1.5 channel and its associated current ($I_{Kv1.5}/I_{Kur}$) are crucial for regulating atrial action potentials and hence normal cardiac rhythm. Since cardiac ischemia precipitates both AF (Sinno et al., 2003) and protease upregulation (Muller et al., 2013), we sought to examine the relationship between proteases and Kv1.5, which is a channel of unique interest in AF-related research. Furthermore, secreted serine proteases have been implicated in regulating the epithelial sodium channel (Donaldson et al., 2002). While previous work has reported that Kv1.5 channels are susceptible to PK treatment (Choi et al., 2005), the subsequent effects of proteolysis on Kv1.5 function have not been investigated. Until now, enzymatic cleavage of ion channels and confirmation of such cleavage by Western blot analysis have been presumed to correspond to an elimination of channel activities. In light of preliminary observations, I aim to explore the effects of proteolytic digestion on Kv1.5 expression and function through testing the following hypotheses:

1. Mature (75 kDa) Kv1.5 channels expressed in a stable human embryonic kidney (HEK) 293 cell line are sensitive to cleavage by extracellular serine proteases, such as PK.
2. PK-generated Kv1.5 C-terminal fragments (pore-containing) are able to conduct $I_{Kv1.5}$ despite a lack of domains that might be required for channel
stability and current production (eg. S1). In addition, this phenomenon is unique to the Kv1.5 channel.

3. Kv1.5 protein fragments, produced by digesting membrane-bound Kv1.5 channels, are physically independent from one another and are stable on the plasma membrane.

To evaluate these hypotheses the following objectives were completed:

1. Assess the effects of PK treatment on Kv1.5 channel expression and function in a Kv1.5-HEK stable cell line. Compare the effects of PK on Kv1.5 with another PK-sensitive cardiac K⁺ channel, hERG.

2. Identify the position(s) of proteolytic cleavage within the Kv1.5 channel sequence.

3. Eliminate the possibility that immature (68-kDa) Kv1.5 proteins are involved in rescuing $I_{Kv1.5}$ after PK treatment.

4. Determine if the PK-produced Kv1.5 fragments are membrane localized and fully-separated from adjacent channel fragments.
Chapter 2: Materials and Methods

Molecular Biology and Cell Culture

Human Kv1.5 cDNA was provided by Dr. Michael Tamkun (Colorado State University, Fort Collins, CO). hERG cDNA was provided by Dr. Gail A. Robertson (University of Wisconsin-Madison, Madison, WI). HEK 293 cell lines stably expressing Kv1.5 (Kv1.5-HEK) or hERG (hERG-HEK) were established using G418 (Geneticin selective antibiotic) for selection (1 mg/ml) and maintenance (0.4 mg/ml). Kv1.5-HEK, hERG-HEK, and wild type (WT) HEK 293 cells were grown in polystyrene disposable Petri dishes containing 5 mM K⁺ minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, and 1% minimal essential amino acids at 37°C and 5% CO₂. Upon reaching 80% confluency (in 35, 60, or 100 mm culture dishes) the cells were used for experiments. Cells were passaged every 2-3 days to maintain adequate growth conditions and to prevent cellular senescence. For passaging, cells were washed three times with pre-warmed phosphate-buffered saline (PBS) prior to the addition of 0.25% trypsin for one minute, which breaks down the adhesive bonds that join cells as well as between the culture plate and the cells. Trypsin was then removed and the singularized cells were re-suspended in supplemented MEM.

Transfection

All experiments were conducted with a HEK 293 cell line stably expressing Kv1.5 or hERG channels, except for the single voltage-clamp experiment with PK-cleaved HEK cells transiently expressing Kv1.5 (data not shown). HEK 293 cells were passaged and seeded into individual 35 mm Petri dishes 24 h prior to transfection. Lipofectamine
2000 reagent was employed to transfect 2 µg of Kv1.5 DNA plasmids into the cells. Transfection was conducted in the presence of Opti-MEM (reduced serum media) over a course of 4-6 h before re-incubating the cells in standard serum-supplemented MEM for approximately 24 h. Empty pcDNA3 vector was used as the control during transfections. Cells were co-transfected with GFP plasmid (at a ratio of 1:4 relative to Kv1.5 plasmid) to ensure selection and study of only successfully transfected cells.

**Cleavage of Cell-surface Proteins**

Live HEK cells stably expressing Kv1.5 or hERG channels were washed with PBS and treated with PK (200 µg/ml) in a buffer solution (10 mM HEPES, 150 mM NaCl, and 2 mM CaCl₂, pH 7.4) at 37°C for 20 min. PBS containing 6 mM phenylmethylsulfonyl fluoride (PMSF) and 25 mM EDTA was used to quench the reaction. Biochemical and electrophysiological experiments were performed either immediately after PK digestion (0 h post-cleavage) or after re-culture of PK-treated cells in fresh supplemented MEM for various periods (2, 4, or 6 h).

**Western Blot Analysis**

Cells were washed twice with ice-cold 1x PBS, dislodged from the culture dishes with sterile rubber scrapers, collected into eppendorf tubes with PBS, and centrifuged at 1,000 RPM for 4 min at 4°C. The ensuing cell pellets were lysed using high-frequency sonication in ice-cold Nonidet P40 lysis buffer supplemented with 1% PMSF and 1% of a protease inhibitor cocktail. Lysates were centrifuged at 10,000 RPM for 10 min and the supernatants containing whole-cell proteins were extracted from the remaining insoluble debris fraction. The protein concentrations were determined using a Detergent Compatible Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) that is based on the Lowry assay.
The optical absorbance of each lysate is compared to a set of bovine serum albumin (BSA) standards to determine protein concentration.

15 µg/50 µL samples were made by diluting whole-cell protein with double distilled water, and 5x Laemmli sample loading buffer containing 5% β-mercaptoethanol was added to each sample. Following a 5 min boiling period in a water bath, the proteins were separated by size via 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The BLUeye Prestained Protein Ladder was used to help distinguish protein molecular weights (FroggaBio, Toronto, ON). Subsequently, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA). The membranes were then blocked with 5% non-fat skim milk in 1x Tris-buffered saline and 0.1% Tween 20 (TBST) for 1 hour to prevent non-specific binding of antibodies to the membrane. Proteins of interest were probed for 1 h with the appropriate primary antibodies that were suspended in 5% non-fat milk and 1x TBST in 1:1000 dilutions. Corresponding (matched by species) horseradish peroxidase-conjugated (HRP) secondary antibodies in 1x TBST at a dilution of 1:20,000 were employed to label the target proteins for 1 h. Following the primary and secondary antibody incubation steps, membranes were washed 3 times for 10 min with 1x TBST to elute any remaining antibody. A rocking platform shaker was used for all membrane blotting and washing. Lastly, an enhanced chemiluminescence detection kit (GE Healthcare, Little Chalfont, United Kingdom) was used for blot visualization. The chemiluminescent blots were exposed to medical x-ray films (Fujifilm, Tokyo, Japan) for varying amounts of time to obtain optimal band densities on the films. β-actin was detected as a protein loading control in blots that were to be quantified by densitometry.
**Co-immunoprecipitation (co-IP)**

Kv1.5-HEK cells were treated with PK (200 µg/ml) for 20 min. The live intact cells were re-cultured for 2 h in supplemented MEM. For each co-IP sample, 0.5 mg whole-cell protein in 0.5 mL lysis buffer was incubated with N-terminal specific anti-Kv1.5 primary antibody at 4°C overnight. As a negative control, GAPDH was precipitated with rabbit anti-GAPDH primary antibody. Protein A/G Plus-agarose beads were added to the protein complexes at 4°C for 4 h prior to precipitation by centrifugation at 10,000 x g for 1 min. An end over end rotating mixer was used during antibody and agarose bead incubation periods. The immunoprecipitates were washed four times with ice-cold Nonidet P40 lysis buffer to remove unbound protein. 2x Laemmli sample loading buffer was added to resuspend the pelleted immunoprecipitates. The samples were boiled for 5 min in a water bath and centrifuged at 18,000 x g for 5 min. The supernatants were subjected to 8% SDS-PAGE and Western blotting. C-terminal recognizing anti-Kv1.5 antibody was utilized to immunoblot PVDF membranes to test for interactions between immunoprecipitated N-terminal Kv1.5 fragments and C-terminal Kv1.5 fragments following proteolytic cleavage.

**Electrophysiological Recordings**

All currents were recorded in the whole-cell voltage-clamp configuration using the Axon Axopatch 200B amplifier, the Axon Digidata 1440A digitizer, and the pClamp 10.3 acquisition software. Borosilicate glass pipettes (World Precision Instruments, Sarasota, FL) were fire polished with a micropipette puller (Sutter Instruments, Novato, CA) to possess resistances of 2-4 MΩ. Micropipette resistance and capacitance were compensated for prior to rupturing the cell membrane. Membrane resistance and capacitance were compensated for upon establishing a gigaseal with the cell. The pipette
solution contained (in mM) 135 KCl, 5 EGTA, 1 MgCl₂, and 10 HEPES (pH 7.2 with KOH). The bath solution contained (in mM) 135 NaCl, 5 KCl, 10 HEPES, 10 glucose, 1 MgCl₂, and 2 CaCl₂ (pH 7.4 with NaOH). Leak subtraction was not used when recording currents, but was employed digitally afterwards if necessary. All patch-clamp experiments were performed at room temperature (22 ± 1°C).

\( I_{Kv1.5} \) was evoked in the Kv1.5-HEK stable cell line or in transiently transfected WT HEK 293 cells from the holding potential of -80 mV by depolarizing steps from -70 to +50 mV in 10 mV increments for 200 ms. A repolarizing step to -50 mV was applied before returning the membrane voltage to the holding potential of -80 mV. Current values at the end of the 50 mV depolarizing step were used to analyze \( I_{Kv1.5} \) amplitudes. For recording \( I_{Kv1.5} \) from WT HEK cells transiently expressing Kv1.5 (single experiment, data not shown), only GFP positive cells were selected (GFP and Kv1.5 plasmid co-transfection). The entirety of the voltage-clamp data shown was obtained using stable HEK cell lines. For Kv1.5 current recordings, data were sampled at 20 kHz and low pass filtering was conducted at 5 kHz. For recording \( I_{hERG} \), currents were elicited by depolarizing steps from -70 to +70 mV in 10 mV increments for 4 s from the command voltage of -80 mV. hERG tail currents upon a 5 s repolarizing step to -50 mV after a depolarization to +50 mV were used to analyze \( I_{hERG} \) amplitudes. For hERG current recordings, data were sampled at 1 kHz and were filtered at 5 kHz using a low pass filter.

**Isolation of Cell-Surface Proteins**

A Cell Surface Protein Isolation Kit (Pierce, Waltham, MA) was used as per the manufacturer’s instructions. Kv1.5-HEK cells were grown in 100 mm culture dishes to 90% confluence. Cells were used as a control group or were treated with PK (200 µg/ml) for 20
min and re-cultured for 2 h in supplemented MEM. Cell-surface proteins with extracellulary exposed primary amines were labeled with Sulfo-NHS-SS-biotin (250 µg/ml), a membrane impermeant thiol-cleavable amine-reactive biotinylation reagent, for 30 min at 4°C. The reaction was terminated via addition of the Quenching Solution. Biotin-labeled cells were then collected and lysed with lysis buffer containing 1% protease inhibitor cocktail. Following a 10,000 x g centrifugation step for 2 min at 4°C, biotin-tagged proteins were isolated with Immobilized NeutrAvidin Gel. After washing away the cytosolic proteins (non-biotinylated proteins), cell-surface proteins were eluted by incubating the gel in a tris buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol) containing 50 mM DTT. The purified cell-surface proteins were analyzed via Western blotting. To ensure equal protein loading, Na⁺/K⁺ ATPase expression was detected using a mouse anti-Na⁺/K⁺-ATPase α1 antibody and the corresponding goat anti-mouse HRP-conjugated secondary antibody.

**Immunofluorescence Microscopy**

Kv1.5-HEK cells were treated with cycloheximide (10 µg/ml) for 24 h to eliminate 68-kDa Kv1.5 channels via inhibition of protein biosynthesis. The cells were treated with PK (200 µg/ml) at 37°C for 20 min to cleave cell-surface channels. The PK-treated cells were then seeded onto glass coverslips and cultured in the presence of cycloheximide (10 µg/ml) for 2 h. Live cell membranes were stained using red-fluorescent Texas Red Wheat Germ Agglutinin (5 µg/ml) for 1 min in Hank’s Balanced Salt Solution. The cells were fixed with 4% ice-cold paraformaldehyde in 1x PBS for 15 min and washed three times with PBS. Following fixation, the cells were permeabilized with 0.1% Triton X-100 for 10 min at room temperature. The fixed and permeabilized cells were washed three times with 1x PBS before incubation with 5% BSA in PBS for 1 h to block non-specific antibody-protein
interactions. Kv1.5 channels were labeled with N- or C-terminal recognizing rabbit anti-Kv1.5 primary antibodies (1:100). Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody was then used to stain Kv1.5 channels (1:200). The coverslips were mounted onto glass slides using Prolong Gold Antifade, which also protects from photobleaching effects. Images were acquired using a Leica TCS SP2 Multiphoton confocal microscope (Leica, Heidelberg, Germany).

Reagents and Antibodies

MEM, FBS, trypsin, sodium pyruvate, minimal essential amino acids, Lipofectamine 2000, Opti-MEM, red-fluorescent Texas Red Wheat Germ Agglutinin, Hank’s Balanced Salt Solution, and Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody were obtained from Invitrogen, Waltham, MA. G418, PMSF, protease inhibitor cocktail, β-mercaptoethanol, proteinase K, Triton X-100, BSA, Prolong Gold Antifade mountant, tunicamycin, cycloheximide, monoclonal mouse anti-actin (AC-10) antibody, and all chemicals/electrolytes used in the patch clamp experiments were purchased from Sigma-Aldrich, St. Louis, MO. Nonidet P40, rabbit anti-Kv1.5 (H-120, N-terminal specific), goat anti-hERG (N-20, N-terminal specific), rabbit anti-GAPDH, mouse anti-Na+/K+-ATPase α1, goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP, mouse anti-goat IgG-HRP, and Protein A/G Plus-agarose for immunoprecipitation assays were purchased from Santa Cruz Biotechnology, Dallas, TX. Rabbit anti-Kv1.5 (C-terminal specific) polyclonal primary antibodies were acquired from Alomone labs, Jerusalem, Israel. Paraformaldehyde was obtained from Alfa Aesar, Ward Hill, MA.
Densitometry and Data Analysis

Developed Western blot x-ray films were digitized with the Epson Perfection V700 Photo scanner using the professional mode. Adobe Photoshop CS3 Extended and Origin 6.0 (OriginLab) were used to quantify protein band intensity on the x-ray films. The film images were changed to grayscale and the colour was inverted. The bands of interest on the films were quantified by selecting the entire region of a protein band and measuring the mean intensity value. Measured protein band intensities were subtracted by the immediate background intensity and normalized to the corresponding β-actin band intensity. To obtain relative band intensities, the protein bands were divided by their controls (eg. PK 0h). Data was inputted into Origin 6.0 for statistical calculations and graphical summarization.

The Clampfit 10.3 software was used to analyze whole-cell voltage clamp data. All data are expressed as the mean ± the standard error of the mean (S.E.M) of at least three individual experiments (n ≥ 3). For determining statistical significance between control and test groups, a two-tailed Student’s t- test was used. A p-value of 0.05 or less was deemed statistically significant.
Chapter 3: Results

The Kv1.5 Channel is Uniquely Affected by Extracellularly Applied PK

In order to examine the impact of serine proteases on Kv1.5 channels, we bathed Kv1.5-HEK cells in a buffer containing active PK (200 μg/ml) for 20 min at 37°C. Western blot analysis was conducted to study the effects of PK on the Kv1.5 expression profile. On a standard Western blot, Kv1.5 channels are represented by two bands that correspond to proteins with molecular masses of 68 kDa and 75 kDa (Ding et al., 2014; Ficker et al., 2003). The 68-kDa band signifies the core-glycosylated immature Kv1.5 protein that is localized in the endoplasmic reticulum (Ficker et al., 2003). The 75-kDa band corresponds to the mature form that has trafficked through the Golgi apparatus to become fully-glycosylated prior to insertion into the plasma membrane for K⁺ ion conduction (Ficker et al., 2003). As depicted in Figure 5A, extracellular addition of PK to live cells selectively cleaved the 75-kDa Kv1.5 channels. Probing the PK-cleaved cell lysates with N-terminal recognizing anti-Kv1.5 antibodies revealed that digestion of mature channels yields fragments of approximately 42 kDa (Figure 5A). Whole-cell voltage clamp recording was utilized to test Kv1.5 function in response to the same PK treatment protocol. Removal of the functional 75-kDa Kv1.5 channels is typically expected to produce an abolishment of $I_{Kv1.5}$. Surprisingly, PK treatment had no effect on the amplitude of recorded Kv1.5 currents (Figure 5B).

To further study the consequences of proteolysis on vulnerable potassium channels, we examined the outcome of PK treatment on hERG channels that were also stably expressed in HEK 293 cells. Consistent with Kv1.5 channels, hERG proteins on a Western blot display two distinct bands. The lower band possesses a molecular weight of
Figure 5. The effects of PK treatment on Kv1.5 channel expression and function when stably expressed in HEK 293 cells. A. Representative Western blot demonstrating the consequences of PK treatment (200 µg/ml) on Kv1.5 protein expression (left). N-terminal specific anti-Kv1.5 antibodies were used. The relative band intensities (Intensity-Rel) of the 75 kDa mature channels from PK-treated cells were compared to the upper bands from control (Ctrl) cells and summarized (right) (n=11). B. Representative current traces depicting the impact of PK cleavage on $I_{Kv1.5}$ (left). The voltage protocol used is displayed above the traces. Summarized current amplitudes in control (Ctrl) and PK-treated cells are shown on the right. The numbers in parentheses denote the number of cells recorded from at least 3 separate experiments. **$P$<0.01 vs. Ctrl.
135 kDa and corresponds to the core-glycosylated immature channels that are retained inside the cell (Gong et al., 2002; Guo et al., 2009). The 155-kDa upper band represents the fully-glycosylated mature channels situated within the plasma membrane (Gong et al., 2002; Guo et al., 2009). Similar to Kv1.5, PK cleavage of hERG was positive but limited to the mature (155-kDa) form (Figure 6A). PK-induced elimination of the 155-kDa band was accompanied by the appearance of a 70-kDa fragment, when blotting with N-terminal specific anti-hERG antibodies (Figure 6A). In contrast to Kv1.5, PK treatment of hERG-HEK cells completely abolished I_{hERG} (Figure 6B). The ability of PK to eliminate I_{hERG} has been reported (Rajamani et al., 2006), while the lack of effect on I_{Kv1.5} has not.

PK-mediated Proteolysis of Kv1.5 Occurs in the S1-S2 Linker

To investigate the peculiar nature of I_{Kv1.5} maintenance despite the effective cleavage of mature channel proteins by PK, we first aimed to determine the proteolytic site(s) within Kv1.5 that might be targeted by PK. Since extracellular proteases like PK cannot permeate lipid bilayers, they may only cleave substrates with suitable sequences that are exposed to the extracellular milieu. Transmembrane segments buried in the plasma membrane and cytosolic domains are shielded from external digestive enzymes. Thus, PK can theoretically digest Kv1.5 by targeting the S1-S2 linker, the S3-S4 linker, or the S5-S6 linker (Figure 3).

To identify the external linker(s) (S1-S2, S3-S4, and/or S5-S6) that is degraded by PK, we compared the sizes of the degradation products using two sets of antibodies that target epitopes on opposing intracellular ends of Kv1.5 proteins. Identical to the Western blot in Figure 5A, with an N-terminal anti-Kv1.5 antibody (raised against amino acids within
Figure 6. The impact of PK treatment on hERG channel expression and function in a stable HEK 293 cell line. **A**. Representative Western blot depicting the effect of PK treatment (200 µg/ml) on hERG protein expression (left). N-terminal recognizing anti-hERG antibodies were used. The relative band intensities (Intensity-Rel) of the 155 kDa mature channels from PK-treated cells were compared to those from control (Ctrl) cells and summarized (right) (n=8). **B**. Representative $I_{\text{hERG}}$ traces (left) as well as the summarized current amplitudes (right) are shown in both control (Ctrl) and PK-treated cells. The voltage protocol employed is displayed above the traces. The numbers in parentheses denote the number of cells recorded from at least 3 separate experiments. **P<0.01 vs. Ctrl.**
position 1-120), a 42-kDa fragment was produced in response to PK-mediated cleavage of the mature 75-kDa protein (Figure 7A). Upon probing the same cell lysates with anti-Kv1.5 antibodies (raised against amino acids within position 513-602) that have affinity for the C-terminus of Kv1.5, disappearance of the 75-kDa channels was accompanied by detection of 33-kDa fragments (Figure 7B). By combining our observed Kv1.5 degradation products (42 and 33 kDa) with molecular mass prediction information obtained using ExPASy-ProtParam tool analysis, we were able to conclude that PK most likely cleaves Kv1.5 at a single locus within the extracellular S1-S2 linker.

The sole site for glycosylation attachment in the Kv1.5 channel has been shown to be N290 located within the external S1-S2 linker (Schwetz et al., 2010). In our Kv1.5 construct (obtained from Dr. Michael Tamkun), which differs from that used by Schwetz et al. by only a few nucleotides, this N-glycosylation position corresponds to N297. Since the oligosaccharide structure is in the same linker that is targeted by PK, it can be used as a means of further refining the PK digestion site.

To determine which PK-generated product contains N297, we compared the sizes of the N- and C-terminal fragments in Kv1.5-HEK cells with or without N-linked glycans. Tunicamycin is a mixture of homologous antibiotics that has been shown to inhibit enzymes required for N-glycosylation (Gong et al., 2002; Mahoney & Duksin, 1979). Incubating Kv1.5-HEK cells in the presence of tunicamycin (10 µg/ml) for 48 h eradicated the 75-kDa Kv1.5 band, indicating that N-glycosylation was entirely blocked (Figure 7). The single prominent Kv1.5 band (65 kDa) in tunicamycin-treated cells represents both Kv1.5 forms, the immature intracellular protein and the mature cell-surface channels. Kv1.5 channels without N-glycans can still travel to the plasma membrane since
tunicamycin-treated cells still display robust \( I_{Kv1.5} \) (data not shown). Via Western blotting with polar opposite anti-Kv1.5 antibodies, PK digestion of tunicamycin-treated cells led to the appearance of 32 kDa N-terminal fragments and 33 kDa C-terminal products (Figure 7). Hence, removal of Kv1.5 N-glycosylation prior to PK digestion selectively decreased the N-terminal fragment and left the C-terminal fragment unchanged. This stipulates, as illustrated in Figure 8 for clarity, that the PK target site in Kv1.5 is situated towards the carboxyl-end of the S1-S2 linker relative to the N-linked oligosaccharide anchoring position (N297).

**68-kDa Immature Kv1.5 Proteins are not Responsible for the Resistance of \( I_{Kv1.5} \) to PK**

As mentioned, cleavage assessment by Western blot analysis shows that PK fragments mature 75-kDa Kv1.5 channels into two pieces (Figure 7). Intriguingly, such severance does not affect \( I_{Kv1.5} \) (Figure 5B). A possible explanation for this, although contrary to the K\(^+\) channel maturation dogma, is that the 68-kDa core-glycosylated channels may produce \( I_{Kv1.5} \) after PK treatment. To confirm that immature channels have no role in conducting \( I_{Kv1.5} \), we cultured Kv1.5-HEK cells with the translation inhibiting antibiotic cycloheximide (10 \( \mu \)g/ml). As demonstrated in Figure 9A, cycloheximide caused a time-dependent reduction in the expression of 68-kDa Kv1.5 protein. After incubating Kv1.5-HEK cells in MEM containing cycloheximide for 24 h, the 68-kDa fraction was abolished, while the 75-kDa channels were still abundantly expressed (Figure 9B). Cleavage of cycloheximide-treated cells with PK gave rise to the familiar two Kv1.5 fragments: 42-kDa N-terminal fragments and 33-kDa C-terminal fragments (Figure 9B). In this case, as evidenced in Figure 9B, the fragments were the sole detectable Kv1.5 species. Moreover, in cycloheximide pre-treated (24 h, 10 \( \mu \)g/ml) cells that were treated
Figure 7. PK cleaves Kv1.5 in the extracellular S1-S2 linker between the glycan attachment site and S2. A and B. Representative Western blots portraying Kv1.5 channel expression in Kv1.5-HEK cells pre-cultured with or without tunicamycin (10 µg/ml, 48 h) and treated with PK (200 µg/ml). Nascent and PK-cleaved Kv1.5 proteins were detected with N-terminal (A) and C-terminal (B) specific anti-Kv1.5 antibodies.
Figure 8. The putative PK cleavage of Kv1.5 channels. Scheme illustrating that PK targets and cleaves mature Kv1.5 at the extracellular S1-S2 linker to produce a 42 kDa N-terminal fragment and a 33 kDa C-terminal fragment. The use of distinct antibodies to detect the fragments via Western blotting is conveyed. The N-linked oligosaccharide attachment position in the S1-S2 linker relative to the PK severance site and the pore region is also shown.
with PK or left undigested, $I_{Kv1.5}$ was still prominent (Figure 9C). Due to the absence of core-glycosylated immature Kv1.5 in cycloheximide pre-treated cells, $I_{Kv1.5}$ after subsequent PK cleavage must have been supplied by the pore-containing C-terminal fragment or through some type of collaborative effort between the two fragments.

Proteolytically-induced Kv1.5 Fragments Retain Plasma Membrane Localization

Functional ion channels that transport electrolytes into or out of cells, thereby producing current, must be integrated perpendicularly across the plasma membrane. By this logic, Kv1.5 channels and presumably active fragments of Kv1.5 must also be situated in the membrane. To study the plasma membrane expression of fragmented Kv1.5 channels, we utilized biotin to label cell-surface proteins so they could be extracted from whole-cell lysates. HEK 293 cells stably expressing Kv1.5 were treated with PK and re-cultured for 2 h in standard 5 mM K$^+$ supplemented MEM to allow potential internalization of fragmented Kv1.5 channels. A 2 h re-culture period was used in light of the documented 4 h half-life for Kv1.5 turnover (Takimoto et al., 1993). Total and plasma membrane Kv1.5 protein expression was then analyzed by Western blotting with N-terminal and C-terminal anti-Kv1.5 antibodies. As shown in the representative images from Figure 10, PK digestion entirely eliminated the expression of 75-kDa Kv1.5 channels, while producing N-terminal (42-kDa) (Figure 10A) and C-terminal (33-kDa, pore-containing) (Figure 10B) fragments. Consistent with our hypothesis that the cleaved channels somehow generate $I_{Kv1.5}$, both fragments were still strongly expressed on the plasma membrane 2 h after PK treatment (Figure 10).

To reinforce our claim that the Kv1.5 fragments are stable enough in the plasma membrane to conduct current after the PK cut, we investigated the localization of the
Figure 9. 68-kDa immature Kv1.5 channels are not responsible for mediating $I_{Kv1.5}$ following PK treatment. 

A. Representative Western blot depicting Kv1.5 protein expression after blockade of protein biosynthesis with cycloheximide (CHX, 10 µg/ml) for 3, 6, and 9 h. 

B. Representative Western blots illustrating Kv1.5 expression using N-terminal specific (left) and C-terminal specific (right) anti-Kv1.5 antibodies following incubation of Kv1.5-HEK cells with cycloheximide (CHX, 10 µg/ml) for 24 h prior to PK-mediated cleavage. 

C. $I_{Kv1.5}$ traces obtained from cells pre-cultured in the presence of cycloheximide (CHX, 10 µg/ml) for 24 h with or without (control, Ctrl) subsequent PK treatment (upper). Summarized current amplitudes are shown in the bar graph below. The numbers in parentheses denote the number of cells tested from 3 independent experiments.
Figure 10. The PK-generated N- and C-terminal Kv1.5 fragments retain stable plasma membrane expression. A and B. Biotinylation-based isolation of cell-surface proteins from PK-treated (200 µg/ml) Kv1.5-HEK cells that were re-cultured for 2 h following cleavage. N-terminal Kv1.5 fragments (A) and C-terminal Kv1.5 fragments (B) are stably expressed on the plasma membrane. In control cells (Ctrl), 75-kDa Kv1.5 channels (not 68-kDa channels) are present on the cell-surface as expected.
Kv1.5 hemi-proteins using immunofluorescence microscopy. First, Kv1.5-HEK cells were treated with cycloheximide (10 µg/ml) for 24 h to selectively expunge internal immature Kv1.5 protein, as performed in the experiments for Figure 9B and C. The cells were then treated with PK to cleave the 75-kDa Kv1.5 channels prior to a 2 h re-culture period in MEM containing cycloheximide. Live intact cell membranes were stained before cell fixation, permeabilization, labeling with N- or C-terminal binding anti-Kv1.5 antibodies, and mounting. The results in Figure 11 indicate that the N- and C-terminal Kv1.5 fragments appear to retain stable membrane expression after PK-proteolysis. These data support our belief that PK treatment gives rise to fragments that competently preserve $I_{Kv1.5}$.

The N- and C-terminal Kv1.5 Fragments are Independent and do not Interact

We tested for an association between the PK-induced 42-kDa (N-fragment) and 33-kDa (C-fragment) Kv1.5 forms using co-IP to assess the physical nature of the fragment-mediated $I_{Kv1.5}$. Kv1.5-HEK cells were PK-cut and subsequently re-cultured in standard supplemented MEM for 2 h. To reiterate, the re-culture period is to allow for potential internalization of unstable Kv1.5 fragments. Following cell collection, the whole-cell lysates were immunoprecipitated with anti-Kv1.5 N-terminal antibodies. Isolated Kv1.5 protein was analyzed by Western blotting with either C-terminal or N-terminal recognizing Kv1.5 antibodies. As portrayed in Figure 12, proteins immunoprecipitated with an N-terminal specific Kv1.5 antibody could be employed to detect the 42-kDa N-terminal fragments with the same Kv1.5 antibody. This was done as a control to illustrate successful PK cleavage and immunoprecipitation. Conversely, the presence of 33-kDa C-terminal Kv1.5 fragments could not be detected in the precipitates obtained.
Figure 11. Confocal immunofluorescence microscopy images showing that the PK-induced N- and C-terminal Kv1.5 fragments colocalize with the plasma membrane. Kv1.5-HEK cells were treated with cycloheximide (10 µg/ml) for 24 h to eliminate intracellular Kv1.5 channels. Cells were then treated with PK (200 µg/ml) prior to a 2 h re-culture in MEM containing cycloheximide (10 µg/ml). The live cell membranes were stained red with fluorescent wheat germ agglutinin (5 µg/ml). The N- and C-terminal Kv1.5 fragments were labeled in separate cells using their respective anti-Kv1.5 antibodies and Alexa Fluor 488-conjugated (green) donkey anti-rabbit antibodies.
via pull down with N-terminal anti-Kv1.5 antibodies (Figure 12B). These results imply that there is no association between the 42-kDa and 33-kDa Kv1.5 fragments that are directly produced by the PK treatment.

To justify our belief that the PK-mediated N-terminal and C-terminal Kv1.5 fragments are no longer interacting, we assessed the two fragment degradation rates since non-association would presumably result in discrepant protein turnover rates. Kv1.5 expressing stable HEK 293 cells were treated with PK and re-cultured in MEM for 2, 4, or 6 h. Western blot analyses of the whole-cell lysates using N-terminal and C-terminal specific anti-Kv1.5 antibodies revealed that the C-terminal fragments degrade slightly slower than the N-terminal counterparts (Figure 13). Whole-cell electrophysiological recordings were performed using Kv1.5-HEK cells from the same time course experiment. Our data show that the $I_{Kv1.5}$ amplitude 2 h after PK treatment was smaller than control (although not significantly) and returned to the control measures by 4 h post-PK cleavage (Figure 14). The small dip in $I_{Kv1.5}$ amplitude at the 2 h time point is most likely a reflection of slow Kv1.5 C-terminal fragment (pore-containing) degradation. Despite the slow destruction of functionally competent C-terminal fragments, uninhibited synthesis and forward trafficking leads to gradual recovery of the 75 kDa Kv1.5 channels, accounting for the relatively constant $I_{Kv1.5}$ amplitude following PK application (Figure 13 and 14).

PK Treatment does not Impact Kv1.5 Activation or Inactivation

When considered in its entirety, our data indicate that the PK-induced C-terminal fragment (pore-containing) must at the very least participate in the production of $I_{Kv1.5}$. It might be expected that a channel lacking S1 and the entire cytosolic amino terminus
Figure 12. Co-IP analyses demonstrating that the N- and C-terminal fragments generated by PK cleavage of mature Kv1.5 channels are not associated. A and B.

Following PK treatment (200 µg/ml), Kv1.5-HEK cells were re-cultured in standard supplemented 5 mM K+ MEM for 2 h. N-terminus targeting rabbit anti-Kv1.5 antibody was utilized to precipitate N-terminal Kv1.5 fragments and any associated proteins from the whole-cell lysates. N-terminus specific Kv1.5 antibodies also precipitate immature Kv1.5 (68 kDa) and non-cleaved mature Kv1.5 (75 kDa). N-terminal Kv1.5 fragments were immunoprecipitated (IP) and then detected via immunoblotting (IB) (A). C-terminal Kv1.5 fragments could not be detected with C-terminus recognizing anti-Kv1.5 antibodies in the whole-cell lysates precipitated with N-terminal specific anti-Kv1.5 antibodies (B). A standard Western blot (WB) sample depicting Kv1.5 expression is shown as a control.
Figure 13. The N- and C-terminal Kv1.5 fragments have unique degradation rates.

Kv1.5-HEK cells were treated with PK (200 µg/ml) and were re-cultured in supplemented MEM for 2, 4, or 6 h. Kv1.5 channel expression was detected with N-terminal (upper Western blot) or C-terminal (lower Western blot) binding anti-Kv1.5 antibodies. The protein band intensities corresponding to the fragments 2, 4, and 6 h after PK cleavage were normalized to the corresponding fragment band intensity at 0 h (immediately following PK treatment) and summarized below the Western blot images (n=4). *P<0.05 vs. Ctrl.
Figure 14. The effects of cell culture duration on $I_{Kv1.5}$ following PK digestion.

Representative $I_{Kv1.5}$ traces following cleavage of Kv1.5-HEK cells with PK (200 µg/ml) and re-culturing in supplemented MEM for 2, 4, or 6 h (upper). Summarized current amplitudes are shown in the bar graph below the current traces. The numbers in parentheses denote the number of cells recorded in each condition from 4 independent experiments.
would gate differently from the intact native channel that exists *in vivo*. To examine whether the PK-cleaved channel has gating properties that differ from the control channel, we plotted current-voltage relationships as well as current activation time constant-voltage relationships. No dissimilarity was identified between the two groups (Figure 15).
Figure 15. PK-fragmented Kv1.5 channels do not have altered gating properties compared to WT channels. A. The current-voltage relationship from control (Ctrl) Kv1.5-HEK cells as well as from cells treated with PK (200 µg/ml) and re-cultured for 2 h. Currents measured at the end of the depolarizing steps were plotted against the corresponding depolarizing voltage (n=8 for control and 6 for PK-digested cells). B. The activation time constant-voltage relationship from control (Ctrl) cells and PK-treated Kv1.5-HEK cells that were re-cultured for 2 h. The rising phase of currents upon depolarizing steps was fitted to a single exponential function. The averaged time constants from each set of cells was plotted against the respective depolarizing voltages (n=5 for control and 4 for PK-cut cells).
Chapter 4: Discussion

Kv1.5 channel-mediated $I_{Kur}$ is essential for repolarization of atrial myocyte action potentials (Fedida et al., 1993; Wang et al., 1993). Over the past decade, the atria specific nature of $I_{Kur}$ (Wang et al., 1993) and the growing proportion of individuals with AF (Kannel & Benjamin, 2008) have led to a significant increase in the volume of work concerning Kv1.5 molecular biology as well as its potential for anti-AF therapies. Pathophysiological conditions such as atrial ischemia are correlated with the emergence of AF (Sinno et al., 2003). It has also been shown that ischemia and hypoxia drive an increase in protease activities (Muller et al., 2013). Hence, the objective of the present thesis was to study the relationship between proteases and Kv1.5, which may be implicated in AF-causing diseases.

As a starting point and for the duration of this study, we investigated the impact of PK on Kv1.5 function and expression. PK is a relatively non-specific serine protease with a broad spectrum of substrates that is widely used in assessing plasma membrane expression of ion channels (Choi et al., 2005; Eldstrom et al., 2010; Manganas & Trimmer, 2000; Rajamani et al., 2006). Extracellular application of PK or other concentrated proteases to our HEK stable cell lines for extended periods can result in uncontrolled digestion or cell lethality. In our conditions, PK-digested HEK cells were intact and viable for experiments. The results reveal that PK treatment selectively cleaved the mature forms (upper bands) of Kv1.5 and hERG potassium channels (Figure 5A and 6A). Indeed, PK-induced cleavage of both Kv1.5 and hERG proteins have been previously observed by other groups (Choi et al., 2005; Rajamani et al., 2006). Interestingly, while it has been reported that PK cleavage of hERG leads to an
absence of \( I_{\text{hERG}} \) (Rajamani et al., 2006), the effects of PK treatment on \( I_{\text{Kv1.5}} \) have not been published. Here, our findings indicate that PK digestion abolishes \( I_{\text{hERG}} \) (Figure 6B), but does not affect \( I_{\text{Kv1.5}} \) whatsoever (Figure 5B).

To commence examining how \( I_{\text{Kv1.5}} \) can be immune to PK proteolysis even though the channel protein is severed, we assessed the sizes of the Kv1.5 fragments and identified the cleavage position. As mentioned previously, Kv1.5 proteins subjected to Western blot analysis display two bands with molecular masses of 68 kDa and 75 kDa. The lower 68-kDa band represents core-glycosylated immature proteins and the upper 75-kDa band corresponds to fully-glycosylated Kv1.5 channels (Ding et al., 2014).

Importantly, under normal culture conditions and unimpeded channel glycosylation, solely the 75-kDa Kv1.5 channels are localized in the cell membrane, as revealed by our Western blot results of biotinylation-isolated membrane proteins (Figure 10). Following external application of PK, the 75-kDa Kv1.5 protein was entirely separated into 42-kDa N-terminal fragments and 33-kDa C-terminal fragments (Figure 7). These distinct Kv1.5 hemi-proteins were detected by Western blot analysis with two antibodies that recognize an epitope on opposing intracellular terminals of the channel. Using the ExPASy-ProtParam tool to compare the sizes of our observed N- and C-terminal products with theoretical sizes, we were able to determine that PK targets and cleaves Kv1.5 in the extracellular S1-S2 linker (Figure 8). This finding is in agreement with a previous study that defined the PK target site within Kv1.5 (Choi et al., 2005).

Prevention of glycosylation with the antibiotic tunicamycin converts both populations of Kv1.5 into a single form of approximately 65-kDa (Figure 7). Glycosylation is not required for Kv1.5 expression on the cell-surface since PK still
cleaves membrane-bound non-glycosylated channels (Figure 7), and Kv1.5-HEK cells cultured in the presence of tunicamycin still display robust $I_{\text{Kv1.5}}$ (data not shown). By comparing the fragment sizes of non-glycosylated and PK-cleaved Kv1.5 proteins with the sizes of glycosylation intact Kv1.5 fragments, we were able to conclude that PK targets the Kv1.5 S1-S2 linker between the carboxyl side of the glycosylation position (N297) and the S2 transmembrane domain (Figure 7 and 8).

For Kv1.5 channels, although the PK cleavage position is near the S2 domain, the long cytoplasmic NH$_2$ terminal domain accounts for the large size of the N-terminal fragment (42 kDa) relative to the C-terminal counterpart (33 kDa). Conversely, cleavage of hERG by PK has been reported to be within the functionally critical pore-containing S5-S6 linker, which logically may account for the eradication of $I_{\text{hERG}}$ (Rajamani et al., 2006). hERG serves as a reasonable comparative channel since it is similar to Kv1.5 in many aspects. Like Kv1.5, hERG channels are glycosylated during maturation, are sensitive to digestion by PK, and conduct a repolarizing potassium current in the human heart, $I_{\text{Kr}}$ (Sanguinetti et al., 1995). However, due to inherent differences in the three extracellular linkers of Kv1.5 and hERG with regards to sequence, length, and 3-dimensional structure, PK influences channel expression and function differently in the two channels. PK cleaves hERG in the S5-S6 linker and eliminates $I_{\text{hERG}}$, while digestion of Kv1.5 is limited to the S1-S2 linker and does not hinder channel function.

To omit the possibility that the 68-kDa protein conducts $I_{\text{Kv1.5}}$ and rescues channel function following PK treatment, we performed experiments in which the immature (68 kDa) channels were selectively eliminated. As shown in Figure 9A, administration of the biosynthesis inhibitor cycloheximide produced a selective and
gradual abolishment of the 68-kDa Kv1.5 fraction. After treatment of Kv1.5-HEK cells with cycloheximide for 24 h, the 75-kDa mature channels (upper band) were the only detectable Kv1.5 protein (Figure 9B). Consistent with prior experiments, PK cleavage of cells pre-cultured in cycloheximide (24 h) separated the 75-kDa channels into N-terminal (42-kDa) and C-terminal (33-kDa) fragments (Figure 9B), and the cleavage event did not affect recorded $I_{Kv1.5}$ (Figure 9C). These findings indicate that cycloheximide pre-treated and PK-cleaved Kv1.5-HEK cells only express N-terminal fragments and the pore-containing C-terminal fragments, yet still produce large $I_{Kv1.5}$.

If the Kv1.5 fragments underlie production of $I_{Kv1.5}$, they must be present in the cell membrane. Western blot analysis of biotinylated membrane proteins and immunofluorescence microscopy images show that after complete cleavage of 75-kDa Kv1.5, the resultant two fragments remained stable on the cell surface (Figure 10 and 11). The results amassed can only be interpreted in one of two ways: i) the C-terminal fragment alone of Kv1.5 conducts current following proteolysis or ii) the two fragments function together as a unit (resembling an intact Kv1.5 channel) to conduct current.

To resolve this dichotomy co-IP analysis was employed using N- and C-terminal anti-Kv1.5 antibodies to probe for an interaction between the two Kv1.5 fragments in the whole-cell lysates. As shown in Figure 12, the PK-generated Kv1.5 N-terminal and C-terminal fragments do not associate. Furthermore, Western blot time course experiments suggest that the degradation rates of the two fragments are slightly different (Figure 13). These data imply that the PK-induced N- and C-terminal fragments may not be physically connected. This supports the possibility that the pore-containing fragment of Kv1.5 itself may be sufficient to conduct $I_{Kv1.5}$ after PK treatment.
From the opposing perspective, protein subunits can properly assemble and form functional units (Glowatzki et al., 1995; Inagaki et al., 1996). In addition, under the correct conditions, certain proteins can reform their native structures after denaturation (Shortle, 1996; Ybarra & Horowitz, 1995). Thus, it is conceivable that the two Kv1.5 fragments created by PK treatment recombine or do not dissociate from each other completely, permitting the “cleaved” channel to function as a 75-kDa mature channel. Harsh experimental steps involved in immunoprecipitation and Western blot analysis could be responsible for breakage of the PK-weakened S1-S2 linker. Importantly, similar to the results from Figure 5B using stable Kv1.5-HEK cells, $I_{\text{Kv1.5}}$ from transiently transfected WT HEK cells that were treated with PK was unchanged relative to control (data not shown). This indicates that the potential recombination of Kv1.5 fragments, which is more probable at higher membrane channel density, may not be occurring.

Our data demonstrate that PK treated Kv1.5 channels have currents that are identical to uncleaved (control) Kv1.5 proteins (Figure 15). The indistinguishable kinetics of the whole and fragmented Kv1.5 channels may be a consequence of using HEK 293 cells as the heterologous expression system, since these cells lack endogenous Kvβ-subunits (Uebele et al., 1996). In vivo, cytosolic ancillary β-subunits bind a conserved intracellular N-terminal region of Kv1.5 α-subunits to influence channel regulation and gating (Ravens & Wettwer, 2011; Sewing et al., 1996). Without co-expression of β-subunits in Kv1.5-HEK cells, investigating the effects of cleaving cell-surface Kv1.5 channels may be limited to assessing the presence of robust current, and not the changes in gating that may occur under such circumstances.
In summary, the experiments performed throughout this thesis revealed the novel observation that cleavage of membrane-bound ion channels, assessed by Western blot analysis, does not necessarily correspond to an elimination of channel function. This information extends our knowledge of ion channel biophysics and also offers insight into unique features of ion channel regulation. Although PK digests hERG at the S5-S6 linker and eliminates \( I_{\text{hERG}} \), it cleaves Kv1.5 at the S1-S2 linker without consequence to recorded \( I_{\text{Kv1.5}} \). The significance of the resistance of \( I_{\text{Kv1.5}} \) to serine protease destruction is not immediately obvious and requires further investigation. However, this preservation of \( I_{\text{Kv1.5}} \) despite channel cleavage may represent a built-in protective mechanism to ensure Kv1.5 function under conditions with enhanced protease activities, such as atrial ischemia.

**Future Directions**

The current study postulates that the Kv1.5 C-terminal fragment (S2-COOH) independently conducts current following PK-mediated cleavage of mature Kv1.5 channels. Although, as previously alluded to, it may be the case that the N-terminal and C-terminal fragments are still interacting in some manner to produce \( I_{\text{Kv1.5}} \). Constructing a mutation that truncates the N-terminus and S1 to mimic PK cleavage may represent one method to test this possibility. Since the cytosolic amino terminus of Kv1.5 contains the T1 domain that is critical for assembly of Kv1.5 α-subunits (Shen *et al.*, 1993), the practicality of this approach requires further investigation. In addition, the amino terminus of Kv1.5 is critical for caveolin-mediated trafficking to cell-surface lipid raft microdomains, and disruption of this pathway results in substantial Kv1.5 intracellular retention (McEwen *et al.*, 2008). However, an N-terminal deletion (Kv1.5Δ209) that
preserves the S1 transmembrane domain has been shown to encode measurable currents (Eldstrom et al., 2002), which supports our notion that the PK-generated N-terminal fragment is not a necessity for function. To irrefutably determine that the PK-generated C-fragment is functionally independent from the N-terminal fragment, other approaches may be required. It would be of interest to insert purified PK-induced C-terminal Kv1.5 fragments (S2-COOH) into an artificial bilayer setup and investigate $I_{Kv1.5}$ functionality. A more simplified approach could involve the use of quinidine after PK treatment to concomitantly block and induce Kv1.5 internalization (Schumacher et al., 2009), making the study of discrepant N- and C-terminal fragment degradation rates more apparent.

The entirety of this endeavour was conducted utilizing HEK 293 cells as a heterologous expression system. Immortalized cell lines, such as HEK cell lines are robust and have low background noise during electrophysiological recordings, making them valuable models for preliminary studies. Nonetheless, results cannot be evaluated for significance in the context of health and disease until the experiments are replicated in native tissue. For instance, whether $I_{kur}$ in atrial cardiac myocytes is also resistant to proteolytic digestion in the same manner as $I_{Kv1.5}$ in Kv1.5-HEK cells should be addressed. Dissecting specific currents from others with similar voltage ranges using pharmacology, differences in gating kinetics, and/or varying electrolyte content in recording solutions represents an additional obstacle.

Finally, since PK is a fungal-derived protease, these findings are interesting from a biophysics standpoint but lack a high degree of physiological significance. Future research can be aimed at elucidating if any other extracellular proteases, such as
MMPs or secreted calpain, can produced the same effects that we have described here. Moreover, whether proteases that are upregulated during ischemia cleave Kv1.5 without consequence to $I_{Kv1.5}$ needs to be examined. Lastly, whether this information can eventually translate into clinical practice requires further consideration.


Isoform-specific localization of voltage-gated K\(^+\) channels to distinct lipid raft 

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