The Role of SUMO Modification in the Regulation of Kv1.5, a Major Cardiovascular Potassium Channel

by

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To my family

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CHAPTER I: INTRODUCTION

OVERVIEW

Atrial fibrillation (AF) is characterized by the uncoordinated beating of the atria of the heart in response to disorganized electrical activity. AF leads to hemodynamic alterations that significantly impair cardiac output and are conducive to thrombus formation (1,2). AF is of significant clinical importance not only because it is the most common chronic arrhythmia in patients over the age of 60, but also because of the associated morbidity and nearly two-fold increased risk of mortality. Atrial fibrillation currently affects nearly 10% of the United States population over the age of 85, and it is forecasted to affect nearly 2% of the *general* population by 2050 (3,4). Current strategies for treatment of atrial fibrillation focus on three major goals: 1) The acute reversion back to normal sinus rhythm using either synchronized electrical or pharmacological cardioversion, 2) Maintained prevention of systemic embolization using anticoagulation therapy with warfarin, and 3) Maintained ventricular rate control to avoid hemodynamic instability and tachycardia-induced cardiomyopathy (usually using agents to slow AV nodal conduction such as beta blockers or digoxin).

Although acute cardioversion is generally effective, only 20-30% of cardioverted patients maintain normal sinus rhythm for more than one year. Given that patients with a prior AF episode are at significantly increased risk for recurrence of atrial fibrillation, there is strong rationale to suggest a fourth major goal of therapy, that is, to maintain prophylactic *rhythm* control (5-7). Although effective for acute cardioversion, currently available Class I and III antiarrhythmics are not suitable for long-term use due to the high

rate of proarrhythmic ventricular side effects. Since the risk of proarrhythmic, potentially lethal side-effects outweighs the benefit of maintained rhythm control in all but the most severe grades of AF (8,9), this has prompted a joint panel of the American Academy of Family Physicians (AAFP) and the American College of Physicians (ACP) to take the position that maintained antiarrhythmic therapy is generally not recommended in the treatment of atrial fibrillation. There is thus a significant impetus for the development of new, longer-term antiarrhythmic therapies that can be used to successfully maintain normal sinus rhythm and prevent the recurrence of atrial fibrillation without risking the occurrence of potentially life-threatening, proarrhythmic ventricular side effects.

Kv1.5 is a voltage-gated potassium channel that has emerged as an extremely promising target for such a ventricular-sparing antiarrhythmic therapy. Kv1.5 mediates the ultrarapid delayed rectifier (I_{kur}) current in human atrial myocytes. This current is a major contributor to atrial repolarization. The absence of I_{kur} in ventricular myocytes (10) is consistent with the selective expression of Kv1.5 in the atria but not ventricular tissue (11,12). This raises the intriguing possibility that inhibition of Kv1.5 function, and therefore repolarization, could serve to selectively lengthen the action potential duration in atrial myocytes. The concomitant prolongation of atrial refractoriness would prevent the generation and propagation of extra or disorganized electrical activity in the atria while sparing ventricular function. Very recent work has provided a handful of candidate drugs that are able to inhibit Kv1.5 activity with varying degrees of specificity (4,11,13-16). Although the exact mechanism of inhibition is largely unknown, these drugs do indeed serve to lengthen atrial action potentials and refractoriness without affecting ventricular refractoriness, ECG intervals, heart rate, or blood pressure (11,13,14). While very promising, none of these drugs has progressed to clinical use, presumably due to unanticipated side effects related to their incomplete specificity for Kv1.5. Based on these developments, it is clear that a better understanding of the basic mechanisms that

cells utilize to regulate the functional properties of Kv1.5 are likely to provide novel insights and therapeutic opportunities for the treatment of AF.

At the outset, the overall goal of this dissertation was therefore to explore post-translational mechanisms that can regulate the activity of Kv1.5. As a result of these efforts, we have identified that Kv1.5 contains two putative target sequences for posttranslational modification by members of the Small Ubiquitin-like Modifier (SUMO) family of proteins. I have found that these sequences are functional, and that Kv1.5 is reversibly modified by multiple members of the SUMO family. Furthermore, loss of SUMOylation, either by disruption of the conjugation sites or expression of the SUMO-deconjugating protease SENP2, leads to a selective hyperpolarizing shift in the voltage-dependence of steady-state inactivation of the channel in a heterologous system without affecting other properties of the channel such as total current density or the voltage-dependence of activation.

Using whole-cell patch-clamp electrophysiological studies of acutely-dissociated murine and human cardiomyocytes, I find that it is possible to acutely modulate the voltage-dependence of steady-state inactivation of endogenous I_{kur} current by loading purified recombinant SUMO1 or SENP2 proteins into the pipette solution and allowing them to diffuse into the patched cardiomyocyte during the recordings. Consistent with our studies in the heterologous system, diffusion of exogenous SENP2 into cardiomyocytes led to a depolarizing shift in the voltage-dependence of steady-state inactivation of endogenous I_{kur} current, while diffusion of exogenous SUMO1 led to a hyperpolarizing shift in the voltage-dependence of steady-state inactivation. These effects of SUMO1 and SENP2 were specific, as the current densities of the major outward potassium currents I_{to} and I_{ss} were unaffected by this treatment. Furthermore, the ability of SENP2 to acutely modulate Kv1.5-mediated current was dependent on the presence of intact SUMOylation motifs on Kv1.5 as diffusion of SENP2 into patched HL-1 atrial myocytes transfected with wild-type Kv1.5 led to a similar hyperpolarizing

shift in the voltage-dependence of steady-state inactivation of the channel, but had no effect on a SUMOylation-motif-deficient mutant form of Kv1.5.

In contrast to the studies in heterologous systems, analysis of the effects of alterations in SUMOylation in cardiomyocytes revealed that infusion of either SENP2 or a dominant-negative form of SUMO1 (SUMO1-ΔGG) that cannot be covalently conjugated to target proteins led to a reduction in the total I_{kur} current density. Since I_{kur} is the major potassium current responsible for regulating the duration of the plateau phase of the cardiac action potential (12), I also explored the effect of SUMOylation on the action potential duration (APD). While diffusion of wild-type SUMO1 into acutely-dissociated murine cardiomyocytes had no significant effect on the action potential duration, initial findings suggest that diffusion of SENP2 preparations into cardiomyocytes led to a specific prolongation of the plateau phase of the action potential and a significant increase in the APD. Notably, a similar effect was observed for SUMO1-ΔGG.

Taken together, these data provide strong evidence that Kv1.5 is indeed post-translationally modified by SUMO, and that this regulation has important functional consequences on both the magnitude and inactivation properties of the I_{kur} current, and by extension, on the duration of cardiomyocyte action potentials. These findings are significant as they identify SUMO modification as a new point of regulation of Kv1.5 function and thereby potentially add a level of specificity for the development of antiarrhythmic medications designed to modulate I_{kur} current. The recent development of novel Kv1.5-targeted antiarrhythmic medications indicates that the strategy of selectively modulating Kv1.5 current is an effective method for achieving ventricular-sparing maintained rhythm control in patients with atrial fibrillation. The discovery that Kv1.5 is a target of SUMOylation and that this modification has important and specific regulatory roles indicates that manipulation of this control mechanism may open novel opportunities for atrial-specific antiarrhythmic therapy.

The findings described in this thesis are also significant as they represent, to our knowledge, the first report of the SUMO modification of a voltage-gated ion channel and one of the first examples of how SUMO modification can regulate the function of a protein located on the plasma membrane. As such, the findings expand the list of known functions of SUMOylation and reveal that, in addition to its more established roles in the regulation of nuclear proteins, this modification plays important roles at the plasma membrane

In the following three sections of this chapter, I introduce the structure and function of Kv channels, describe our current understanding of the function and regulation of Kv1.5, and present an overview of the process of SUMOylation and its regulation.

VOLTAGE-GATED POTASSIUM (KV) CHANNELS

Introduction

Voltage-gated potassium (Kv) channels form aqueous pores that mediate the highly selective and regulated passage of K^+ ions across the hydrophobic core of the plasma membrane of electrically excitable cells. The resting membrane potential of myocytes is approximately -70 mV, and under these conditions, it has been estimated that the probability of opening for a single Kv channel is less than 10^{-9} (17). However, upon depolarization of the plasma membrane, Kv channels rapidly open to allow for the flow of approximately 10^6-10^8 ions/second for each channel with over a 100 times more selectivity for K^+ over Na^+ (18,19). The regulation of this K^+ conduction is critical for establishing the resting membrane potential and dictating the duration and frequency of action potentials in the heart and peripheral vascular beds and is thought to be a function of both the number of active channels expressed on a cell's surface as well as the

properties of each channel's activation and inactivation gates. Defects that modulate the expression and/or gating properties of these important channels are thus linked to a wide range of cardiovascular diseases including long Q-T syndrome, chronic atrial fibrillation, chronic hypertension, and pulmonary arterial hypertension (20-24).

Structure

Kv channels are composed of four α-subunits, each of which contains 500-1000 amino acids and six transmembrane domains (S1-S6) with both N- and C-terminal sequences residing in the cytoplasm (Figure I-1). Kv channel α-subunits assemble into homotetramers in the endoplasmic reticulum through their membrane-spanning domains as well as via an N-terminal tetramerization (T1) domain (25). Heterotetrameric complexes composed of different α-subunits have unique properties and can be detected both *in vitro* and *in vivo* (26). Multiple Kv channel gene families (Kv1-Kv9) have been identified which encode more than 40 different mammalian α-subunit isoforms. Auxiliary cytosolic β-subunits can associate with Kv channel α-subunits and alter both membrane expression and channel properties. Co-assembly between α- and β-subunits takes place in the endoplasmic reticulum as an early step in the biosynthetic pathway (27,28), and since only specific α /β subunit combinations are permissible, this interaction appears to be selective (29,30). At least four Kv β-subunit gene families have been identified, with alternative mRNA splicing in the Kv1 and Kv2 β-families yielding additional diversity (31). X-ray crystallographic data reveals that β-subunits tetramerize with four-fold symmetry similar to that of the α-subunits and interact directly with the similarly-organized T1 tetramerization domain of the α-subunits (32).

Structure/function studies have characterized amino acids in Kv α-subunits involved in ion selectivity, activation and inactivation gating, and neurotoxin binding (33). The determination of the atomic structures of K^+ channels from bacteria, as well as the recent

determination of the structure of rat Kv1.2 (34), support much of the structure/function data derived from multiple studies (35,36). The S1-S6 segments of each α subunit arrange with four-fold symmetry around a central axis that forms the conducting pore of the channel (Figure I-1). S5-S6 are in direct contact with this conducting pore and contain the activation and inactivation gates as well as the selectivity filter, while S1-S4 are arranged around the perimeter of the channel and contain the voltage-sensor.

Voltage-Dependence and Gating

Based on the crystal structures of bacterial potassium channels trapped in the closed conformation (KcsA and KirBac1.1) and open conformation (MthK and KvAP), it has become apparent that the activation gate is contained on S6 (36-39). In the closed conformation, the S6 segments from each α subunit form a bundle crossing at the plasma membrane/cytoplasm interface that occludes access of K^+ (or quaternary ammonium ions such as the classic internal Kv channel blocker tetraethyl ammonium (TEA^+)), to a large, almost 10Å-wide, aqueous inner cavity of the channel (18). Upon activation, the S6 helices bend at a well-conserved "PxP" motif (where P stands for proline and x any amino acid), and this inner cavity becomes accessible to the cytoplasm (40). "Deactivation," which is distinct from "inactivation" (discussed below) is the re-closing of this activation gate.

The selectivity filter of the channel is located along the extracellular third of the conduction pore and is composed of the so-called TXGYG "signature sequence" in the S5/S6 loop of Kv channels (41,42). By lining the conduction pore with the backbone carbonyl oxygen atoms of the residues in this sequence spaced by the geometrical constraints of the glycine and threonine residues, the selectivity filter is able to provide four octahedral binding sites that are able to coordinate K^+ ions with almost the exact same geometry of the water molecules that surround the hydrated potassium in the inner

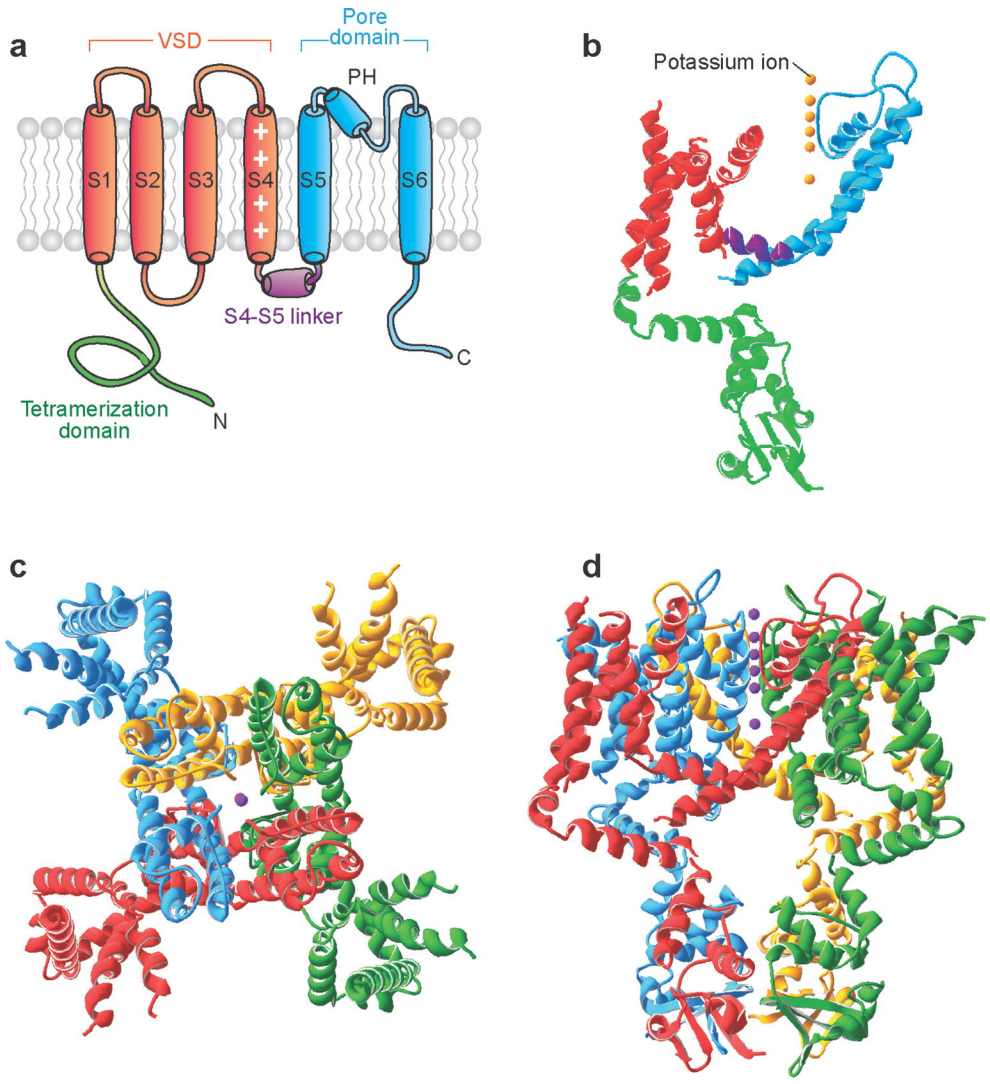

Figure I-1. The Structure of Kv Channels

(A) A schematic representation of a single Kv channel α subunit. (B) Structural model of a Kv channel α subunit using the coordinates of Kv1.2 (34) color coded as in A. (C) Extracellular view of tetramerized channel color coded as in A. A potassium ion is shown in purple. (D) Side view of fully assemble channel as in C. Adapted from (19).

cavity of the channel (36,41). Thus, the selectivity filter is able to effectively dehydrate K^+ ions from the inner cavity and allow these ions to pass through the filter and on to the extracellular side of the channel where the K^+ is again re-hydrated. Due to the size constraints of the octahedral binding sites, however, the filter is unable to effectively dehydrate the smaller Na^+ ions also present in the inner cavity.

The ability of Kv channels to sense changes in transmembrane potential is thought to be primarily due to the location of four, key arginine residues located on the S4 transmembrane helix (43). The positively-charged guanidinium groups of these arginines provide a "gating charge" that, due to their location within the membrane, respond to changes in the local transmembrane electrical field and lead to the displacement of the S4 helix (44). The exact nature of the movement of the S4 helix upon this displacement is heavily debated with helix motion ranges between 2 and 20 Å. Based on the crystal structure of Kv1.2 and scanning mutagenesis studies, there is general agreement, however, that this movement is transmitted to the S5/S6 pore-forming region of the neighboring α subunit via direct contact with the linker that connects the displaced S4 to S5 (19).

As mentioned above, the current through Kv channels is regulated by the collective effects of two gates: the activation and inactivation gates. Upon membrane depolarization, Kv channels rapidly transition from a closed, non-conducting conformation to an open conformation via a change in the S6 transmembrane segment as already described. If the membrane is repolarized, conduction through the channel stops due to the re-closing of this gate through the process of "deactivation." However, for many Kv channels, if the membrane continues to be depolarized, channels enter a stable, non-conducting state in a process referred to as "inactivation." That is to say, upon sustained depolarization, channels activate to produce an outward potassium current. Over time, this current begins to decay due to the transition of channels from open to inactivated states. The kinetics of this decay can vary widely between channels and can

be critical to the function of the channel. For example, while both Kv1.5 and Kv4.2 activate during the initial depolarization phase of the cardiac action potential, the very fast inactivation of Kv4.2 limits its effects to early (notch) repolarization, while the slow inactivation of Kv1.5 allows its effects to be distributed through the entire plateau phase of repolarization.

Two major mechanisms of inactivation have been described in Kv channels. The first, or "N-type inactivation," has rapid kinetics (on the order of tens of milliseconds). This process is dependent on a region of the channel consisting of ~10 hydrophobic residues followed by a series of positively-charged amino acids. This "inactivating peptide" is contributed by the extreme N-terminus of a subset of Kv channel α subunits or by specific accessory β subunits (45,46). Upon activation of the channel, these residues enter the conducting pore leading to direct occlusion of the conduction path. It is hypothesized that the hydrophobic residues directly interact with hydrophobic residues in the pore of the channel. The positively-charged residues are thought to interact with acidic residues located on the S1/T1 linker and position the inactivating peptide in a location that facilitates its entry into the pore (47-49). N-type inactivation is not directly voltage-sensitive, depending instead only on whether the channel is open or closed (50). This "ball and chain" model of inactivation was originally described for the squid axon sodium channel (51), but is present in Kv channels that contain such an N-terminal "ball" (*Shaker*, Kv1.4, Kv3.4, and Kv4 family members) and Kv channels that associate with cytoplasmic accessory subunits that contain a "ball" (Kvβ1.1 and Kvβ1.3)(45,46).

Interestingly, N-type inactivation has been found to be highly regulated by certain forms of posttranslational modification. Protein kinase C phosphorylation of the N-terminus of Kv3.4 (52,53), protein kinase A phosphorylation of the N-terminus of Kvβ1.3 (54), and Src tyrosine kinase phosphorylation of the N-terminus of *Shaker* (55) have all been found to have inhibitory effects on N-type inactivation. The current view is that the addition of negative charge to the "ball" interrupts the stabilizing interactions

with the acidic residues in the S1/T1 linker (45). Consistent with this idea, PKA phosphorylation of the C-terminus of *Shaker* (which is located near the S1/T1 linker (see the crystal structure of Kv1.2 in Figure I-1)) and protonation of an N-terminal histidine in Kv1.4 both stimulate N-type inactivation (56,57). Presumably, both of these modifications could serve to stabilize the interaction between the basic N-terminal "ball" and the acidic S1/T1 linker.

The second major type of inactivation is called "C-type" inactivation and follows much slower kinetics than that of N-type with time constants in the range of 10^2-10^3 milliseconds (45). While C-type and N-type inactivation can exist simultaneously in some Kv channels, C-type inactivation persists after the deletion of the N-terminal "ball" in channels with N-type inactivation (58). C-type inactivation is thought to be due to local conformational changes in the selectivity filter and outer pore of the channel. Studies based on the accessibility of engineered cysteine residues around the outer pore of the channel to thiol-reactive reagents (59) or on the fluorescence properties of cysteine-reactive dyes targeted to such cysteines (60-62), have demonstrated that upon sustained depolarization, the four α-subunits in this outer region undergo a localized conformational rearrangement that partially collapses the conducting pore across the selectivity filter. The rearrangement of the selectivity filter during C-type inactivation is also manifested as a drop in the specificity of Kv channels for K^+ over Na^+ during C-type inactivation. Thus, when K^+ is replaced by Na^+ in the recording solution, small Na^+ currents can be detected in Kv channels that have undergone C-type inactivation (63). High extracellular K^+ or TEA^+ concentrations inhibit C-type inactivation by a so-called "foot in the door" mechanism. These ions are able to bind the extracellular pore of the channel and prevent this conformational change (46).

In contrast to studies of N-type inactivation, fewer studies describe modulation of C-type inactivation by forms of posttranslational modification. One exception to this is the finding that the epidermal growth factor receptor (EGFr)-linked tyrosine kinase

phosphorylation of Kv1.3 significantly increases the rate of C-type inactivation (64). However, the mechanism by which this occurs is still poorly understood and may not be due to direct phosphorylation of Kv1.3, as mutation of tyrosine residues within consensus sequences for tyrosine kinases did not interrupt the effect of EGFr on Kv1.3 inactivation (45).

In contrast to N-type inactivation, C-type inactivation is voltage-dependent. However, the mechanism of coupling to the S4 voltage sensor is not as well understood as in the case of activation. The development and recovery from inactivation are sensitive to the transmembrane potential during times of sustained depolarization (58,65,66). This voltage dependence, however, is limited to a narrow range of potentials (46). For most Kv channels, the time constant of inactivation is sensitive to voltage only at potentials where the channel is partially activated (46). This has led to the speculation that movement of the S4 activation sensor may be a required first step to drive the channel into a state where the conformational changes associated with C-type inactivation are then possible. The conformational changes of C-type inactivation may not, by themselves, be intrinsically voltage-dependent, but may only be enabled after the voltage-dependent movement of the S4 sensor (46).

In whole-cell patch-clamp studies, the steady-state voltage-dependence of inactivation can be measured by using a two-pulse protocol. First, a prolonged depolarizing pulse is used to drive channels into a steady-state between the open and inactivated states. This inactivating pulse is then immediately followed by a fast, strongly depolarizing "test pulse" to sample the number of channels that remain in the open, conducting conformation. The relationship between the potential of the inactivating pulse and the number of channels that occupy the inactivated state at steady state represents the voltage-dependence of steady-state inactivation and can be described by the Boltzmann equation. Importantly, mutations on the S4 of Kv1.4 that affect the gating charge of the voltage sensor have been found to shift the voltage-dependence of

steady-state inactivation, suggesting that the process is coupled to the movement of the S4 sensor, as well (65). Despite the imprecise understanding of the atomic basis for the voltage-sensitivity of C-type inactivation, it is clear that this process is a major mechanism of regulation central to the physiological role of Kv channels.

Surface expression, heterotetramerization, co-assembly with accessory subunits, posttranslational modifications, sensitivity to drugs, the kinetics of activation and inactivation, and the voltage-dependence of activation and inactivation are thus all critical parameters to defining a Kv channel, and differences in these parameters allow Kv channels to play important roles in a remarkably diverse array of physiological contexts. During my thesis, I have focused on the study of the effects of SUMOylation on the *Shaker*-family-related Kv channel Kv1.5 (Figure I-2). In the following two sections, I cover the salient properties of Kv1.5 and subsequently describe the process of SUMOylation.

KV1.5, A CARDIOVASCULAR POTASSIUM CHANNEL

Cloning and Characterization

Kv1.5 is an approximately 600 amino acid Kv channel α subunit that was simultaneously cloned by many laboratories using homology screening with *Drosophila Shaker* cDNA from rat heart (originally named "RK4") (67), rat brain ("Kv1") (68), a human ventricular library ("HK2") (69), and a human insulinoma cell line ("hPCN1") (70). Northern blot analysis revealed that, in rat, Kv1.5 was expressed in the heart, aorta, and skeletal muscle, but was absent from the liver and only weakly expressed in the brain (67). While mRNA expression in the ventricle and atrium was found to be comparable in the rat, Northern blot analysis of human tissue revealed that Kv1.5 mRNA was vastly more abundant in the atrium than the ventricle (69).

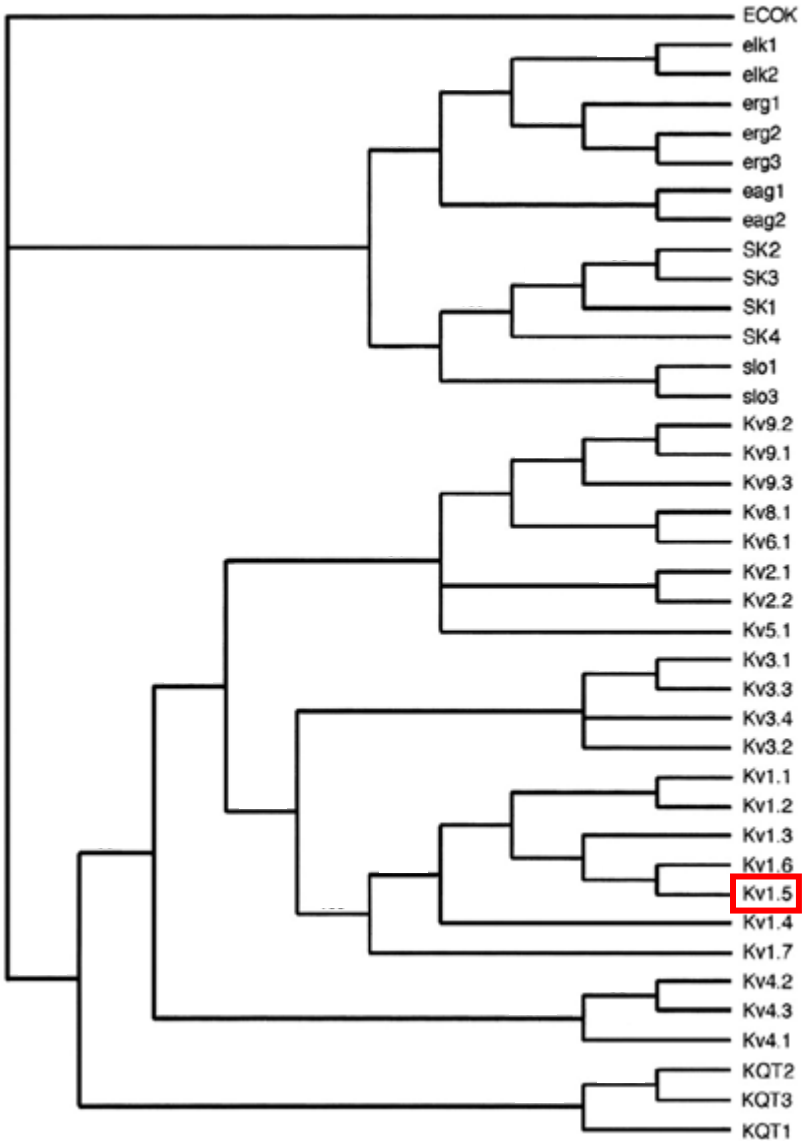

Figure I-2. Phylogenetic Tree of Six Transmembrane Voltage K^+ Channels

Adapted from Coetzee et al. (1999) Ann NY Acad Sci 868, 233-85.

Cloned hPCN1 expressed in *Xenopus laevis* oocytes demonstrated that Kv1.5 encoded a rapidly activating (time constant of activation was 8.33 milliseconds at 0 mV), slowly inactivating (time constant of inactivation was 1155 milliseconds at 30 mV), K^+ selective, rectifying, outward current (70). This current was found to be sensitive to 4-AP in a use-dependent manner and insensitive to TEA^+. The threshold for activation was -25mV (with a V_{50} of activation of -6.0 mV), and the steady-state inactivation became apparent at voltages above -50mV (with a V_{50} of steady-state inactivation of -25 mV)(70). Similar properties were determined in Ltk- cells stably expressing HK2 (71) with the added findings that Kv1.5-mediated currents were sensitive to the antiarrhythmic quinidine in a state-dependent manner, as well as to the neurotoxin dendrotoxin. As Kv1.5 lacks an N-type inactivation peptide sequence on its N-terminus, it does not display N-type rapid inactivation in either oocytes or Ltk- cells. However, coexpression of Kv1.5 with Kv 1.1 or Kv 1.3, both of which contain N-type inactivation peptides, can confer N-type rapid inactivation to Kv1.5.

Kv1.5 Mediates the I_{kur} Current in Cardiomyocytes

Since Kv1.5 is abundantly expressed in the heart and is inhibited by the clinically-prescribed antiarrhythmic quinidine, it was suggested that Kv1.5 may play a part in shaping the cardiac action potential. To determine if a cloned channel mediates a specific current in a cardiac cell, it has been proposed that a certain set of criteria be met (72,73). These criteria are as follows:

1) The basic biophysical properties (kinetics and their voltage-dependence, conductance, rectification, ion selectivity) should be in reasonable agreement.
2) The pharmacology of compounds known to interact directly with the channel pore should be similar.

3) Immunohistochemistry with isoforms-specific antibodies made against the cloned subunits should confirm that the channel protein is present in the cardiac myocytes.

4) Affinity-purification from native tissue should confirm the protein composition of the native channel in terms of accessory subunits and heterotetramer formation.

5) Deletion of the cloned channel using *in vitro* antisense approaches or *in vivo* suppression should further confirm the identity of the current that this gene supports.

The cardiac action potential is mediated by the coordinated effects of Na^+, K^+, Cl^-, and Ca^{2+} channels (Figure I-3). Upon the initial Phase 0 depolarization mediated by rapid inward Na^+ current, fast-activating Kv channels mediate an initial early repolarization (the Phase 1 "notch"). The following plateau phase (Phase 2) is mediated by the balanced outward repolarizing currents of Kv channels and depolarizing, inward currents of Ca^{2+} and Na^+. The balance of these opposing currents, as well as the extent of the initial Phase 1 repolarization dictate the duration of the action potential and are thus critical to dictating the contraction properties of the cell as well as the frequency of the action potentials.

Using different pulse protocols and pharmacological methods in whole-cell voltage-clamp studies of acutely dissociated myocytes from both mice (74) and humans (12), three distinct K^+ currents have been identified and correlated to the cardiac action potential. These are the I_{to} current, which is a rapidly-activating (<2 ms), fast inactivating (time constant of inactivation is 20-35 milliseconds at +30 mV), 4-AP insensitive current that mediates the initial Phase 1 early repolarization of action potential. This current is thought to be mediated by Kv4 family members. I_{kur} (or "Isus" as described by Wang, et al (12)) is a rapidly-activating, delayed rectifier, K^+ current that is sensitive to micromolar concentrations of 4-AP, and, as will be described below, is thought to be

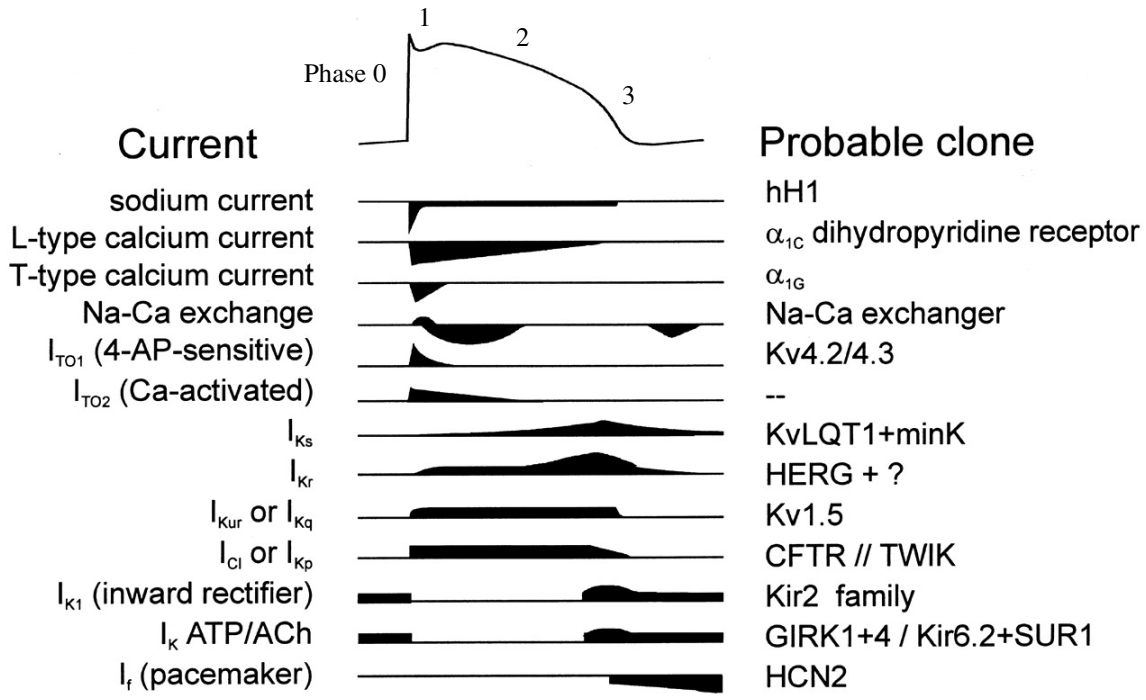

Figure I-3. The Underlying Na^+, K^+, Ca^{2+}, and Cl^- Currents of the Cardiac Action Potential

Adapted from Snyders D. (1999) Cardiovasc Res. 42, 377-90.

mediated by Kv1.5. Inhibition of this current by 4-AP causes a selective lengthening of the Phase 2 plateau of the cardiac action potential. Finally, I_{ss} is a 4-AP insensitive, slow activating, non-inactivating K^+ current that is thought to be mediated by Kv2.1 and/or HERG (73). These channels are important in Phase 3, late-stage repolarization.

Careful characterization of the biophysical and pharmacological properties of I_{kur} seems to fulfill criteria 1 and 2 as listed above for assigning Kv1.5 to the I_{kur} current. In human atrial myocytes (12), I_{kur} (or "Isus") activates very rapidly (time constant of activation is 2.1 ms at +50 mV), has a threshold for activation near -30 mV, and has a steady-state half activation of -4.3 mV. I_{kur} inactivates very slowly (the rate of inactivation was not reported) with a steady-state half inactivation of -48.3 mV in mouse myocytes (74). I_{kur} was found to be highly sensitive to 4-AP (IC_{50} of 49 micromolar) and open-channel block by quinidine (IC_{50} of 6 micromolar), but insensitive to TEA^+, Ba^{2+}, and dendrotoxin. These biophysical and pharmacological properties are very similar to those described for cloned Kv1.5 in oocytes and Ltk- cells, as described above.

Toward filling criteria 3 listed above for assigning Kv1.5 as the molecular correlate to I_{kur} current, Mays et al. (75) successfully used two antibodies directed against the N-terminus and S1/S2 extracellular loop of Kv1.5 to determine that Kv1.5 protein is expressed in human atrial and ventricular myocytes. Furthermore, using immunocytochemistry studies, they were able to localize Kv1.5 to the intercalated disks of these myocytes. Interestingly, these immunofluorescent data are in contrast to northern blot data that suggest that Kv1.5 mRNA is preferentially expressed in atrial myocytes over ventricular myocytes in humans. Mays et al. suggest that careful quantitation of expression levels by Western blotting might help to determine exactly what the difference of Kv1.5 protein expression might be between atrial and ventricular myocytes in humans, but report that they were unable to successfully conduct these studies. At any rate, this finding that Kv1.5 protein is present in cardiomyocytes satisfies criteria 3 for assigning Kv1.5 to I_{kur} current.

Finally, studies in which cultured human atrial myocytes were incubated in medium containing antisense oligodeoxynucleotides directed against segments of the Kv1.5 mRNA coding sequence, determined I_{kur} current to be specifically and significantly reduced by approximately 50%, while I_{to} and I_{ss} currents were left unaffected (76). Furthermore, I_{to} and I_{ss} were unaffected in ventricular myocytes that were exposed to similar Kv1.5 antisense oligodeoxynucleotides. Similarly, studies that used gene targeting to replace Kv1.5 with 4-AP-insenstive Kv1.1 in transgenic mice, determined that the 4-AP sensitive component of ventricular myocyte outward potassium current was specifically dependent on Kv1.5 expression, and that Kv1.5 replacement had no effect on either the I_{to} or I_{ss} currents (77). Finally, electrophysiological studies of right atrial appendages taken during coronary artery bypass graft surgery from patients with chronic atrial fibrillation found a significant reduction in the magnitude of I_{kur} in these patients that was concomitant with a reduction in Kv1.5 protein expression (20). This effect was specific, as protein levels of Kv2.1 were found to be unaffected. These findings satisfy criteria 5 as listed above for assigning Kv1.5 to I_{kur} current.

Taken together, the similarities in the biophysical and pharmacological properties of Kv1.5 and I_{kur}, the determination that Kv1.5 is expressed in human atrial myocytes, and the finding that the elimination of the Kv1.5 channel can knock down I_{kur} current in atrial myocytes, provide strong evidence that Kv1.5 mediates the I_{kur} repolarizing current critical to action potential phase 2 plateau repolarization in human atrial myocytes.

Defects in Kv1.5 Are Associated with Cardiovascular Disease

Due to its important role in action potential repolarization, it is not surprising that defects in Kv1.5 are associated with the life-threatening arrhythmia chronic atrial fibrillation (20). Chronic atrial fibrillation is the most common chronic arrhythmia in patients over the age of 60 and is important clinically because of its associated morbidity

and increased risk of mortality (1.5-1.9-fold risk in the Framingham study) (1,2). Electrical remodeling in chronic and paroxysmal atrial fibrillation involves changes in Kv1.5 expression. Study of right atrial appendages taken during coronary artery bypass surgery from patients suffering from persistent or paroxysmal atrial fibrillation determined that while the mRNA levels of Kv1.5 were unaffected compared to control groups, Kv1.5 protein expression was significantly decreased in these groups (78). In a similar study (20), whole-cell patch clamp study of acutely dissociated atrial myocytes taken from the right atrial appendage of patients in chronic atrial fibrillation revealed that this observed reduction of Kv1.5 protein expression was paralleled by a dramatic decrease in I_{kur} current as compared to patients in sinus rhythm. This effect was specific, as levels of Kv2.1 were unaffected between the two groups.

Recently, genomic DNA screening of a patient diagnosed with lone atrial fibrillation refractory to treatment with flecainide (which is a class Ic antiarrhythmic that blocks both Na^+ and K^+ channels) and dofetilide (which is a new-generation class III antiarrhythmic that specifically blocks K^+ channels with no associated Na^+ channel blocking activity) revealed a heterozygous nonsense mutation in Kv1.5 (E375X) that resulted in a non-functional truncation mutant of the channel (79). This mutation was also identified in the proband's two siblings, both of whom suffered from either atrial fibrillation or paroxysmal atrial fibrillation, and was absent in the screening of 540 unrelated, healthy controls. The E375X mutation truncated Kv1.5 immediately after the S3 region of the channel, resulting in a non-conducting mutant that was still expressed and, presumably through successful tetramerization with WT channel mediated through its intact N-terminal TI tetramerization domain, exerted a dominant-negative effect on WT Kv1.5 current.

Together, these findings establish a strong association between defects in Kv1.5 function and atrial fibrillation. In addition to its important role in cardiac myocytes, Kv1.5 also plays a central role in excitation-contraction coupling in the smooth muscle

cells of a wide number of vascular beds including the aorta, portal vein, cerebral arteries, mesenteric artery, and the coronary, renal, and pulmonary vasculature (80-87). When Kv channels are activated in these cells, the concomitant increase in K^+ efflux causes voltage-activated calcium channels to open, leading to a decrease in intracellular calcium and vasodilation. By this mechanism, Kv1.5-mediated current is critical to regulating vascular tone and blood vessel diameter. Furthermore, because of the high electrical input resistance of smooth muscle cells, the opening or closing of only a few Kv channels leads to large changes in the contractile state of the vessels (88). Defects in Kv1.5 can lead to decreased Kv-mediated currents in pulmonary arterial smooth muscle cells and are associated with chronic hypoxic pulmonary arterial hypertension (PAH) (89), a clinically serious condition due to its increased risk for life threatening right ventricular failure. Kv1.5 has been shown to participate in oxygen-sensitive channel complexes (86,87), and thus abnormalities in Kv1.5 and its associated role in excitation-contraction coupling in pulmonary arterial smooth muscle cells is thought to be an important predisposing factor that leads to PAH (90).

Regulation of Kv1.5

Because defects in Kv1.5 expression levels and function can have such significant physiological and pathophysiological consequences, there has been substantial interest in understanding the factors that regulate Kv1.5. Recent studies have begun to identify regulatory mechanisms throughout the lifecycle of the channel—from its transcription, to its trafficking, to its insertion and removal from the plasma membrane. I have outlined the major points of Kv1.5 regulation that have been identified below. I will briefly describe these points and then focus on what is known about the posttranslational modification of Kv1.5, as this has been the focus of my work.

Regulation during development.

Kv1.5 mRNA is first detected in rat ventricular myocytes at approximately postnatal day 15 (91). This correlates with whole cell patch clamp studies that detected no I_{kur} current in developing mouse ventricular myocytes until about postnatal day 20, at which point there was approximately 10% of the I_{kur} current density present in adult ventricular myocytes (92). Kv1.5 protein expression does not seem to follow this pattern, however. In the same study that followed mRNA levels of Kv1.5, protein levels of Kv1.5 in rat ventricular myocytes were detected at postnatal day 5 and remained unchanged through adulthood. The authors speculate that this may suggest a role for the post-transcriptional regulation of Kv1.5.

Transcriptional control

A number of studies have begun to identify roles for the glucocorticoid receptor (GR), thyroid hormone receptor (TR) , transcription factor Sp1, and CREB/ATF nuclear DNA-binding proteins CREB and CREM in regulating Kv1.5 expression on the transcriptional level. Reduction of endogenous glucocorticoids in adrenalectomized rats resulted in a ~8-fold reduction in Kv1.5 mRNA and a ~6-fold reduction in Kv1.5 protein in ventricular myocytes compared to that of WT rats (93). Furthermore, injection of dexamethasone (a glucocorticoid receptor agonist) rescued this loss, and in fact led a ~50-fold increase in Kv1.5 mRNA levels and ~20-fold increase in Kv1.5 protein levels within one day of administration. This regulation by dexamethasone was specific for Kv1.5, as mRNA and protein levels of Kv1.4 and Kv2.1 were unaffected by this treatment. Interestingly, as well, this regulation by the GR appeared to be extremely tissue specific, as neither the adrenalectomy nor the administration of dexamethasone affected mRNA or protein levels of Kv1.5 in atrial myocytes. Similarly, Kv1.5 mRNA levels were found to be modulated by TR as Kv1.5 mRNA levels were decreased in

hypothyroid rats, whereas Kv1.5 mRNA levels were greatly increased in hyperthyroid rats (94).

Investigation of the 5' flanking region of the Kv1.5 gene has revealed both a cAMP response element (CRE) consensus sequence and Sp1 transcription factor binding motif (95,96). These binding motifs are functional as cAMP was found to induce a 6-fold increase in Kv1.5 mRNA expression in primary neonatal rat atrial myocytes, and reporter constructs driven by the Kv1.5 5' flanking regions were found to bind CRE-binding protein (CREB) and CRE modulator protein (CREM)(96). Furthermore, inhibition of Sp1 using a dominant-negative form of Sp1, decreased Kv1.5 promoter activity (as measured using a reporter construct under the control of the Kv1.5 5' flanking region), while Sp1 over-expression increased promoter activity (95).

Assembly and Trafficking

As already mentioned, Kv1.5 is assembled into tetramers concurrent with synthesis in the endoplasmic reticulum and then trafficked to the Golgi via a "VXXSN" forward trafficking signal located on the C-terminus of the channel (97). For many Kv channels, this ER-to-Golgi transport has been found to be dependent on interactions with accessory proteins such as the β-subunits, the K^+ channel associated protein KChAP, and the K^+ channel interacting protein KChIP (98). Consistent with a chaperone-like role of KChAP for ER-to-Golgi trafficking of Kv1.5, KChAP has been shown to interact with the N-terminus of Kv1.5, but this interaction does not affect the properties of Kv1.5 currents (99). Importantly, these interactions with chaperone proteins are not necessary for Kv1.5 trafficking to the plasma membrane since the absence of either β-subunits or KChAP does not affect Kv1.5 current density. As will be discussed later in this introduction, KChAP has also been identified as a member of the SUMO E3 ligase family (100). This raises the interesting possibility these effects of KChAP on Kv channels could be, in part, due to modulation of the SUMO state of Kv channels.

Insertion into the Plasma Membrane

The trafficking of Kv1.5 from the Golgi to the plasma membrane has recently been shown to provide another opportunity for regulation. It has been shown that Kv1.5, but importantly not other closely-related Kv channel members such as Kv2.1, is targeted to specific plasma membrane microdomains such as caveolar-lipid rafts (101). Depletion of cellular cholesterol and sphingolipids located in these lipid rafts leads to a hyperpolarizing shift in the voltage-dependence of activation and inactivation of Kv1.5, while not effecting non-raft-associated Kv channels such as Kv4.2, providing strong evidence that localization to these rafts has important consequences on the biophysical properties of the channel (101).

Targeting of Kv1.5 to these lipid rafts is contingent on interactions with trafficking proteins such as caveolin (102). In a caveolin-null cell line, Kv1.5 did not associate with membrane lipid rafts. However, transfection of caveolin-1 back into these cells rescued the association of Kv1.5 with these rafts. Similarly, expression of a caveolin-deletion mutant that has reduced ability to traffic to the plasma membrane led to the accumulation of intracellular Kv1.5 and a loss in plasma membrane expression. Consistent with the findings described above that disruption of lipid raft cholesterol and sphingolipids leads a *hyperpolarizing* shift in the voltage dependence of activation and inactivation of Kv1.5, expression of caveolin-1 led to a *depolarization* shift in the voltage-dependence of Kv1.5 activation and inactivation (102).

Membrane-Associated Scaffolding Complexes

The idea that Kv1.5 organizes into specific microdomains of the plasma membrane, allowing for local interactions to help regulate the activity of the channel, is supported by the finding that Kv1.5 interacts with the PDZ domain-containing proteins SAP97 and PSD95 (103). SAP97 is a membrane-associated guanylate kinase (MAGUK) that is localized in lipid rafts and directly interacts with caveolin-3 . Interestingly, SAP97

and caveolin-3 have both been shown to interact directly with the C- and N-termini of Kv1.5, respectively, and all three proteins have been shown to form a tripartite complex (103). The fact that MAGUKs contain numerous protein interaction motifs (in addition to PDZ domains, they also contain the Src homology-3 (SH3) and guanylate kinase (GUK)-like domains) has prompted the speculation that the interaction between SAP97, caveolin-3, and Kv1.5 may serve as a nucleation site for the assembly of larger, macromolecular, regulatory complexes around the channel (103). SAP97 and caveolin-3 have both been shown to have important regulatory functions on the current density and voltage-dependence of activation of Kv1.5 in a heterologous system (103,104). Similarly, PSD95, which binds Kv1.5 via the T1 tetramerization domain of the channel, has been found to mediate the clustering of both the NMDA receptor and Kv1.4 to specific plasma membrane microdomains in neurons (104).

Interactions with Other Signaling Pathways

Activation of the receptors for platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), human thrombin, or rat 5-HT1c (serotonin) leads to a reduction in Kv1.5 current density in *Xenopus oocytes* with a $t_{1/2}$ of approximately 20 minutes (105). Because the downstream signaling pathways of each of these receptors involves the upregulation of phospholipase C (PLC) activity, it has been hypothesized that this effect on Kv1.5 current is mediated by PLC (105). In support of this idea, a mutation that specifically prevents the FGF receptor from being able to activate PLC ablates this effect on Kv1.5, and simultaneous injection of IP3 and superfusion with a DAG analog (IP3 and DAG are the products of PLC hydrolysis of PIP2) reproduces the effect. Interestingly, in addition to the production of IP3 and DAG, depletion of PIP2 via PLC activity has been suggested to play a role in the regulation of other ion channels, including KCNQ channels.

The alpha and beta adrenergic G protein-coupled receptors have also been shown to modulate I_{kur} current in both human (106) and canine (107) atrial myocytes. Activation of beta-receptors (via isoproterenol) was found to increase I_{kur} current, while activation of alpha-receptors (via phenylephrine given in the presence of propralol to block beta-receptors) decreased I_{kur} current in human atrial myocytes. These effects were inhibited by blocking the activities of protein kinase C (PKC) and protein kinase A (PKA), which are downstream effectors of the alpha- and beta-adrenergic receptors, respectively. As Kv1.5 contains numerous consensus sites for phosphorylation by both PKA and PKC, it has been speculated that this adrenergic modulation may be mediated via posttranslational modification of the channel (106), although, as described below, this has not yet been directly demonstrated.

Endocytosis and Recycling

Work that I was involved with in the Martens laboratory (I helped generate an extracellular-tagged Kv1.5 that contains GFP inserted into first S1/S2 extracellular loop of the channel) has identified that endoyctosis also plays a critical regulatory role in Kv1.5 function (108). Kv1.5 internalizes in a dynein-dependent manner to perinuclear endosomal compartments. At 37 °C during basal conditions, this internalization occurs with a $t_{1/2}$ of approximately 11 minutes and results in a reduction of surface-expressed channel. Furthermore, a population of internalized Kv1.5 is recycled back to the plasma membrane in a Rab4- and Rab11-GTPase-dependent manner. This defines an important role for internalization and recycling in the regulation of steady-state levels of surface-expressed Kv1.5, and identifies trafficking proteins such as the Rab-GTPases as potential points of regulation of Kv1.5 current.

Posttranslational Regulation

As previously mentioned, although the notion that posttranslational modification may play an important role in regulation of Kv1.5 function was noted when the channel was first cloned and sequence analysis revealed the presence of putative PKC and PKA phosphorylation sites, as well as a glycosylation site (70), sources of posttranslational modification of Kv1.5 are just beginning to emerge.

Kv1.5 has not directly been demonstrated to be phosphorylated by PKA or PKC, however PKA and PKC-mediated phosphorylation of Kvβ accessory subunits has been shown to effect Kv1.5 / -subunit interactions and Kv -mediated inactivation properties (54,109). Activation of PKA led to a reduction in Kvβ1.3-induced inactivation of Kv1.5 that was dependent on a PKA phosphorylation site located on the N-terminus of Kvβ1.3. Similarly, inhibition of PKC prevented Kvβ1.2 or Kvβ1.3 from conferring N-type inactivation properties to Kv1.5, or from shifting the voltage-dependence of activation of Kv1.5 (54).

Kv1.5 has been shown to be directly phosphorylated by Src tyrosine kinase, and this phosphorylation dramatically inhibited Kv1.5 current density (110). Src tyrosine kinase was shown to bind to Kv1.5 via two, repeated Src homology 3 (SH3) domains on the N-terminus of Kv1.5, and this association resulted in the phosphorylation of the channel. Furthermore, co-expression of Src tyrosine kinase almost completely inhibited total outward current of Kv1.5 in transfected HEK-293 cells.

In addition to phosphorylation, Kv1.5 currents are posttranslationally modified by N-linked glycosylation (103) and S-acylation (111). Mutation of serine 292 in human Kv1.5 results in a loss of channel glycosylation along with a depolarizing shift in the voltage-dependence of activation and reduction in current density of Kv1.5 (103). These findings support the hypothesis that glycosylation in the Golgi regulates successful expression of Kv1.5 on the plasma membrane. S-acylation involves the posttranslational,

reversible linkage of fatty acids to target proteins via a thioester bond. Kv1.5 contains numerous intracellular cysteine residues and has been found to be S-acylated on both its N- and C-termini. Pharmacological inhibition of this modification resulted in intracellular accumulation of Kv1.5 and targeting of the channel for proteasomal degradation, along with a dramatic reduction in total Kv1.5 current density. From pulse-chase experiments, this S-acylation was found to take place early in the biosynthetic pathway before the channel was glycosylated. These findings present the possibility that S-acylation may play an important role in regulating the trafficking of only properly-assembled Kv1.5 to the plasma membrane (111).

Together, the above findings demonstrate that Kv1.5 current is carefully regulated throughout the lifecycle of the channel, and that modulation of these regulatory mechanisms can have important functional consequences on Kv1.5-mediated currents. Thus, these points of regulation offer potential targets for pharmacological control of Kv1.5. As introduced above in the Overview and as detailed in Chapter II, we have identified that a number of Kv channels, including Kv1.5, contain evolutionarily conserved, canonical receptor sites for posttranslational modification by Small Ubiquitin-like Modifier (SUMO) proteins. Based on sequence analysis and reports that the Kv1.5-interacting protein KChAP belongs to the PIAS family of SUMO E3 ligases (100), we suspected that these canonical motifs were functional, and that posttranslational modification by SUMO may offer a previously unrecognized source of Kv1.5 regulation. In the following section, I will thus introduce some fundamental aspects of post-translational modification by SUMO proteins.

SUMO MODIFICATION

Introduction

Small Ubiquitin-like Modifier (SUMO) proteins are approximately 11 kDa proteins that share a similar structural fold and enzymological pathway of conjugation to that of ubiquitin (112-115). Although posttranslational modification of target proteins by SUMO was only recently discovered in 1996 (116,117), it has rapidly been found to be an essential process in *S. cerevisiae (118)*, *C. elegans (119)*, *Arabidopsis thaliana (120)*, and mice (121) and has served as the founding class of a growing group of ubiquitin-like (Ubl) proteins including Nedd8, Apg12, Hub1, FAT10, and ISG15 (122). The amino acid sequence, surface charge distribution, and functions of SUMO are distinct from those of ubiquitin and other Ubl proteins, however, and represent a unique and widespread form of posttranslational regulation.

Four members of the SUMO family (SUMO-1-4) have been identified in humans. While SUMO-1-3 are ubiquitously expressed, SUMO-4 is limited mainly to the kidney, lymph nodes, and spleen (123). All four SUMO members are initially expressed in an immature pro-form that cannot be conjugated to target proteins. SUMO-specific proteases, which are discussed below, cleave a 2-11 amino acid sequence on the extreme C-terminus of this pro-form to expose a di-glycine motif that is necessary for conjugation. Interestingly, it appears that SUMO-4 may be unable to be processed to its mature, conjugation-competent form, instead persisting in its pro-form (124). The functional significance of SUMO-4 thus remains to be understood.

Conjugation Pathway

Preparation of SUMO for modification of proteins involves two steps carried out by specific E1 activating (SAE1/SAE2) (125) and E2 transfer (UBC9) (126) enzymes. Although analogous to ubiquitin E1 and E2s, SAE1/SAE2 and UBC9 are SUMO-specific, and there is no functional overlap with the ubiquitin conjugation pathway. After processing, SAE1/SAE2 adenylates the C-terminal glycine of SUMO in an ATP-dependent manner, which can then be linked to a catalytic cysteine residue on SAE2 via a high-energy thioester bond. The SAE2-loaded SUMO is then transferred to the catalytic cysteine residue of UBC9 via the formation of a new thioester linkage. UBC9 interacts directly with the core SUMOylation consensus sequence in the target protein to catalyze the formation of an isopeptide bond between the C-terminal SUMO glycine and the ε-amino group of the target lysine (Figure I-4).

Although SUMOylation can be achieved without E3 activity *in vitro* (unlike ubiquitination), proteins like RanBP2 (127,128), the polycomb family member Pc2 (129), and members of the PIAS family (PIAS-1, -2, -3, -x-α, -x-β, and –y) (127,130-134) have E3-like activity and enhance SUMO conjugation, potentially by stabilizing the tripartite interaction between Ubc9, activated SUMO, and the target protein. Interestingly, and as mentioned above, the K^+ channel associated protein KChaP, which interacts directly with the N-terminus of Kv1.5 and serves to modulate the current density of many Kv channels (99), has recently been identified as PIAS3β (100). This clearly raises the intriguing possibility that PIAS3 may serve to regulate the SUMO modification of Kv1.5 described in this thesis.

As with other ubiquitin-like proteins, SUMOylation is reversible, and specific cysteine proteases such as Ulp-1 and -2 in yeast, and SENP-1-3, and -5-8 in mammals release the SUMO moiety (135-137). It appears that the subcellular localization of these proteases may vary, potentially allowing each to regulate distinct processes in the cell.

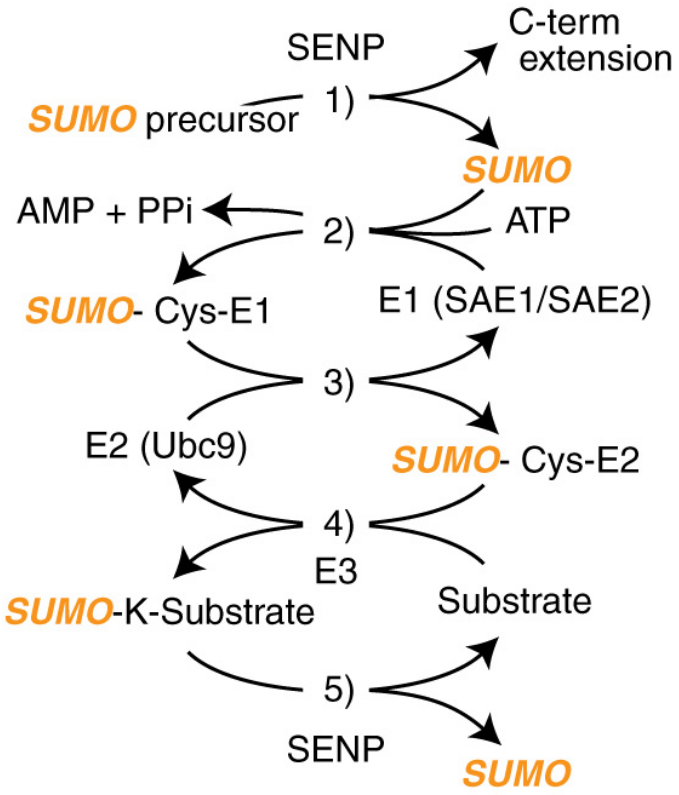

Figure I-4. The SUMO Conjugation Pathway

SENP-1, for example, can traffic between the nucleus and cytoplasm (135,138); SENP-2 is detected at nuclear pores, at nuclear bodies, or in the cytoplasm (136,139,140); SENP-3 is nucleolar (141); and SENP-6 is both nuclear and cytoplasmic (137,142). In addition to the isopeptidase activity of SUMO-specific proteases, SENPs also have C-terminal hydrolase activity necessary for the processing of immature pro-form SUMO to conjugation-competent SUMO (122). While E1 and E2 conjugating enzymes are distributed in all tissues, differential expression of E3s and SENPs has been hypothesized to offer an additional layer of regulation of SUMO modification.

The Consensus SUMOylation Motif

Through the analysis of multiple SUMO modified proteins, a minimal consensus SUMOylation motif has been identified as ψ-K-X-E/D (where ψ is a large, hydrophobic residue and X is a non-conserved residue) (143). Interestingly, a detailed description of these properties, including the additional feature that this core sequence is often flanked by proline or glycine residues, was already established in the definition of Synergy Control Motifs (SCMs). These regulatory motifs, found in many transcription factors, were initially defined by our group (144) and soon afterwards shown to function as sites of SUMOylation (145,146). Many groups, including ours, have shown that SUMOylation can be abolished by a lysine-to-arginine mutation and drastically reduced by mutations to the rest of this consensus sequence (134,146). We have further determined in our analysis of transcription factor SUMOylation that substitution of the Pro/Gly residues flanking the core SUMOylation sites with amino acids other than glycine leads to a reduced interaction with Ubc9 and a concomitant reduction in SUMOylation (unpublished data). Structural analysis indicates that these residues likely serve to facilitate exposure of the core SUMOylation site to the conjugation machinery. Interestingly, this idea that flanking residues to the core SUMOylation motif can play

important roles in facilitating Ubc9 binding appears to be a common theme. Analysis of the transcription factors ELK1 (147), MEF2A (148), and GATA1 (148), has revealed that inclusion of a local negative charge just downstream of the core motif may serve to stabilize interactions with Ubc9, as well. In the case of ELK1, this negative charge is produced by a cluster of glutamic acid and aspartic acid residues, whereas in the case of MEF2a and GATA1, this charge is provided by the phosphorylation of a serine residue just C-terminal to the core motif.

The Function of SUMOylation

As with other posttranslational modifications, the functional consequence of SUMOylation varies greatly depending on the specific target protein. Since the discovery of SUMO just over ten years ago, well over 100 targets have now been identified, and this modification has been found to alter properties as diverse as the stability, subcellular localization, trafficking, and activity of its substrates. Recently, it has begun to emerge that a common mechanism by which SUMOylation is able to regulate such a diverse array of effects may be by its ability to modulate inter- and intramolecular interactions (122). In addition to the covalent interaction between its C-terminal glycine and the target lysine of its substrate, structural and scanning mutagenesis analyses by our group and others have revealed that a well-conserved pocket contributed by residues emanating from the β2 strand and α1 helix of SUMO provides a surface for interactions with many other target proteins. The structures of a PIASx peptide (149), thymine DNA glycosylase (150), and RanBP2 (151) in complex with SUMO via this surface have all recently been solved. Furthermore, a comprehensive alanine-replacement analysis of SUMO2 surface residues by our group (152) has revealed that disruption of four key basic residues (K33, K35, K42, and R50) that surround this pocket dramatically affects the ability of SUMO2 to inhibit transcription. This is a key finding,

as it suggests that the functional consequence of SUMO on transcriptional inhibition is dependent not on the intrinsic properties of SUMO itself or on the SUMO-modified transcription factor, but instead on the successful recruitment of other regulatory complexes to the SUMO-modified factor.

It should be mentioned that there are examples of SUMOylation inducing conformational changes in its covalently-bound target protein. The most well-defined example of this is the SUMOylation of the DNA repair enzyme thymine DNA glycosylase (TDG). Upon SUMO modification, TDG undergoes a conformational change that alters its affinity for DNA and thereby allows it to be released from its bound DNA. This conformational change, however, is mediated by intra-molecular binding of a region of TDG to the β2-strand/α1-helix groove of the covalently-conjugated SUMO (150). Thus, electrostatic interactions with this hydrophobic groove seem to be critical to SUMO function both in recruiting intra- and intermolecular binding partners.

SUMO-interacting/binding motifs (SIM/SBMs) are just beginning to be recognized in proteins that are able to interact with this effector groove on SUMO. Analysis of p73α (153), PML (149,153,154), Daxx (155), SAE2 (149), PIAS E3 (149), RanBP2 (149,151), thymine DNA glycosylase (150), and RNF4 (156), among others, has revealed that one consensus SIM/SBM motif is the loosely-defined sequence V/I-X-V/I-V/I (149). In many proteins, this critical hydrophobic core is also surrounded by acidic residues. Importantly, disruption of SIM/SBMs in these SUMO-interacting proteins has been found to interrupt function. For example, disruption of the SIM/SBM in PML has been found to interrupt the formation of PML bodies (154). Clearly, this short, degenerate sequence can be found in numerous proteins, so future work to determine added specificity of SIM/SBMs will be needed. However, the idea that SUMO-interacting candidate proteins might soon be able to be identified through a bioinformatics screen is exciting. It has long been known that similar, non-covalent interactions are critical to the function of posttranslational modification by ubiquitin. As

currently there are over 16 distinct ubiquitin-binding domain (UBDs), it is clearly very possible that many more SIM/SBMs will soon be determined (157,158).

Extranuclear Targets of SUMOylation

The overwhelming majority of targets of SUMOylation that have been described to date are located in, or near the nucleus. These proteins regulate transcription, DNA repair, RNA processing, genome integrity, chromatin modification, and nucleocytoplasmic transport, among many other functions. Recently, however, a role for SUMO modification outside of the nucleus has begun to emerge. Numerous global proteomics analyses have repeatedly identified non-nuclear targets for SUMOylation. Furthermore, the SUMOylation conjugation machinery has long been known not to be limited to the nuclear compartment. Recent reports have identified important roles for SUMOylation in the cytoplasm, mitochondria, endoplasmic reticulum, and plasma membrane (122,159). Clearly, of particular interest to me during this thesis has been the idea that the SUMOylation machinery is functional and plays an important regulatory role for integral membrane proteins at the plasma membrane.

The glucose transporters GLUT1 and GLUT4 were the first plasma membrane proteins shown to be SUMOylated (160). Using a yeast-two-hybrid assay, it was initially found that Ubc9 interacts with the intracellular C-terminus of GLUT4. This interaction was verified using an Ubc9 pulldown assay for both GLUT1 and GLUT4 transporters and localized to an 11-amino acid sequence on the C-termini of both transporters. Both transporters were later found to be SUMO modified using Western Blotting techniques. Interestingly, stable overexpression of Ubc9 in skeletal muscle cells, resulted in a 65% reduction in the expression of GLUT1 and an almost 8-fold increase in the expression of GLUT4. This led to the important functional consequence of decreased basal glucose transport, which is mediated by the GLUT1 transporter, and significantly increased

insulin-stimulated glucose transport mediated by the GLUT4 transporter. Also interesting from this study was the finding that in 3T3-L1 adipocytes, L6 skeletal muscle myoblasts, and terminally differentiated myotubes, endogenous Ubc9 was present (although to differing extents) in subcellular fractions from the plasma membrane, high- and low-density microsomal membranes, nuclei/mitochondria, and (to a very low extent) the cytosol. This report established that Ubc9 is present at the plasma membrane, and that SUMO modification can have important functional consequences for plasma membrane proteins.

Following this initial discovery, multiple metabotropic glutamate receptor subunits and the ion channels K2P1, Kv1.5 (from the work in this thesis), GluR6, and GluR7a/b have been identified as targets for SUMO modification. Metabotropic glutamate receptor (mGluR) subunits are a family of G-protein coupled receptors (GPCRs) that bind glutamate and modulate the activity of nearby ion channels to effect pre- or postsynaptic activity in neurons. To date, mGluR2, 4, 7a, 7b, 8a, 8b (161,162), and the excitatory amino acid transporter 2 (EAAT2) (163) have all been shown to be SUMO modified. The Group III presynaptic mGluR8a and b were the first of these receptor subunits found to be SUMOylated (162). Using a yeast-two-hybrid assay of an adult rat brain cDNA library, it was identified that the cytoplasmic C-termini of mGluR8a and b interact with SUMO1, Ubc9, PIAS1, PIASγ, and PIASxβ. While verification of these interactions using recombinant protein pull-down assays proved difficult, recombinant PIAS1 was found to bind mGluR8a and b robustly, and this interaction was mapped to a consensus SUMOylation motif on the C-termini of both receptor subunits. Interestingly, PIAS1 was also found to bind to all six members of the Group III glutamate receptor subunits (mGluR4, -6, -7a/b, and -8a/b). Using Western Blot analysis, mGluR8a and b were found to be SUMO modified, and this modification could be disrupted by mutation of the target lysine in the identified consensus SUMOylation motifs. Although not as extensively characterized, mGluR2, 4, 7a, and 7b have since been shown to serve

as substrates of SUMO modification using a bacterial SUMOylation assay. The functional consequences of the SUMO modification these metabotropic glutamate receptor subunits is still unknown and awaits further study.

Recently, a C-terminal fragment of the EAAT2 has been shown to be SUMO modified in a mouse model of amyotrophic lateral sclerosis (ALS) (163). EAAT2 is a Na^+-dependent glutamate transporter in glial cells that has been found to have decreased activity in patients with ALS or in a mutant superoxide dismutase 1 (SOD1) mouse model of ALS. Interestingly, a source of this inhibition has been found to come from the caspase-3 cleavage of EAAT2 at a defined locus on the C-terminus of the transporter in response to mutant SOD1-induced oxidative stress. Western Blot analysis of this C-terminal fragment of EAAT2 (called the CTE) in spinal cord homogenates of mutant SOD1 mice revealed that the fragment migrated at a higher molecular weight than expected. Based on the observation that the CTE contained a consensus SUMOylation motif and that this high migration seemed not to be due to oligomeric aggregation, the CTE was tested for SUMO modification. The CTE could be immunoprecipitated from mutant SOD1 mouse spinal cord homogenates using a monoclonal anti-SUMO1 antibody. Additionally, the CTE was found to interact with Ubc9 and SUMO1 in a yeast-two-hybrid assay, and to be SUMOylated *in vitro*. Furthermore, SUMOylated CTE, but not unmodified CTE was targeted to promyelocytic leukemia (PML) nuclear bodies. Based on this observation and that PML bodies are known to regulate transcription, the authors propose the idea that EAAT2, through SUMO-mediated targeting of the CTE to PML bodies, may contribute to the pathology of ALS. While this example of the SUMO modification of a glutamate transporter is unique, it is clear that SUMOylation of metabotropic glutamate receptors, in general, is emerging as a widespread and likely important regulatory mechanism in pathological and pathophysiological states.

SUMOylation of Ion Channels

Four ion channels, including the K^+ leak channel K2P1 (164), Kv1.5 as described in this thesis (165), the kainate receptor subunit GluR6 (166), and the ionotropic glutamate receptor subunits GluR7a/b (161) have been shown to be SUMO modified. While GluR7a/b has simply been shown to serve as a substrate for SUMO modification using a bacterial SUMOylation assay, SUMOylation of K2P1, Kv1.5, and GluR6 has been characterized to various extents and shown to have important regulatory consequences on the currents mediated by these channels.

The potassium leak channel K2P1 was the first ion channel found to be SUMO modified (164). K2P channels are a family of potassium-selective pores that influence the resting membrane potential and activity of excitable cells by remaining open across a physiological range of transmembrane potentials. Over fifteen K2P channels have been identified and characterized since the original discovery of K2P0 in *Drosophila melanogaster*. These channels have the common feature of being composed of four transmembrane segments with two pore-forming "P" loops. While many members of the K2P family do not encode measurable currents in over-expressed systems, many others have been found to encode outward K^+ currents dynamically regulated by a number of mechanisms including phosphorylation, pharmacological outer pore blockers, lipid interactions, G-protein interactions, and mechanical stretch. A major unanswered question in the field involved the observation that while mRNA encoded by the founding mammalian member of these channels, K2P1, could be detected in the heart, brain, and kidney, currents from this channel could not be measured. In 2005, Rajan et al. reported that while exogenous K2P1 was abundantly expressed on the plasma membrane of *Xenopus* oocytes and Cos7 cells, current through the channel could not be detected due to constitutive SUMOylation of lysine 274 on the C-terminus of the channel. Upon disruption of this SUMOylation, either by mutation of the receptor lysine, or treatment

with SENP, a K^+-selective, pH-sensitive, openly rectifying, macroscopic current could be detected.

Confocal microscopy of oocytes overexpressing GFP-tagged Ubc9 revealed that GFP-Ubc9 displayed a uniform, non-polarized distribution on the plasma membrane of these cells. Interestingly, when co-expressed with K2P1, which was found to have expression polarized to the plasma membrane of the animal pole of oocytes, GFP-Ubc9 expression reorganized and was restricted only to the animal pole, suggesting an interaction between the channel and Ubc9. Endogenous Ubc9 was found to display a similar, uniform expression on the plasma membrane that could be polarized upon expression with K2P1. Using a yeast-two-hybrid assay, Ubc9 was confirmed to interact with the C-terminus of K2P1. These observations are striking because they clearly demonstrate that components of the SUMO conjugation machinery are present at the plasma membrane of oocytes, and suggest that targets of SUMO modification may be able to actively recruit this machinery while at the membrane.

Co-expression of HA-tagged SUMO1 and K2P1 in oocytes revealed that K2P1 appears to be a target for SUMO modification. Immunoprecipitation of K2P1 and subsequent separation by SDS-PAGE and Western Blotting revealed the presence of ~45 kDa protein species that was immunoreactive to antibodies directed against K2P1, HA-SUMO1, and endogenous SUMO1. Furthermore, disruption of K2P1 lysine 274 shifted the migration of this species to an apparent lower molecular weight and disrupted the immunoreactivity to either anti-HA or anti-SUMO1 antibodies. This suggests that K2P1 can be SUMO modified, and that this modification takes place at acceptor lysine 274. Similarly, treatment of immunoprecipitated K2P1 with wt SENP-1 caused the same shift in molecular weight, and the channel was no longer found to be immunoreactive to anti-HA-SUMO1 antibodies. This effect was shown to be dependent on the catalytic activity of SENP, as a C603S mutation that disrupts the activity of the protease, had no effect on

K2P1, but surprisingly, it was not investigated whether this effect of SENP was dependent on the presence of K2P1 lysine 274.

Disruption of K2P1 SUMOylation, either by mutation of acceptor lysine 274 or co-expression of wt channel with SENP-1, was further found to unmask the silenced current of K2P1 and reveal a K^+-selective, open rectifying, macroscopic current. This was observed both in oocytes and Cos7 cells, and found to be dependent on the catalytic activity of SENP as C603S SENP-1 mutant had no effect on K2P1 current. In a striking set of experiments, this effect of SUMOylation was shown to be acutely reversible. Single-channel currents could be recorded from patches of membrane from oocytes co-expressing WT K2P1 and SENP-1. When these patches were excised in inside-out mode and inserted into naïve oocytes expressing endogenous SUMOylation machinery, these single-channel currents were suppressed within 13 seconds, suggesting that acute exposure of the channel to SUMO could serve to block channel conductance. When this same patch was withdrawn from the cell and re-inserted into an oocyte over-expressing SENP-1, single-channel current could be restored within 18 seconds, arguing that the effects of SUMOylation are also acutely reversible. Furthermore, K274E K2P1 current was not inhibited by insertion into naïve cells, suggesting that the suppressive effect of these cells on K2P1 current was dependent on an intact SUMOylation motif. Similarly, insertion of wt channel in oocytes expressing C603S SENP-1 did not induce current, suggesting that the activating effects of SENP were dependent on its catalytic activity. These sets of experiments were remarkable as they dramatically presented that SUMOylation could acutely control the function of ion channels expressed on the plasma membrane.

It should be mentioned at this point, that many of the observations in this initial study have since been challenged and are highly intriguing. Specifically, Feliciangeli et al (167), were unable to detect any SUMOylation of K2P1 using similar Western Blotting techniques, instead detecting only the lower migrating K2P1 species that Rajan et al.

identify as unmodified channel. Additionally, while Feliciangeli et al. found that the K274E mutation increased K2P1 current as described, they found that the K274R mutation, which should also inhibit channel SUMOylation while preserving the basic charge of this residue, was unable to unmask K2P1 macroscopic currents. These studies were undertaken with a slightly different construct of K2P1 that had a *Heteractis crispa* red (HcRed) fluorescent protein fused to the N-terminus of the channel. However, the authors argue that there is no evidence that this should affect the results of the study or the ability of the channel to be SUMO modified. In their original report, Rajan et al. described, but did not present, data in which they found that mutations of lysine 274 to arginine, glutamine, alanine, and cysteine all led to macroscopic current in oocytes. They also presented data from Cos7 cells that demonstrate that the K274R mutation leads to the induction of K2P1 macroscopic current, although only to about one-fourth the extent of that induced by the K274E mutation. This point is critical, as if the effects of the K274E mutation can be assigned to disruption of the SUMOylation of the channel, the K274R mutation must also be found to be able to recapitulate this effect. However, if the effect of the K274E mutation on the channel is due instead to a charge effect by replacing the positively-charged lysine with a negatively-charged glutamic acid, the K274R mutation would be predicted to have a much less severe effect on K2P1 current density. Future experiments will clearly have to be conducted to resolve this issue.

Several other aspects of the SUMOylation pattern of K2P1 are highly intriguing. The overwhelming majority of known SUMOylation targets are modified with very low stochiometries. A common feature of SUMOylation appears to be that this modification can have significant functional effects on target proteins even though only 1-5% of the total cellular target protein normally appears to SUMO modified at any given time. It has been speculated that SUMOylation of target proteins may be a very transient process, thus allowing for only a very small percentage of target protein to be captured in the modified state using biochemical techniques. However, this phenomenon represents a

major, unanswered conundrum in the SUMOylation field. The finding that K2P1 appears *exclusively* SUMOylated at any given time during basal conditions would thus be exceptional. The observation that Ubc9, even when over-expressed, can be completely redistributed to membrane K2P1 would imply that the high rate of steady-state K2P1 SUMOylation may be a result of an unequal balance between Ubc-9-mediated SUMOylation and low SENP-mediated deconjugation. What is surprising about this, however, is that the same quantitative SUMOylation of K2P1 was observed in Cos7 cells, in which numerous targets of SUMOylation, including the plasma membrane-expressed Kv1.5 described in this thesis, are found to have low stochiometries of SUMOylation. This would imply that the complete SUMOylation of K2P1 is not merely caused by a lack of SUMO proteases either in the cell or at the plasma membrane, specifically. As the conjugation of SUMO to target proteins is an ATP-dependent process, this finding that K2P1 is quantitatively SUMOylated, presumably due to a steady-state of active SUMOylation and deSUMOylation, raises the interesting point that this process would require a significant expenditure of energy on the part of the cell. Feliciangeli et al. have noted this unusual finding and have reported that, in their hands, again using the slightly different HcRed-K2P1 fusion construct, they are unable to detect any SUMOylation of the channel, let alone stochiometric amounts of it.

One last unique finding of K2P1 SUMOylation involves the identified SUMOylation motif. The motif was identified as L-K-K-F, which does not conform to the canonical ψ-K-X-E/D description of SUMOylation motifs (where ψ is a hydrophobic residue, and x is any residue). While there are numerous examples of proteins being targeted by SUMOylation at non-canonical SUMOylation motifs, this observation begs the question if there is any significance to the fact that this is an altered motif. The properties of the sequence surrounding the acceptor lysine in SUMOylation motifs are thought to affect Ubc9 binding dynamics to the target protein. Is it thus possible that this non-canonical motif may increase the affinity of Ubc9 for K2P1 and provide a possible

mechanism for the observed quantitative SUMOylation of the channel? The authors make the point that numerous proteins in the nucleus are SUMOylated at non-canonical motifs, thus suggesting that this is not a distinctive feature of extra-nuclear or plasma membrane-expressed target of SUMOylation. However the significance of this newly-identified motif remains to be more fully understood.

While there are a number of distinct features of the reported K2P1 SUMOylation, and the SUMOylation patterns and role of the K274E mutation clearly remains controversial, this report was the first to identify a potential role for the SUMOylation of ion channels. This report also laid the groundwork for further exploration of ion channel SUMOylation by confirming the presence of components of the SUMOylation machinery at the plasma membrane. As an interesting note, this original report was published as we were actively investigating the role of SUMOylation in regulating the currents of Kv1.5. We reported our findings, which represented the first description of the SUMOylation of a voltage-gated ion channel, in 2006, and then, soon after, in 2007 the GluR6 kainate receptor was reported to be regulated by SUMO modification, as described below.

The kainate receptor GluR6 subunit is widely expressed throughout the brain, being most enriched in the hippocampus. By regulating neuronal excitability at both pre- and post-synaptic sites, GluR6 is thought to play important roles in synaptic plasticity, learning and memory, and epilepsy. As is the case with other kainate receptor (KAR) subunits, GluR6 subunits are composed of an extracellular N-terminus, three transmembrane segments and a P loop, and an intracellular C-terminus. These subunits can homo- or heterotetramerize to form a functional channel that is able to conduct Na^+ or K^+ ions in response to extracellular binding of the excitatory neurotransmitter glutamate.

GluR6 was initially identified as a potential substrate of SUMOylation when it was found to interact with Ubc9 and PIAS3 in a yeast-two-hybrid screen (166). These interactions were verified using co-immunoprecipitation studies and mapped to a 13-

amino acid sequence that contains a consensus SUMOylation motif on the C-terminus of the channel. Interestingly, this consensus SUMOylation motif was fond to be absent in all other KARs subunits. In rat brain extracts, a slow-migrating, GluR6-immunoreactive band was detected by Western Blot analysis and found to be sensitive to the SUMO protease inhibitor N-ethylmaleimide (NEM). Furthermore, GluR6 was shown to co-immunoprecipitate with SUMO1 in cultured hippocampal rat neurons, and WT, but not K886R GluR6 was able to serve as a substrate for SUMO modification in a bacterial SUMOylation assay.

GluR6 is known to internalize from the cell surface in response to stimulation by kainate or N-methyl-D-aspartate (NMDA) (168). This agonist-mediated endocytosis was found to be dependent on SUMO modification of the channel (166). Application of kainate or glutamate led to the internalization of GluR6, as expected, but also led to a rapid increase in the amount of channel that could be recovered in the SUMOylated state. This agonist-induced stimulation of GluR6 SUMOylation occurred rapidly, as it could be detected in less than one minute and reached maximal conjugation (at three times basal levels) within ten minutes. The effect was specific to GluR6 as kainate did not induce global increases in cellular SUMOylation. Similarly, the effect was also agonist-specific as NMDA was able to induce strong GluR6 internalization, but was found to have no effect on channel SUMOylation.

In immunocytochemical studies of cultured hippocampal neurons, co-localization of internalized GluR6 with SUMO-1 dramatically increased with time when treated with kainate, but showed no effect when treated with NMDA. Moreover, transduction of these cells with SENP-1 dramatically inhibited this kainate-induced GluR6 internalization, but had no effect on NMDA-induced internalization. Importantly, a catalytically-inactive mutant (C603S) of SENP-1 was found to have no effect on either kainate- or NMDA-induced endocytosis. Consistent with the idea that agonist-induced SUMOylation of GluR6 leads to the rapid internalization of the channel, SUMOylation of surface-

expressed GluR6 could not be detected unless KAR endocytosis was inhibited by treatment with sucrose. Furthermore, disruption of the consensus SUMOylation motif with the K886R mutation led to a dramatic loss of kainate-induced internalization in Cos7 cells.

The SUMO modification of GluR6 subunits was found to have important functional consequences on the channel. KAR-mediated excitatory postsynaptic currents (KAR-EPSCs) were recorded from CA3 neurons at the mossy fiber synapse in hippocampal slices. When recombinant SUMO-1 was loaded in the recording pipette and allowed to diffuse into the patched neurons, the amplitude of KAR-EPSCs was found to decrease rapidly, reaching maximal inhibition within approximately five minutes of break-in. Conversely, when SENP-1 was allowed to diffuse into these CA3 neurons, KAR-EPSC amplitude rapidly increased within a similar time period. Importantly, no changes were detected in KAR-EPSC amplitudes when either C603S SENP-1 or conjugatable-deficient SUMO-1-ΔGG was diffused into these cells, and AMPA receptor-mediated EPSC amplitudes recorded from the same cells were found to be unaffected by SUMO-1 or SENP-1 treatment. Taken together, these data provide strong evidence that upon kainate or glutamate-induced stimulation, GluR6 is SUMOylated and subsequently internalized. This SUMO-mediated removal of channel from the plasma membrane surface has important functional consequences on the post-synaptic amplitude of KAR-mediated EPSCs, and thus may have a potential role in the processes of learning and memory.

There are some important observations that can be made from these first reports of SUMO modified ion channels. The first observation involves the finding that the SUMO conjugation machinery appears accessible to integral membrane proteins on the plasma membrane. As already mentioned, studies of GLUT1 and GLUT4 transporters, as well as of the K2P1 channel identified that Ubc9 is expressed at the plasma membrane. Additionally, immunocytochemical studies of cultured hippocampal neurons in the

GluR6 study, demonstrated that endogenous SUMO, Ubc9, and PIAS3 are all present not only in the nucleus, but also throughout dendrites and at PSD95-positive synapses. Future studies that characterize the subcellular localization of the E3 ligases and SENP proteases may offer insights into additional layers of regulation of SUMO modification at the membrane. For example, SENP-1 has previously been found to be primarily expressed in the nucleus. It will thus be interesting to determine if similar studies using other SENP isoforms that are known to be cytoplasmic might acutely modulate GluR6 and K2P1 in different ways.

The finding that both K2P1 and GluR6 can be acutely regulated by SUMO and SENP may also provide some information as to where in the cell ion channel SUMOylation occurs. One could imagine that SUMOylation of K2P1 or GluR6 could take place at the plasma membrane or during the synthesis, trafficking, or recycling of the channel. Transient SUMOylation of GluR6 during its synthesis, for example, could induce conformational changes or promote assembly with other binding partners that could persist throughout the lifecycle of the channel and affect its function at the plasma membrane. This does not seem to be the case, however, since SUMO and SENP were found to be able to regulate both GluR6 and K2P1 channel activity within seconds to minutes of application. According to this time frame, it seems more likely that SUMOylation affects channels in or near the plasma membrane where this modification can have immediate effects on function.

It has been suggested that SUMOylation may serve to inhibit ion channel activity (122) since SUMOylation reduced current in both K2P1 and GluR6, while SENP served to increase these currents. This generalization seems somewhat premature, however, since the mechanisms by which SUMO inhibits K2P1 and GluR6 are very different. Thus, while GluR6 current was inhibited by SUMOylation-promoted endocytosis, internalization does not appear to be involved in SUMOylation-induced inhibition of K2P1-mediated currents. This suggests that SUMO may be acting by different

mechanisms in each channel. Indeed, as described in this thesis, SUMO does not seem to inhibit the activity of Kv1.5 directly, but rather to protect the channel from inactivation. Thus, while further work may reveal a common mechanism for channel SUMOylation—by promoting interactions with other binding partners, or inducing conformational changes, or modulating the local electrostatic environment, for example—the functional consequences of SUMOylation will most likely be dependent on the individual target channel.

Finally, these studies provide some insight into the regulation of SUMO modification of ion channels. GluR6 SUMOylation was clearly found to be induced by treatment with certain known agonists of the channel. The mechanism by which agonist binding regulated SUMOylation of the channel is not clear, however. It seems reasonable that conformational changes induced by ligand binding might serve to make the SUMOylation motif of the channel more accessible to SUMO conjugation machinery. Alternatively, such a conformational change could serve to stabilize transient SUMOylation of the channel by inhibiting access by SUMO proteases. Interestingly, treatment of oocytes with a broad range of volatile anesthetics, long-chain free fatty acids, lysophospholipids, and classical regulators of kinases and phosphatases was shown to have no effect on the constitutive SUMOylation status of K2P1. This suggests that regulation of channel SUMOylation is specific and potentially dependent on distinct conformational changes that modulate the accessibility of the SUMOylation motif by the SUMO machinery. Clearly, further work is needed to fully understand this regulatory mechanism.

In summary, although only recently appreciated, reversible control of ion channels through SUMOylation constitutes a unique and likely widespread mechanism for adaptive tuning of the electrical excitability of cells. In the following chapters, I will describe our findings that Kv1.5 serves as a target for SUMOylation, and that this

modification can regulate the inactivation properties and amplitude of the channel in heterologous systems and isolated mouse and human primary cardiomyocytes.

CHAPTER II: SUMO MODIFICATION REGULATES INACTIVATION OF THE VOLTAGE-GATED POTASSIUM CHANNEL Kv1.5[1]

INTRODUCTION

The voltage-gated potassium (Kv) channel Kv1.5 mediates the I_{Kur} repolarizing current in human atrial myocytes (12) and regulates vascular tone in multiple peripheral vascular beds (80-87). Understanding the complex regulation of Kv1.5 function is of substantial interest since it represents a promising pharmacological target for the treatment of atrial fibrillation and hypoxic pulmonary hypertension.

Using a bioinformatics approach, we identified that a number of Kv channels contain sequences that conform well to SUMOylation motifs. Kv1.5 contains two consensus SUMOylation sequences that are highly conserved across species and that are located on cytoplasmic regions of the channel. Concurrent with this observation, the Kv associated protein KChAP, which is known to modulate the surface expression and whole-cell current densities of several Kv channels, was identified as a member of the PIAS family of SUMO E3 ligases. This raised the intriguing possibility that the consensus SUMOylation sites that we identified in Kv1.5 may be functional and contribute to a novel form of Kv channel regulation.

Herein, we demonstrate that Kv1.5 interacts specifically with the SUMO conjugating enzyme Ubc9 and serves as a target for post-translational modification by SUMO1, 2, and 3 in Cos7 cells. In addition, purified recombinant Kv1.5 serves as a

[1] With the exception of Figure II-5 and Figure II-6, the data in this chapter have been previously published as "Mark D. Benson, Quiju Li, Katherine Kiekhafer, David Dudek, Mathew Whorton, Roger K. Sunahara, Jorge A. Iñiguez-Lluhí and Jeffrey R. Martens (2007). SUMO Modification Regulates Inactivation of the Voltage-Gated Potassium Channel Kv1.5. Proc Natl Acad Sci USA. 104(6): 1805-10"

substrate in a minimal *in vitro* reconstituted SUMOylation reaction. The SUMO-specific proteases SENP2 and Ulp1 efficiently deconjugate SUMO from Kv1.5 in Cos7 cells and *in vitro*, and disruption of the two identified target motifs results in a loss of the major SUMO-conjugated forms of Kv1.5. Selective analysis of surface-exposed proteins revealed that Kv1.5 is SUMO modified at the plasma membrane with a stochiometry comparable to that of the total cellular pool. Moreover, exposure of Cos7 cells expressing SUMO3, Ubc9, and Kv1.5 to increasing concentrations of hydrogen peroxide demonstrates that oxidative stress may serve an important regulatory role in controlling Kv1.5 SUMOylation. Functional analysis in whole-cell patch clamp electrophysiology revealed that loss of Kv1.5 SUMOylation, either by disruption of the conjugation sites or expression of the SUMO protease SENP2, leads to a selective ~15 mV hyperpolarizing shift in the voltage dependence of steady-state inactivation with no associated effects on the voltage-dependence of activation or total current density.

Together, these data identify a novel role for reversible, post-translational modification by SUMO in the regulation of Kv1.5 inactivation and represent the first report of SUMOylation of a voltage-gated ion channel. Given that a number of voltage-gated ion channels contain well-conserved, consensus SUMOylation motifs, SUMO modification may constitute a unique and widespread mechanism for adaptive tuning of the electrical excitability of cells.

EXPERIMENTAL PROCEDURES

Expression plasmids, cell culture, and in vivo SUMOylation assays

The expression vectors pCDNA3.1 hKv1.5 V5-His and pCDNA3.1 hKv1.5 N556 V5-His consist of the full-length or the first 556 residues of human Kv1.5 inserted at the *Eco*RI and *Xho*I sites of pCDNA3.1 V5-HIS A (Invitrogen). Derivatives bearing K221R,

or K536R substitutions alone or in combination were generated by PCR. pCMV FLAG SENP2 (71-590) was a gift of Dr. Mary Dasso (NIH) and pEGFP-C1 was from Clontech. Other vectors are described in (134). COS-7 cells were maintained ad transfected as described (146). For *in vivo* SUMOylation assays, 1.2 x 10^5 cells were seeded in 6-well plates and transfected with the indicated amounts of expression plasmids. The total amount of DNA per transfection was supplemented to 1.3 µg/well with pBSKS (-). For the data in Figure II-3A, 2 x 10^6 cells were seeded in 10 cm plates and the total amount of DNA was 12 µg/plate. *In vivo* SUMOylation assays were adapted from (134). Cells were harvested 48 h after transfection and homogenized in 300 µl CHAPS lysis buffer (50 mM Na phosphate pH 8.0, 500 mM NaCl, 10 mM imidazole, 1% CHAPS, 20 mM NEM, supplemented with complete EDTA-free miniprotease inhibitor tablets (Roche), 1 tablet/10 ml). After centrifugation (16,000 x g, 15 min), solublized proteins were incubated(1h, 25 °C) with 0.1 ml of Ni-NTA-agarose (Qiagen). The resin was sequentially washed (2 ml, thrice) with buffer 1 (50 mM Na phosphate pH 6.5, 400 mM NaCl, 10 mM imidazole, 0.1% CHAPS), buffer 2 (buffer 1 with 150 mM NaCl, no imidazole, and 8 M urea) and buffer 3 (50 mM Na phosphate pH 8.0, 50 mM NaCl, 0.1% CHAPS). Ulp1 treatment was carried out between washes 1 and 2 and consisted of 1h incubations at 30 °C with 242 pmol of purified GST or GST-Ulp1 after equilibration in buffer 2 without urea. For the data in Figure II-3, the cells were lysed in 750 µl urea lysis buffer (CHAPS lysis buffer with 8 M urea instead of 1% CHAPS and no NEM) and washed in urea buffer 1 (wash buffer 1 with 8 M urea and 0.2% NP-40 instead of 0.1% CHAPS) and NP-40 buffer 2 (wash buffer 2 with no urea and 0.2% NP-40 instead of 0.1% CHAPS). Proteins were eluted by incubating (55 °C, 20 min) in SDS Sample Buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 3.3 mM EDTA, 0.0075% bromphenol blue, 6.5 mM dithiothreitol), resolved by SDS PAGE (7.5%) and processed for immunoblotting using anti-V5 (Invitrogen), anti HA (HA-11, Covance), or anti-FLAG (Sigma) mouse monoclonal antibodies. For surface biotinylation studies, cells

transfected with HA-SUMO3, Ubc9, and Kv1.5-V5-His6 variants as described were washed with PBS at 4 °C (3 times) to remove amine-containing culture media and cells were treated with 2 mM Sulfo-NHS-biotin reagent (Pierce, Rockford, IL) for 1 hour at 4°C with gentle rocking. After the cells were washed thoroughly (3 times) with PBS + 100 mM glycine (to quench and remove excess biotin reagent), cells were lysed in CHAPS lysis buffer and sonicated. After centrifugation (16,000 x g, 15 min), soluble protein was loaded onto either streptavidin-agarose (Pierce) or Ni^{2+}-agarose beads (Qiagen) and incubated at 4°C for 1 hr with rotation. For sequential purifications, the streptavidin resin was washed three times with wash buffer 1 and then eluted with wash buffer 1 + 20 mM DTT for 30 minutes at 4°C. Eluted samples were alkylated by addition of NEM, and loaded onto Ni^{2+}-agarose beads and washed as described above. For oxidative stress studies, 1.2 x 10^5 Cos7 cells were seeded in 6-well plates and transfected with 0.2 μg HA-SUMO3, 0.1 μg Ubc9, and 0.2 μgs WT or K221/536R Kv1.5 as indicated. Forty-eight hours after transfection, cells were treated with 0, 0.1, or 1 mM H_2O_2 in phenol-red-free DMEM media as indicated for 2 hours at 37 °C (5% CO2). Cells were lysed in CHAPS lysis buffer and processed as above. Detection was achieved with goat-anti-mouse IgG peroxidase conjugate (Bio-Rad) and Super Signal West Femto substrates (Pierce). All experiments were performed at least thrice with similar results.

Purification of hKv1.5, protein-protein interaction and in vitro SUMO conjugation assays

Purification of recombinant hKv1.5 was carried out as follows by David Dudek in the Martens laboratory. A recombinant baculovirus for the expression of V5/His-hKv1.5 was generated using the Bac-to-Bac expression System (Invitrogen). Sf9 cells were grown at 27 °C in SF-900 II media and harvested 60 hours after infection. After nitrogen cavitation lysis and clearing of cell debris by centrifugation (1780 x g, 10 min), a membrane fraction containing His-tagged hKv1.5 was isolated by ultracentrifugation at

100,000 x g for 35 min and extracted with 1% dodecyl maltoside (DDM). Solublized membrane proteins were recovered by ultracentrifugation (100,000 x g, 35 min.) and loaded onto a Ni^{2+}-NTA sepharose column equilibrated with 50 mM Tris, pH 7.7, 50 mM NaCl, 0.1% DDM, and protease inhibitors. After washing with 20 column volumes of 20 mM Hepes, pH 8.0, 300mM NaCl, 0.1% DDM, protease inhibitors, and increasing concentrations of imidazole (5, 10, 20, and 40mM), hKv1.5 was eluted with 20 mM Hepes, pH 8.0, 50mM NaCl, 150mM imidazole, 0.1% DDM and protease inhibitors. Fractions containing hKv1.5 were pooled, diluted into 20mM Hepes, pH 8.0 1mM EDTA, 0.1% DDM, and protease inhibitors (Buffer A), and applied to a source Q (HR5/5, Amersham) FPLC anion exchange column. hKv1.5 was eluted using a linear gradient of NaCl in buffer A, concentrated using a MiniCon (Millipore) concentrator and applied to a Superdex 200 HR 10/30 (Amersham) gel filtration column in 20mM Hepes, pH 8.0, 1mM EDTA, 50 mM NaCl, 0.1% DDM, and protease inhibitors. Peak protein fractions were pooled, and hKv1.5 was detected by immunoblot probing for the V5 epitope.

For protein-protein interaction assays, 2 x 10^6 cells were seeded in 10 cm plates and transfected with 5 μg of either pCDNA3.1 Kv1.5 V5-His or pCDNA3.1 Kv1.5 N556 V5-His. Cells were harvested 48 h post-transfection in 750 l CHAPS lysis buffer sonicated, and centrifuged (16,000 x g, 15 min, 4 °C). 4.5 l of supernatant was processed as described (134) using buffers supplemented with 0.1% DDM. Samples were resolved by SDS-PAGE and processed for V5 epitope immunoblotting. *In vitro* SUMOylation assays were conducted as described (146) using purified hKv1.5 V5-His preparations as substrate. Reactions were carried out at 30 °C for 2h. Samples were resolved by SDS-PAGE, and processed for V5 epitope immunoblotting.

Electrophysiology

For electrophysiological studies, 1 x 10^6 cells were seeded in 35 mm plates and co-transfected with 0.1 μg each of pEGFP-C1 and the indicated pCDNA3.1 hKv1.5 plasmid. Electrophysiological recordings and analysis were conducted as previously described (86). The current-voltage (I-V) relationships and activation curves were measured using 250 ms voltage-clamp pulses applied in 10 mV steps between -80 mV and + 60 mV. Current was measured at the end of the 250 ms depolarization to obtain the steady-state I-V relationships. Tail current amplitude immediately following the capacitive transient was measured to obtain the voltage dependence of activation curves. Inactivation was measured using a 5s conditioning pulse applied in 10 mV steps from -60 mV to +40 mV followed by a 500 ms test pulse at +30 mV. Current was measured at the end of the 500 ms test pulse. Normalized activation and inactivation curves were fitted to the Boltzmann equation and results are expressed as mean +/- standard error. Comparison of V_{50} values was carried out with an F test applied to global fits of pooled vs separate sets.

RESULTS

Kv1.5 harbors conserved SUMOylation motifs and interacts with Ubc9

Scanning of the human Kv1.5 sequence using a search profile based on functionally characterized synergy control motifs in transcription factors identified two high-scoring sequences. The first, centered on Lys 221, lies between the T1 tetramerization domain and the first transmembrane segment. The second, centered on Lys 536, is located in the C-terminal tail just beyond the sixth transmembrane segment (Figure II-1A). Structural modeling of Kv1.5 using the recent crystal structure of Kv1.2 (34), places the motifs of each -subunit (colored red in Figure II-1B) in close proximity

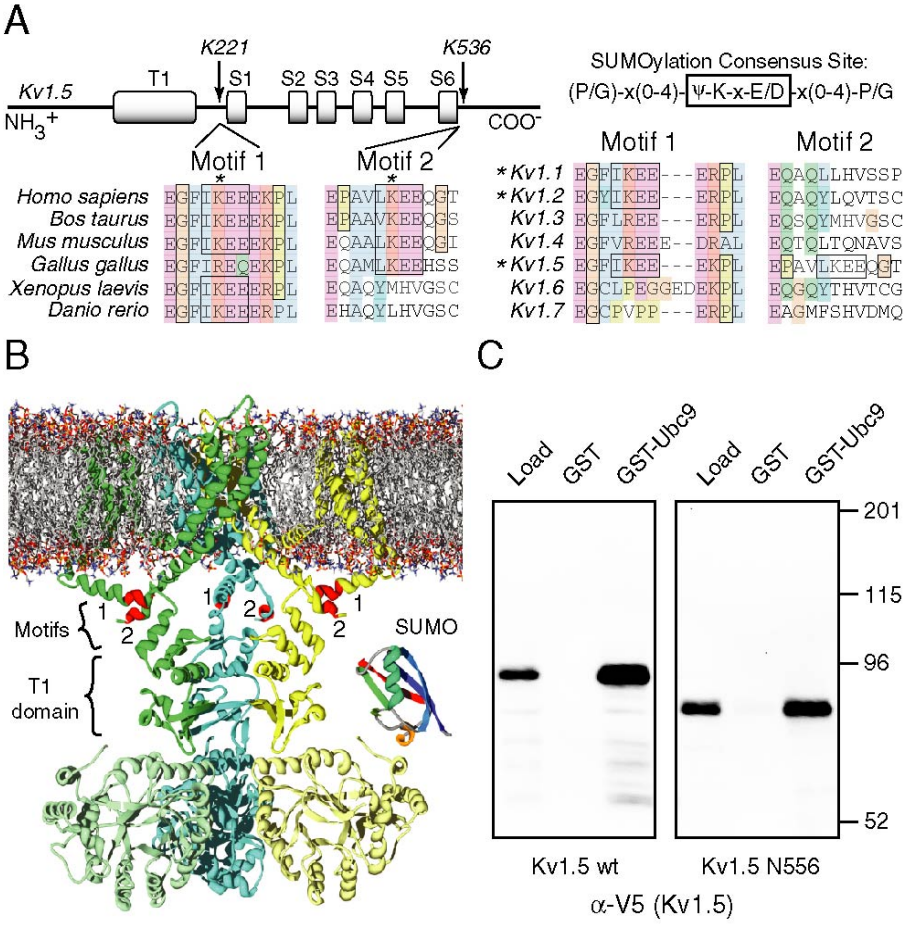

Figure II-1. Kv1.5 contains two conserved consensus SUMOylation motifs and interacts with Ubc9.

A) A schematic representation of Kv1.5. T1: tetramerization domain. S1-6: transmembrane domains. Aligned vertebrate Kv1.5 sequences centered on human K221 (Motif 1) and human K536 (Motif 2) are on the left, whereas the corresponding regions in human Kv channels are at right. The core and flanking Gly/Pro residues of the motif are boxed. Asterisks indicate channels with predicted SUMOylation motifs. A SUMOylation consensus is shown above. B) Structural model of Kv1.5 using the coordinates of Kv1.2-β2 (24). Modeled consensus SUMOylation motifs on each of the three depicted α-subunits are highlighted in red. SUMO-2 is shown for comparison. C) *In vitr*o interaction between Kv1.5 and Ubc9. Kv1.5 N556 corresponds to a C-terminal truncation lacking the last 57 amino acids but retaining both SUMOylation motifs. Load corresponds to 10% of applied material.

to each other exposed to the side portals that provide cytoplasmic access to the pore of the channel. The model suggests that SUMO attachment to either K221 or K536 can be accommodated without undue alterations of the overall structure. The predicted SUMOylation sequences (Figure II-1A) conform to the canonical -K-X-E/D pattern and the core sequence is flanked by nearby proline or glycine residues, a criterion included in our search model. Sequence comparison across vertebrate species revealed that they lie within regions of high local conservation since surrounding sequences, especially those C-terminal to the second motif, are far more divergent (not shown). Interestingly, whereas both sites are conserved in mammalian sequences, only one of the sites is present in avian, amphibian and fish species. Comparison to other human Kv family members reveals that the first motif is intact in Kv1.1 and Kv1.2.

To determine if Kv1.5 can interact with the E2 SUMO-conjugating enzyme Ubc9, we subjected extracts from cells expressing V5/His epitope-tagged hKv1.5 to affinity chromatography using immobilized recombinant GST-Ubc9 or GST. Kv1.5 was retained efficiently on the GST-Ubc9 resin but failed to interact with GST alone (Figure II-1C). Titration of the GST-Ubc9 fusion indicates that the affinity of interaction is ~400 nM (not shown). Consistent with the location of the two predicted modification sites, a 57-amino acid C-terminal truncation of Kv1.5 (N556) which preserves both motifs also interacts efficiently (Figure II-1C right). The presence of putative modification sites and the interaction with Ubc9 makes Kv1.5 a likely target of SUMOylation.

Kv1.5 is SUMO modified *in vivo* and *in vitro*

To evaluate if Kv1.5 is SUMOylated in live cultured cells (*in vivo*), we isolated V5/His-tagged hKv1.5 from cells co-expressing HA-SUMO-3 and Ubc9 by Ni^{+}-chelate chromatography under denaturing conditions. Western blot analysis of the preparations using anti-HA antibodies (Figure II-2A) indicates that two major HA-immunoreactive

bands (marked with arrows) are detected only in samples derived from cells co-expressing SUMO and Kv1.5. These bands correspond to SUMO-modified Kv1.5 since they are also visible as minor V5-immunoreactive species (arrows on anti-V5 blot). As in the case of most SUMOylated proteins (112), the extent of Kv1.5 modification appears to be relatively low (~1%). For the N556 C-terminal deletion mutant of Kv1.5, we detected a corresponding shift in the mobility of the HA- and V5-immunoreactive species confirming that the SUMO-conjugates do indeed derive from Kv1.5 and that the C-terminal region is dispensable for SUMOylation. Omission of N-Ethylmaleimide (NEM), an inhibitor of SUMO-specific deconjugating enzymes, during sample preparation led to reduced recovery of both HA- and V5-immunoreactive SUMO-modified Kv1.5 species (Figure II-2B). Likewise, exposure of Kv1.5 to purified recombinant Ulp1, a yeast SUMO isopeptidase (169) during the Ni^{+}-chelate chromatography released substantial amounts of the SUMO moiety from Kv1.5 (Figure II-2B).

To examine whether Kv1.5 can serve as a substrate for SUMO modification *in vitro*, we obtained purified preparations of recombinant WT V5/His-tagged hKv1.5 after baculovirus expression in Sf9 cells. Incubation of this material in a reconstituted SUMOylation reaction containing homogenous, recombinant E1 activating (SAE1/SAE2) and E2 conjugating (Ubc9) enzymes along with purified SUMO-2 and an ATP regenerating system led to the appearance of slow-migrating, SUMO-2 conjugated forms of Kv1.5 (Figure II-2C, lane 1). The two lower bands (marked by arrows) correspond in size to the species seen in panels A and B. The additional higher MW species likely represent multiply SUMOylated forms due to the abundant SUMO chain formation that occurs under these *in vitro* conditions. Although not as extensive, a similar pattern of conjugation is observed with SUMO-1 (lane 6). Notably, when any one component is omitted from the reaction, no SUMO-modified species could be detected. These results provide strong evidence that Kv1.5 is covalently modified by SUMO both *in vivo* and *in vitro*.

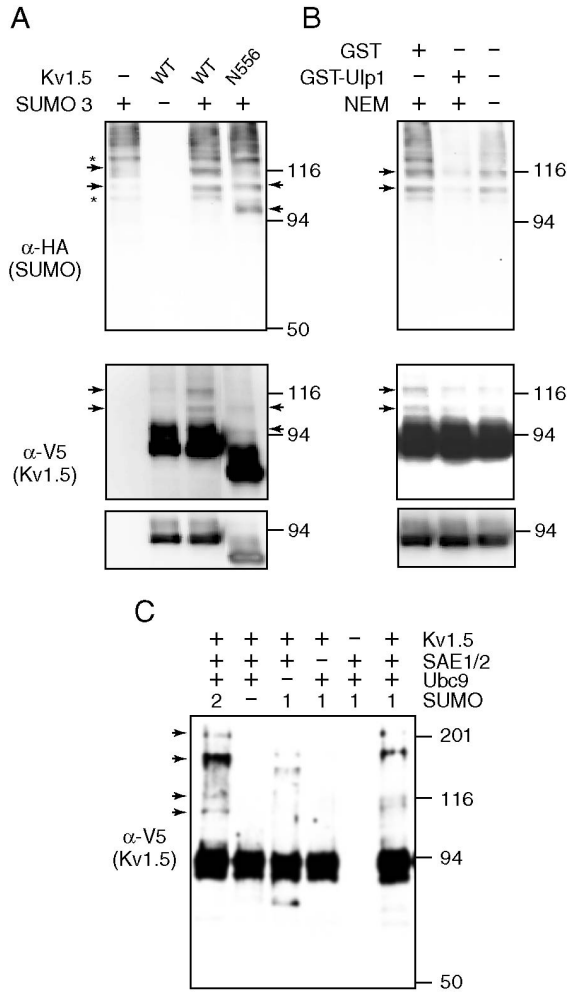

Figure II-2. Kv1.5 is SUMO modified in vivo and in vitro.

A) Cos-7 cells were co-transfected as described in *Experimental Procedures* with 200 ng pCDNA3 Ubc9 and as indicated, 200 ng pCDNA3 HA-SUMO3, 200 ng pCDNA3.1 hKv1.5 V5-His, 160 ng pCDNA3.1 hKv1.5 N556 V5-His or an equimolar amount of the corresponding empty vector. His-tagged proteins were purified and detected by immunoblotting with anti-HA (top panel) and anti-V5 (middle and bottom panels) antibodies. The middle panel is an over-exposed version of the lower panel. SUMO modified species of Kv1.5 are indicated with arrows and nonspecific bands with asterisks. B) Samples were transfected and processed as in A) except cells were harvested in the presence or absence of NEM as indicated, and samples were treated with GST or the SUMO-specific protease GST-Ulp1. Arrows indicate SUMO modified species of Kv1.5. C) Purified hKv1.5 was incubated with the indicated SUMO conjugation machinery components as described in *Experimental Procedures*. Arrows indicate SUMO-modified species of Kv1.5.

Modification of Kv1.5 by multiple SUMO isoforms requires intact SUMOylation motifs.

In humans, three SUMO isoforms competent for conjugation have been identified. Whereas SUMO-2 and SUMO-3 are nearly identical, they share only 50% amino acid identity with SUMO-1 and there is mounting evidence for isoform-selective subcellular localization (112) and function (146). We find that each of these SUMO isoforms can be conjugated to Kv1.5 *in vivo*; however, they do so to different extents (Figure II-3A). In contrast to SUMO-3 modified forms, SUMO-2 and especially SUMO-1 conjugates are substantially less abundant and require longer exposures to be detected. Although this preference may be intrinsic to Kv1.5, it also reflects the relative levels of expression of the isoforms since free SUMO-3 accumulates to substantially higher levels than SUMO-2 or SUMO-1. To determine if the two putative SUMOylation motifs that we identified serve as sites of conjugation, we replaced the predicted target lysines with non-SUMOylatable arginine residues. Mutation of the first (K221R) or second (K536R) motifs leads to a preferential loss of the higher and lower migrating SUMO conjugated species respectively (Figure II-3B, arrows). Although we have not assessed the stoichiometry or topology of the linkage within individual species, modification at different positions may lead to distinct migration patterns. The double mutant (K221/536R) however, leads to loss of the two major SUMO-modified Kv1.5 species. Consistent with the SUMOylation consensus, replacement of the conserved Glu 538 at the fourth position of the second motif by Arg (in the context of K221R) or mutation of the first hydrophobic position of both motifs to Asn (I220N/L535N) also severely compromised Kv1.5 SUMOylation (Figure II-8). In contrast, disruption of the motifs with the K221/536R mutations did not alter the recovery of ubiquitinated species in the presence of a proteasomal inhibitor (Figure II-4). These findings indicate that the proposed motifs in Kv1.5 serve as the major sites of SUMO conjugation.

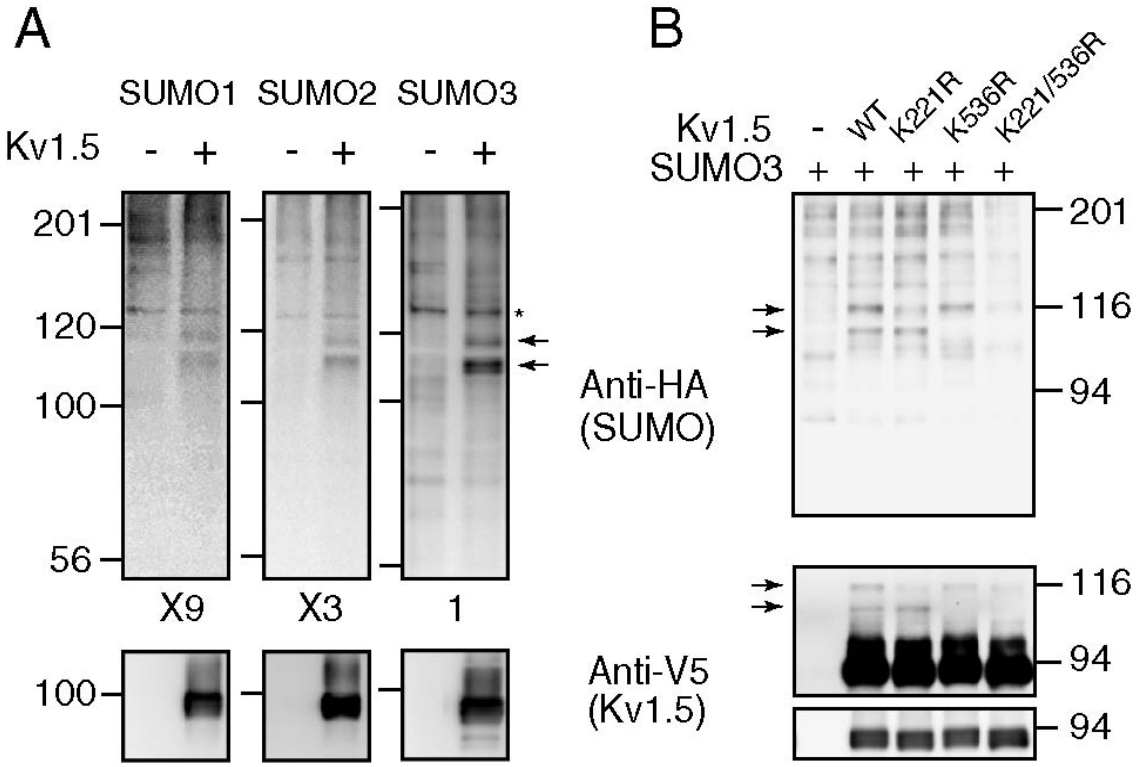

Figure II-3. Modification of Kv1.5 by multiple SUMO isoforms requires intact SUMOylation motifs.

A) Cos-7 cells were co-transfected with 3 μg each of pCDNA3 Ubc9, pCDNA3 HA-SUMO1/2/or 3, and 1μg pCDNA3.1 hKv1.5 V5-His or an equimolar amount of the corresponding empty vector. Samples were analyzed as in Figure II-2A. Relative exposure times of the anti-HA immunoblots are indicated below each blot. Arrows indicate SUMO modified species of Kv1.5. B) Cos-7 cells were transfected with pCDNA3 based vectors for the indicated proteins. Transfection and sample analysis was as in Figure II-2A. SUMO-modified species are indicated with arrows, and non-specific bands observed occasionally are indicated with an asterisk.

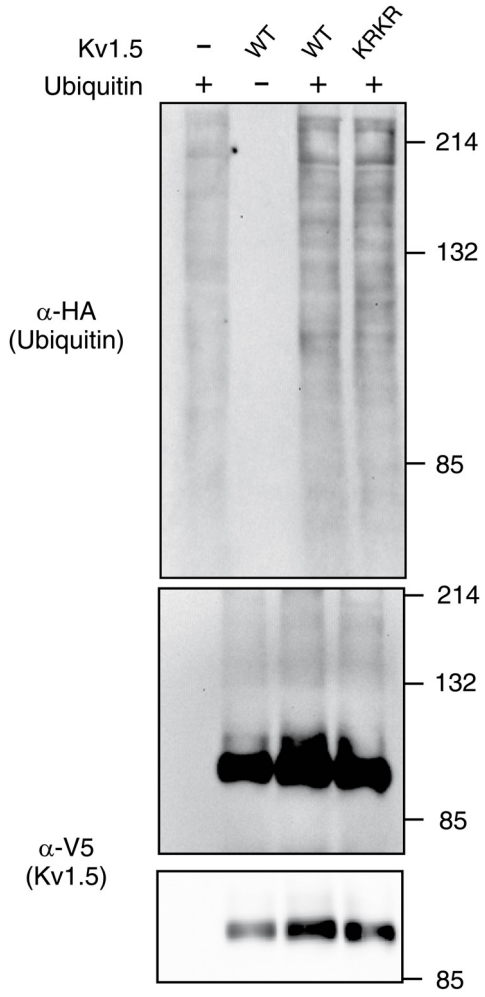

Figure II-4. Effect of Disruption of SUMOylation Motifs on Kv1.5 Ubiquitination.

Cos-7 cells were co-transfected as described in *Experimental Procedures* with 200 ng pCDNA3 HA-ubiquitin, 200 ng of the indicated pCDNA3.1 hKv1.5 V5-His plasmid, or an equimolar amount of the corresponding empty vector. Cells were treated with 10 μM lactacystin (Boston Biochem) for 1 hr at 37 °C. Subsequent lysis in the presence of 20 mM NEM and protease inhibitors was as described in Figure II-2. His-tagged proteins were purified and detected by immunoblotting with anti-HA (top panel) and anti-V5 (middle and bottom panels) antibodies as in Figure II-2. The middle panel is an over-exposed version of the lower panel.

Kv1.5 is SUMOylated at the plasma membrane.

To probe if the stoichiometry of SUMOylation of Kv1.5 depends on the subcellular localization of the channel, we isolated channel expressed at the plasma membrane using a surface biotinylation approach and compared the SUMO modification of this pool of channels to that of total cellular Kv1.5. As shown in Figure II-5A, treatment of Cos7 cells co-expressing HA-SUMO3, Ubc9, and either WT or SUMOylation-deficient K221/536R Kv1.5-V5-His6 with the cell-impermeant biotinylation reagent EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL) resulted in the selective retention of Kv1.5 on streptavidin-agarose resin (lanes 4-6). Notably, the enrichment of the glycosylated species of Kv1.5 (marked with an asterisk) is detected in plasma membrane samples compared to those from the whole cell. This is consistent with a preferential isolation of surface channel since membrane insertion occurs after the channel has traversed the ER and Golgi compartments where glycosylation takes place. The slower migrating species corresponds to glycosylated Kv1.5 since this form is sensitive to glycanase treatment (Figure II-5B). A similar collapse to a single form is also observed for the total cellular Kv1.5. To examine the SUMOylation state of the cell-surface pool of Kv1.5, cells co-expressing HA SUMO3 and either WT or the SUMOylation deficient K221/536R mutant were surface biotinylated and the complement of modified plasma membrane proteins was purified by streptavidin-affinity chromatography. Kv1.5 was isolated from this pool via a second, Ni^{2+}-chelate chromatography under denaturing conditions. As seen in Figure II-5C, we detect two HA-immunoreactive species (lane 5) with similar gel migration pattern and molecular weights to those derived from SUMO-modified Kv1.5 in total cellular samples (first two lanes). These species are not detected in preparations derived from cells expressing the SUMOylation-deficient K221/536R Kv1.5, further indicating that they represent SUMO-modified forms of Kv1.5 derived from the plasma membrane. As in the case of total

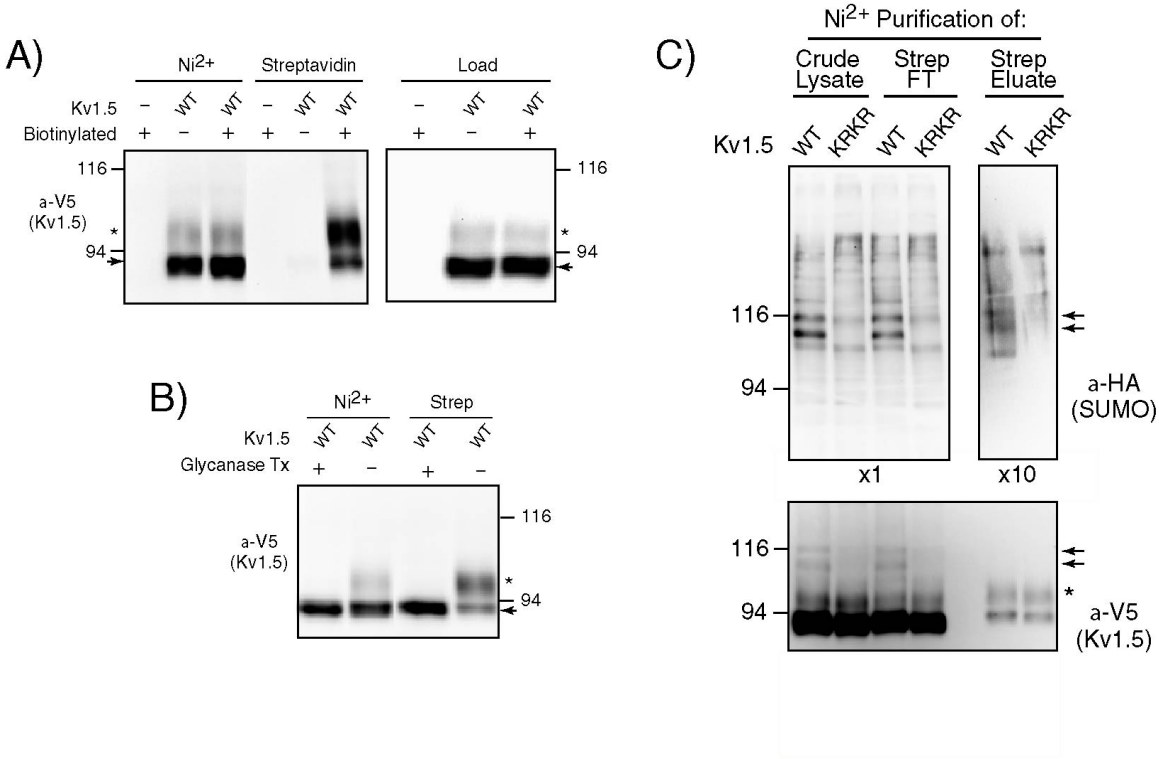

Figure II-5. Kv1.5 is SUMOylated at the Plasma Membrane.

(A) Forty-eight hours after transfection with HA-SUMO3, Ubc9, and Kv1.5-V5-His6 variants, Cos7 cells were surface biotinylated using a cell-impermeant, amine-reactive ester biotinylation reagent and lysed in the presence of 1% CHAPS. Whole cell lysates were centrifuged at 16,000 x g for 15 min, and soluble protein was loaded onto either a $Ni2^{+}$-agarose or streptavidin-agarose resin and visualized by Western blotting. Kv1.5-V5-His6 was only retained by streptavidin-agarose resin after surface biotinylation. The asterisk indicates glycosylated Kv1.5; the arrowheads indicate unmodified Kv1.5. B) Lysates prepared as in A were loaded onto either a $Ni2^{+}$ resin or streptavidin-agarose resin and visualized by Western Blotting. The higher-migrating species of V5-immunoreactive Kv1.5 (marked with an asterisk) was sensitive to treatment with glycanase (incubated for 30 minutes at 37 deg C with the agarose resin immediately before elution). Arrowheads indicate unmodified Kv1.5. C) Lysate prepared as in A was subjected to sequential purification by streptavidin-affinity chromatography followed by $Ni2^{+}$-chelate chromatography as described in *Experimental Procedures* in the absence of glycanase. Arrowheads indicate SUMO modified species of Kv1.5; the asterisk indicates glycosylated Kv1.5.

cellular Kv1.5, the extent of SUMO modification of surface-expressed Kv1.5 appears to be relatively low (~ 1%). Notably, even after loading the equivalent of ~ 40 times more starting material, we recover only a very small fraction of total cellular Kv1.5 on the plasma membrane (visible on the anti-V5 blot in Figure II-5C). In future studies, increasing the ratio between surface-expressed and total cellular channel may enable us to better visualize the SUMO-modified species of Kv1.5 derived from the plasma membrane. These findings indicate that Kv1.5 is SUMO modified at the plasma membrane with approximately the same stochiometry as that for total cellular channel.

Treatment of Cos7 cells with hydrogen peroxide regulates the SUMO modification of Kv1.5

Oxidative stress is a widespread regulator of protein SUMOylation. Moreover, recent data indicate that hydrogen peroxide can have direct effects on the biophysical properties of Kv1.5 (170). To determine the effect of oxidative stress on the SUMOylation status of Kv1.5, we exposed Cos7 cells expressing HA-SUMO3, Ubc9, and either WT or SUMOylation-deficient K221/536R Kv1.5-V5-His6 to increasing concentrations of hydrogen peroxide. As can be seen in Figure II-6, exposure of cells for 120 minutes, led to a shift of the Kv1.5 SUMOylated species that we normally detect (marked with arrowheads) to higher molecular mass SUMO conjugates (indicated by a bracket). Such higher-order species may represent SUMO chain formation on Kv1.5. This increase in overall modification and in high-molecular weight conjugates is dose-dependent and absent in the SUMOylation-deficient K221/536R Kv1.5 mutant (last lane). These data provide preliminary evidence that oxidative stress may serve an important regulatory role in controlling SUMOylation of Kv1.5.

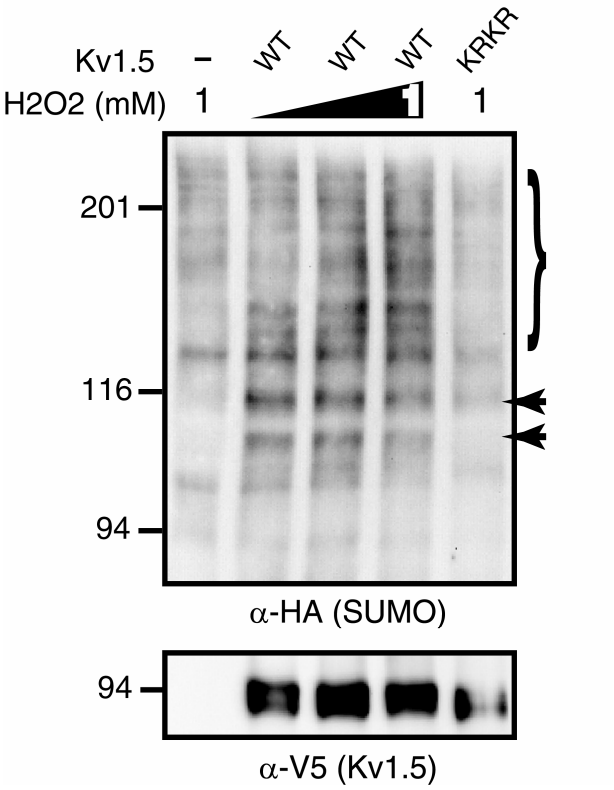

Figure II-6. Effect of Oxidative Stress on Kv1.5 SUMOylation.

Cos7 cells expressing Kv1.5-V5-His6, HA-SUMO3, and Ubc9 were treated with 0, 0.1, 0r 1 mM H2O2 in phenol red free DMEM media as indicated for 2 hours at 37 °C. Lysates were enriched for Kv1.5 by Ni^{2+}-chelate chromatography and probed with HA (top) and V5 (bottom) antibodies. Arrowheads indicate SUMOylated Kv1.5 species. Bracket indicates higher-order SUMOylated Kv1.5.

Disruption of SUMOylation motifs alters Kv1.5 function

To probe the functional role of the SUMOylation motifs in Kv1.5, we characterized the voltage-dependent potassium currents elicited by expression of Kv1.5 forms harboring intact or disrupted SUMOylation motifs. Analysis of whole-cell patch-clamp recordings indicated that the K221/536R substitutions had no effect on the total current density (Figure II-7A), nor on the voltage-dependence of channel activation (Figure II-7B). In contrast, the substitutions led to a specific ~15 mV hyperpolarizing shift in the half-maximal voltage of inactivation (V_{50}) from the WT value of 0.33 +/- 1.2 to -14.8 +/- 1.4 mV ($p<0.0001$). This shift occurs without appreciable alterations in the extent of inactivation (53.8 and 50.9% for WT and K221/536R respectively). Notably, reintroduction of the WT sequence at either position returned the V_{50} value to nearly WT levels (Figure II-7C). This indicates that the motifs are functionally equivalent and that the presence of a single intact motif is sufficient to exert a nearly complete effect. Moreover, mutation of other residues within the motifs also had a functional effect. In the context of the K221R mutant, addition of the E538R substitution in the second motif led to a significant shift in V_{50} from -4.0 +/- 0.9 to -8.8 +/- 1.2 mV ($p<0.0005$). Likewise, mutation of the first position in both motifs to Asn (I220N/L535N) led to a shift indistinguishable from that of the K221/536R mutant (-17.44 +/- 1.6 vs -14.8 +/- 1.4 mV $p = 0.32$) (Figure II-8B). Taken together, these data suggest that although not essential for assembly of functional Kv1.5 channels, the motifs selectively regulate their biophysical properties.

SUMOylation regulates Kv1.5 inactivation

The concurrent loss of SUMO modification and shift in the voltage dependence of inactivation in multiple SUMOylation motif mutants argues strongly in favor of SUMOylation (over other lysine-directed modifications) as the mechanism responsible

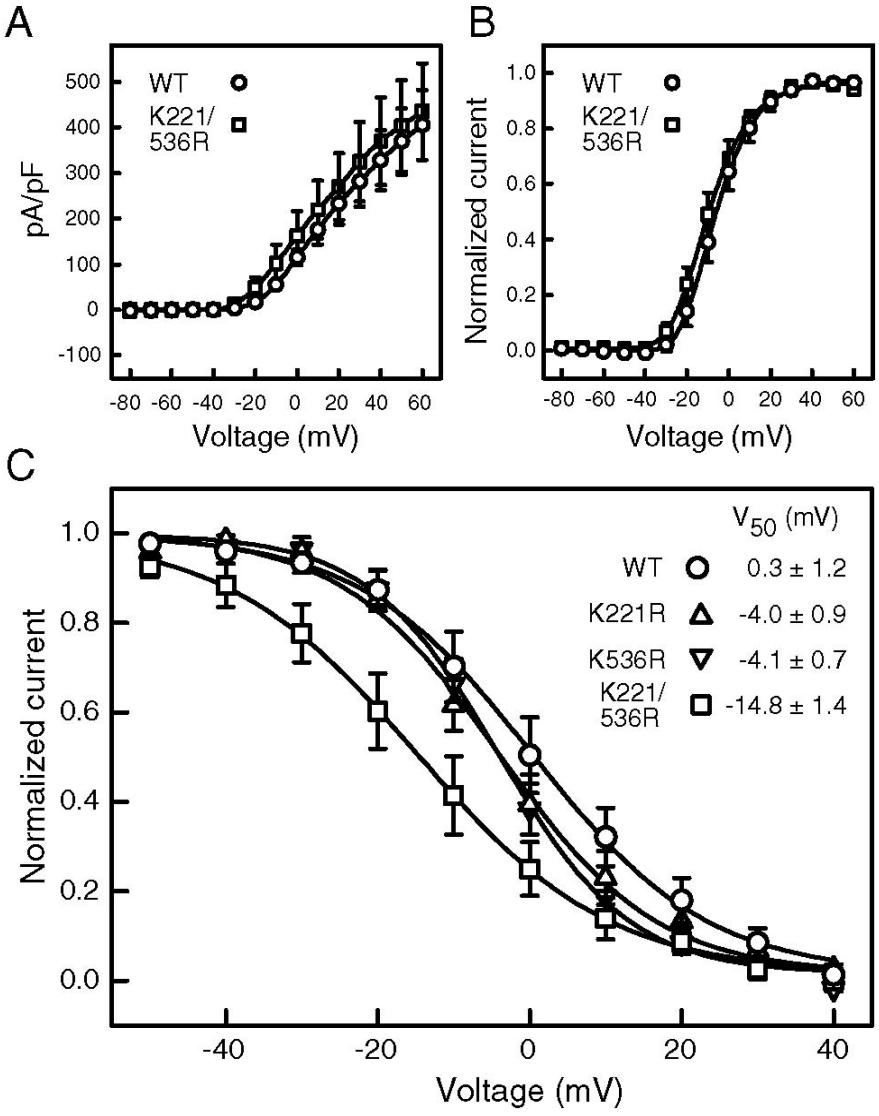

Figure II-7. Disruption of SUMOylation motifs alters Kv1.5 inactivation.

Cos-7 cells were co-transfected with 0.1 µg each of pEGFP-C1 (to identify transfected cells) and a pCDNA3 based vector for the expression of the indicated Kv1.5 variant. Recordings were obtained as described in *Experimental Procedures*. A) I/V curves. Data represent average +/- SEM of 11 WT and 11 K221/536R cells. B) Voltage-dependence of steady-state activation. (n= 11 and 10 for WT and K221/536R). C) Voltage-dependence of steady-state inactivation (n= 7-10 for each construct). The inset shows calculated V_{50} values.

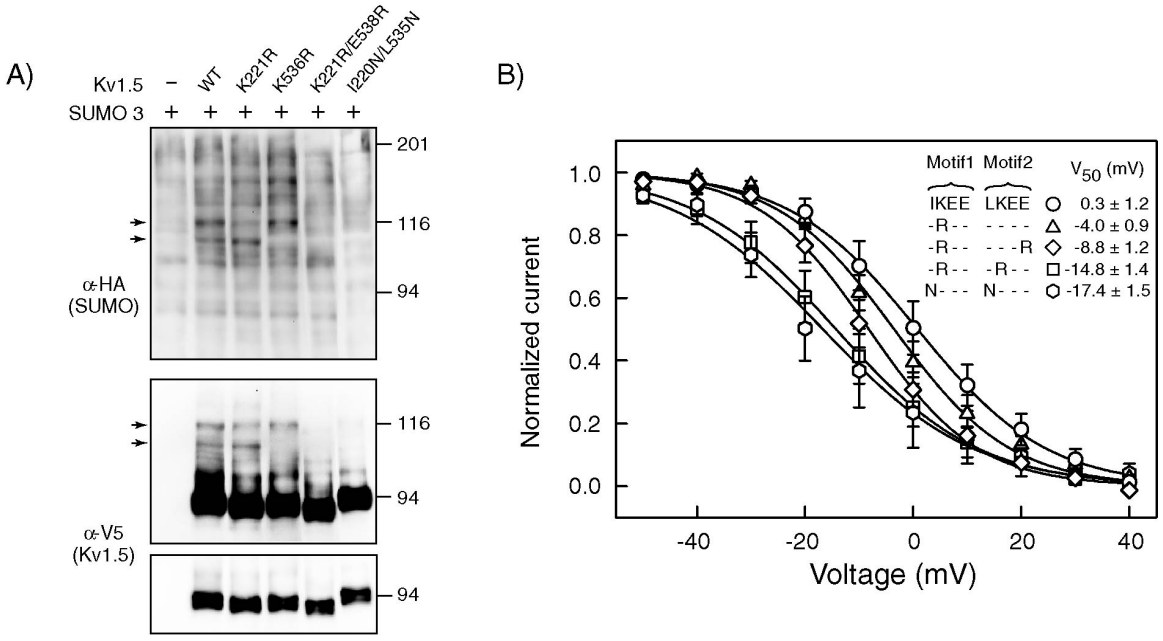

Figure II-8. Lysine-Sparing Disruptions of SUMOylation Motifs Alter Kv1.5 Inactivation.

A) Cos-7 cells were co-transfected and processed as in Figure II-2A. Arrows indicate SUMO modified species of Kv1.5 in the anti-HA and anti-V5 immunoblots. B) Cos-7 cells were co-transfected with 0.1 μg of the indicated Kv1.5 plasmid and 0.1 μg pEGFP-C1 as in Figure II-7. Voltage dependence of steady-state inactivation was obtained as in Figure II-7C (n= 6 and 5 for K221R/E538R and I220N/L535N, respectively; data from WT, K221R, and KRKR are from Figure II-7). The inset shows a schematic of each SUMOylation motif variant and the corresponding inactivation V_{50} values.

for the functional effects exerted by the motifs. If this is indeed the case, reducing the levels of SUMOylation from WT Kv1.5 should lead to a similar alteration in the voltage dependence of inactivation. To test this hypothesis, we took advantage of a variant of the SUMO protease SENP2 lacking the first 70 N-terminal residues (FLAG-SENP2 71-590). This form displays enhanced activity against multiple SUMOylated substrates (136) and localizes to the cytoplasm (140). Co-expression of this SUMO protease led to the complete loss of Kv1.5 SUMO conjugates (Figure II-9A). In parallel, co-expression of SENP2 with WT Kv1.5 produced a ~14 mV hyperpolarizing shift in the voltage dependence of steady-state inactivation, altering the V_{50} from 0.33 +/- 1.2 mV in its absence to -13.5 +/- 0.9 mV in its presence ($p<0.0001$) (Figure II-9B). This effect is indistinguishable from that of the K221/536R mutant (V_{50} = -14.8 +/- 1.4 mV, $p>0.4$). Importantly, the effects of SENP2 require the integrity of the SUMOylation motifs in Kv1.5 since co-expression of SENP2 did not alter the V_{50} of the SUMOylation deficient K221/536R mutant (V_{50} = -12.3 +/- 0.9 mV, $p>0.16$). Taken together, these findings demonstrate that loss of SUMO modification of Kv1.5, either by point mutations at the SUMOylation motifs or co-expression with the SUMO-specific protease SENP2, leads to a significant hyperpolarizing shift in the voltage-dependence of inactivation without altering either the total current density or voltage-dependence of activation of the channel.

DISCUSSION

The present study identifies SUMOylation as a novel form of posttranslational modification of Kv1.5 and establishes that it exerts a selective regulatory function. While this is the first member of the voltage-gated ion family found to serve as a target for SUMO modification, multiple Kv channel α subunits, including Kv1.1 and Kv1.2, contain similarly located consensus SUMOylation motifs. SUMOylation may thus be a widespread mechanism of channel regulation. The recent description of SUMOylation of

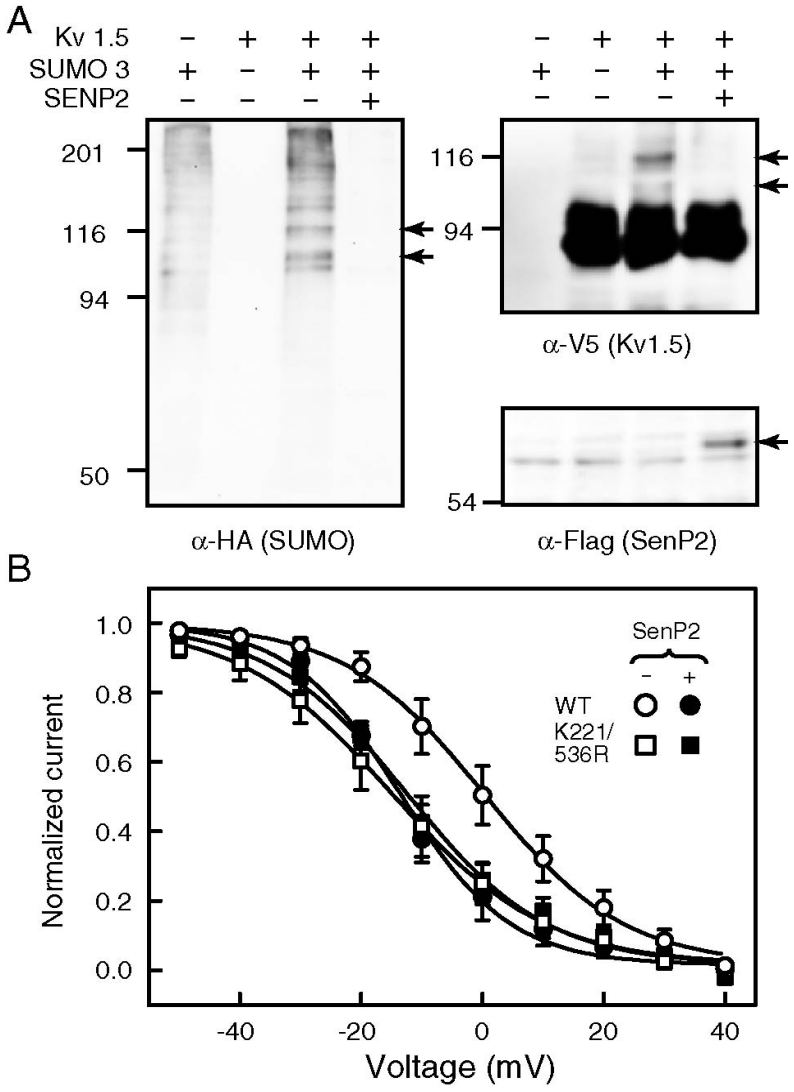

Figure II-9. SENP2-mediated loss of Kv1.5 SUMOylation alters inactivation.

A) Cos-7 cells were co-transfected as in Figure II-2A with an additional 200 ng of pCMV or pCMV FLAG SENP2 (71-590). Samples were analyzed as in Figure II-2A. Whole-cell lysates were resolved by SDS-PAGE and probed with anti-FLAG antibodies (bottom right immunoblot). Arrows indicate SUMO modified species of Kv1.5 in the anti-HA and anti-V5 immunoblots and SENP2 in the anti-FLAG blot. B) Cos-7 cells were co-transfected with 0.1 µg of the indicated Kv1.5 plasmid, 0.1 µg pEGFP-C1 and 0.5 µg pCMV FLAG SENP2 (71-590). Voltage dependence of steady-state inactivation in the presence of SENP2 was obtained from 7 and 6 cells for WT and K221/536R respectively. Data in the absence of SENP2 are from Figure II-7C.

the glucose transporters GLUT1 and GLUT4 (160,171), the two-pore potassium leak channel K2P1 (164) and the G-protein-coupled receptor mGluR8 (162) further supports the view that SUMOylation is an important regulatory mechanism for integral membrane proteins.

In this report, we have demonstrated that disruption of Kv1.5 SUMO modification has a significant effect on the biophysical properties of the channel. Notably, loss of SUMOylation elicits a substantial phenotype even though only a small fraction of the total cellular Kv1.5 appears to be SUMOylated. Interestingly, neither Ubc9 nor SUMO appear to be limiting, since overexpression of SUMO3 and Ubc9, did not shift the V_{50} of WT Kv1.5 in the depolarizing direction. Furthermore, although SUMOylation of channel expressed at the plasma membrane was detected, this population did not appear selectively targeted for SUMOylation on the basis of our surface biotinylation assays. Given the oligomeric structure of the channel modification of only one of the eight sites per tetramer may be sufficient to exert a functional effect. Alternatively, transient SUMOylation may be sufficient to establish a long-lived assembly or functional state of the channel. The ability of SUMO to exert substantial regulatory effects even at low stoichiometries is also observed for other target proteins such as transcription factors and appears to be more the norm than the exception. There are however, proteins that are modified at high levels such as the K2P1 channel (164), RanGap (116) and the A40R vaccinia virus protein (172). For this second class, SUMO may be functioning as an essential structural assembly element as opposed to serving a transient modulatory one. Thus, in the case of the K2P1 channel, SUMOylation is an absolute gatekeeper preventing any access to conducting states of the channel (164), whereas in Kv1.5 this modification plays a more modulatory role.

Although the basis for the effects of SUMOylation on inactivation of Kv1.5 is not fully understood, the juxtamembrane location of the modification sites may be instructive. The electrostatic environment in this region is thought to influence the

voltage-sensitivity of Kv channels (45). The surface of SUMO is highly charged. Notably, one hemisphere is rich in basic residues whereas the opposite face is predominantly acidic. Conjugation of SUMO may modulate the local electrostatic environment and thereby influence voltage-sensitive steps. The selective effects of SUMOylation on the voltage dependence of inactivation, but not activation however, argue that SUMOylation affects steps other than the voltage sensitive transitions along the usual path towards activation. Certain Kv channel -subunits confer rapid N-type inactivation to Kv1.5 by providing an N-terminal inactivation peptide. Intriguingly, the first SUMOylation site (K221) is three residues downstream from an acidic region in the T1-S1 linker that is thought to facilitate N-type inactivation by positioning the inactivating peptide in close vicinity to the pore (32). SUMOylation could regulate -subunit-dependent N-type inactivation through direct steric hindrance or by altering the local electrostatic environment. The current experiments were conducted in cells that do not express -subunits so exploring the role of SUMO modification of Kv1.5 in the context of various / -subunit combinations will likely be informative. The effects of SUMO modification in contexts such as transcriptional regulation are likely due to the promotion of protein-protein interactions (146,153,173). Notably, a critical surface in SUMO essential for transcriptional inhibition (152) is the site of interaction for I/L/V rich SUMO-interacting motifs present in multiple proteins (149,174), including some associated with membrane proteins (171). Since Kv1.5 functions as part of a multi-protein/lipid complex comprised of proteins with scaffolding, cytoskeletal and enzymatic activities, SUMO modification of Kv1.5 may function by modulating these interactions.

Given the functional effects of SUMOylation, SUMO proteases or E3 enzymes that target Kv1.5 are likely to exert important regulatory functions. In this regard, the K^+ channel associated protein KChAP (99) has been shown to be a member of the PIAS family of proteins originally identified as negative regulators of several transcriptional responses (175). PIAS proteins possess intrinsic SUMO-specific E3 activity (133) and

most of their transcriptional effects depend on this function. While the regulatory consequences of KChAP association with Kv1.5 remain to be determined, the effects on surface expression observed for other Kv channels occur in a transcription-independent manner (175). In addition, since the SUMOylation pathway is sensitive to redox signals (176), SUMOylation may contribute to the oxygen-sensing properties of Kv1.5 in the pulmonary vasculature (87). Clearly, alterations in the SUMOylation of Kv1.5 have the potential to alter the excitability of both atrial myocytes and vascular smooth muscle cells by modulating either the action potential duration or resting membrane potential. Thus, mechanisms that regulate Kv1.5 SUMOylation afford a novel link between signaling pathways and cellular excitability.

CHAPTER III: ACUTE AND BIDIRECTIONAL EFFECTS OF SUMO MODIFICATION ON THE Kv1.5-MEDIATED I_{KUR} CURRENT IN CARDIOMYOCYTES[2]

INTRODUCTION

In the human heart, Kv1.5 is selectively expressed in the atria (69) and mediates the ultrarapid K^+ current (I_{kur}) critical to action potential phase II repolarization (12). These properties have identified Kv1.5 as a promising target for the development of novel antiarrhythmic medications intended to increase atrial refractoriness in patients with atrial fibrillation, while sparing the risk of potentially life-threatening, ventricular side-effects.

In the previous chapter, we identified that Kv1.5 is post-translationally modified by all three isoforms of SUMO at two conserved consensus SUMOylation motifs centered on lysines 221 and 536, and that loss of SUMOylation, either by disruption of these motifs or expression of the SUMO-specific protease SENP2, leads to a selective ~15 mV hyperpolarizing shift in the voltage-dependence of steady-state inactivation of the channel. These findings identify SUMO modification as a new point of regulation of Kv1.5 function and strongly suggest a potential role for SUMOylation in the regulation of Kv1.5-mediated I_{kur} current in cardiomyocytes.

It is important to note that the studies in heterologous systems described in Chapter II involved complete and persistent loss of SUMOylation through mutations or co-expression of SENP2, which could reflect stable alterations in the biosynthesis and

[2] The data from this chapter are planned for publication as "Benson, M, Kaur K, McEwen D, Martens JR, Iñiguez-Lluhí JA. The Acute and Bidirectional Effects of SUMO Modification on Kv1.5-Mediated I_{kur} Current in Cardiomyocytes."

assembly of Kv1.5 complexes. Thus, these experiments did not address whether SUMOylation could serve as an acute and rapid regulatory mechanism acting on pre-existing channels at the plasma membrane. Furthermore, the experiments did not address whether this regulatory mechanism is operational in the native cellular context of cardiomyocytes where Kv1.5 serves its physiological functions, or whether SUMOylation has additional roles in the regulation of Kv1.5-mediated currents.

In the work described in this chapter, we aimed to probe the role of SUMO modification on the regulation of Kv1.5-mediated I_{kur} current in cardiomyocytes and to examine whether the effects of Kv1.5 SUMOylation can be regulated acutely. As described below, we show that loss of SUMOylation of Kv1.5, by either disruption of the SUMOylation motifs or inclusion of purified, recombinant SENP2 in the patch pipette during our electrophysiological recordings, leads to a significant, hyperpolarizing shift in the voltage-dependence of steady-state inactivation of expresssed Kv1.5 in HL-1 atrial myocytes. This acute effect of SENP2 on Kv1.5 is dependent on the presence of intact SUMOylation motifs since SENP2 has no effect on the inactivation of the SUMOylation-deficient Kv1.5 K221/536R mutant. Furthermore, we find that inclusion of SENP2 in the patch pipette during whole-cell electrophysiological recordings of acutely-dissociated mouse ventricular myocytes leads to a hyperpolarizing shift in the voltage-dependence of steady-state inactivation of *endogenous* I_{kur} current. Conversely, inclusion of SUMO1 in the patch pipette during these recordings leads to an opposite, depolarizing shift in the voltage-dependence of inactivation of I_{kur} current in these cells. We find that this regulatory mechanism extends to human cardiomyocytes, as inclusion of SUMO1 in the patch pipette during recordings from acutely-dissociated human atrial myocytes results in a similar depolarizing shift in the voltage-dependence of steady-state inactivation of I_{kur}. To probe the mechanism by which infused SUMO leads to this shift, we took advantage of a SUMO1-ΔGG mutant. This form lacks the C-terminal di-glycine motif required for formation of the isopeptide bond between SUMO and its substrate, but retains an intact

binding surface known to facilitate the formation of non-covalent inter- or intra-molecular interactions. Inclusion of this SUMO1-ΔGG mutant in the pipette solution recapitulated the effects of SENP2 and led to a significant hyperpolarizing shift in the voltage-dependence of steady-state inactivation of I_{kur} current, suggesting that the mechanism of action by which SUMO regulates I_{kur} current may be through the facilitation of inter- or intra-molecular interactions with the channel. Importantly, while we found that infusion of SUMO1, SENP2, or SUMO1-ΔGG had no effect on the current densities of other outward K^+ currents such as I_{to} or I_{ss}, infusion of either SENP2 or SUMO1-ΔGG led to a ~ 40% reduction in I_{kur} current density. Consistent with the role of I_{kur} in cardiac repolarization, this treatment with SENP2 or SUMO1-ΔGG led to a significant prolongation of the plateau phase of the action potential without affecting the resting membrane potential or the amplitude of the action potential.

Together, these findings provide strong evidence that SUMO modification acutely regulates the voltage-dependence of inactivation of endogenous I_{kur} current in mouse and human cardiomyocytes. Moreover, we find that modulation of the SUMOylation pathway can lead to significant functional consequences on the shape and duration of the cardiac action potential.

EXPERIMENTAL PROCEDURES

Expression plasmids and cell culture

The expression vectors pCDNA3.1 hKv1.5 V5-His and pCDNA3.1 hKv1.5 K221/536R V5-His have been previously described (165). pET28b expression vectors bearing the N-terminal His-tagged human Senp2 catalytic domain (364-489) and C548S mutant were generous gifts of Christopher D. Lima (177) (Structural Biology Program, Sloan-Kettering Institute, New York, NY 10021, USA). pET15b His6-SUMO1 was

prepared by inserting a full-length, human SUMO1 fragment generated by PCR into the NdeI/BamHI restriction sites of pET15b (Novagen, Gibbstown, NJ). The ΔGG mutant was generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). HL-1 cells were transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) and maintained on gelatin/fibronectin-coated wells in Claycomb medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 0.1 mM norepinephrine, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin as described (178).

Expression and purification of recombinant proteins

BLR(DE3) pLysS cells (Novagen) containing pET15b His6-SUMO1, pET15b His6-SUMO1 ΔGG, pET28b His6-SENP2, or pET28b His6-SENP2 C548S were grown at 37 °C in LB medium containing 25 μg/ml chloramphenicol, 12 μg/ml tetracycline, and 50 μg/ml of either carbenicillin (for SUMO1 constructs) or kanamycin (for SENP2 constructs). Cultures in the logarithmic growth phase were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h at 37 °C. Cells were harvested by centrifugation at 8,000 × *g* for 15 min at 4 °C and lysed in either Buffer A (50 mM NaH2PO4, 300 mM NaCl, 10 % glycerol, 10 mM imidazole, 5 mM β-mercaptoethanol, EDTA-free Complete Miniprotease Inhibitor mixture tablets (1 tablet/10 ml), pH 8.0) for SUMO1 constructs, or Buffer A containing no protease inhibitors for SENP2 constructs. After lysozyme treatment (40 μg/ml for 30 min) and sonication at 4 °C, the suspension was centrifuged at 35,000 rpm at 4 °C for 30 minutes, and the supernatant was incubated with Ni^{2+}-NTA agarose beads (Qiagen) with rocking for 60 minutes at 4 °C. The resin was washed with 10 bed volumes of Buffer A, followed by 2 bed volumes of Buffer A containing 20 mM imidazole. Protein was eluted in Buffer A containing 250 mM imidazole. All proteins were exchanged into Buffer C (50 mM NaH2PO4 (pH 8.0), 50 mM NaCl, 5 mM β-

mercaptoethanol, 10% glycerol) via gel filtration, quantitated using a Bradford assay, and stored at -80 °C.

Isolation of murine ventricular myocytes

Single ventricular myocytes were isolated from adult (2-3 months old), male C57/black mice as previously described (179) by Dr. Kuljeet Kaur in the laboratory of Dr. Rick Neubig in our department. Briefly, and in accordance with the University of Michigan University Committee on the Use and Care of Animals, mice were anesthetized by pentobarbital, heparinized, and killed by cervical dislocation. Hearts were rapidly removed and retrogradely perfused using a modified Langendorff apparatus with calcium-free perfusion buffer (120.4 mM NaCl, 14.7 KCl, 0.6 mM KH2PO4, 0.6 mM Na2HPO4, 1.2 mM MgSO4-7H2O, 10 mM Na-HEPES, 4.6 mM NaHCO3, 30 mM taurine, 10 mM 2,3-butanedione monoxime (BDM), 5.5 mM glucose, pH adjusted to 7.0 with HCl, continually maintained at 37°C and bubbled with 100% O_2) for 4 minutes at approximately 4 ml/min. The heart was then enzymatically digested by perfusion with Mycoyte Digestion Buffer (Perfusion Buffer + 2.4 mg/ml collagenase II (Worthington Biochemical Co, Lakewood, NJ)) for 3 minutes at 4 ml/min. Subsequently, $CaCl_2$ was added to a final concentration of 40 μM, and perfusion was continued until the heart appeared pale, swollen, and fibrillar (~8 minutes). The heart was removed from the cannula, and the right and left ventricles were cut into small pieces in Myocyte Stopping Buffer (Perfusion Buffer + 10% calf serum + 12.5 μM $CaCl_2$). The pieces were gently triturated until they were dissociated into rod-shaped, single cells. ATP was added to a final concentration of 2 mM, and Ca^{2+} was gradually reintroduced in a four-step process over ~8 minutes until the calcium concentration was 1.2 mM. Isolated myocytes were stored in Myocyte Culture Medium (DMEM, 0.1% BSA, 100 U/ml penicillin, 2 mM glutamine) at 25°C until use.

Isolation of human atrial myocytes

A specimen of atrial pectinate muscle was obtained in accordance with the University of Michigan Institutional Review Board from a donor heart that failed criteria for successful cardiac transplantation. Single atrial myocytes were isolated using a modified protocol as previously described for the isolation of human ventricular myocytes (180) by Dr. Dyke McEwen in the laboratory of Dr. Jeffrey Martens. Briefly, tissue that had been maintained on ice was transferred to ice-cold Storage Buffer (100 mM NaCl, 5.4 mM KCl, 1.5 mM $MgSO_4$, 10 mM NaOAc, 1.5 mM NaH_2PO_4, 15 mM $NaHCO_3$, 10 mM glucose, 10 mM taurine, 10 mM HEPES, pH 7.4), and pectinate muscle from the right and left atria were collected and minced into small pieces using a scalpel and fine scissors. These pieces were incubated in Wash Buffer (Storage Buffer + 5 mM nitrilotriacetic acid (NTA) + 15 mM BDM, pH 7.0) for 10 minutes, rinsed briefly in Storage Buffer, digested for 30 minutes in Protease Buffer (Storage Buffer + 4 U/ml Protease Type XXIV (Sigma-Aldrich), 400 U/ml Collagenase Type II (Worthington Biochemical Co), 15 mM BDM, 1.25 μM $CaCl_2$, pH 7.4), and further digested for approximately 60 minutes in Hylauronidase Buffer with occasional, gentle trituration (Storage Buffer + 198 U/ml hylauronidase (Sigma-Aldrich), 400 U/ml Collagenase Type II, 15 mM BDM, 1.25 μM $CaCl_2$, pH 7.4) until fully-dissociated, rod-shaped cells were apparent. All incubations were conducted at 37°C with moderate shaking (500 rpm) and bubbled 100% O_2. Cells were resuspended in Incubation Buffer (Storage Buffer + 15 mM BDM + 2 % BSA), and calcium was gradually reintroduced in a six-step process over ~ 60 minutes until the calcium concentration was 1.7 mM. Cells were maintained at 25°C until use.

Electrophysiology

Whole-cell patch clamp recordings were conducted using a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, USA) as previously described (74,86). For experiments in the HL-1 cell line, cells transfected with 0.5 μg pEGFP (Clontech) and 0.5 μg of either pCDNA3.1 Kv1.5 V5-His or pCDNA3.1 Kv1.5 KRKR V5-His were trypsinized 24 h after transfection and resuspended in HL-1 supplemented medium using established protocols (178). A 200 μl aliquot of these cells was placed in a recording chamber mounted on the stage of an inverted microscope and allowed ~ 30 minutes to settle before being superfused with Bath Solution (in mmol/l): NaCl 110, KCl 4, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 10, and glucose 1.8; adjusted to pH 7.35 with NaOH. Fluorescent cells with 2-6 nA of total current were selected for analysis. For primary cardiomyocytes, a 200 μl aliquot of cells was placed in the recording chamber, allowed five minutes to settle, and continuously superfused with Bath Solution. Only non-beating, single, rod-shaped cells were selected. All experiments were conducted at room temperature. Patch pipettes were pulled from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL, USA) and had resistances in the range of 2-5 MΩ when filled with pipette solution containing (in mmol/l): potassium aspartate 110, KCl 20, NaCl 8, HEPES 10, K_4BAPTA 10, K_2ATP 4, $MgCl_2$ 1, $CaCl_2$ 1; adjusted to pH 7.2 with KOH. Pipette series resistance in the whole-cell mode was in the range of 2–10 MΩ. Capacitave surface area, access resistance, and input impedance were determined based on the capacitave transients elicited by symmetrical 10 mV voltage clamp steps from -80 mV. A holding potential of -80 mV was maintained in all experiments.

For HL-1 cells, the protocol to obtain steady-state inactivation curves consisted of a 5 second conditioning pulse applied in 10 mV steps as indicated followed by a 500 ms test pulse at +30 mV. Peak current amplitude at each test pulse was plotted as a function of conditioning pulse potential and fit to a Boltzmann equation using Prism 5 software

(GraphPad). Data from each cell was normalized to both maximal and minimal current values derived from the fit.

For isolated primary cardiomyocytes, I_{kur} was calculated as the difference between peak current and the current at the end of a 3-second test pulse to +30 mV. A 100 ms prepulse to -40 mV was included to inactivate I_{to} (74). I_{ss} was determined as the current remaining at the end of the test pulse, and I_{to} was calculated as the difference in current between the peak of the test pulse and the peak of a corresponding test pulse lacking the -40 mM prepulse (74). The protocol to measure the steady-state inactivation of I_{kur} consisted of a 5-second conditioning pulse applied in 10 mV steps as indicated, followed by a 100-ms prepulse to -40 mV, and a 3-second test pulse to +30 mV (74). The I_{kur} amplitude after each conditioning pulse was normalized to the maximal amplitude of this current. I_{kur} values as a function of conditioning potential were fit to the Boltzmann equation. The resting membrane potential was determined in current clamp mode and nulling all currents. Action potentials were elicited using a 2 ms trigger pulse of 2 nA and monitored at 0.1 Hz. The magnitude of the action potential was calculated as the difference between the peak of the action potential and the resting membrane potential.

For infusions of purified, recombinant proteins, protein samples prepared as described above were thawed on the day of the experiment, exchanged into fresh pipette solution using a Bio-Spin 6 gel filtration column (Biorad, Hercules, CA), and quantitated using a Bradford assay. Samples were diluted into pipette solution to achieve the indicated concentrations, and loaded directly into the pipette with no back-filling.

Statistical analysis

Statistical analyses were performed using Sigma Plot 9.0 (Systat Software) and Prism 5 (GraphPad). Data are expressed as mean +/- s.e.m. Unpaired Student's *t*-tests

were performed as indicated, and statistical significance was assigned to $p < 0.05$. Comparison of V_{50} values was carried out with an F test applied to global fits of pooled vs separate sets using Prism5 software.

RESULTS

SUMO modification acutely regulates Kv1.5 inactivation in HL-1 atrial myocytes

To probe the functional role of the SUMOylation motifs in Kv1.5 in atrial cardiomyocytes, we characterized the voltage-dependence of steady-state inactivation elicited by expression of Kv1.5 forms harboring intact or disrupted SUMOylation motifs in HL-1 immortalized mouse atrial myocytes. As seen in Figure III-1, disruption of the Kv1.5 consensus SUMOylation motifs with double K221/536R substitutions, led to a significant, ~ 6.5 mV hyperpolarizing shift in the half-maximal voltage of steady-state inactivation (V_{50}) from the WT value of -10.02 +/- 1.82 (n=10) to -16.59 +/- 1.26 mV (n=9) ($p < 0.0001$). This shift occurred without significant alterations in the extent of inactivation (35.3 +/- 3.9 and 35.5 +/- 2.4 % for WT and K221/536R, respectively).

Our previous findings, (described in Chapter II) that loss of SUMOylation induces a hyperpolarizing shift in the voltage-dependence of steady-state inactivation of Kv1.5 in heterologous systems, argue strongly in favor of SUMOylation (rather than other lysine-directed modifications) as the mechanism responsible for the functional effects of the motifs in HL-1 cells. However, to test this hypothesis, we made use of a variant of the SUMO-specific protease SENP2 (His6-SENP2 364-489) that displays enhanced activity against multiple SUMOylated substrates and localizes to the cytoplasm. For simplicity, we refer to this form as SENP2 in the remainder of the thesis. Similar to our approach in Cos7 cells, we reasoned that reducing the levels of SUMOylation of WT channel by exposure to SENP2 should lead to a similar shift in the voltage-dependence of steady-

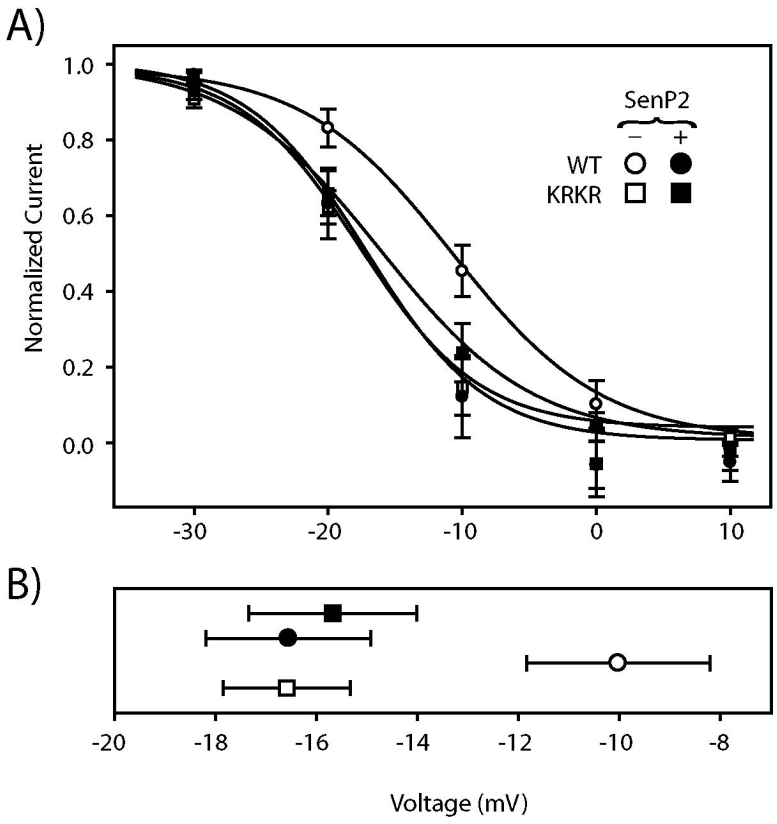

Figure III-1. SUMO Modification Acutely Regulates Kv1.5 Inactivation in HL-1 Atrial Myocytes.

HL-1 cells were co-trasnsfected with 0.5 g each of pEGFP-C1 (to identify transfected cells) and a pCDNA3.1-based vector for the expression of the indicated Kv1.5 variant and whole whole-cell patch-clamp recordings were conducted as described in Experimental Procedures. 333 nM purified, recombinant SENP2 was included in the patch pipette, as indicated. Data represent average +/- SEM. (A) The voltage-dependence of steady-state inactivation was fit to a Boltzmann equation. (B) The V_{50}s of steady-state inactivation are plotted from the fit in Panel A.

state inactivation. In contrast to our studies in Cos7 cells in which we co-expressed SENP2 with WT channel, we chose to probe the acute effects of SENP2 on Kv1.5 regulation by generating purified, recombinant forms of the protein and including the preparations into the pipette solution during our electrophysiological recordings. As can be seen in Figure III-1, inclusion of SENP2 in the pipette solution produced a significant ~ 6.5 mV hyperpolarizing shift in the voltage-dependence of steady-state inactivation in HL-1 cells. The treatment shifted the V_{50} from -10.03 +/- 1.82 (n=10) to -16.55 +/- 1.63 mV (n=5) ($p < 0.0001$) with no significant alteration in the extent of inactivation (43.4 +/- 4.7% (n=5) ($p = 0.23$). This shift was indistinguishable from that of the K221/536R mutant ($p = 0.91$), and importantly, was dependent on the integrity of the SUMOylation motifs in Kv1.5 since inclusion of SENP2 in the pipette solution did not alter the V_{50} of the SUMOylation-deficient K221/536R mutant (V_{50} = -15.68 +/- 1.67 mV (n=8); $p = 0.24$). The shift induced by SENP2 was detectable immediately at the initiation of recordings (which were started ~ 1 minute after establishment of the whole-cell configuration) and were stable for the duration of the recording (> 10 minutes). Infusion of a purified, recombinant form of SUMO1 (His6-SUMO1) had no effect on the voltage-dependence of steady-state inactivation (V_{50} = -10.22 +/- 1.49 mV (n=3)), consistent with our observation that co-expression of WT SUMO3 had no effect on the voltage-dependence of inactivation of Kv1.5 in Cos7 cells.

Taken together, these findings provide strong evidence that loss of SUMOylation, by either point mutations at the SUMOylation motifs or acute exposure to the SUMO-specific protease SENP2, leads to a hyperpolarizing shift in the voltage-dependence of steady-state inactivation in immortalized HL-1 atrial myocytes in a manner similar to that observed in Cos7 cells.

Isolation of I_{kur} current in acutely-dissociated mouse ventricular myocytes

Our characterization of the specific effects of SUMO modification on the isolated properties of Kv1.5 coupled with the ability to acutely modulate SUMOylation allowed the opportunity to examine the consequences of altering SUMOylation on the electrophysiological properties of acutely dissociated myocytes. In these cells, Kv1.5 is embedded in its natural context and mediates I_{kur}, a well-characterized outward current important for repolarization and establishment of the action potential duration. By taking advantage of the characterized effects of SUMOylation on Kv1.5 this approach can not only reveal effects of SUMOylation on Kv1.5 in its native context, but also can reveal the full impact of this regulatory mechanism on the endogenous complement of proteins that give rise to the specific electrophysiological properties of cardiac myocytes.

To this end, we conducted whole-cell patch-clamp studies of acutely dissociated mouse ventricular myocytes. These cells are useful model system because in rodents, Kv1.5 is expressed in both atrial and ventricular myocytes. Moreover, the ultrarapid-delayed rectifier potassium current (I_{kur}) in these cells has been extensively characterized. This current is mediated by Kv1.5 (74) and can be isolated from other outward currents based on its specific kinetic and pharmacologic properties (12,74).

As shown in Figure III-2A), and consistent with previous studies (74), whole-cell voltage-clamp recordings of mouse ventricular myocytes revealed that sustained depolarization to +30 mV from a holding potential of -80 mV yielded a rapidly-activating, slowly inactivating, outward current. The inactivation of this current displays three major kinetic components. The rapidly ($\tau \sim 17$ ms) inactivating component (I_{to}) is known to be mediated by members of the Kv4 family (181). A second component that inactivates more slowly (I_{kur}) is mainly mediated by Kv1.5 (Figure III-2A). A third, non-inactivating component (I_{ss}), is revealed at the end of the trace and is mediated by Kv2.1 and HERG family members (73).

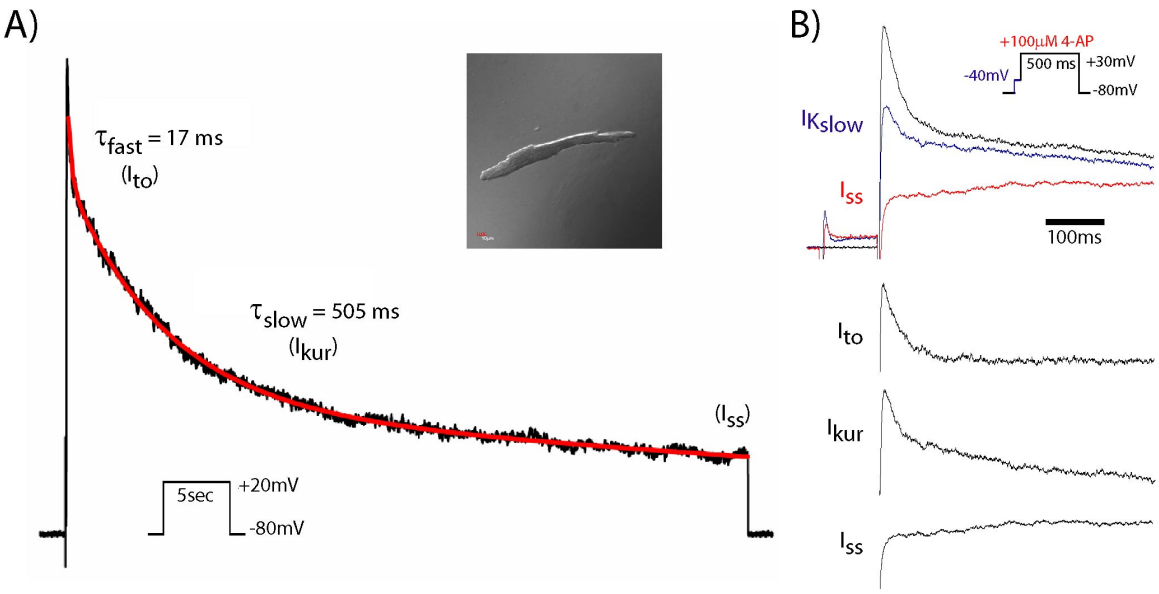

Figure III-2. Isolation of I_{kur} Current in Acutely-Dissociated Mouse Ventricular Myocytes.

(A) I_{to}, I_{kur}, and I_{ss} were isolated from total, outward K^+ current in acutely-dissociated ventricular myocytes as described in the text. I_{to} was calculated as the difference between the total outward current and I_{Kslow}. I_{kur} was calculated as the difference between I_{Kslow} and I_{ss}. And I_{ss} was the current remaining after treatment with 100 M 4-AP. (B) A raw trace of the total outward K^+ current in these cells obtained by depolarization from a resting potential of -80 mV to +30 mV. Inset shows an isolated mouse ventricular myocyte.

I_{to}, I_{kur} and I_{ss} can be separated using established protocols as shown in Figure III-2B (74). A brief (100 ms) depolarizing prepulse to -40 mV is sufficient to inactivate the I_{to} component without affecting I_{kur} or I_{ss}. A depolarization to +30 mV that incorporates a 100 ms pre-pulse to -40 mV thus reveals a current carried exclusively by I_{kur} and I_{ss}. This component is referred to as I_{kslow}. As had been previously described (74), we confirmed that by the end of a 3-second test pulse to +30 mV, I_{kur} current inactivates completely and only noninactivating I_{ss} persists. In this manner, I_{to} can be revealed as the difference between the total outward current and I_{kslow}. Using the two pulse protocol, the peak amplitude of I_{kur} can thus be defined as the peak I_{kslow} amplitude – I_{ss}.

Consistent with the pharmacological properties of the underlying channels, (12) treatment of the cells with 100 M of the potassium channel blocker 4-aminopyridine (4-AP) inhibits the I_{kur} component without affecting I_{to} or I_{ss} (Figure III-2B). Using the above two-pulse protocol, it is thus possible to isolate I_{kur} and study its inactivation properties.

SUMO modification acutely regulates inactivation of I_{kur} in mouse ventricular myocytes.

Given that SUMOylation alters the inactivation properties of Kv1.5, we focused initially on this property of isolated I_{kur} (Figure III-3). Using the two-pulse protocol, we measured the voltage-dependence of steady–state inactivation of I_{kur} as has been previously described (74), we confirmed that by the end of the 3-second test pulse, I_{kur} current had inactivated completely and only 4-AP-insensitive I_{ss} persisted (Figure III-3A). This enabled us to calculate the voltage-dependence of steady-state inactivation by measuring the difference in current between the peak (I_{Kslow}) and the end (I_{ss}) of the test pulse ($I_{Kslow} - I_{kss} = I_{kur}$) and plotting this value as a function of the potential of the conditioning pulse. Two representative traces from the voltage-dependence of steady-state inactivation protocol described above are shown in Figure III-3. The trace in panel

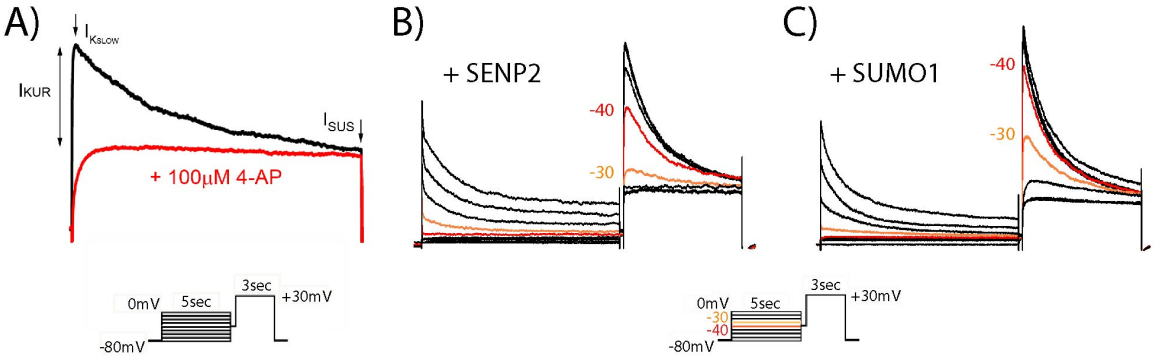

Figure III-3. The Voltage-Dependence of Steady-State Inactivation of Endogenous I_{kur} Current in Mouse Ventricular Myocytes.

The voltage-dependence of steady-state inactivation of endogenous I_{kur} current in acutely-dissociated ventricular myocytes could be determined using a 5-second conditioning pulse in 10 mV intervals from -80 mV to +20 mV, followed by a 100 ms "inactivating pre-pulse" to -40 mV to inactivate I_{to}, followed by a 3 sec test pulse to +30 mV. (A) The 3 second test pulse from the protocol for the voltage-dependence of steady-state inactivation is shown. Treatment with 100 M 4-AP demonstrates that I_{kur} has completely inactivated by the end of the pulse and only 4-AP-insensitive I_{ss} remains. I_{kur} was calculated as the difference in current between the peak (I_{Kslow}) and end (I_{ss}) of this test pulse. (B) A raw trace from the protocol for the voltage-dependence of steady-state inactivation with 2.5 M SENP2 included in the pipette solution. Conditioning pulses to -40 and -30 are indicated in red and orange, respectively. (C) A raw trace with 20 M SUMO1 included in the pipette solution.

B was collected with 2.5 M SENP2 included in the pipette solution, and the trace in Panel C was collected with 20 M SUMO1 included in the pipette solution. Comparison of these traces shows that while I_{kur} has undergone substantial inactivation after a conditioning pulse of -40 mV (highlighted in red) in the presence of SENP2, significantly less inactivation is observed at this potential in the presence of SUMO1. Quantitation of these results (Figure III-4), shows that inclusion of SENP2 in the pipette solution leads to a small, but statistically significant ~ 2.4 mV shift in the voltage-dependence of steady-state inactivation relative to control recordings in the absence of the protein. SENP2 shifted the V_{50} from -35.97 +/- 0.78 mV (n=17) in its absence to -38.31 +/- 0.81 mV (n=6) in its presence ($p = 0.016$). Conversely, inclusion of SUMO1 in the patch pipette during these recordings led to a small but significant ~ 2.1 mV depolarizing shift in the voltage-dependence of steady-state inactivation of I_{kur}, shifting the V_{50} from -35.97 +/- 0.78 mV (n=17) in its absence to -33.81 +/- 0.74 mV (n=19) in its presence ($p < 10^{-4}$). The ~ 4.5 mV shift in V_{50}s induced by infusion of SENP2 compared to infusion with SUMO1 was highly significant ($p < 10^{-4}$). The effects of these proteins appear to be restricted to I_{kur}, since we did not observe significant effects on I_{ss} current density. Moreover, SUMO1 had no significant effect on I_{to} current density (data on the effect of SENP2 on I_{to} was not collected) (Figure III-4, inset).

Consistent with our studies of Kv1.5 in Cos7 and HL-1 cells, these data provide evidence that loss of SUMOylation in acutely-dissociated mouse ventricular myocytes leads to a hyperpolarizing shift in the voltage-dependence of steady-state inactivation of endogenous I_{kur} current. In contrast, infusion of purified SUMO1 into these cells leads to a significant depolarizing shift in the voltage-dependence of steady-state inactivation of I_{kur} current. Notably, this effect of SUMO1 was not detected in HL-1 cells suggesting that the basal SUMOylation state of Kv1.5 may be lower in ventricular myocytes compared to HL-1 cells. Importantly, these preparations do not appear to have global

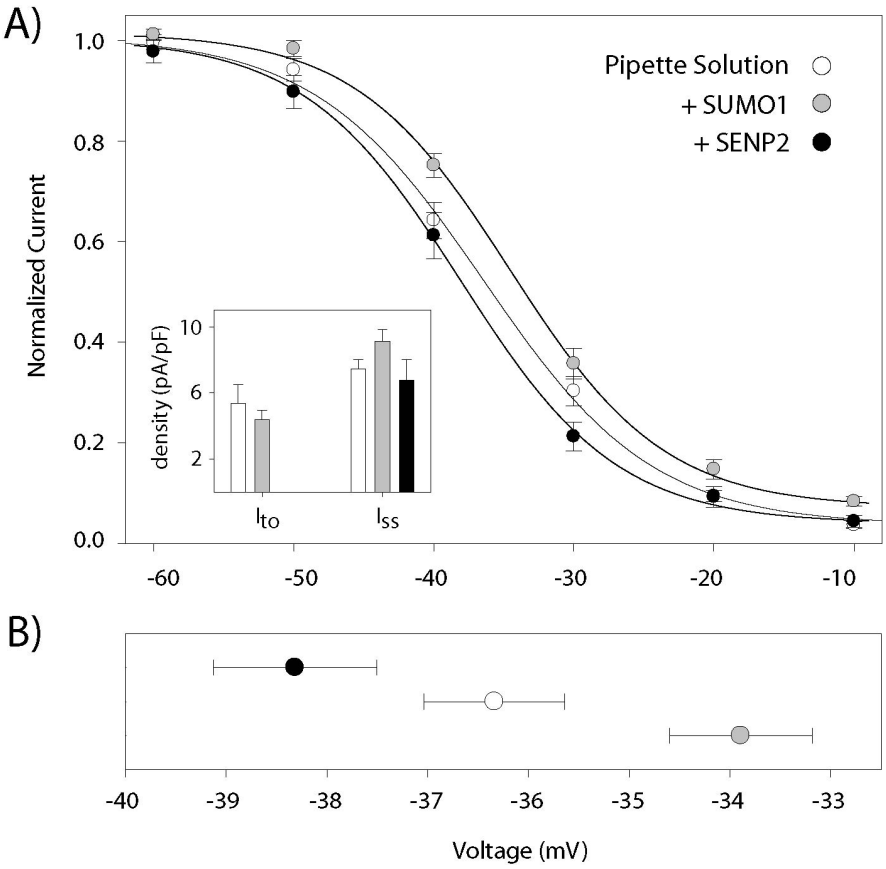

Figure III-4. SUMO Modification Acutely and Reversibly Regulates Inactivation of I_{kur} in Mouse Ventricular Myocytes.

(A) The voltage-dependence of steady-state inactivation of endogenous I_{kur} in acutely-dissociated mouse ventricular myocytes was recorded with either 3 M SENP2 or 20 M SUMO1 included in the patch pipette, as indicated. (B) The V_{50}s of inactivation as calculated from the fits in A are plotted.

effects on outward currents since the current densities of I_{to} and I_{ss} are unaffected by this treatment.

SUMO modification acutely regulates endogenous I_{kur} current in human atrial myocytes

As mentioned in Chapter I, Kv1.5 is an attractive target for atrial fibrillation therapy because of its functional role and atrial specific expression. In an effort to determine if the regulatory effects of SUMOylation on the inactivation of I_{kur} current are present in human cardiac tissue, we conducted whole-cell patch clamp studies of acutely-dissociated human atrial myocytes. The cells were derived from a single human donor heart that failed criteria necessary for successful cardiac transplantation. As shown in Figure III-5, inclusion of SUMO1 preparations in the pipette solution during these recordings led to a ~5.7 mV depolarizing shift in the voltage-dependence of steady-state inactivation of I_{kur} current, altering the V_{50} from -44.56 +/- 2.67 mV (n=5) in its absence to -38.83 +/- 1.85 mV (n=3) in its presence ($p<0.02$). Inclusion of SENP2 in the patch pipette during these recordings had no appreciable effect on the voltage-dependence of inactivation -43.98 +/- 3.21 mV (n=2) ($p=0.87$). These initial findings are consistent with the effects of SUMOylation on the properties of Kv1.5 and indicate that the regulatory effects of SUMO modification on I_{kur} current are indeed operational in the human heart. The fact that infusion of SUMO1 rather than SENP2 alters the inactivation of I_{kur} argues that the basal SUMOylation state of Kv1.5 may be lower in the human atrial preparation than in mouse ventricular myocytes.

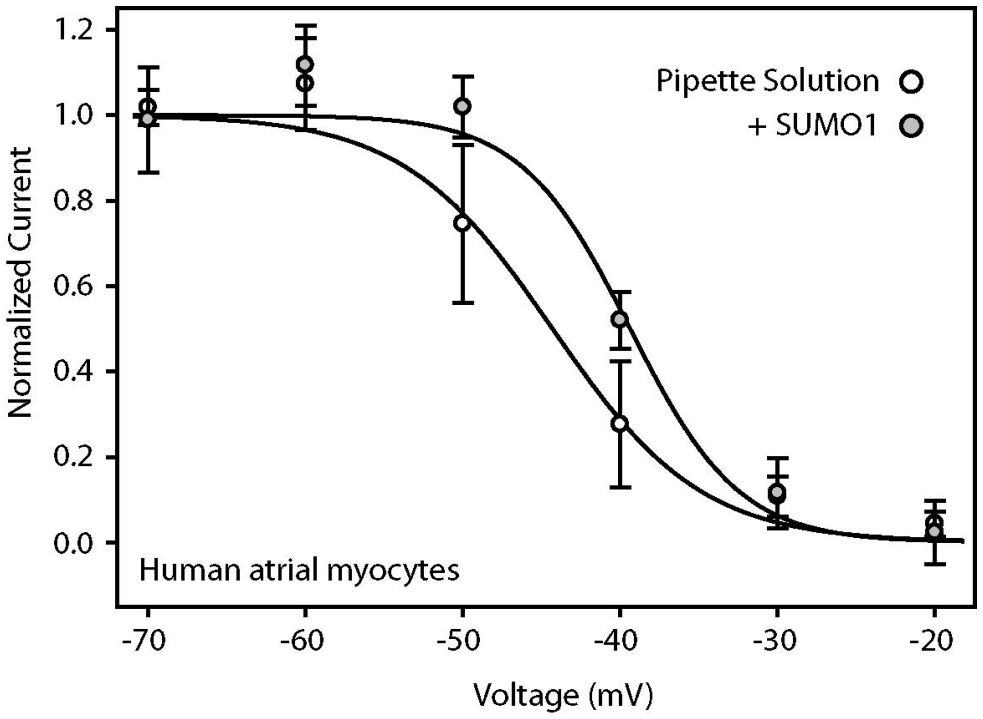

Figure III-5. SUMOylation Acutely Regulates Endogenous I_{kur} Current in Human Atrial Myocytes.

The voltage-dependence of steady-state inactivation of endogenous I_{kur} current from acutely-dissociated human atrial myocytes. Recordings were in the presence of 14 M SUMO1, as indicated.

A conjugation-deficient SUMO functions in a dominant-negative manner to regulate I_{kur} current in mouse ventricular myocytes

To probe the mechanism by which infused SUMO1 regulates the inactivation properties of the Kv1.5-mediated I_{kur} current in cardiomyocytes, we took advantage of a conjugation-deficient variant of SUMO1 (SUMO1-ΔGG). This form lacks the C-terminal di-glycine motif required for activation and conjugation to target proteins. Nevertheless, this form bears an intact effector binding surface required for non-covalent interactions with SUMO-binding partners (Figure III-6). If SUMO1 regulates I_{kur} current in cardiomyocytes by providing a binding surface for the formation of inter- or intra-molecular interactions, an excess of non-conjugatable form of SUMO may compete with the SUMO moiety attached to the channel and prevent the establishment of the relevant interactions. We would therefore predict that SUMO1-ΔGG may act in a dominant-negative manner to interfere with the normal function of Kv1.5 SUMOylation. In this view, this protein may exert effects similar to SENP2 on the I_{kur} current.

As seen in Figure III-7, inclusion of SUMO1-ΔGG in the pipette solution during whole-cell patch clamp studies of acutely-dissociated mouse ventricular myocytes led to a significant, ~ 3.5 mV hyperpolarizing shift in the voltage-dependence of steady-state inactivation of I_{kur}, altering the V_{50} from -35.97 +/- 0.78 mV (n=17) in its absence to -39.41 +/- 0.57 (n=11) in its presence ($p < 10^{-4}$). This shift was indistinguishable from that caused by infusion of SENP2 (-38.32 +/- 0.81 mV (n=6); $p = 0.06$) and occurred without significant effects on the current densities of I_{to} or I_{ss} (Figure III-9). The ~ 5.6 mV difference between the V_{50}s of inactivation in the presence of WT- and ΔGG-SUMO1 was highly significant ($p < 10^{-4}$).

Consistent with a dominant-negative mechanism, these findings indicate that inclusion of SUMO1-ΔGG in the patch pipette recapitulates the effects of SENP2 on the voltage-dependence of steady-state inactivation of endogenous I_{kur} current. Furthermore,

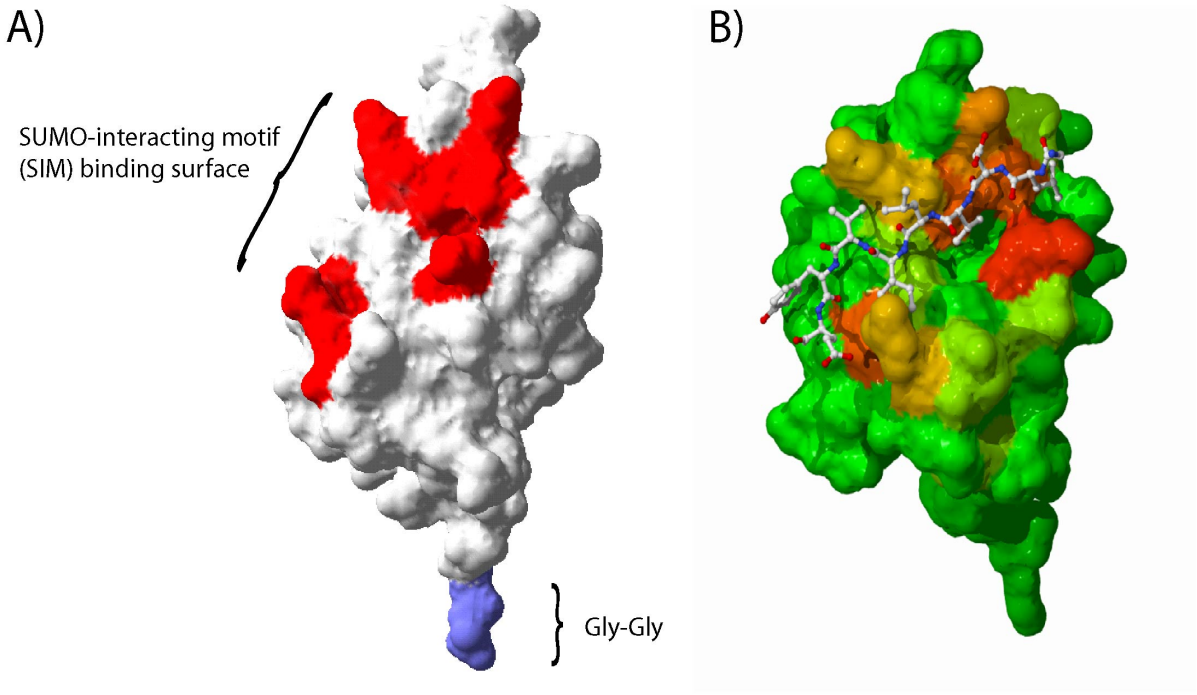

Figure III-6. SUMO Contains a Binding Surface that Facilitates the Formation of Inter- and Intra-Molecular Interactions with SIM-Containing Proteins.

(A) Homology modeling of SUMO2 was carried out using Swiss PDB viewer software (SPDBV V3.7) using the crystal structure coordinates of SMT3 (residues 20 to 98) as a template. The critical residues K33, K35, K42, R50, V30, I34, and T38 that make up this binding surface are highlighted in red. The C-terminal, di-glycine motif that is necessary for the covalent interactions of SUMO are highlighted in blue. (B) A model of the SUMO binding motif ([D] [VI] [LQE] [VIE] [V]) of RanBPII in complex with the binding surface of SUMO2 based on the crystal structure of the RanBPII/SUMO2 complex (Reverter and Lima (2005) Nature 435(7042): 687-92).

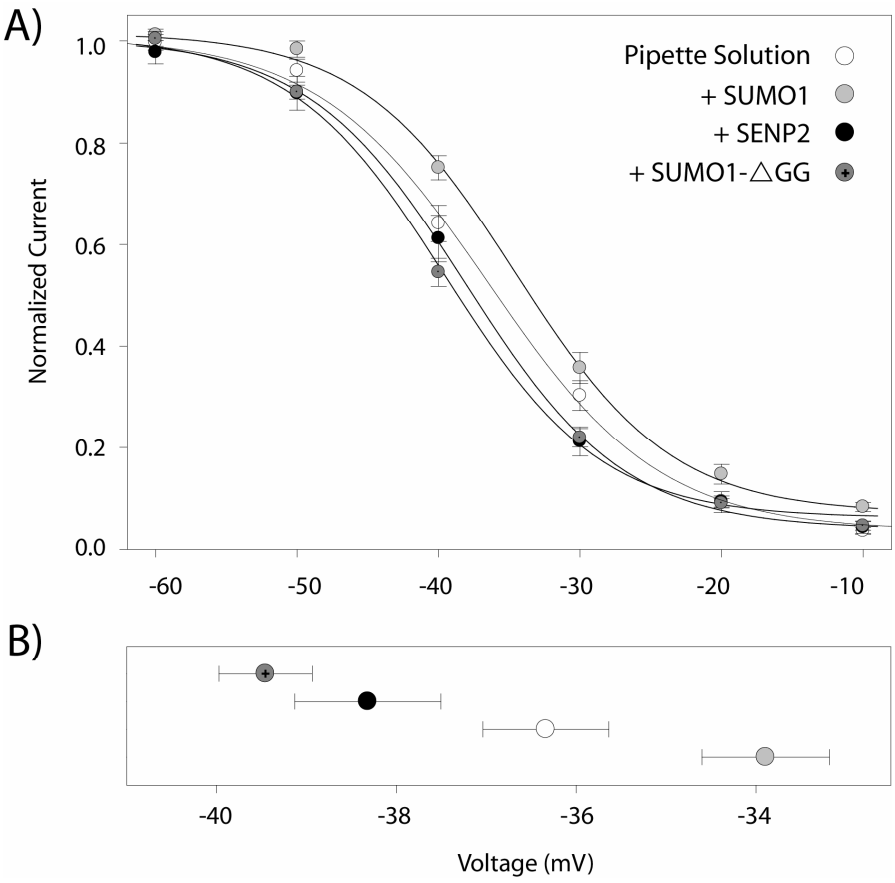

Figure III-7. Conjugation-Deficient SUMO1-ΔGG Functions as a Dominant-Negative Mutant and Regulates I_{kur} Current in Mouse Ventricular Myocytes.

(A) The voltage-dependence of steady-state inactivation of endogenous I_{kur} current in acutely-dissociated mouse ventricular myocytes was recorded in the presence of 20 M SUMO1-ΔGG, as indicated. Data from SENP2 and SUMO1 are from Figure 3.4. (B) The V_{50}s of steady-state inactivation as calculated from the Boltzmann plot in A.

they suggest that the mechanism of action by which SUMOylation regulates I_{kur} current may involve the facilitation of inter- or intra-molecular interactions with the channel.

Analysis of the effects of SUMO on the kinetics of I_{Kur} inactivation

The experiments described above demonstrate that SUMOylation affects the voltage-dependence of steady-state inactivation. This process depends on multiple kinetic parameters governing both the onset and recovery from inactivation. As an initial step to examine the mechanism of SUMOylation on the inactivation of I_{kur}, we examined in more detail the kinetics of inactivation after a strong depolarizing pulse to +30 mV where reliable estimates of kinetic parameters can be obtained.

The time course of inactivation of I_{kur} after a strong depolarizing pulse is complex. Previous characterization of Kv1.5 inactivation has revealed that the time course of inactivation can be well-described by fitting to a bi-exponential function with a fast time constant on the order of 10^2 ms and a slower time constant of approximately 1-2 seconds (182).

Using a similar protocol to that described above for the voltage-dependence of inactivation, I_{kur} current was accessed using a three-second depolarizing pulse to +30 mV from a holding potential of -80 mV immediately following a 100-ms pre-pulse to -40 mV to inactivate I_{to} (74). This current (I_{kslow}) is composed of I_{kur} and the non-inactivating component I_{ss}. During the +30 mV pulse, I_{kur} decays until only I_{ss} remains.

In control recordings, two populations of cells could be distinguished based on the kinetics of I_{kur} inactivation. In more than half of the cells, (12 of 19 or 63%) both a fast and a slow component could be discerned. The fast component had an average time constant of 179 ± 43 ms whereas the value for the slow component was 1242 ± 119 ms (See Figure III-8A). In the rest of the cells, only a slow component could be resolved.

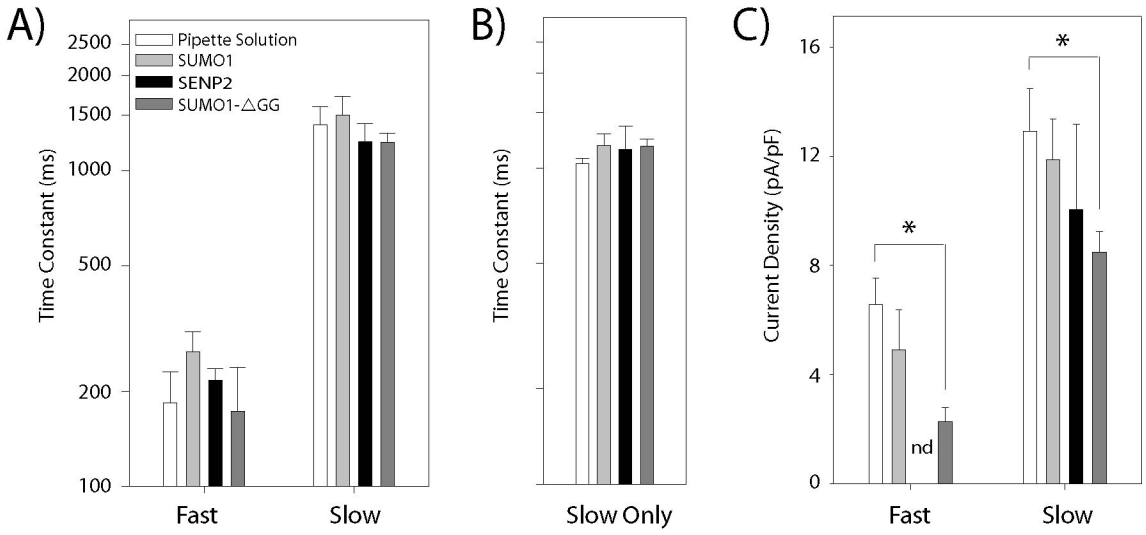

Figure III-8. Analysis of the effects of SUMO on the kinetics of I_{Kur} inactivation

The inactivating trace of I_{kur} at a sustained depolarizing pulse to +30 mV was fit to either a single- or bi-exponential equation. A) Time constants of inactivation for recordings best fit by a bi-exponential equation. B) Time constants of inactivation for recordings best fit to a single-component. C) Current densities of the fast and slow components. The current density of the slow component includes all cells described by either a single- or bi-exponential equation. Asterisk denotes $p<0.05$; n.d. denotes not determined.

The time constant in these cells was indistinguishable form the value of the slow component observed in cells that displayed a fast component (Figure III-8B).

Notably, inclusion of either SUMO1, SENP2 or SUMO1-ΔGG preparations in the recording pipette solution did not alter the time constants of inactivation of either the fast or slow component (Figure III-8A,B). It is important to note however, that the above measurements were obtained in response to a strong depolarizing pulse. It remains possible that SUMOylation affects these parameters at potentials closer to the V_{50} of steady-state inactivation. Closer inspection of the data however, revealed that although the proportion of cells displaying both fast and slow components was comparable for control and SUMO1 treated cells (63 vs 50%), this ratio was reduced to 31 and 33% in the case of SENP2 and SUMO1-ΔGG preparations, respectively. This suggested that the amplitude of the fast component may be affected by loss or interference with SUMOylation. Especially since a reduction in the amplitude may preclude its detection in this type of analysis. We therefore examined the effect of the proteins on the amplitude of both the fast and slow components. As can be seen in Figure III-8C, analysis of untreated cells revealed values of 6.6 ±0.99 and 12.9±1.6 pA/pF for the fast and slow components respectively. Notably, although inclusion of SUMO1 preparations in the recording pipette did not significantly alter the amplitude values, inclusion of SUMO1-ΔGG led to a significant reduction in the amplitude of both the fast and slow components ($p=0.03$ and 0.03 respectively). A reduction in the amplitude of the slow component was also observed for SENP2 but did not reach statistical significance. Notably, the relative contribution of each component to the total current was unaffected with 29 ± 3 % corresponding to the fast component.

Taken together, these results indicate that at +30 mV, altering SUMOylation does not influence the time constants of inactivation of I_{kur}. It is important to note, that A more extensive characterization of the effects of SUMOylation on the inactivation of I_{kur} will require a detailed analysis of the kinetics of onset of inactivation as well as its

recovery but also an examination of the voltage-dependence of the relevant rate constants. Importantly, the analysis did reveal that alterations in SUMOylation may influence the amplitude of I_{kur}. Given that we did not observe changes in current density upon *persistent* disruption of Kv1.5 SUMOylation in Cos7 cells (Chapter II), this suggests that in the native context, *acute* alterations in SUMOylation may have additional regulatory effects on I_{kur} beyond alterations in the voltage dependence of inactivation.

Manipulation of the SUMOylation pathway acutely regulates the current density of I_{kur} current in mouse ventricular myocytes.

The kinetic analysis described above indicated that alterations in SUMOylation likely influence the current density of I_{kur}. We therefore examined this possibility directly. As can be seen in Figure III-9, we find that inclusion of WT SUMO1 preparations in the recording pipette did not alter the I_{kur} amplitude. In contrast, inclusion of SUMO1-ΔGG led to a 48% reduction in I_{kur} current (9.17 +/- 0.88 pA/pF (n=13) relative to the control cells 17.53 +/- 1.84 pA/pF (n=17) (p= 0.001). Likewise, inclusion of SENP2 caused a 42% reduction to 10.21 +/- 3.08 pA/pF (n=5). This difference however fell short of statistical significance (p = 0.067) likely due to the relatively limited number of cells analyzed. Importantly, these effects were specific, as WT SUMO1, SENP2, and SUMO1-ΔGG had no significant effects on I_{ss} current densities, and neither WT SUMO1 nor SUMO1-ΔGG had a significant effect on I_{to} current density. This data indicates that in addition to regulating the inactivation properties of I_{kur} current, alterations in SUMOylation also influence overall I_{kur} current density. Since SUMO1-ΔGG and SENP2 but not SUMO1 inhibit I_{kur} current density, the data are consistent with the view that reducing or antagonizing SUMOylation has a negative impact on I_{kur} density and argues that in the native context, acute alterations in SUMOylation could affect electrophysiological properties that depend on the amplitude of I_{kur}, such as the action potential duration.

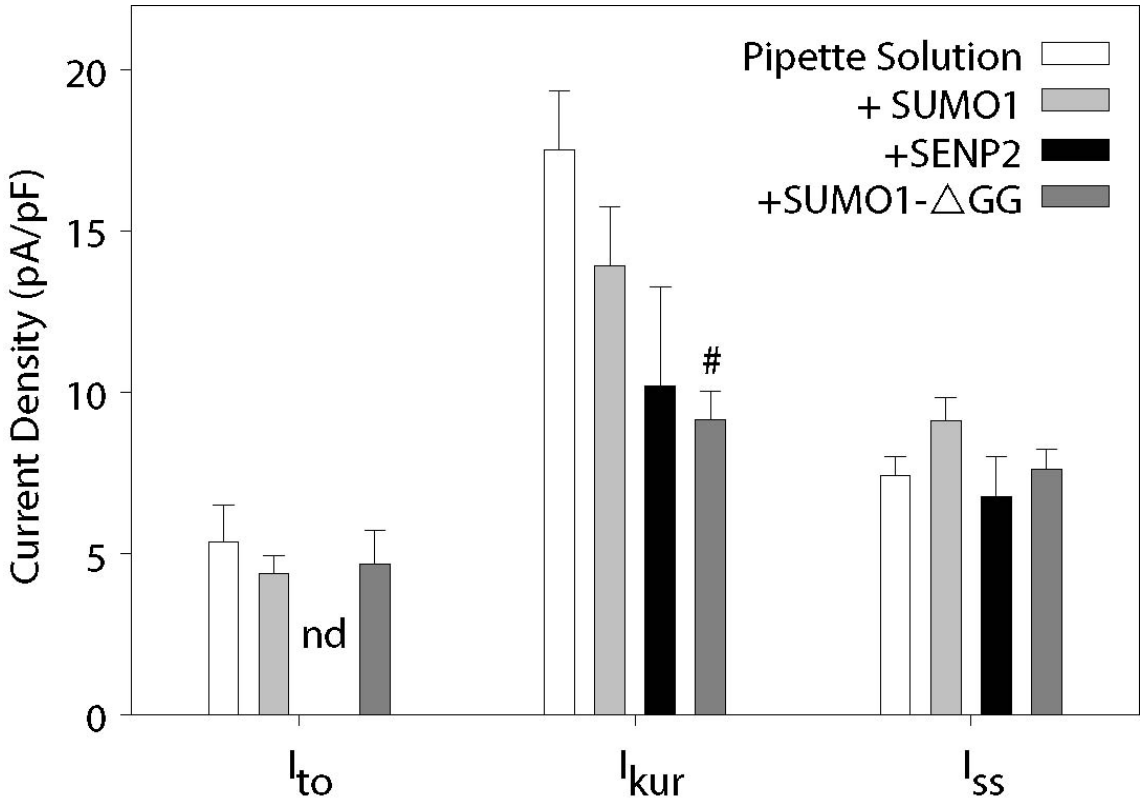

Figure III-9. SUMO Modification Acutely Regulates the Current Density of I_{kur} Current in Mouse Ventricular Myocytes.

Current densities of I_{to}, I_{kur}, and I_{ss} were calculated as described in *Experimental Procedures*. Data needed to calculate the current density for I_{to} in the presence of SENP2 were not collected. The # sign indicates statistical significance.

SUMOylation regulates the plateau phase of the action potential in mouse ventricular myocytes.

I_{kur} current is the main outward potassium current that mediates phase-two repolarization in the action potential in mouse cardiomyoytes (74). The findings that SENP2 and SUMO1-ΔGG acutely modulate the voltage-dependence of steady-state inactivation and the total current density of I_{kur} in mouse ventricular myocytes, prompted us to probe the effects of SUMO modification on the action potential of these cells. Whole-cell current-clamp electrophysiological recordings of acutely–dissociated mouse ventricular myocytes indicated that inclusion of SUMO1 in the pipette solution did not have a major effect on the shape or duration of the action potential (Figure III-10A). In contrast, inclusion of SENP2 or SUMO1-ΔGG preparations in the patch pipette during these recordings, led to a specific prolongation of the plateau phase of the action potential. This shift in the action potential duration (APD) was detectable soon after establishment of the whole-cell configuration and continued to develop over a period of approximately 10 minutes, at which point the APD remained constant Figure III-10B.

The effects of SENP2 or SUMO1-ΔGG are most evident in the plateau phase of the action potential. As shown in Figure III-10C, this is reflected as a 3.0- and 4.0-fold increase in APD_{50}, respectively. The effect on the APD_{10}, reflective of the notch phase, was considerably less dramatic. The final stages of repolarization are also affected to a lesser extent since the fold increase in APD_{80} is comparable to the effects on APD_{50}. Notably, the effects of the SUMO1-ΔGG preparation on action potential duration are dependent on a native protein structure since heat treatment of the preparation for 10 minutes at 95 °C inactivated the ability of the preparation to affect APD (last bars in Figure III-10C). Consistent with the previous data, the effects of these proteins appear restricted to properties affected by I_{kur}. None of the proteins altered

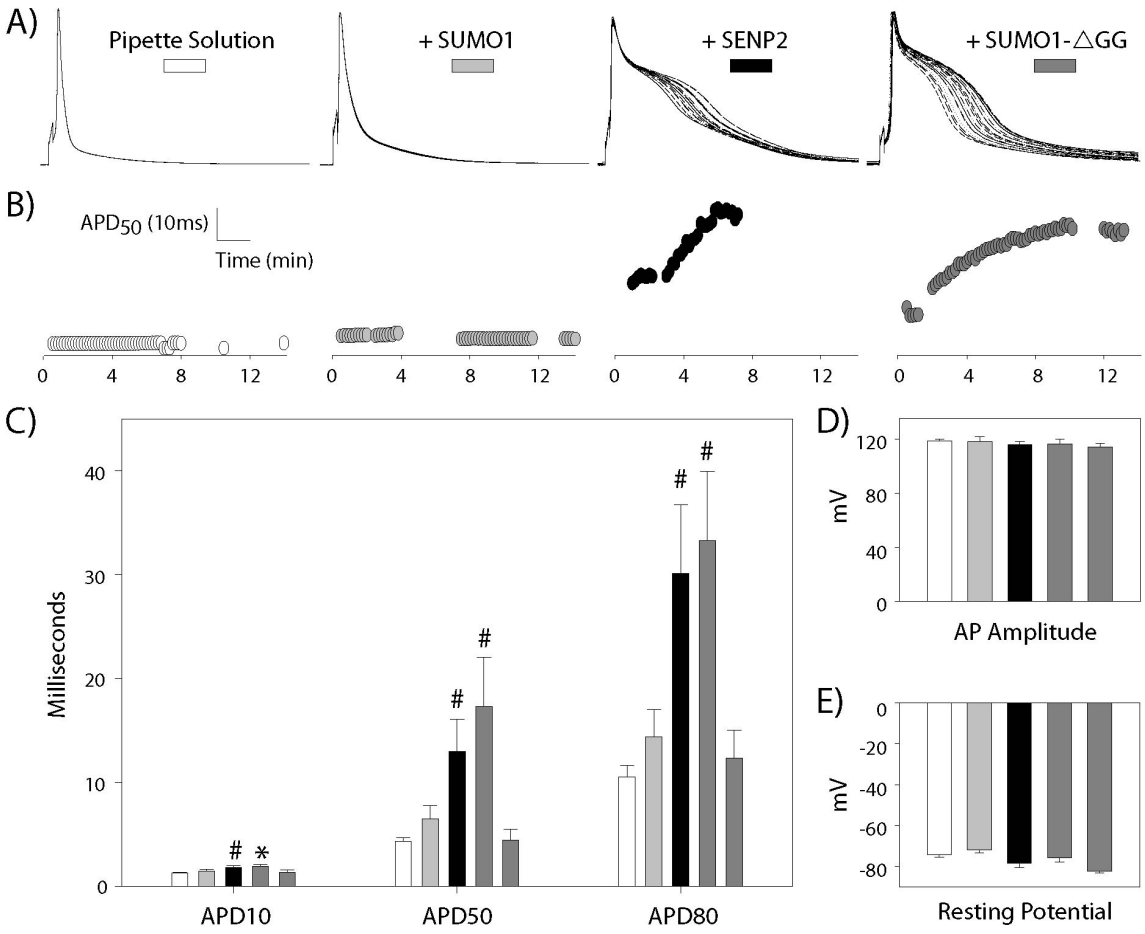

Figure III-10. SUMOylation Leads to a Specific Prolongation of the Plateau Phase of the Action Potential in Mouse Ventricular Myocytes.

(A) Raw traces from whole-cell current-clamp recordings of acutely-dissociated mouse ventricular myocytes in the presence of 20 M SUMO1, 2.5 M SENP2, or 20 M SUMO1-ΔGG, as indicated. (A) A 2- ms trigger current of 2 nA was used to induce the action potential at a frequency of 0.1 Hz. (B) The duration of the APD50 (in milliseconds) was monitored over successive recordings (0.1 Hz) from establishing whole-cell configuration (C) The quantitation of data from A and B as described in the text. The last, dark gray bars represent boiled SUMO1-ΔGG. The # sign indicates statistical significance. (D) The amplitude of the action potential was calculated as the difference between the peak of the action potential and the resting membrane potential. (E) The resting membrane potential was recorded while holding the cells at 0 pA in current-clamp mode.

appreciably the amplitude of the action potential (Figure III-10D) or the resting membrane potential (Figure III-10E).

Taken together, these initial findings provide exciting evidence consistent with the view that manipulation of the SUMOylation pathway can *acutely* regulate the duration of the plateau phase of the action potential. This reveals SUMOylation as a novel potential target for the development of antiarrhythmic medications aimed to prolong atrial refractoriness.

DISCUSSION

The studies presented in this chapter revealed that the Kv1.5-mediated I_{kur} current in cardiomyocytes can be acutely modulated by SUMO modification both at the level of its inactivation properties and its magnitude. We find that infusion of purified, recombinant SENP2 into HL-1 cells and mouse ventricular myocytes leads to a hyperpolarizing shift in the voltage-dependence of steady-state inactivation of Kv1.5-mediated current, while infusion of purified SUMO1 leads to an opposite, depolarizing shift in the V_{50} of inactivation of I_{kur} current in both mouse and human cardiomyocytes.

Interestingly, SENP2 infusion has a significant effect on modulating the V_{50} of inactivation of over-expressed Kv1.5 current in HL-1 cells, while infusion of SUMO1 has very little effect. Conversely, we find that in mouse and human cardiomyocytes, SUMO1 has a more prominent effect than SENP2 on modulating the V_{50} of inactivation of endogenous I_{Kur} current. This may suggest a difference in the basal SUMOylation "tone" between these cell types. In this view, Kv1.5 may be more highly SUMOylated in Cos7 and HL-1 cells under basal conditions, and thereby more susceptible to the effects of SENP2. The tone of Kv1.5 SUMOylation may be lower in endogenous cardiomyocytes, enabling I_{kur} to be more responsive to the effects of exogenous SUMO1. Differences in the SUMOylation tone of these cells could be due to differential expression of the SUMO conjugation machinery, accessory proteins that modulate Kv1.5 SUMO modification

(such as Kv accessory subunits), or additional targets of SUMOylation that compete for the conjugation machinery.

It is interesting to note that the value of the V_{50} of steady-state inactivation of Kv1.5 is different in cardiomyocytes than in Cos7 cells. While the V_{50} of inactivation of WT human Kv1.5 was 0.33 +/- 1.2 mV in Cos7 cells, it was found to be -14.88 +/- 1.4 mV in HL-1 atrial myocytes. Likewise, while we detected an ~15 mV shift in the voltage-dependence of steady-state inactivation upon disruption of the SUMOylation motifs or treatment with SENP2 in Cos7 cells, we consistently detected this shift to be ~5 mV in HL-1 cells, mouse ventricular myocytes, and human atrial myocytes. As described in Chapter I, the biophysical properties of Kv channels are often dictated not only by the subunits, themselves, but also by interactions of these subunits with other cellular components. In this regard, accessory cytoplasmic subunits, lipid interactions, additional posttranslational modifications, and interactions with scaffolding proteins are all known to modulate the properties of Kv subunits. Thus, the finding that the V_{50}s of steady-state inactivation of Kv1.5 are different in Cos7 cells and cardiomyocytes may imply the presence of regulatory effectors in cardiomyocytes that are absent in Cos7 cells.

Kv accessory subunits are one such potential regulator. These accessory subunits are known to be expressed in cardiomyocytes (183), but not in Cos7 cells, and have important effects on the inactivation properties of Kv1.5 (184). It seems possible that SUMO modification could serve to modulate interactions of these accessory subunits with the channel. It will be interesting in future experiments to probe whether interactions with Kv accessory subunits, as well as other potential, as-of-yet un-identified protein partners present in cardiomyocytes, are responsible for the effects of SUMOylation on Kv1.5 function.

SUMO modification not only has important regulatory functions on the inactivation properties of Kv1.5-mediated I_{kur} current in mouse ventricular myocytes, but

also modulates the amplitude of this current. Whereas infusion of SUMO1 has no appreciable effect on the density of I_{kur} current in these cells, agents that reverse (SENP2) or antagonize the effects of SUMOylation (SUMO1-ΔGG) leads to a substantial reduction in I_{kur} amplitude. Notably, these effects are restricted to I_{kur}, since no effects on I_{to} or I_{ss} total current density could be detected. As I_{kur} amplitude is a critical factor in establishing the duration of the plateau phase of the action potential, this finding could have important clinical implications, especially since extending the refractory period is one of the goals of long-term atrial fibrillation therapy. The initial studies presented in this chapter indicate that in mouse cardiomyocytes, both SENP2 and SUMO1-ΔGG preparations led to significant and specific prolongation of the plateau phase of the action potential. In contrast, SUMO1 had no effect. The observed prolongation is remarkably similar to the effects of clinically-used, Class III antiarrythmic drugs. Given the selective expression of Kv1.5 in human atrial tissue, strategies that specifically inhibit the SUMOylation of this channel may thus provide an independent and chamber-selective mechanism to delay repolarization. Such strategies could act synergistically with currently available pore-blocking drugs.

The observation that the effects of SUMOylation on the total current density of Kv1.5-mediated I_{kur} current were limited to cardiomyocytes and not visible in Cos7 cells, suggests that the mechanism responsible for this regulation may be distinct from that underlying the effects of SUMOylation on the inactivation properties of Kv1.5. Our finding that the effect of infused SENP2 on the voltage-dependence of steady-state inactivation of Kv1.5 in HL-1 cells was dependent on the presence of intact SUMOylation motifs in Kv1.5 provides strong evidence that SUMOylation is likely acting on the channel directly to regulate the inactivation properties. However, our studies have not addressed whether the effects of SUMOylation on I_{kur} amplitude are dependent on SUMOylation of Kv1.5 directly. As discussed in more detail in Chapter IV, it is possible that SUMOylation may regulate I_{kur} current by altering other

components of the cell that indirectly regulate Kv1.5. For example, SUMO modification may regulate the delivery of Kv1.5 to the plasma membrane or its internalization. The Martens laboratory has recently shown that Kv1.5 current density is tightly regulated by the dynamic processes of dynein-mediated endocytosis and recycling in HL-1 cardiomycytes (108). It will therefore be very interesting in future studies to probe the role of SUMOylation on the regulation of Kv1.5 internalization and transport.

The finding that SUMO1-ΔGG recapitulates the effects of SENP2 on both the amplitude and inactivation properties of the I_{kur} current in cardiomyocytes is consistent with the view that inter- or intra-molecular interactions are critical to the functional consequences of Kv1.5 SUMOylation. As with most other forms of post-translational regulation, the effects of SUMO modification vary greatly depending on the target protein. It has begun to emerge that a common mechanism by which SUMOylation is able to regulate such a diverse array of functional effects may be by its ability to modulate inter- and intramolecular interactions (122). In addition to the covalent interaction between its C-terminal glycine and the target lysine of its substrate, structural and scanning mutagenesis analyses (152) have revealed that an evolutionarily conserved surface contributed by the β2 strand and α helix of SUMO provides a surface for interactions with many target proteins. The structures of a PIASx peptide (149), thymine DNA glycosylase (150), and RanBP2 (151) in complex with SUMO via this surface have all recently been solved. As SUMO1-ΔGG is selectively missing the C-terminal di-glycine motif required for formation of covalent interactions with target proteins, but retains an intact effector surface required for non-covalent interactions, this protein provides a unique tool to probe the functional importance of this functional surface. The finding that SUMO1-ΔGG has similar effects on I_{kur} as SENP2, suggests that this variant may be serving as a dominant-negative to endogenous SUMO by competing for inter- and intra-molecular interactions. As discussed below in Chapter IV, a subset of proteins associated with the plasma membrane contain I/L/V-rich sequences resembling the

SUMO-interacting motifs (SIMs) known to interact with the effector surface of SUMO (171). It will be very interesting in future studies to investigate whether such SIM-containing candidate proteins contribute to the functional effects of Kv1.5 SUMOylation and to determine whether the same or distinct components mediate the effects on inactivation and current density.

In contrast to our previous studies in Cos7 cells, in the present study we identify that the effects of SUMOylation on Kv1.5 and I_{kur} current can be modulated *acutely*. The effects of SENP2 and SUMO1-ΔGG on the action potential duration could be detected within ~ 1 minute of establishment of the whole-cell configuration and developed relatively quickly. Although in most cases the effects of SUMO1, SENP2, and SUMO1-ΔGG on the voltage-dependence of steady-state inactivation were recorded after at least 5 minutes, occasionally, we were able to obtain data early enough to monitor a shift in the V_{50} of inactivation in the same cell. In this regard, future experiments in which protein diffusion is slowed by back-filling the recording pipette may prove useful to further characterize the onset of the effects. The acute effects we have observed are consistent with the studies of K2P1 (164) and GluR6 (166) SUMOylation. In inside-out, single-channel studies of the K2P1 channel, exposure of K2P1 to the SUMO conjugation machinery suppressed channel activity within seconds, while subsequent exposure to SENP1 restored channel activity in a similar time frame (164). Similarly, in whole-cell patch-clamp studies of the GluR6 kainate receptor, infusion of SUMO1 into CA3 neurons caused a rundown of kainate-receptor-mediated excitatory postsynaptic currents (KAR-EPSCs). This effect was half-maximal at ~ 1-2 minutes after gaining access to the cell. These results strongly suggest that SUMO modification of Kv1.5 and other ion channels occurs while the channel is at the plasma membrane. This is consistent with the findings that Ubc9 can be detected in subcellular fractions from the plasma membrane in numerous, mammalian cell types. This is also consistent with our findings described in

Chapter II, that surface-biotinylated Kv1.5 is SUMO modified with approximately the same stochiometry as that for total cellular Kv1.5.

It is important to note that given the use of purified preparations, we cannot formally rule out the possibility that minor components in these preparations may have unsuspected effects. This seems to be unlikely, however, since we have taken reasonable steps to insure the purity of the preparations. Moreover, the preparations were purified in parallel, yet display opposing effects. Furthermore, the effects are fully consistent with the results obtained with the expression approaches described in Chapter II.

In conclusion, the findings presented in this chapter extend our initial studies in heterologous systems by demonstrating that alterations in SUMOylation modulate Kv1.5 inactivation in the native context of cardiomyocytes, and that they can occur acutely. The studies in this natural context also revealed additional effects on Kv1.5-mediated currents that were absent in simpler model systems. Thus, the data indicate that reversing or antagonizing SUMOylation leads to a significant reduction in I_{kur} current density and a concomitant prolongation in the action potential duration. Moreover, the opposing effects of agents that promote or antagonize SUMOylation suggest that cells have an intrinsic Kv1.5 SUMOylation "tone" that can be manipulated bidirectionally. Taken together, the data raise the intriguing possibility that manipulation of the SUMOylation of Kv1.5 may provide a novel target for the highly specific pharmacological manipulation of I_{kur} current in patients with atrial fibrillation.

CHAPTER IV: CONCLUSION

Through the work of this thesis, we have established that Kv1.5, which encodes the major outward K^+ current responsible for phase-two repolarization in human atrial cardiomyocytes, serves as a target for reversible, posttranslational modification by small ubiquitin-like modifier (SUMO) proteins. We demonstrated that this modification occurs at two conserved consensus SUMOylation sequences centered on lysines 221 and 536, and that this modification has important functional consequences on the inactivation properties of the channel. Furthermore, we have found that this novel form of Kv channel regulation is intact in mouse and human cardiomyocytes. Through the use of purified, recombinant components of the SUMOylation conjugation machinery, we show that alterations in SUMOylation result in the specific regulation of the voltage-dependence of steady-state inactivation and total current density of native Kv1.5-mediated I_{kur} current in cardiac myocytes. Consistent with the role of Kv1.5 in controlling the shape and duration of the action potential, we find that reversing SUMOylation or antagonizing its effects leads to reductions in the amplitude of I_{kur} and as a consequence, specifically prolongs the plateau phase of the action potential in a manner that mimics the pharmacologic blockade of I_{kur} by antiarrythmic drugs.

These findings are significant as they represent the first report of the SUMO modification of a voltage-gated ion channel and only one of a handful of reports demonstrating the SUMOylation of a protein located at the plasma membrane. Kv1.5 represents a promising target for the development of ventricular-sparing, antiarrhythmic medications for treatment of atrial fibrillation. These findings have substantial implications for the development of novel therapeutic modalities since they reveal a

hitherto unrecognized regulatory mechanism that can serve as a potential new, highly-specific target for the pharmacological manipulation of I_{kur} current.

As described in the Introduction, at the onset of this thesis in 2004, numerous global proteomic analyses had repeatedly identified non-nuclear targets for SUMOylation, and the SUMOylation conjugation machinery had long been known not to be limited to the nuclear compartment. However, the overwhelming majority of well-described targets of SUMOylation were located in, or near the nucleus. The only proposed targets for SUMO modification at the plasma membrane were the GLUT1 and GLUT4 glucose transporters (160). This initial study was important not only for providing the first example of SUMOylation of integral membrane proteins, but also for establishing that the SUMO E2 Ubc9 could be detected in subcellular fractions from the plasma membrane of 3T3-L1 adipocytes, L6 skeletal muscle myoblasts, and terminally-differentiated myotubes. Following this initial report, it was not until 2005 that the potassium leak channel K2P1 (164) and the metabotropic glutamate receptor mGluR8 (162) were shown to serve as targets for SUMOylation. The K2P1 study again demonstrated that Ubc9 could be detected at the plasma membrane (this time in *Xenopus oocytes*). Our report of the SUMO modification of Kv1.5 was published in 2006. Since then, it has recently been shown that the ionotropic kainate receptor GluR6 (166) and numerous metabotropic glutamate receptor (mGluR) subunits (159) are targets for SUMO modification.

SUMO MODIFICATION OF PROTEINS AT THE PLASMA MEMBRANE

Together, these reports clearly identify an emerging role for the SUMO modification of ion channels at the plasma membrane. The extent to which this regulatory mechanism contributes to membrane protein function however, remains to be established. In an initial analysis, we have examined the complement of SUMO modified proteins present at the plasma membrane. As can be seen in Figure IV-1, SUMOylation of multiple

proteins is readily detected and the pattern is distinct to that observed for soluble proteins. This indicates that analysis of SUMOylation in this compartment is likely to be quite revealing. Although the identity of the target proteins remains to be determined, scanning of the human ProSite database using a search profile based on functionally characterized synergy control motifs in transcription factors (144) and a description filter of "channel," identifies that numerous ion channels contain high-scoring motifs (Figure IV-2). Included in these are multiple members of the voltage-gated K^+, Ca^{2+}, and Na^+ families. Interestingly, while members of the Kv4 family known to mediate I_{to} current in cardiomyocytes are absent from this list, the HERG channel (Kv11.1), which mediates the I_{ss} current in cardiomyocytes (73), contains a high-scoring motif on its cytoplasmic N-terminus (Figure IV-2). While we find that infusion of SUMO1, SENP2, or SUMO1-ΔGG has no effect on the current density of HERG-encoded I_{ss}, this clearly raises the possibility that SUMO modification may have other effects on the biophysical properties of this important channel.

Intriguingly, as well, a recent genotyping screen has revealed the occurrence of a naturally-occurring missense mutation in a patient with Long-QT syndrome that disrupts a flanking proline residue in a putative SUMOylation motif in HERG (P114S) (185). This finding is very interesting in light of a recent report of a similar nonsynonymous polymorphism in Kv1.5 (P532L) that maps to the well-conserved upstream proline of the second Kv1.5 SUMOylation motif. Remarkably, this polymorphism, which is carried by 1.1% of African Americans, confers a substantial reduction in the ability of quinidine to block Kv1.5 (186,187). As described in Chapter I, the conservation of Pro/Gly residues flanking the core SUMOylation motifs reflects their role as terminators of secondary structure elements and suggests that they facilitate exposure of the SUMOylation site to the conjugation machinery. Consistent with this idea, analysis of transcription factor SUMOylation by our group indicates that substitution of the proline residues flanking the core SUMOylation sites with amino acids other than glycine leads to a reduced

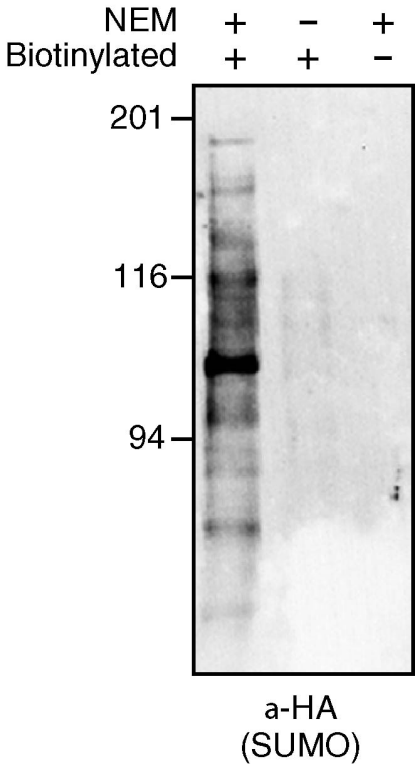

Figure IV-1. Multiple Plasma Membrane Surface Expressed Proteins in Cos7 Cells Appear to be Targets for SUMOylation.

Forty-eight hours after transfection with HA-SUMO3, Cos7 cells were surface biotinylated (as indicated) using a cell-impermeant, amine-reactive ester biotinylation reagent and lysed in the presence of 1% CHAPS and 20 mM NEM, as indicated. Following sonication, whole cell lysates were centrifuged at 16,000 x g for 15 min, and soluble protein was loaded onto either a streptavidin-agarose resin. After sequential washes with Buffer 1, 2, and 3 (as described in Chapter II *Experimental Procedures*), protein was eluted in SDS loading buffer, separated by SDS-PAGE, and visualized by Western blotting using an HA antibody. HA-immunoreactive bands represent NEM-sensitive species (lane 2) that were surface biotinylated (note absence of species in lane 3).

interaction with the SUMO E2 conjugating enzyme Ubc9 and a concomitant reduction in SUMOylation (unpublished data). An examination of whether the putative SUMOylation site in HERG is indeed functional and the potential relation with the P114S mutation associated with Long-QT syndrome is likely to be revealing.

It is also interesting to note that the Kv accessory proteins KChIP1, KChIP4, and Kv 4.1 all contain high-scoring SUMOylation motifs. While little is known about the interactions or functional effects of Kv 4.1, the other two proteins, KChIP1 and KChIP4 are well-known to interact with members of the Kv4 family and to alter the current densities and biophysical properties of these channels (188). As Kv4 family members mediate the I_{to} current in cardiomyocytes, this raises the possibility that while Kv4 family members do not appear to contain SUMOylation motifs, critical regulators of these pore-forming subunits may be subject to this form of regulation.

A less stringent scan of the human ProSite database using a profile containing only the core SUMOylation motif ([ILV]-K-X-[ED]) and a description filter of "channel" yielded many additional hits not identified in the above search (205 hits in 123 sequences). Included in these hits were multiple isoforms of serotonin receptors, ATP-binding cassette (ABC) transporters, cyclic nucleotide-gated (CNG) channels, inward rectifier potassium (Kir) channels, ryanodine receptors (RyR), and two-pore potassium channels (among many other families of membrane-associated proteins). Many of these identified motifs appeared to be located in regions of the proteins accessible to the cytoplasmic SUMO conjugation machinery.

These intriguing observations coupled with our analysis of Kv1.5 support the view that SUMO modification is likely to be an important and widespread regulatory mechanism at the plasma membrane and that it can have a significant impact on the control of the electrical properties of excitable cells and in particular in the heart.

Acc # | Symbol | (Size aa)	Description	SUMOylation Motif
|Q09470|KCNA1| (495)	Voltage-gated potassium channel subunit Kv1.1	135 - 145: Gf...IKeEerplP
		190 - 200: Pe...LKdDkdftG
|P16389|KCNA2| (499)	Voltage-gated potassium channel subunit Kv1.2	131 - 141: Gy...IKeEerplP
|P22460|KCNA5| (613)	Voltage-gated potassium channel subunit Kv1.5	218 - 228: Gf...IKeEekplP
		532 - 540: Pav..LKeEq...G
|Q03721|KCNC4| (635)	Voltage-gated potassium channel subunit Kv3.4	20 - 32: PsktcLKeEmak.G
|Q12809|KCNH2| (1159)	Voltage-gated potassium channel subunit Kv11.1	114 - 120: P....VKnEd...G
|Q9ULD8|KCNH3| (1083)	Voltage-gated potassium channel subunit Kv12.2	115 - 121: P....IKnEk...G
		786 - 797: GragaLKaEag..P
|Q9UQ05|KCNH4| (1017)	Voltage-gated potassium channel subunit Kv12.3	115 - 121: P....IKnEm...G
|Q9H252|KCNH6| (994)	Voltage-gated potassium channel subunit Kv11.2	114 - 120: P....VKnEd...G
|Q9NS40|KCNH7| (1196)	Voltage-gated potassium channel subunit Kv11.3	1093-1105: Peas.IKtDrsfsP
|Q96L42|KCNH8| (1107)	Voltage-gated potassium channel subunit Kv12.1	115 - 121: P....IKnEk...G
|Q9UNX9|IRK14| (436)	ATP-sensitive Inward rectifier K(+) channel Kir2.4	50 - 57: Grf..VKkD....G
|Q9NZI2|KCIP1| (227)	Kv channel-interacting protein 1 (KChIP1)	175 - 182: Pv...LKeDt...P
|Q6PIL6|KCIP4| (250)	Kv channel-interacting protein 4 (KChIP4)	198 - 205: Pv...LKeDa...P
|Q9Y244|POMP| (141)	Voltage-gated K channel beta subunit 4.1	34 - 44: Gfsc.VKnEll..P
|Q8IZS8|CA2D3| (1091)	Voltage-gated calcium channel subunit alpha-2/delta-3	225 - 235: Pg...IKwEpdenG
|Q15878|CAC1E| (2312)	Voltage-gated calcium channel subunit alpha Cav2.3	852 - 860: Ggs..LKgDg...G
|O43497|CAC1G| (2377)	Voltage-gated calcium channel subunit alpha Cav3.1	653 - 663: Pc...LKaDsgacG
|O95180|CAC1H| (2353)	Voltage-gated calcium channel subunit alpha Cav3.2	938 - 948: GckfsLKtDt...G
|Q9P0X4|CAC1I| (2223)	Voltage-gated calcium channel subunit alpha Cav3.3	1932-1945: PvrswLKhDssqaP
|Q13698|CAC1S| (1873)	Voltage-gated calcium channel subunit alpha Cav1.1	1472 - 1481: GtalkIKtE....G
|Q92736|RYR2| (4967)	Cardiac muscle ryanodine receptor-calcium channel (RyR2)	4381 - 4389: Gld..LKrEg...G
|A8K7I4|CLCA1| (914)	Calcium-activated chloride channel family member 1) (hCLCA1)	754 - 766: GqitdLKaEihg.G
|Q9UQC9|CLCA2| (943)	Calcium-activated chloride channel family member 2) (hCLCA2)	662 - 670: Gadv.IKnD....G
|Q9Y696|CLIC4| (253)	Intracellular chloride ion channel protein p64H1	6 - 17: Plng.LKeEdke.P
|P35498|SCN1A| (2009)	Voltage-gated sodium channel subunit alpha Nav1.1	1748-1759: Pgss.VKgDcgn.P
|Q99250|SCN2A| (2005)	Voltage-gated sodium channel subunit alpha Nav1.2	1738-1749: Pgss.VKgDcgn.P
|Q9NY46|SCN3A| (2000)	Voltage-gated sodium channel subunit alpha Nav1.3	1733-1744: Pgss.VKgDcgn.P
|P35499|SCN4A| (1836)	Voltage-gated sodium channel subunit alpha Nav1.4	1560-1571: Pgts.VKgDcgn.P
|Q9UQD0|SCN8A| (1980)	Voltage-gated sodium channel subunit alpha Nav1.6	1134-1147: GstidIKpEveevP
|P51172|SCNND| (638)	Nonvoltage-gated sodium channel 1 subunit delta) (SCNED)	580-592: Gass.IKpEasqmP
|Q8IWT1|SCN4B| (228)	Sodium channel subunit beta-4 precursor	82 - 91: Gt...VKnEksd.P
|Q9BX84|TRPM6| (2022)	Melastatin-related TRP cation channel 6	1995-2006: Gle..IKiEsaeeP
|Q9Y5S1|TRPV2| (764)	osm-9-like TRP channel 2) (TrpV2)(OTRPC2)	421 - 428: Ph...LKaEv...G
|Q9BQR3|PRS27| (290)	Channel-activating protease 2 (CAPH2)(Marapsin) (Pancreasin)	205 - 216: Pkt..IKnDmlcaG
|Q8TDI7|TMC2| (906)	Transmembrane channel-like protein 2 (cochlear-expressed)	7 - 15: G....LKeEarg.G

Figure IV-2. Identification of Membrane Ion Channels that Contain Synergy Control Motif SUMOylation Sequences.

Using the ExPASy: ScanProsite Tool, the UniProtKB/Swiss-Prot protein database was scanned using the sequence [PG]-X(0,4)-[ILV]-K-X-[ED]-X(0,4)-[PG] with the species filter "human" and the description filter "channel" and the match mode "greedy/overlaps/no includes." All 38 hits on 35 sequences are shown.

MULTIPLE EFFECTS OF SUMO ON KV1.5 MEDIATED CURRENTS

In mouse ventricular myocytes, we find that SUMOylation has important regulatory effects on at least two properties of native I_{kur} current. In addition to the bi-directional effects of SUMO1 and either SENP2 or SUMO1-ΔGG on the voltage-dependence of steady-state inactivation of I_{kur}, we find that infusion of SENP2 or SUMO1-ΔGG leads to a substantial decrease in I_{kur} total current density. Importantly, these multiple effects are restricted to I_{kur} current since SUMO1, SENP2, and SUMO1-ΔGG infusion had no effect on current densities of I_{to} or I_{ss}.

It is interesting to note that we detect differences in how SUMOylation modulates these two properties of Kv1.5 in different cell types. For example, whereas SUMO modification regulates the voltage-dependence of steady–state inactivation of Kv1.5-mediated current in Cos7 cells, HL-1 cells, mouse ventricular myocytes, and human atrial myocytes, we find that SENP2 regulates Kv1.5-mediated I_{kur} current density in mouse ventricular myocytes, but not in Cos7 cells. As discussed further below, this difference suggests that the regulation of Kv1.5 inactivation and amplitude by SUMOylation may be mediated by distinct mechanisms. While effects of SUMOylation on the voltage-dependence of steady-state inactivation of Kv1.5 are visible in all cell types we have examined, the dominant direction and magnitude are characteristic of different cells. Likewise, the impact of SUMO modification in the regulation of Kv1.5 mediated current density appears restricted to cardiomyocytes.

Furthermore, whereas we find that the voltage-dependence of steady-state inactivation of I_{kur} current in mouse ventricular myocytes is bi-directionally regulated by the opposing effects of SUMO1 and either SENP2 or SUMO1-ΔGG, the total density of I_{kur} current is affected only by agents that reverse SUMOylation (SENP2) or antagonize its effects (SUMO1-ΔGG), but not by SUMO1 itself. This may suggest that the voltage-

dependence of steady-state inactivation and the current density of I_{kur} have different sensitivities to regulation by SUMOylation. While the voltage-dependence of steady-state inactivation is responsive to increases or decreases in the SUMOylation state of the cell, the regulation of I_{kur} amplitude by SUMOylation may have reached saturation under the basal "tone" of SUMOylation in these cells, and thus may not be sensitive to exogenous SUMO. In this view, Kv1.5 amplitude might be expected to become sensitive to the effects of added SUMO1 in cells with low basal SUMOylation "tone." Therefore, it might be very interesting to probe the effects of SUMOylation on I_{kur} current density in human atrial cells. Although the scarcity of cells prevented us from accurately assessing the effects of SUMOylation on I_{kur} amplitude, our findings that SUMO led to a depolarizing shift in the V_{50} of steady state inactivation of I_{kur} in these cells while SENP2 had no effect, suggest that the basal SUMOylation tone is likely to be lower than in mouse ventricular cells.

Together, these observations suggest that the mechanisms by which SUMOylation regulates the voltage-dependence of steady-state inactivation and current density of I_{kur} current in cardiomyocytes are likely distinct. In the following section, I therefore describe potential mechanisms that may mediate the effects of SUMOylation on Kv1.5 inactivation and current density.

POTENTIAL BASIS FOR THE EFFECTS OF SUMOYLATION ON THE DENSITY AND INACTIVATION PROPERTIES OF THE KV1.5 MEDIATED I_{KUR} CURRENT

The effects of SUMO modification vary greatly depending on the target protein and range from changes in subcellular localization, to protein stability, to trafficking, to protein-protein interactions. Recently, it has begun to emerge that a common mechanism by which SUMOylation is able to regulate such a diverse array of functional effects may be through its ability to modulate inter- and intra-molecular interactions (122). In addition to the covalent interaction between its C-terminal glycine and the target lysine of

its substrate, a distinct effector domain on SUMO provides a surface for interactions with proteins that contain so-called SUMO-interacting motifs (SIMs). While SIMs are just beginning to be recognized in proteins that are able to interact with SUMO, a number of proteins, including p73α (153), PML (149,153,154), Daxx (155), SAE2 (149), PIAS E3 (149), RanBP2 (149,151), thymine DNA glycosylase (150), and RNF4 (156), have been identified to interact with SUMO through a loosely-defined V/I-X-V/I-V/I (149) hydrophobic sequence. In many proteins, this hydrophobic core has been found to be surrounded by acidic residues, which likely serve to further stabilize interactions with SUMO.

Because Kv1.5 functions as part of a multi-protein/lipid complex comprising proteins with scaffolding, cytoskeletal, and enzymatic activities, SUMO modification of Kv1.5 may function by modulating these interactions. This model would be consistent with our observations, since the cell-type dependence of some of the effects of SUMOylation may be due to the expression in each cell of a different complement of potential interacting partners. Indeed, our studies with the non-conjugatable SUMO1-ΔGG indicate that this variant may be functioning in a dominant-negative manner by competing for these interactions with endogenously SUMOylated channel.

To identify potential binding partners within the Kv1.5/SUMO complex that may be facilitated by the effector surface of SUMO, we conducted a scan of the human ProSite database using a search sequence based on the identified SIM/SBMs from PIASx, PML, SAE2, Daxx, RanBP2, and thymine DNA glycosylase (149,151,155). Comparison of these sequences revealed that the core hydrophobic motif could be described using the sequence [VI]-X-[VIE]-[VIL]. Additionally, in each of these cases, this motif was immediately flanked by either an N-terminal aspartic acid residue or a C-terminal aspartic acid/serine residue. The N-terminal Asp or the C-terminal Asp/Ser residues were included in the search since these features have been found to be critical to binding of SIM/SBMs to SUMO (158). This search, conducted with the descriptive filter “channel,”

yielded 154 hits in 101 sequences, and included multiple voltage-gated K^+, Na^+, and Ca^{2+} channels. However, careful inspection of these hits revealed that, given the hydrophobic nature of this motif, many of them were located in the transmembrane domains of the channels, making them less likely candidates. For example, Kv1.5 contains an I-A-I-V-S motif in the first six residues of the S1 transmembrane domain (residues 252-256) just 30 residues downstream from the first SUMOylation site. While it is potentially possible that this SIM/SBM could facilitate an intra-molecular interaction within Kv1.5, the detection of this sequence may be simply due to the enrichment of hydrophobic residues in transmembrane domains.

Potentially more interesting, however, is the finding that the Kv 1.3 and Kv 2.1 subunits each contain potential SIM/SBMs. These sequences are located on the C-termini of the subunits (400-404 and 348-393, respectively). This suggests that a potential consequence of SUMOylation of Kv1.5 may be to modulate interactions with accessory Kv subunits. This may be particularly significant since recruitment of Kvβ subunits can influence the voltage dependence of steady-state inactivation of the channel.

As more SUMO-interacting proteins are identified, and the definition of SIM/SBMs becomes more refined, it will be interesting to expand this initial search to include a larger pool of proteins not restricted to channel proteins (without the "channel" filter, the search yields more than one thousand hits). It seems likely that potential candidates for SIM/SBM-mediated interactions may include adapter, scaffolding, and trafficking proteins. Notably, the membrane-associated adaptor protein Daxx, which contains a SIM/SBM known to be critical to its role in transcriptional regulation, was found to interact with GLUT4 in a yeast-two-hybrid screen (171). It remains to be determined, however, whether this particular interaction is dependent on GLUT4 SUMOylation. We anticipate that relevant SIM/SBM-mediated interactions with SUMOylated membrane proteins will be identified in the near future.

In addition to promoting the formation of inter- or intra-molecular interactions with Kv1.5, SUMO modification could potentially alter channel properties through modulating the local electrostatic environment of the channel. As described above, this mechanism of action would be particularly attractive for describing the effect of SUMOylation on the regulation of I_{kur} inactivation, since we find this regulation to be largely independent of cell-type. Both of the identified SUMOylation motifs in Kv1.5 are located very near the transmembrane/cytosolic interface of the channel. The electrostatic environment of this region is thought to influence the voltage-sensitivity of Kv channels (45). The surface of SUMO is highly charged and has a significant dipole moment, with one hemisphere of the protein being rich in basic residues while the opposite face is predominantly acidic. Conjugation of SUMO to the channel could thus serve to affect the juxtamembrane, electrostatic environment of the channel and influence voltage-sensitive steps. The selective effects of SUMOylation on the voltage-dependence of inactivation, but not on activation (at least in Cos7 cells), however, argues that SUMOylation affects steps other than the voltage-sensitive transitions along the usual path toward activation.

Similarly, and also consistent with a possible cell-type-independent mechanism for regulation by SUMOylation on the voltage-dependence of steady-state I_{kur} inactivation, SUMOylation could serve to induce specific conformational changes in Kv1.5. As mentioned in Chapter I, SUMOylation has been shown to lead to conformational changes in target proteins. The most well-defined example of this is the SUMOylation of the DNA repair enzyme DNA glycosylase (TDG). Upon SUMOylation, TDG undergoes a conformational change induced by the formation of an intra-molecular interaction between the effector surface of SUMO and a SIM-containing domain of TDG. This conformational change alters the affinity of TDG for DNA and thereby allows it to be released from its bound DNA (150). Such a conformational change could be induced in Kv1.5 upon SUMOylation either through the formation of

similar intra-molecular interactions (with the SIM contained in Kv1.5 described above, for example), or through allosteric changes within the channel. In either case, this effect of SUMOylation would be largely independent of interactions with other components of the cell.

Although the effects of SUMOylation machinery components on the current density in cardiomyocytes appears restricted to I_{kur}, the specific target(s) responsible for these effects may extend beyond Kv1.5. Thus, SUMOylation may not only be acting on Kv1.5 directly, but may also affect other proteins in cardiomyocytes that indirectly regulate I_{kur} function. Our finding that the effect of infused SENP2 on the voltage-dependence of steady-state inactivation of Kv1.5 in HL-1 cells was dependent on the presence of intact SUMOylation motifs in Kv1.5, provides strong evidence that SUMOylation is likely acting on the channel directly to regulate the inactivation properties. However, while the effects of SENP2 and SUMO1-ΔGG on total current density were specific for I_{kur} in mouse ventricular myocytes and did not affect other channels, our experiments did not address whether these effects were due exclusively to Kv1.5 SUMOylation.

Interestingly, it is beginning to emerge that SUMOylation may play an important role in regulating the retrograde transport of proteins in cells. The RNA-binding protein La has recently been shown to be SUMO modified in rat sensory neurons. Whereas SUMOylated La binds dynein and shows retrograde transport from the axonal process back to the cell body, SUMOylation-motif-deficient La (K41R) binds kinesin and only displays anterograde transport. This provides a striking view that SUMOylation may directly facilitate the binding of cargo proteins to the dynein motor protein responsible for retrograde transport. Consistent with this, and as described in the Introduction, SUMOylation of the ionotropic kainate receptor GluR6 has recently been shown to facilitate kainate-evoked endocytosis of the receptor, while reduction of SUMOylation using SENP1 prevents this internalization. As the dynein motor system is critical to

facilitating endocytosis, this suggests a further role for SUMOylation in facilitating dynein-mediated, retrograde transport. Recent data from the group of Dr Martens has demonstrated that the steady-state current density of Kv1.5 in HL-1 cells is dependent on a dynamic balance between dynein-mediated internalization and delivery to the plasma membrane of recycled or newly synthesized channel (108). In future studies, it will be very interesting to probe the role of SUMOylation in the regulation of Kv1.5 retrograde and anterograde transport.

REGULATION OF THE SUMO MODIFICATION OF KV1.5-MEDIATED I_{KUR} CURRENT

SUMO E3 ligases and proteases.

At steady-state, the status of Kv1.5 SUMOylation is dependent on the balance between the forward conjugation of SUMO to the channel and the reverse de-conjugation of the SUMO moieties. We have identified Kv1.5 as a target of SUMOylation but the endogenous components responsible for the SUMOylation and de-SUMOylation of the channel remain to be defined. As described in Chapter I, the conjugation of SUMO can be enhanced through the action of proteins with SUMO specific E3 ligase activity. In this regard, the K^+ channel-associated protein (KChAP) may participate in this role. KChAP is a 574-amino acid, cytosolic accessory protein known to couple with the functional Kv1, Kv2.1, and Kv4.3 channels (99,175). It has recently been identified that KChAP is a splice variant of PIAS3, making it a member of the protein inhibitor of STAT (PIAS) gene family. This group of proteins was originally identified as negative regulators of the JAK/STAT signaling pathway. However, members of this family, including PIAS3, have been shown to possess SUMO-specific E3 ligase activity towards transcription factors (99,100,133). KChAP associates only transiently with Kv channels through the N-terminus of α-subunits and the C-terminus of β-subunits (99).

Overexpression of KChAP can lead to increases in total expression of Kv1 α-subunits and an enhancement of Kv1 total current density without apparent changes in the gating of these channels (99). The exact role and mechanism of action of this protein in Kv channel function and regulation remains ill-defined. However, as PIAS proteins possess intrinsic SUMO specific E3 activity and can enhance the conjugation of SUMO to multiple substrates (133), these findings raise the intriguing possibility that the effects that KChAP have on the expression and electrophysiology of Kv channels may be related to the regulation of SUMO modification. It will clearly be very interesting in future studies to determine if KChAP can serve as a functional SUMO E3 ligase and enhance Kv1.5 SUMO modification.

It will also be very interesting to investigate the role of SUMO-specific proteases in regulating the SUMOylation of Kv1.5. We have demonstrated in Cos7 cells, HL-1 cells, and mouse ventricular myocytes, that Kv1.5-mediated current is sensitive to the effects of a catalytic fragment of SENP2. Whether endogenous SENP2 plays a role in Kv1.5 regulation remains to be established. As described in Chapter I, multiple members of the SENP family have been identified (SENP-1-3, and -5-8 in mammals) (135-137). It is beginning to emerge that each of these members may display distinct subcellular and tissue distribution. This has raised the interesting possibility that differential expression of SENP family members may play an important role in the regulation of SUMOylation. It will thus be very interesting in future studies to characterize the expression of the SENP family members in human atrial and ventricular cardiomyocytes and to examine the association of such isoforms with the plasma membrane compartment. Given that proteases can be targeted by small molecule inhibitors, the identification of the relevant SUMO proteases could be instrumental in designing therapeutic strategies targeted at modulating the SUMOylation of Kv1.5.

Oxidative Stress

The factors regulating the modification of target proteins by SUMO are only beginning to be appreciated. Recent data suggest that SUMOylation is a developmentally-regulated process (189). Moreover, various cellular stresses such as heat shock and osmotic stress have all been shown to lead to an increase of SUMOylation in many target proteins (113). Intracellular oxidative stress, however, has emerged as perhaps the most powerful regulatory force to enhance the SUMOylation of a number of target proteins (113,190,191). Our laboratory and several others have found that proteins that are SUMO modified often show an increase in high molecular mass SUMO conjugates (especially when modified by SUMO-2 or SUMO-3) when exposed to reactive oxygen species. Presumably, these high molecular mass conjugates correspond to the formation of SUMO chains. Cellular oxidative stress also can lead to the global redistribution of SUMO to different target proteins (192). In *Saccharomyces cerevisiae*, for example, the presence of reactive oxidative species has been shown to distinctively shift the profile of SUMO conjugated species in a reproducible manner. Although the exact mechanisms involved are not fully understood, recent data indicate that oxidative stress may directly modulate the machinery responsible for SUMO conjugation and deconjugation (191).

Using a Cos7 cell system, we have identified that SUMOylation of Kv1.5 is induced by oxidative stress in a dose-dependent manner *in vitro*. As described in Chapter II, we observe that exposure of Cos7 cells to H_2O_2 leads to a shift from the Kv1.5 SUMOylated species that we normally detect to higher molecular mass SUMO conjugates. This type of experiment can provide preliminary evidence that the SUMO modification of Kv1.5 may be sensitive to oxidative stress. It is important to note in this regard that Kv1.5 also plays an important role in the vasculature, particularly in the pulmonary circulation where it contributes to the vasoconstriction elicited by hypoxic

conditions (89). SUMOylation of Kv1.5 may also contribute to this regulatory mechanism.

An important and well-studied source of pathophysiological oxidative stress in the heart occurs during ischemia/reperfusion injury following a heart attack (193). Although reperfusion following ischemia-induced damage of myocardium is essential to overall survival of the cells, oftentimes this re-oxygenation of damaged tissue can actually result in further insult due to the introduction of reactive oxygen metabolites (193). In an initial exploratory experiment, we find that global protein modification by SUMO1, as well as SUMO2/3, is greatly induced *in vivo* in living rabbit hearts that undergo a period of transient ischemia (30 minutes of left anterior descending (LAD) coronary artery occlusion) followed by four hours of reperfusion. For this experiment, tissue from the region of heart that underwent ischemic injury (marked "Ischemia") or from the surrounding region of the heart that was not supplied by the LAD coronary artery (marked "Normal") was homogenized and lysed in the presence of the SUMO protease inhibitor NEM. Lysates were then resolved by SDS-PAGE and probed with antibodies against either SUMO1 or SUMO2/3. As shown in Figure IV-3, we detect a significant increase in total SUMOylated species. This is consistent with our findings that oxidative stress can up regulate SUMOylation *in vitro* as described above, as well as a very recent report in the literature that transient ischemia can induce SUMOylation in cerebral tissue in a model for stroke (194). In future studies, it will be very interesting to determine at what stage is SUMOylation upregulated and to probe the role of SUMOylation in the pathophysiological response to ischemia/reperfusion.

CODA

In conclusion, the work in this thesis has identified the novel regulation of Kv1.5 by members of the SUMO family, and has determined that this regulation has important functional consequences on the properties of Kv1.5-mediated current in heterologous

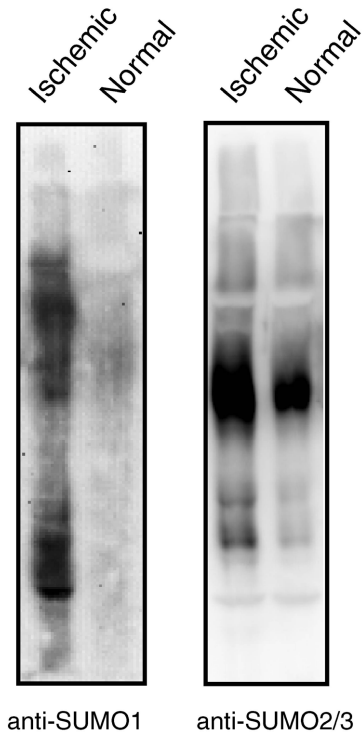

Figure IV-3. Global Protein SUMOylation is Induced in Living Rabbit Hearts Following Ischemia/Reperfusion Injury.

A period of transient ischemia induced in living rabbits by 30 minutes of left anterior descending (LAD) coronary artery occlusion was followed by four hours of reperfusion. Tissue from the region of heart that underwent ischemic injury (marked "Ischemia") or from the surrounding region of the heart that was not supplied by the LAD coronary artery (marked "Normal") was homogenized and lysed in the presence of the SUMO protease inhibitor NEM, and this lysate was separated by SDS-PAGE and blotted with antibodies against either SUMO1 or SUMO2/3. The tissue specimens were collected by Erin Booth and Adam Lauver in the laboratory of Dr Ben Lucchesi at our institution.

model systems, as well as in acutely dissociated mouse and human cardiomyocytes. This raises the exciting possibility that the SUMOylation of Kv1.5 may provide a novel, highly-specific target for the pharmacological manipulation of I_{kur} current in patients with atrial fibrillation. Furthermore, as we have identified that numerous other voltage-gated ion channels contain consensus SUMOylation motifs, SUMOylation may constitute a widespread mechanism for the adaptive tuning of electrically-excitable cells. Indeed, we have collected data that indicate that infusion of purified SENP2 or SUMO1-ΔGG into mouse cardiomyocytes leads to a selective decrease in I_{kur} current density and a prolongation of the plateau phase of the action potential. These findings suggest that disruption of SUMO modification of Kv1.5 in cardiomyocytes may lead to important physiological and pathophysiological consequences.

Although much more work clearly remains to be done to fully understand the role of SUMO modification in the regulation of Kv1.5, I feel fortunate to have been able to contribute to this exciting and emerging field.

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