Functional Consequences of Cantu Syndrome Associated Mutations in the ATP Sensitive Potassium Channel

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Functional Consequences of Cantu Syndrome Associated Mutations in the ATP Sensitive Potassium Channel
by
Paige E. Cooper

A dissertation presented to the Graduate School of Arts & Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT OF THE DISSERTATION

Functional Consequences of Cantu Syndrome Associated Mutations in the ATP Sensitive Potassium Channel

by

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Doctor of Philosophy in Biology and Biomedical Sciences

Molecular Cell Biology

Washington University in St. Louis, 2015

Professor Colin G. Nichols, Chair

ATP-sensitive potassium (K\textsubscript{ATP}) channels are composed of inward-rectifying potassium channel pore-forming subunits (Kir6.1 and Kir6.2, encoded by \textit{KCNJ8} and \textit{KCNJ11}, respectively) and regulatory sulfonylurea receptor subunits (SUR1 and SUR2, encoded by \textit{ABCC8} and \textit{ABCC9}, respectively). These channels couple metabolism to excitability in multiple tissues. Mutations in \textit{ABCC9} have been linked to Cantú syndrome (CS), a multi-organ disease characterized by congenital hypertrichosis, distinct facial features, osteochondrodysplasia, and cardiac defects. Additionally, two \textit{ABCC9} mutation-negative patients, exhibiting clinical hallmarks of CS, have been identified as having \textit{KCNJ8} mutations. This body of work is focused on determining the functional consequences and molecular mechanisms of documented CS-associated \textit{ABCC9} and identified \textit{KCNJ8} mutations. These mutations were engineered into recombinant cDNA clones and expressed as functional channels. Macroscopic rubidium (\textsuperscript{86}Rb\textsuperscript{+}) efflux assay experiments demonstrate that K\textsubscript{ATP} channels formed with SUR2 and Kir6.1 mutant subunits both result in gain of function in K\textsubscript{ATP} activity. Inside-out patch-clamp electrophysiological experiments show that there are at least 3 mechanisms by which these mutations alter K\textsubscript{ATP} channel activity,
including: 1) an SUR2 mutation that indirectly decreases ATP sensitivity by allosteric changes, 2) a Kir6.1 mutation that increases channel-open probability, and thereby indirectly decreases ATP sensitivity, and 3) mutations in SUR2 that increase channel activation in response to MgADP. Taken together, CS-associated $\textit{ABCC9}$ or $\textit{KCNJ8}$ mutations result in enhanced $K_{\text{ATP}}$ activity that underlies the cardinal features of Cantu Syndrome.
CHAPTER 1: INTRODUCTION

Inwardly-rectifying potassium (Kir) channels are a class of channels generated by 7 gene sub-families. All Kir channels are made of tetramers of Kir subunits and all require phosphatidylinositol 4,5-bisphosphate (PIP$_2$) for channel activity (Huang et al., 1998; Zhang et al., 1999). These channels are expressed widely throughout the human body and play important roles in regulating membrane potential. Disruption in Kir channel activity is linked to a variety of diseases (Pattnaik et al., 2012). The Kir6.X sub-family members generate the pores of adenosine triphosphate (ATP) sensitive potassium channels, which is the focus of this project.

The ATP-sensitive potassium current (I$_{\text{K,ATP}}$) was first described, in cardiomyocytes, as an outward current that is activated during metabolic poisoning and inhibited by ATP (Noma, 1983). Over the next several years, additional tissues were found to have I$_{\text{K,ATP}}$ (Cook and Hales, 1984; Spruce et al., 1985). Capitalizing on the sensitivity of the current to sulfonylurea drugs, Aguilar-Bryan et al. identified and then purified the heteromeric complex of pore and auxiliary subunits that comprise the ATP-sensitive potassium (K$_{\text{ATP}}$) channel (Aguilar-Bryan and Bryan, 1999). The genes that encode the pore and auxiliary subunits are found in pairs on two different human chromosomes. On human chromosome 11p at position 15.1, $KCNJ11$, which encodes the pore-forming Kir6.2 subunit, is directly downstream of $ABCC8$, which encodes the auxiliary subunit SUR1 (Aguilar-Bryan et al., 1995). Conversely, on human chromosome 12p at position 11.23, $KCNJ8$, which encodes the pore-forming subunit Kir6.1, is located downstream of $ABCC9$, which encodes the auxiliary subunit SUR2 (Figure 1.1) (Chutkow et al., 1996; Inagaki et al., 1995b).
Figure 1.1- The genes encoding K\textsubscript{ATP} channel proteins. Cartoon representation of human chromosomes 11 and 12, where the genes encoding K\textsubscript{ATP} channels are found. Each box represents the gene denoted in italics and the name of the encoded protein is indicated above.
1.1 \textbf{\textit{K}}\textsubscript{ATP} Channel Protein Topology

The pore-forming subunit proteins Kir6.2 and Kir6.1, which are encoded by \textit{KCNJ11} and \textit{KCNJ8}, respectively, are members of the inward-rectifying potassium (Kir) channel family. Crystallization of the bacterial KcsA potassium channel provided a structural model for the Kir6 family (\textbf{Figure 1.2A}). The N and C termini orient within the cytoplasm with two transmembrane helices connected by a short loop (P-loop), which forms the pore and ion selectivity filter (Doyle et al., 1998). The two Kir6.X family member proteins share \textasciitilde70\% sequence identity (Inagaki et al., 1995a).

\textit{K}ATP channels formed with either Kir6.2 or Kir6.1 exhibit distinct gating and conductance. Kir6.2 channels spontaneously activate in the absence of ATP; however, Kir6.1 channels require the application of activating nucleotides (Kondo et al., 1998). Second, the single-channel conductance for Kir6.2, is double that of Kir6.1 conductance (Inagaki et al., 1995a; Isomoto et al., 1996; Sakura et al., 1995; Yamada et al., 1997). In chimera studies, replacing the Kir6.2 extracellular linker and the p-loop with that of Kir6.1 had no effect on gating but lowered the unitary conductance, implying that this region determines the unitary conductance (Kondo et al., 1998; Kono et al., 2000; Repunte et al., 1999). Conversely, additional chimera studies demonstrate that the unique N-terminus and C-terminus of Kir6.2 are necessary for spontaneous channel activity.

The sulfonylurea receptor (SUR) 1 and 2, the \textit{K}ATP auxiliary subunit protein isoforms encoded by \textit{ABCC8} and \textit{ABCC9}, respectively, are members of the ATP-binding Cassette (ABC) family (\textbf{Figure 1.1}). SUR1 and SUR2 share \textasciitilde68\% sequence identity homology (Inagaki et al., 1996) and are oriented in the membrane with an extracellular N terminus and a cytoplasmic C terminus (\textbf{Figure 1.2A}). As first predicted by hydrophobicity mapping and later confirmed using
Figure 1.2- Topology and stoichiometry of the K$_{ATP}$ channel complex. A cartoon of a K$_{ATP}$ channel Kir6.X (blue) and a SURX (yellow) subunit orientation within the membrane. The P-loop and the nucleotide binding domains (NBDs) are boxed in red and green, respectively (A). The 4:4 stoichiometry of Kir6.X to SURX proteins which form the K$_{ATP}$ channel complex is depicted in (B).
biotinylation assays and immunofluorescence, the SUR protein contains 17 transmembrane helices grouped into 3 domains (TMD0-2) connected and followed by cytoplasmic loops that form two nucleotide-binding domains (NBD) (Figure 1.2A) (Conti et al., 2001; Tusnady et al., 1997). SUR2 has two major splice variants, SUR2A and SUR2B (Inagaki et al., 1996; Isomoto et al., 1996). Both SUR1 and SUR2 have additional splice sites, but the resulting variants are poorly understood (Shi et al., 2005).

### 1.2 K\textsubscript{ATP} Channel Stoichiometry/Trafficking/Subunit Pairing

The size of the purified K\textsubscript{ATP} channel complex suggested that each K\textsubscript{ATP} channel is formed by four Kir6.X and four SURX subunits. 1:1 Kir6.2-SUR1 fusion proteins forming functional channels provided supporting evidence (Clement et al., 1997) for this hypothesis. The current-voltage profiles of channels formed with various heteromeric molar ratios of wild-type Kir6.2 were compared with a strongly rectifying Kir6.2 mutant. This work provided direct evidence that four Kir6.X subunits form the pore (Shyng and Nichols, 1997). The electrophysiological properties of K\textsubscript{ATP} channels produced from an engineered fusion construct made of one Kir6.2 and one SUR1 were identical to those produced by co-expression of Kir6.x and SURX subunits, thus confirming a 4:4 stoichiometry of Kir6.x and SURX subunits forming K\textsubscript{ATP} channels (Figure 1.2B) (Shyng and Nichols, 1997).

The short Arg-Lys-Arg endoplasmic reticulum (ER) retention signals in Kir6.X at the C-terminus and in SURX between TMD1 and NBF1 are mutually masked in the full complex, allowing for translocation of these subunits to the plasma membrane (Zerangue et al., 1999). The retention signal on each Kir6.X and SURX subunit also obligates correct subunit pairing and stoichiometry before trafficking to the plasma membrane (Zerangue et al., 1999). However, removing the last 30 amino acids from the C-terminus of Kir6.2, which includes the ER-retention
signal, allows Kir6.2 to traffic and function alone on the plasma membrane (Tucker et al., 1997). As both pore and auxiliary subunits come in multiple isoforms, the resulting $K_{ATP}$ channels can be formed with multiple subunit combinations. Studies in heterologous expression systems have shown that all combinations are able to traffic and function at the cell membrane (Shi et al., 2005). Table 1.1 lists subunit combinations that have been identified in native tissues.

1.3 $K_{ATP}$ Channel Activity Regulation

$K_{ATP}$ channels couple cell metabolism and excitability in multiple cell types. Importantly, there are a variety of physiological and pharmacological modulators of $K_{ATP}$ channels, some of which are discussed in detail below.

1.3.1 Physiological Modulators

1.3.1.i Nucleotides

In addition to forming the pore of the channel, the Kir6.X subunits bind inhibitory ATP and adenosine diphosphate (ADP) (Mg-free) (Figure 1.3A) (Tucker et al., 1997). Truncation and mutagenesis experiments demonstrate that both the N- and C-termini of Kir6.X contribute to the ATP-sensitivity (Drain et al., 1998; Koster et al., 1999; Tucker et al., 1998). Adenylylimidodiphosphate (AMP-PNP), a non-metabolizable form of ATP, produces an inhibitory response identical to that of ATP, indicating that binding, not hydrolysis, of ATP is sufficient for $K_{ATP}$ inhibition (Inagaki et al., 1995a). The specificity of ATP binding and inhibition was validated in photoaffinity labeling experiments. Additionally, numerous triphosphates, including: guanine, inosine, cytidine or uridine, fail to inhibit $I_{K_{ATP}}$ currents in both recombinant systems and in native tissues, further supporting the specificity of the $K_{ATP}$
<table>
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<th>TISSUE</th>
<th>KATP Channel COMPOSITION</th>
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Figure 1.3- Nucleotide Binding and Regulation of the K\textsubscript{ATP} channel.
The Kir6.X is represented in blue and the SURX subunit is represented in yellow. ATP and ADP (No Mg\textsuperscript{2+}), which both bind to the Kir6.X subunits to inhibit the channel, are represented by red and green circles, respectively. MgADP binds to the Nucleotide Binding Domains (NBDs) within the SURX subunit (A). The NBD region is enlarged in the square region and the Walker A and Walker B motifs, which are within the NBDs, are represented by purple and gray regions, respectively. In these Walker motifs ATP and MgADP have been shown to bind. Mg\textsuperscript{2+} is represented by light blue hexagons attached to ADP (B).
channel to regulation by ATP (Koster et al., 1999; Lederer and Nichols, 1989; Tanabe et al., 2000; Tucker et al., 1998).

Channels composed of Kir6.2 alone, which traffic after truncation of the ER retention signal, have decreased sensitivity to ATP inhibition compared to WT channels composed of Kir6.2 and SUR1 (Tucker et al., 1997). This finding suggests that SUR subunits also play a role in regulating channel activity. Photoaffinity labeling using 8-azido-[α-32P]ATP or 8-azido-[γ-32P]ATP in cells overexpressing SUR1 demonstrated that ATP binds the SUR subunit directly (Ueda et al., 1997). MgADP works at the level of the NBDs in SUR to activate the channel (Nichols et al., 1996). Nucleotide binding folds (NBFs), which are conserved in all ABC proteins including SURX subunits, contain Walker A and Walker B nucleotide-binding motifs (Figure 1.3B) (Higgins, 1992). Nucleotide binding sites in SURX were identified by mutagenesis of the conserved Walker A and Walker B regions in both NBF1 and NBF2. Free ATP binds to NBF1 and MgADP interacts with NBF2, but can also alter ATP binding to NBF1 (Ueda et al., 1997).

Disruption of the NBF2 Walker B motif or linker region within SUR1 completely abolishes the activating effect of MgADP, without altering ATP-sensitivity. Conversely, mutations at the equivalent positions in the NBF1 Walker B motif only alter activation kinetics (Shyng et al., 1997). Expression of SUR1 mutagenized at lysine residue(s) within Walker A motifs in NBF1, NBF2 or both, with Kir6.2-WT, produced $K_{\text{ATP}}$ channels with increased ATP-sensitivity (no $\text{Mg}^{2+}$) and with MgADP inhibition instead of activation (Gribble et al., 1997). Taken together, the Walker A motif in NBF1 and both Walker A and Walker B motifs in NBF2 are required for MgADP activation of $K_{\text{ATP}}$ channels (Figure 1.3B). MgADP stimulation differs among the SUR isoforms, where SUR1-based channels are more stimulated than SUR2A (Masia et al., 2005). The differences in nucleotide-binding affinities among the SUR isoforms may
account for some of the diversity of behavior among the different channel compositions (Matsuo et al., 2000).

1.3.1.ii Phosphorylation

PKC

Direct modulation via either the protein kinase C (PKC) pathway or $K_{ATP}$ channel activity modulates vascular tone. In addition, activation of PKC can decrease $K_{ATP}$ channel activity indirectly to influence tone. The response of $K_{ATP}$ channels to PKC activation could occur because: 1) $K_{ATP}$ channels are directly regulated by PKC signaling or 2) PKC activation inhibits $K_{ATP}$ channel indirectly by depleting PIP$_2$ from the membrane. Stable Kir6.1+SUR2B and Kir6.2+SUR2B cell lines were used to monitor $K_{ATP}$ channel activity response to pharmacological activation of PKC in combination with inhibitors of the PKC signaling cascade, providing some of the first evidence that Kir6.1 based channels are directly regulated by PKC (Quinn et al., 2003).

Additional engineered chimera studies demonstrated that N- and C-termini of Kir6.1 are necessary for PKC regulation. Use of electrophysiology and phosphorylation assays revealed that individual intracellular serine residues, which are unique to the C-terminus of Kir6.1, mutated to alanine do not prevent PKC inhibition. However, mutating these serine residues simultaneously abolishes PKC inhibition of $K_{ATP}$ channels. Taken together, several serine residues in the C-terminus of Kir6.1 are required for direct PKC phosphorylation and inhibition of KATP channel activity (Figure 1.4A) (Shi et al., 2008).

The ability of PKC to inhibit Kir6.1-based $K_{ATP}$ channels might suggest that $K_{ATP}$ channel activation results from dephosphorylation by phosphatases. Electrophysiological data
from isolated rat aortic smooth muscle show that different structural classes of Ca\(^{2+}\)-dependent protein phosphatase type 2B (PP2B) inhibitors can inhibit \(I_{\text{K,ATP}}\). These results suggest that in native tissue, PP2B phosphatase activity can regulate \(K_{\text{ATP}}\) channel activity (Figure 1.4A) (Wilson et al., 2000).

**PKA**

At least two studies have shown that \(K_{\text{ATP}}\) channels composed of Kir6.1+SUR2B are activated by PKA in a cAMP-dependent manner (Quinn et al., 2004; Shi et al., 2007). There is conflicting evidence however about the actual residues phosphorylated by PKA. Quinn et al. concluded that a serine residue in Kir6.1 and/or a serine and a threonine residue in SUR2B are phosphorylation sites for PKA (Quinn et al., 2004). On the other hand, Shi et al. made alanine substitutions at these residues and found no differences in PKA sensitivity of \(K_{\text{ATP}}\) channel currents, leading them to conclude that these sites are not relevant. They went on to identify a single serine residue in SUR2B as the only PKA phosphorylation site, which they confirmed by in vitro phosphorylation assays (Figure 1.4B) (Shi et al., 2007).

Despite the debate over which specific Kir6.1 and/or SUR2B residue(s) PKA phosphorylates (Quinn et al., 2004; Shi et al., 2007), it is agreed that PKA phosphorylates \(K_{\text{ATP}}\) channels directly to regulate vascular tone. There has therefore been an interest in identifying potential phosphatase(s) to counter activation of \(K_{\text{ATP}}\) channel after PKA phosphorylation (Orie et al., 2009). Dephosphorylation of \(K_{\text{ATP}}\) would inhibit \(K_{\text{ATP}}\) channel activity and, noting that Kir6.1+2B activity decreases in high intracellular Ca\(^{2+}\), the Ca\(^{2+}\)-dependent phosphatase calcineurin was tested as a potential \(K_{\text{ATP}}\) channel phosphatase. Multiple chemically unrelated calcineurin inhibitors increase both in vitro phosphorylation of \(K_{\text{ATP}}\) channel subunits and \(K_{\text{ATP}}\)
Figure 1.4- PKA and PKC signaling pathway regulation of the K\textsubscript{ATP} channel. Cartoon of K\textsubscript{ATP} channel regulation by PKC (A) and PKA (B) pathways in which Kir6.1 is depicted in blue surrounded by SUR2B in yellow. Red dots depict phosphorylation sites on Kir6.1 c-terminus or SUR2B as described in the text.
channel activity (Figure 1.4B) (Orie et al., 2009). In the presence of a PKA catalytic domain that is constitutively active, calcineurin has no effect on $K_{ATP}$ channel activity. This observation indicates that calcineurin directly dephosphorylates $K_{ATP}$ channels to regulate channel activity (Orie et al., 2009).

1.3.1.iii pH

Increases in intracellular pH result in increases in $K_{ATP}$ channel current, in the presence or absence of ATP, in isolated rat beta cells (Misler et al., 1989). Further evidence for $K_{ATP}$ channel pH sensitivity has been obtained from studies on inside-out patches from Xenopus oocytes expressing Kir6.2+SUR1 and from isolated mouse cardiomyocytes (Baukrowitz et al., 1999). Histidine is the most common amino acid protonated or deprotonated at physiological pH. By mutating each of the 9 intracellular histidine residues in Kir6.2 to amino acids with non-titratable side chains, a single residue was identified as the pH-sensing residue in $K_{ATP}$ channels (Baukrowitz et al., 1999).

1.3.2 Pharmacological Modulation

In addition to physiological regulation, $K_{ATP}$ channel activity is modulated by distinct pharmacological agents. The molecular basis and action of these modulators are discussed below.

1.3.2.i K channel openers (KCOs)

Diverse classes of drugs called $K^+$ channel openers (KCOs) activate $K_{ATP}$ channels by binding to the SUR subunits (Inagaki et al., 1996). The ability of individual KCOs to activate channels is dependent on the SUR isoform. Whereas SUR1-containing channels are activated specifically by diazoxide (Inagaki et al., 1995a), SUR2A-containing channels are effectively activated by all
KCOs except diazoxide (Inagaki et al., 1996). SUR2B-containing channels respond to all KCOs (Isomoto et al., 1996).

Synthesis and study of SUR chimera proteins has demonstrated that KCOs such as levercromakalim, cromakalim, P1075 and pinacidil appear to bind SUR2 at the intracellular loop between TM13 and TM14, with two additional critical residues in TM17, SUR2-L1249 and SUR2-T1253. These interactions have been confirmed by the observation that disruption to these areas and/or residues alters the activating properties of the KCOs (Moreau et al., 2000; Uhde et al., 1999). Diazoxide diverges structurally from that of the majority of KCOs, and its preferential binding is thought to be at the NBFs (Shyng et al., 1997).

1.3.2.ii Sulfonylureas

A class of drugs called sulphonylureas also specifically binds the SUR subunit of K\textsubscript{ATP} channels but inhibit channel activity (Inagaki et al., 1996). Tolbutamide is an example of a first-generation sulfonylurea that binds at TMD2 (Ashfield et al., 1999). Second and third generation, more potent, sulfonylureas have been developed; an example is glibenclamide. The binding site of glibenclamide also includes TMD2 with additional involvement of a site on the cytoplasmic loop between TMD0 and TMD1 (Mikhailov et al., 2001). In general, sulfonylureas are most effective at inhibiting SUR1-based channels, less so for SUR2B-, and least effective for SUR2A-based channels.

1.4 K\textsubscript{ATP} Channels in Tissue

The variation in nucleotide sensitivity, pharmacology and physiological pairings (Aguilar-Bryan et al., 1995; Inagaki et al., 1996) of the Kir6.X and SURX subunits results in diversity of the
properties and functional roles of K\textsubscript{ATP} channels in the tissues in which they are expressed (Akrouh et al., 2009; Nichols, 2006).

### 1.4.1 Tissue Distribution

RT-PCR data from a large variety of tissues indicates the pore-forming subunit Kir6.1 is very widely expressed (Inagaki et al., 1995c). In contrast, Kir6.2 expression is prominent in pancreatic beta cells, brain, heart and skeletal muscle (Inagaki et al., 1995a). In native tissues, SUR1 subunits only physiologically interact with Kir6.2. Consistent with this finding, SUR1 expression is also in pancreatic beta cells, brain, and heart (Inagaki et al., 1995a). Conversely, the SUR2 subunits pair in native tissue with either Kir6.1 or Kir6.2. The splice variant SUR2A is found in the heart and skeletal muscle where, based on electrophysiology studies, it pairs with Kir6.2. Like Kir6.1, SUR2B subunits are expressed widely, and electrophysiological recordings from K\textsubscript{ATP} channels in vascular smooth muscle cells appear to reflect the co-assembly of SUR2B with Kir6.1 (Table 1.1) (Chutkow et al., 1996; Inagaki et al., 1995c).

### 1.4.2 Role in Tissue Function

#### 1.4.2.i Brain

K\textsubscript{ATP} channel expression and activity has been reported throughout the nervous system including the pituitary gland, basal ganglia, cerebral cortex, hippocampus, basal forebrain, striatum, and brain stem (Liss and Roeper, 2001; Seino and Miki, 2003; Yamada and Inagaki, 2005).

Electrophysiological characterization of K\textsubscript{ATP} channel currents, combined with RT-PCR analysis of K\textsubscript{ATP} channel subunit expression, suggests composition of native channels in various regions of the brain is quite heterogeneous. Based on radiolabeled glibenclamide studies, K\textsubscript{ATP} channels are most abundant in the hypothalamus and substantia nigra pars rectculata (SNr) neurons (Liss
and Roeper, 2001; Seino and Miki, 2003; Yamada and Inagaki, 2005). The roles for K\textsubscript{ATP} channels in the brain will be discussed below in reference to these regions specifically.

**Hypothalamus**

The hypothalamus, which plays a critical role in glucose homeostasis by regulating counter-regulatory hormones such as glucagon and catecholamines, relies on glucose-sensing neurons. Within these neurons, the electrical firing patterns that lead to the autonomic input to release glucagon and catecholamines are regulated by K\textsubscript{ATP} channel activity (Miki et al., 2001). Electrophysiological and RT-PCR analysis indicates that Kir6.2 and SUR1 compose the K\textsubscript{ATP} channels in these glucose-responsive neurons (Ashford et al., 1990; Miki et al., 2001; Spanswick et al., 1997). As brain glucose levels drop, glucose-sensing neurons experience a decrease in the intracellular ATP/ADP ratio that activates K\textsubscript{ATP} channels, thereby hyperpolarizing the plasma membrane and stimulating autonomic input for glucagon release (Figure 1.5). The release of glucagon, from pancreatic alpha cells, stimulates gluconeogenesis and lipolysis to increase blood glucose levels (Seino and Miki, 2003).

**SNr**

The basal ganglion contains SNr neurons, which are implicated in the propagation of seizures. K\textsubscript{ATP} channels are not active in SNr neurons at rest, allowing for active firing (Yamada and Inagaki, 2005). In metabolically stressful conditions, such as hypoxia or hypoglycemia, the activity of K\textsubscript{ATP} channels is thought to be critical for protection from seizures. In metabolically stressful conditions SNr neurons decrease ATP production, reducing the ATP/ADP ratio, activating K\textsubscript{ATP} channels and thereby hyperpolarizing the membrane. Membrane hyperpolarization decreases the firing rate of SNr neurons inhibiting GABA release, which is
Figure 1.5- The role of $K_{ATP}$ channel activity in hypothalamic glucose-sensing neuron function. Illustrated above is the role $K_{ATP}$ channels play in hypothalamic glucose-sensing neurons. The $K_{ATP}$ channel is depicted in green and the voltage gated $Ca^{2+}$ channel is in orange.
hypothesized to slow or prevent propagation (Figure 1.6)(Amoroso et al., 1990; Yamada et al., 2001).

1.4.2.ii Pancreas

Within the pancreas there are clusters of endocrine cells called islets of Langerhans, of which beta cells are the most abundant. Beta cells secrete insulin, an endocrine hormone which helps decrease blood glucose levels. $K_{ATP}$ channels are expressed abundantly on the plasma membrane of beta cells, and are critical for regulating insulin release (Ashcroft and Gribble, 1999). During or after a meal, as blood glucose levels elevate, beta cell metabolism increases, which elevates the intracellular ATP/ADP ratio, inhibiting $K_{ATP}$ channels. Inhibition of $K_{ATP}$ channels leads to membrane depolarization and activation of L-type $Ca^{2+}$ channels, which increases $Ca^{2+}$ entry. The increased flux of calcium promotes fusion and release of insulin (Figure 1.7). Once in the bloodstream insulin helps decrease blood glucose levels by facilitating glucose uptake into muscle (Ashcroft and Gribble, 1999).

1.4.2.iii Heart

In the resting state, cardiomyocytes have elevated intracellular ATP/ADP ratios which leaves $K_{ATP}$ channels generally closed (Koster et al., 2001). While the role of $K_{ATP}$ channels in regulating heart rhythm or contraction is not fully understood, complete activation of $K_{ATP}$ channels in the heart can lead to profound shortening of the action potential (Figure 1.8)(Nichols et al., 1991). During ischemia, in which a lack of oxygen or glucose disrupts the metabolic function of cardiomyocytes, the ATP/ADP ratio decreases, activating $K_{ATP}$ channels. Activation of $K_{ATP}$ channels under these conditions is thought to be cardio-protective since hyperpolarization of the membrane will prevent excessive $Ca^{2+}$ entry, thus preserving cardiomyocyte function and promoting cell survival (Bers, 2008).
Figure 1.6- Hypothesized role of $K_{ATP}$ channels in SNr neuron function. Cartoon of the potential role $K_{ATP}$ channels play in SNr neurons. The $K_{ATP}$ channel is depicted in green, the voltage-gated $Ca^{2+}$ channel is in orange, and yellow circles represent GABA.
Figure 1.7- The role of $K_{ATP}$ channels in beta cell function. Cartoon of the role $K_{ATP}$ channels play in the pancreatic beta cell, where the $K_{ATP}$ channel is depicted in green, the voltage-gated Ca$^{2+}$ channel is depicted in orange and insulin is represented by purple circles.
Figure 1.8- The role of K\textsubscript{ATP} channels in cardiomyocyte function. Cartoon of the role K\textsubscript{ATP} channels play in cardiomyocytes, where the K\textsubscript{ATP} channel is depicted in green and the voltage gated Ca\textsuperscript{2+} channel is depicted in orange.
1.4.2.iv Skeletal Muscle

$\text{K}_{\text{ATP}}$ channels are not thought to play a significant role in regulating the electrical activity of skeletal muscle in the resting state, but may be crucial during muscle fatigue (Cifelli et al., 2008; Gong et al., 2000). $\text{K}_{\text{ATP}}$ channel activity can preserve a polarized membrane potential, preventing voltage-dependent $\text{Ca}^{2+}$ entry and decreasing contractility (Gong et al., 2000; Matar et al., 2000). After treadmill or swim training, Kir6.2$^{-/-}$ mice, but WT, experience extensive fiber damage in muscle. This observation is also consistent with $\text{K}_{\text{ATP}}$ channel activation being protective during fatigue (Kane et al., 2004; Thabet et al., 2005).

There is also evidence that $\text{K}_{\text{ATP}}$ channels play a role in glucose uptake in skeletal muscle: 1) sulfonylureas enhance glucose uptake into isolated skeletal muscle (Miki et al., 1998); 2) Kir6.2$^{-/-}$ mice have increased effectiveness of insulin in lowering blood glucose in the muscle versus WT mice (Miki et al., 2002); and 3) muscle from SUR2$^{-/-}$ mice have enhanced insulin-stimulated glucose transport in vitro compared to WT (Chutkow et al., 2001).

1.4.2.v Smooth Muscle

In smooth muscle cells, $\text{K}_{\text{ATP}}$ channel activity is thought to be important for regulating vascular tone (Chutkow et al., 2002; Miki et al., 2002). Excised patch clamp experiments on vascular smooth muscle cells revealed $\text{K}_{\text{ATP}}$ channels which did not spontaneously open in the absence of ATP, a characteristic of nucleoside-dependent Kir6.1 channels (Beech et al., 1993; Kajioka et al., 1991; Zhang and Bolton, 1996). It is hypothesized that these $\text{K}_{\text{ATP}}$ channels help regulate tone by activating in response to a declining ATP/ADP ratio or input from PKA pathways (Figure 1.4B). Outward $\text{K}^+$ currents through these channels hyperpolarize the membrane, thereby preventing $\text{Ca}^{2+}$-entry and leading to vasodilation of the vessel and decreases in contractility (Figure 1.9). In
support of this proposed mechanism, treatment with the L-type Ca\(^{2+}\)-channel inhibitor, nifedipine, protects against the development of vasospasms in SUR2\(-/-\) animals (Chutkow et al., 2002).

1.5 \(\text{K}_{\text{ATP}}\) Channels in Pathophysiology

\(\text{K}_{\text{ATP}}\) channels are widely expressed and provide a finely tuned link between metabolism and electrical activity in tissues. Dysregulation of \(\text{K}_{\text{ATP}}\) channel activity (gain of function or loss of function) results in pathophysiology. Several examples, in which \(\text{K}_{\text{ATP}}\) channel dysregulation underlies a disease state, are described below.

1.5.1 Persistent Hyperinsulinemic Hypoglycemia of Infancy (PHHI)

The first reported clinical characterization of a child with hypoglycemia that failed to improve without treatment intervention occurred in 1956 (Cochrane et al., 1956). This disease was later termed Persistent Hyperinsulinemic Hypoglycemia of Infancy (PHHI), and is found in 1 of every 50,000 live births or up to 1 in every 3,000 live births in familial cases (Stanley and De León, 2012). Clinically, patients commonly present with increased plasma levels of insulin in hypoglycemic conditions along with decreased levels of ketone bodies and free fatty acids (Aguilar-Bryan and Bryan, 1999).

Mutations in \(KCNJ11\) (Kir6.2) or \(ABCC8\) (SUR1) account for nearly 70% of known PHHI cases, with the majority of mutations found in \(ABCC8\). Every PHHI-associated \(KCNJ11\) (Kir6.2) or \(ABCC8\) (SUR1) mutation leads to a decrease in \(\text{K}_{\text{ATP}}\) channel activity. This keeps the beta cell membrane constantly depolarized and leads to elevated Ca\(^{2+}\) levels and secretion of insulin (Figure 1.10)(Remedi and Koster, 2010).
Figure 1.9- The role of $K_{ATP}$ channels in smooth muscle cell function. Cartoon of the role $K_{ATP}$ channels play in smooth muscle cell function, where the $K_{ATP}$ channel is depicted in green and the voltage gated $Ca^{2+}$ channel is depicted in orange.
There is no single mechanism by which all PHHI-associated $K_{\text{ATP}}$ channel mutations reduce channel activity. The mechanisms identified to date include trafficking defects, increased ATP sensitivity, decreased MgADP activation and Kir6.2 inactivation (Denton and Jacobson, 2012). Patients may be treated with the $K_{\text{ATP}}$ channel opener diazoxide, or high doses of glucose to maintain euglycemia (Pinney et al., 2008). However, in extreme cases in which these treatments are ineffective, partial or complete removal of the pancreas is necessary (De Leon and Stanley, 2007).

### 1.5.2 Neonatal Diabetes Mellitus

Phenotypically the opposite of PHHI is a disease called Neonatal Diabetes Mellitus (NDM). Also a condition commonly diagnosed in infants less than six months old, NDM patients present with hyperglycemia and hypoinsulinemia. NDM incidence is estimated to be ~1 in every 100,000-300,000 births (Naylor et al., 2011). It results from disruption in genes encoding critical proteins for beta cell function including missense mutations in genes encoding proteins in the metabolic pathway or involved in insulin production or secretion (Remedi and Nichols, 2009). The most common monogenic cause of NDM is gain of function in $K_{\text{ATP}}$ channel activity due to mutations in either $KCNJ11$ (Kir6.2) or $ABCC8$ (SUR1) (Naylor et al., 2011). Despite elevated ATP/ADP ratios, which results from increased glucose metabolism, the gain of function in KATP channel activity prevents channel inhibition. Because $K_{\text{ATP}}$ channels remain open, the membrane remains hyperpolarized, leading to cessation of beta cell secretion of insulin (Figure 1.11) (Koster et al., 2000).

NDM-associated $K_{\text{ATP}}$ channel mutations are scattered throughout the Kir6.2 and SUR1 proteins (Denton and Jacobson, 2012), with multiple underlying molecular mechanisms. Kir6.2
Figure 1.10- The role of $K_{ATP}$ channels with decreased activity in the function of beta cells in PHHI patients. Cartoon of the role $K_{ATP}$ channels play in normal beta cell function (A) or with decreased $K_{ATP}$ channel activity as found in PHHI patients (B). The $K_{ATP}$ channel is depicted in green and the voltage-gated $Ca^{2+}$ channel is depicted in orange.
mutations have most commonly been shown to give rise to increased channel activity by increasing the intrinsic open probability of the channel or by decreasing the affinity or binding of inhibitory ATP (Koster et al., 2008b). Similarly, mechanisms for SUR1 mutations also result in overactive $K_{\text{ATP}}$ channels, but enhanced affinity for MgADP and altered nucleotide hydrolysis at the NBDs can additionally be responsible (Denton and Jacobson, 2012).

Originally NDM patients were treated with insulin injections as a means of managing their blood glucose levels, but understanding the molecular basis of $K_{\text{ATP}}$ channel-dependent NDM cases has allowed for better/simpler treatment (Babenko et al., 2006; Sagen et al., 2004; Zung et al., 2004). Now, instead of insulin injections, patients are typically administrated sulphonylureas tablets orally, as an easier and more targeted treatment that directly inhibits the overactive $K_{\text{ATP}}$ channels. Additionally, sulfonylureas typically result in more regulated glycemic control, relative to insulin therapy (Koster et al., 2008a). However, it should be noted that in patients with mutations that result in extremely overactive $K_{\text{ATP}}$ channels sulfonylureas are not always effective (Remedi and Koster, 2010).

1.5.3 Cantu Syndrome

A disease more recently proposed to be associated with defective $K_{\text{ATP}}$ channels is Cantu Syndrome (CS), a rare and complex disorder. In the first description of this disorder, reported by Dr. Cantu in 1982, four patients presented with hypertrichosis and dental abnormalities, comparable to that seen in patients with hypertrichosis universalis and/or congenital hypertrichosis lanuginose. Additional skeletal abnormalities in these patients establish CS as a unique disorder (Cantu et al., 1982). The heritability of CS in both males and females from
Figure 1.11- The role of overactive K\textsubscript{ATP} channels in the beta cell function of NDM patients. Cartoon of the role K\textsubscript{ATP} channels play in normal beta function (A) or the role of overactive K\textsubscript{ATP} channels play in the beta cell function of patients with NDM (B). The K\textsubscript{ATP} channel is depicted in green and the voltage-gated Ca\textsuperscript{2+} channel is depicted in orange.
asymptomatic parents originally suggested that it can be an autosomal recessive disease (Cantu et al., 1982). However, a familial case in which a father with CS passed it to his son, made autosomal dominance more likely (Lazalde et al., 2000).

The number of CS patients described in the literature to date totals ~50 and the clinical hallmarks have expanded to include: hypertrichosis, macrosomia, macrocephaly, coarse facial appearance, cardiomegaly, and skeletal abnormalities (Nichols et al., 2013). Additional symptoms present in subsets of patients includes patent ductus arteriosus, lymphedema, heart valvular anomalies, and congenital hypertrophic cardiomyopathy (Grange et al., 2014). The fact that hypertensive patients treated with (KCOs) such as Minoxidil (Mehta et al., 1975), diazoxide (Goldberg, 1988), or pinacidil (Koblenzer and Baker, 1968) can develop similar features, including hypertrichosis and structural defects in the heart, led to the hypothesis that CS could be a result of dysregulated potassium channel activity (Grange et al., 2006).

It was not until 2012, however, when the genetic analysis of two separate CS patient cohorts determined that the majority have mutations in \textit{ABCC9} (SUR2), that the \textit{K}\textsuperscript{+} channel dysregulation hypothesis was investigated. Harakalova et al. expressed Kir6.2-WT with three of the identified SUR2 mutations and found that \textit{K}_\text{ATP} channel activity was increased (Harakalova et al., 2012). The data presented in the following chapters represent the first reports of \textit{KCNJ8} mutations which result in CS, and the mechanisms underlying the functional effects of these mutations. Additionally included is the first molecular account and detailed mechanistic study of how the gain of function in \textit{K}_\text{ATP} channel activity arises from CS-associated SUR2 mutations.

\textit{K}_\text{ATP} channels are highly regulated and are expressed throughout the body, and these channels primarily function to link cell metabolism and cell excitability. This body of work is
focused on determining the mechanism(s) by which identified mutations in Kir6.1 and SUR2, found in CS-patients, alter $K_{ATP}$ channel activity. CS patients with Kir6.1 or SUR2 mutations have overlapping symptoms, and the results presented imply that the gain of function in $K_{ATP}$ channel activity is causal. This work underscores the intricate role $K_{ATP}$ channels play in tissues and how minor changes in channel activity can have profound effects on disease presentation. Understanding the impact these mutations have on channel activity should provide insight into how a gain of function in $K_{ATP}$ channels activity results in the various pathophysiological consequences of CS and, more importantly, guide future efforts focused on inhibiting these overactive channels as a treatment option for patients.
Mutagenesis and Heterologous Expression of $K_{\text{ATP}}$ Channels

The Quick Change II Site-Directed Mutagenesis kit (Agilent Technologies) was used to engineer SUR2 mutations into ratSUR2A-pCMV6, Kir6.1 mutations into ratKir6.1-pcDNA3.1, and Kir6.2 mutations into mouseKir6.2-pcDNA3.1. Mutations were confirmed by direct sequencing of the SUR2 or Kir6.X coding regions.

For channel expression, COSm6 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum, $10^5$ units/L penicillin, and 100 mg/L streptomycin. At 60-70% confluence, cells were transfected with the relevant plasmids using FuGENE6 transfection reagent (Promega). For experiments with homomeric CS mutant channels, cells were co-transfected with pCDNA3.1-mouseKir6.2-WT (0.6 µg) and wild-type (WT) or mutant pCMV6-ratSUR2A (SUR2) (1 µg). For experiments on heteromeric channels, cells were co-transfected with mouseKir6.2-WT, ratSUR2-WT, and mutant SUR2 at ratios of 0.6:0.5:0.5 (w:w:w). Cells transfected with GFP-pcDNA3.1 served as controls. A small amount of GFP cDNA was included to allow for identification of GFP-expressing transfected cells during electrophysiology experiments.

Macroscopic $^{86}\text{Rb}^+$ Efflux Assays

Cells were incubated overnight at 37°C in DMEM containing 1 µCi/mL $^{86}\text{RbCl}$ (PerkinElmer Life Sciences), and then incubated in Ringers solution (mM: NaCl 118, HEPES 10, NaHCO$_3$ 25, KCl 4.7, KH$_2$PO$_4$ 1.2, CaCl$_2$ 2.5, MgSO$_4$ 1.2, adjusted to pH 7.4 with NaOH) in (1) the absence (basal) or (2) the presence of 2.5 mg/mL oligomycin and 1 mM 2-deoxy-d-glucose (metabolic
(32)
inhibition), (3) the presence of SUR1 or SUR2-specific $K^+$ channel openers diazoxide or pinacidil or (4) a combination of metabolic inhibition and pinacidil, to achieve maximal channel activation. Subsequently, at selected time points (2.5, 5, 7.5, 15, 25, and 40 minutes), the solution was collected and replaced with fresh solution. Upon completion of the assay, cells were lysed with 2% SDS and collected, and the radioactive $^{86}\text{Rb}^+$ in these samples was measured. $^{86}\text{Rb}^+$ efflux is expressed as a fraction of total content. An inactivating, $K_{\text{ATP}}$-independent, conductance was assumed to be present in all $^{86}\text{Rb}^+$ efflux experiments, and apparent rate constants for the $K_{\text{ATP}}$-independent $^{86}\text{Rb}^+$ efflux were obtained from GFP transfected cells using the equation:

$$ [1] \quad \text{Rb efflux} = \left\{ 1 - e^{-(k_1 + k_{-1})t} \right\} $$

where $k_1$ and $k_{-1}$ are the apparent activation and inactivation rate constants. $K_{\text{ATP}}$-dependent $^{86}\text{Rb}^+$ efflux was also assumed to activate and inactivate with time and was obtained using the equation:

$$ [2] \quad \text{Rb efflux} = \left\{ 1 - e^{-(k_1 + k_2 + k_{-1} + k_{-2})t} \right\} $$

where $k_1$ and $k_{-1}$ are the rate constants for $K_{\text{ATP}}$ independent pathways (obtained from GFP-transfected cells by Equation 1), $k_2$ and $k_{-2}$ are the activation ($k_2$) and inactivation ($k_{-2}$) rate constants for $K_{\text{ATP}}$-specific $K^+$ conductance. $k_2$ is then assumed to be proportional to the number of active $K_{\text{ATP}}$ channels.

$^{86}\text{Rb}^+$ efflux experiments were also carried out in the presence of MI with the addition of 10 µM glibenclamide. In these experiments, the $k_{-2}$ parameter was assumed to be the same for each construct and all $K_{\text{ATP}}$-dependent fluxes were fitted simultaneously on any given day and
under any given condition. To account for day-to-day variability, $k_l$ was determined in mock transfected cells for each condition (Basal, MI, PIN, MI+PIN, and MI+GLIB), and incorporated into the equation to estimate the $K_{ATP}$-dependent $k_2$ for each construct.

**Excised Patch Clamp**

After 24-48h, transfected cells that fluoresced green under UV light were selected for analysis by excised patch clamp experiments at room temperature. For experiments, cells were placed in a perfusion chamber that allowed for the rapid switching of solutions. $K_{\text{INT}}$ solution (mM: KCl 140, HEPES 10, and EGTA 1, to pH 7.4) was used as the standard pipette (extracellular) and bath (cytoplasmic) solution in these experiments. ATP or ADP was added as indicated. The appropriate amounts of MgCl$_2$ to be added in each Mg$^{2+}$-nucleotide containing solution to attain 0.5 mM free Mg$^{2+}$ were calculated by means of the CaBuf program (KU Leuven). Membrane patches were voltage-clamped using an Axopatch 1D amplifier (Molecular Devices), and currents were recorded at −50 mV (pipette voltage, +50 mV) in the on-cell and inside-out excised patch configurations. Data were typically filtered at 1 kHz and digitized at 5 kHz with a Digidata 1322A (Molecular Devices) A-D converter. pClamp and Axoscope software (Molecular Devices) were used for data acquisition. For ATP inhibition, [ATP]-response relationships (Fig. 4) were fitted by equation 3:

$$I_{rel} = \frac{1}{1 + \left(\frac{[ATP]}{K_i}\right)^H}$$

where $I_{rel}$ (relative current) is the current in the presence of a given concentration of ATP relative to current in zero ATP, $K_i$ is the apparent ATP inhibition constant, and $H$ is the Hill coefficient. For PIP$_2$ activation experiments, an ammonium salt of L-α-phosphatidylinositol-4,5-bisphosphate from Porcine Brain (Brain PI(4,5)P2) (Avanti Polar Lipids) was dissolved in $K_{\text{INT}}$ to make a 5 µg/µL working solution. For each patch, we estimated the relative $P_o$ by dividing the maximum steady-state current in zero ATP by the maximum steady-state current in PIP$_2$. 

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CHAPTER 3: CANTU SYNDROME RESULTING FROM ACTIVATING MUTATION IN
THE KCNJ8 GENE

Cooper PE et al. 2014

3.1 Introduction

In collaboration with Heiko Reutter we screened KCNJ8 in an ABCC9 mutation-negative patient who also exhibited clinical hallmarks of CS (hypertrichosis, macrosomia, macrocephaly, coarse facial appearance, cardiomegaly, and skeletal abnormalities). A de novo missense mutation encoding Kir6.1[Cys176Ser] was identified in the patient. Kir6.1[p.Cys176Ser] channels exhibited markedly higher activity than wild-type channels, as a result of reduced ATP sensitivity, whether co-expressed with SUR1 or SUR2A subunits. Our results identify a novel causal gene in CS, and also demonstrate that the cardinal features of the disease result from gain of K\textsubscript{ATP} channel function, not from a Kir6-independent SUR2 function.

3.2 Methods

All genetic analysis and patient assessments were carried out in collaboration with Heiko Reutter’s group.

3.3 Results

Case study

The clinical phenotype of the proband at the age of 3 months was originally described by Engels et al. (Engels et al., 2002). Following the realization that this CS patient harbored a distinct molecular basis, we have now carried out a detailed reevaluation of the patient at the age of 13 years. The patient shows key clinical hallmarks of CS, including congenital hypertrichosis, macrosomia at birth, macrocephaly, coarse facial appearance, cardiomegaly, skeletal
abnormalities, and developmental delay. At the time of reevaluation, he also had excessive gingival hyperplasia *(Figure 3.1C).*

Echocardiography at 13 years of age showed normal biventricular function with signs of noncompaction of the left ventricular apical myocardium, sonography of parenchymal abdominal organs was within the normal range, as were ECG and 24 hr blood pressure measurements. The patient's weight was 41 kg (25.–50. centile), height was 144 cm (10.–25. centile) and BMI was 19.5 (+0.3 SDS). He appeared disproportionate with a sitting height of 76 cm, arm span of 151.5 cm (10–25th centile) and arm span: height ratio >97th centile. His chest X-ray showed the same broadening of ribs as initially reported (Engels et al., 2002). X-ray of his left hand showed his bone age to be 10½ years at 13 years of age, corresponding to a delay of 2½ years. Laboratory studies showed essentially undetectable serum IGF-1 levels (<25 ng/ml, Ref. value 131–690) and a relatively low IGFBP3 value of 1.4 μg/ml (Ref. value 2.6–8.9). Arginine tolerance and clonidine test revealed absolute growth hormone (GH) deficiency (basal and all stimulated GH concentrations were below 1.0 ng/ml). GHRH testing resulted in subnormal GH secretion (max. GH 2.8 ng/ml).

Comparison of the present case with recently published CS patients carrying *ABCC9* mutations (Harakalova et al., 2012; van Bon et al., 2012) shows that the present patient exhibits all clinical hallmarks of CS, as well as 10/12 CS associated facial/cranial features; 2/5 cardiac features; 7/17 skeletal abnormalities, visible on radiographic studies; and 3/15 additional previously reported CS-associated features.
Figure 3.1- Gain-of-function $\text{KCNJ8}$ mutation $\text{Kir6.1}[\text{Cys176Ser}]$ underlies Cantu syndrome. A: Sequence chromatogram of patient and unaffected parents’ genomic DNA confirms de novo g.21,919,406A>T transition mutation in the patient, which is absent in the mother and father. B: Ribbon diagram of two of the four Kir6.1 subunits that form the $\text{K}^+ $-selective pore in $\text{K}_{\text{ATP}}$, based on the crystal structures of KirBac1.1 (PDB ID: 1BL8) and cytoplasmic domain of Kir3.1 (PDB ID: 1N9P). Shown are Kir6.1 residues mutated in CS (Cys176Ser), and reported in association with the J-wave syndrome (p.Ser422Leu). C: Clinical phenotype of the patient. Photographs of the patient at 13 years reveal extensive hypertrichosis, macrocephaly, coarse facial appearance, long arm- and torso-to-height ratio, and gingival hyperplasia with thickened lips.
Genetic analysis

To date, mutations in ABCC9 are the only known genetic cause of CS (Czeschik et al., 2013; Harakalova et al., 2012; van Bon et al., 2012). In our initial study, we found that nine out of 12 patients diagnosed with CS carry missense mutations in ABCC9 (van Bon et al., 2012). We performed Sanger sequencing of the three coding exons of KCNJ8 in the three unexplained patients, including the present case. KCNJ8 was chosen as the most promising candidate gene because of the functional considerations stated above. The study was approved by the local ethics committees, and all participating patients gave written informed consent. Parental consent was given on behalf of patients younger than 18 years of age; the study was explained to children capable of giving assent, and they provided oral assent.

In the proband, we identified a mutation Chr12(GRCh37):g.21919406A>T (NM_004982.2:c.526T>A), in the KCNJ8 gene, encoding a missense mutation (Cys176Ser) in the Kir6.1 protein (Figure 3.1). We did not observe the mutation in any of 2,096 in-house exomes, nor is it reported in any of 6,503 individuals from the exome variant server (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) [March 2014]). The variant has been deposited in the LOVD 3.0 (http://databases.lovd.nl/). The mutation is predicted to be “deleterious” by SIFT (score 0.03), and probably damaging by PolyPhen2 (score 1.0). The affected amino acid is fully conserved in both mammalian Kir6.1 and Kir6.2 proteins, suggesting a key role in channel function. Paternity was proven and sample mix-up excluded by STR marker analysis. The mutation was not present in maternal or paternal DNA and hence was presumed to have arisen de novo. Primer sequences and PCR conditions are available upon request.
Functional analysis of KCNJ8 mutations

To assess the effect of the Cys176Ser mutation on channel activity, mutant and wild type KCNJ8 (Kir6.1) cDNAs were co-transfected with ABCC8 (SUR1) or ABCC9 (SUR2A) cDNAs. Channel activity was assessed under normal metabolic conditions, in metabolically inhibited conditions (mimicking tissue ischemia), and in the presence of pharmacological potassium channel openers (KCOs), using $^{86}$Rb$^+$ efflux assays (see Methods). We also examined the channel activity resulting from Kir6.1[Ser422Leu], a mutation reported in several studies to be associated with J-wave abnormalities in the electrocardiogram (Barajas-Martinez et al., 2012; Medeiros-Domingo et al., 2010). As shown in Figure 3.2 and 3.3, very similar fluxes were measured for WT Kir6.1 and Ser422Leu channels. Measurable basal conductance was present for WT Kir6.1 with SUR1, but only MI- or KCO-stimulated fluxes were present for WT plus SUR2A. In contrast, significant fluxes were present for Kir6.1[Cys176Ser] with SUR1, and both MI- and KCO-stimulated fluxes were higher for each Kir6.1[Cys176Ser]/SUR combination. These experiments establish that the Cys176Ser mutation indeed causes a gain-of-function in the Kir6.1 channel, leading to markedly enhanced channel activity even under basal metabolic conditions, in co-expression with either SUR1 or SUR2A subunits.

To gain further insight to the mechanism by which the mutation enhances channel activity, we turned to patch-clamp experiments. The majority of K$_{\text{ATP}}$ channels in vivo are probably formed as homomeric Kir6.1 tetramers or Kir6.2 tetramers, although there is evidence that heteromeric Kir6.1/Kir6.2 combinations may also be present in cardiac (Bao et al., 2011) and smooth (Flagg et al., 2010; Insuk et al., 2003) muscle, for instance. Since the original cloning and expression of K$_{\text{ATP}}$ subunit genes, it has been clear that Kir6.1 channels are experimentally considerably more labile in excised patches than are Kir6.2 channels, and channel
Figure 3.2- Increased channel activity in basal and stimulated conditions in intact cells expressing Kir6.1[C176S]-containing K\textsubscript{ATP} channels. Relative efflux of \textsuperscript{86}Rb\textsuperscript{+} as a function of time in basal conditions (left), in the presence of potassium channel openers (center), or in metabolic inhibition (right) for reconstituted wild-type and mutant K\textsubscript{ATP} channels (with SUR1 (above) or SUR2A (below)), and untransfected controls (mean ± s.e.m., from 4-6 experiments). Data were fit with a double-exponential equation to obtain rate constants for K\textsubscript{ATP}-dependent efflux, \( k_2 \) (see Methods). Lines show mean fitted equations.
Figure 3.3- Increased relative efflux in basal and stimulated conditions in intact cells expressing Kir6.1[C176S]-containing K<sub>ATP</sub> channels. Relative efflux rate constants for K<sub>ATP</sub>-dependent $^{86}$Rb$^+$ efflux ($k_2$) of $^{86}$Rb$^+$ as a function of time in basal conditions (black), in the presence of potassium channel openers (white), or in metabolic inhibition (gray) for reconstituted wild-type and mutant K<sub>ATP</sub> channels (with SUR1 (A) or SUR2A (B)), and untransfected controls in basal conditions relative to metabolic inhibition (MI) for WT and mutant K<sub>ATP</sub> channels (mean ± s.e.m., from 4 to 6 experiments). *P < 0.01 compared with the wild-type K<sub>ATP</sub> by unpaired Student's t-test.
activity rapidly runs down in the absence of Mg-nucleotides, complicating the assessment of both inhibitory nucleotide sensitivity and intrinsic open probability of the channel. In the patient, the mutation is present in only one allele and since the active channels are tetramers, expressed K$_{\text{ATP}}$ channels in native cells will therefore be expected to consist of a mixture of both Cys176Ser and wild-type Kir6.1 or Kir6.2 subunits. To take advantage of this fact and additionally test the effect of heterozygosity, we co-expressed a 1:1 mixture of wild-type or mutant Kir6.1 cDNAs with wild-type Kir6.2 and SUR2A cDNAs (Figure 3.4).

These mixtures generated measurable fluxes in tracer studies and stable currents in excised patches. From these measurements it is evident that enhanced channel activity in intact cells expressing Cys176Ser (Figure 3.4) result from reduced ATP sensitivity (Figure 3.5B and 3.5C). We cannot formally rule out the possibility that altered trafficking and increased plasma membrane channel density may also play a role, but such effects have not previously been described for mutations in Kir channel pore regions. The findings indicate that the heterozygous Cys176Ser mutation in Kir6.1 will over activate any native K$_{\text{ATP}}$ channels in which it is present. The results are very consistent with the effects of the exact equivalent, and well-studied mutation, Cys166Ser in Kir6.2, which reduces ATP sensitivity of expressed channels by $\sim$50-fold (Loussouarn et al., 2000; Tucker et al., 1998). Additional mutations at this residue in Kir6.2, which also cause marked GOF, have been identified as causal in the most severe form of neonatal diabetes that is associated with developmental delay and epilepsy (Gloyn et al., 2006).

3.3 Discussion

CS was first characterized as such by J.M. Cantú in 1982 (Cantu et al., 1982). A genetic cause was recently reported (Harakalova et al., 2012; van Bon et al., 2012), with coding mutations identified in the $ABCC9$ gene, in 25 out of 30 patients. The present study reveals that a heterozygous mutation in $KCNJ8$, encoding the Kir6.1 pore-forming subunit of K$_{\text{ATP}}$ channels, can also cause CS. A recent study identified a different mutation in $KCNJ8$ (encoding Val65Met)
Figure 3.4- Increased channel activity in basal and stimulated conditions in reconstituted heteromeric KATP channels containing Cys176Ser subunits. A) Relative efflux of $^{86}$Rb$^+$ as a function of time in basal conditions (left), in the presence of potassium channel openers (center), or in metabolic inhibition (right) for heteromeric Kir6.1/Kir6.2 or Kir6.1[Cys176Ser]/Kir6.2 plus SUR2A KATP channel-expressing cells, and untransfected control cells (mean ± s.e.m., from 3 experiments). Data were fit with a double-exponential equation to obtain rate constants for KATP-dependent efflux, $k_2$ (see Methods). Lines show mean fitted equations. B) Rate constants for KATP-dependent $^{86}$Rb$^+$ efflux ($k_2$) in basal conditions (black) and in metabolic inhibition (MI) (gray) from COS cells expressing heteromeric Kir6.1/Kir6.2 or Kir6.1[Cys176Ser] (C176S)/Kir6.2 plus SUR2A KATP channels.
Figure 3.5- Reduced ATP-sensitivity of reconstituted heteromeric $K_{ATP}$ channels containing Kir6.1[Cys176Ser] subunits. A: Rate constants for $K_{ATP}$-dependent $^{86}$Rb$^+$ efflux ($k_2$) in basal conditions and in metabolic inhibition (MI) from COS cells expressing heteromeric Kir6.1/Kir6.2 or Kir6.1[Cys176Ser] (C176S)/Kir6.2 plus SUR2A $K_{ATP}$ channels. B: Representative currents recorded from inside-out membrane patches from COS cells expressing heteromeric Kir6.1/Kir6.2 or Kir6.1[Cys176Ser] (C176S)/Kir6.2 plus SUR2A $K_{ATP}$ channels at $-50$ mV in K$_{int}$ solution (see Supplementary information). Patches were exposed to differing [ATP] and baseline current was determined by exposure to 5 mM ATP. C: Steady-state dependence of membrane current on [ATP] (relative to current in zero ATP ($I_{rel}$)) for wild-type and Cys176Ser-containing channels. Data points represent the mean ± s.e.m. ($n = 6–8$ patches). The fitted lines correspond to least squares fits of a Hill equation. *$P < 0.01$ compared with the wild-type $K_{ATP}$ by unpaired Student's $t$-test.
in another CS patient (Brownstein et al., 2013), although there was no functional characterization of mutant channels. The marked GOF in expressed Kir6.1[Cys176Ser] channels (Figures 3.1 and 3.2), which mirrors the well-studied properties of Kir6.2 channels with mutations at the equivalent Cys166 residue (Gloyn et al., 2006; Loussouarn et al., 2000; Trapp et al., 1998; Tucker et al., 1998), clearly establishes Cys176Ser as a severe GOF. Thus GOF mutations in both ABCC9 and KCNJ8 cause essentially the same hallmark CS features in the affected patients indicating that these features all result from GOF of K\textsubscript{ATP} channels formed from these subunits.

This is a subtle but critical conclusion: previous studies (Aggarwal et al., 2010; Stoller et al., 2010) have raised the possibility of Kir6-independent roles of SUR2 proteins and, in the absence of the present findings, some or all of the features of CS could conceivably have arisen from non-channel functions of the SUR2 protein.

The results underscore the key role of K\textsubscript{ATP} channels in coupling cell membrane potential with diverse tissue functions. Our results are consistent with the finding that vascular smooth muscle contractility is markedly decreased in the presence of K\textsubscript{ATP} channel openers (Flagg et al., 2010) and in transgenic mice expressing mutant Kir6.1 subunits with reduced sensitivity to inhibitory ATP (Li et al., 2013). These observations potentially explain some of the key findings in CS, including patent ductus arteriosus, as was present in the patient reported here.

A novel finding is the puzzling combination of biochemical signs of absolute growth hormone (GH) deficiency, yet postnatal growth with height within the normal range. Pituitary GH secretion is mainly regulated by two hypothalamic neuropeptides. GHRH stimulates GH release, whereas somatostatin inhibits GH secretion (Muller et al., 1999). In addition, a number of GH-releasing peptides are able to modulate GH release. The rat pituitary expresses several Kir channel alpha-subunits, including Kir6.1 (Wulfsen et al., 2000). GHRP-2, one member of the
GH-releasing neuropeptides, exerts its GH secretory effect through a reduction in potassium current (Xu et al., 2002). It is therefore tempting to speculate that GOF mutations in pituitary $K_{ATP}$ channels exert an opposite effect on GHRH stimulation, leading to resistance to hypothalamic induction of GH release. On the other hand, GH insufficiency yet normal growth can only be explained by a concurrent stimulation of long bone growth. Previous animal models indicated that a significant part of postnatal growth depends on local growth stimulation in the growth plate rather than on the effect of circulating IGF-1 (Ohlsson et al., 2009; Yakar et al., 1999), although understanding of molecular mechanisms at the growth plate leading to long bone growth is incomplete. It is not yet clear whether $ABCC9$ mutation patients exhibit similar deficiencies, and if not, this finding may be a $KCNJ8$-specific outcome, or may be unrelated to CS.

A common $K_{ATP}$ pathway can account for direct overlap of CS features resulting from mutations in $ABCC9$ and $KCNJ8$, as well as with features resulting from overexposure to minoxidil and other $K_{ATP}$ channel openers (Avatapalle et al., 2012; Kaler et al., 1987; Nguyen and Marks, 2003; Shanti et al., 2009). However, this does not provide an obvious explanation for many CS features. For instance, macrocephaly and characteristic facial features, as well as skeletal abnormalities are present in both $ABCC9$ and $KCNJ8$ patients. However, while $K_{ATP}$ channels generated by these subunits have been reported in human chondrocytes (Mobasheri et al., 2012; Rufino et al., 2013) and osteoblasts (Kawase et al., 1996), their role in maturation and proliferation remains unknown, and the cellular pathway involved is not obvious. Interestingly, minoxidil has been reported to induce pseudoacromegalic features in the absence of elevated GH or IGF-1 (Nguyen and Marks, 2003). Similarly, epicanthal folds and deep plantar creases might result from $K_{ATP}$ gain-of-function, but the underlying cause is unknown. Finally, pericardial
effusion, polydramnios and lymphoedemia, potentially all related problems of membrane barrier breakdown, are unexplained. SUR2/Kir6.1 K\textsubscript{ATP} channels have been reported in epithelial cells (Bardou et al., 2009), but again, cellular pathways are unexplained.

Finally, we would note that several other studies have reported a different mutation (p.Ser422Leu) in the Kir6.1 protein to be associated with ‘early repolarization syndrome’ (ERS), characterized by abnormalities in the J-point of the electrocardiogram (Delaney et al., 2012; Haissaguerre et al., 2009). Recent studies have reported that this p.Ser422Leu mutation enhances channel activity (Medeiros-Domingo et al., 2010), by reducing ATP-sensitivity (Barajas-Martinez et al., 2012). If so, there is a clear inconsistency: neither J-wave abnormalities nor other arrhythmias have been reported in CS patients, and none of the CS features have yet been reported for ERS patients. Our own data (Figure 3.2) indicate that, in recombinant COS cells, p.Ser422Leu does not affect Kir6.1-dependent K\textsubscript{ATP} channel activity, consistent with the recent recognition that this mutation may actually represent a common variant (~4%) in Ashkenazim subjects, and not clearly associated with ERS (Veeramah et al., 2014).
CHAPTER 4: DISEASE CAUSING SLIDE HELIX RESIDUE MUTATION IN Kir6.1 AND Kir6.2

4.1 Introduction

While all previous reports of CS-associated mutations are in the ABCC9 gene, a CS patient with a *KCNJ8* mutation, encoding V64M in the Kir6.1 subunit, was described by Brownstein *et al.* in 2013, although no functional support for this mutation causing any change in channel activity was provided. Interestingly, a previously identified mutation at the equivalent position in Kir6.2 (V64L), was reported to have no effect on *K*<sub>ATP</sub> channel a. Here, I directly compare these two published mutations: valine to methionine and valine to leucine in the equivalent positions in Kir6.1 (V65) and Kir6.2 (V64), co-expressed with SUR1 or SUR2A. Using macroscopic rubidium (*<sup>86</sup>Rb<sup>+</sup>*) efflux assays, I show that, compared to WT channels, both Kir6.1-V65M and Kir6.2-V64M enhance *K*<sub>ATP</sub> activity, but channels formed with Kir6.1-V65L and Kir6.2-V64L are unaffected. Additionally, Kir6.2-V64M-based channels decrease ATP sensitivity and PIP<sub>2</sub> activation, but V64L-based channels do not. Taken together, these data provide functional support for the notion that CS-associated Kir6.1-V65M mutation leads to overactive *K*<sub>ATP</sub> channels. More specifically, the mechanism of this GOF is a result of the valine-to-methionine substitution increasing channel *P*<sub>o</sub> and thereby indirectly decreasing ATP inhibition. These findings are consistent with a causal involvement of this mutation in CS.

4.2 Results

*Kir6.1-V65M, but not Kir6.1-V65L, forms overactive *K*<sub>ATP</sub> channels paired with SUR1 or SUR2*

To examine *K*<sub>ATP</sub> channel activity in intact cells, I performed rubidium (*<sup>86</sup>Rb<sup>+</sup>*) efflux assays (see Methods) under three metabolic conditions: basal; metabolic inhibition (MI), which prevents
ATP production; or in the presence of SURX-specific potassium channel openers diazoxide (SUR1) or pinacidil (SUR2). Kir6.1-WT, Kir6.1-V65M and Kir6.1-V65L were each homomerically expressed with SUR1 (Figure 4.1). Under basal and diazoxide conditions Kir6.1-V65M based channels exhibited significantly higher $^{86}$Rb$^+$ efflux rate compared to WT channels, while Kir6.1-V65L channels efflux rates were comparable to WT (Figures 4.1A and 4.1B). Maximal activation, estimated under MI conditions, comparable for Kir6.1-V65M, V65L and Kir6.1-WT based channels, implying no differences in expressed channel densities (Figure 4.1C).

When paired with SUR2A, basal fluxes through Kir6.1-WT, Kir6.1-V65M and Kir6.1-V65L were undistinguishable (Figure 4.2A), presumably due to lower activation of SUR2 by MgATP or MgADP compared to SUR1 (Masia et al., 2005). However, when K$_{ATP}$- specific fluxes were activated in pinacidil or MI conditions, Kir6.1-V65M but not Kir6.1-V65L fluxes were significantly increased compared to WT channels (Figure 4.2B and 4.2C). These trends are consistent with Kir6.1-V65M causing a GOF, but Kir6.1-V65L having WT-level K$_{ATP}$ channel activity when paired with SUR1 or SUR2 (Figures 4.1 and 4.2).

*Kir6.2-V64 mutated to methionine or leucine produces comparable functional changes to Kir6.1-V65M or L mutations*

Kir6.1 (V65) and Kir6.2 (V64) are localized within an identical sequence region in the slide helix (Inagaki et al., 1995c). Therefore, it is likely the role this valine residue plays in gating is conserved between each channel. In the basal condition, whether expressed with SUR1 or SUR2, Kir6.2-V64M formed channels with increased efflux rates compared to wild type channels, but Kir6.2-V64L-based channels exhibited comparable activity to WT (Figure 4.3A and 4.4A).
Figure 4.1- Homomeric Kir6.1-V65M–but not Kir6.1-V65L–expressed with SUR1 increases $K_{ATP}$ channel activity under basal conditions and in the presence of diazoxide in intact cells. Cumulative $^8$Rb$^+$ efflux, as a function of time, was measured in GFP-transfected control cells (black symbols), and in cells transiently expressing SUR1 plus Kir6.1-WT (white triangles), Kir6.1-V65M (blue squares) or Kir6.1-V65L (light grey circles). Experiments were done in basal conditions (A), in the presence of the K+ channel opener diazoxide (B), or metabolic inhibitors (MI) oligomycin and 2-deoxy-d-glucose (C). The data represents the means ± S.E.M. of 8-18 experiments. Flux data from each condition was fit with a double-exponential equation to obtain the rate constants for $K_{ATP}$-dependent efflux, $k_2$, where lines show mean fitted relationships. Graphical representation of the determined $k_2$ rate constants are shown on the right of the corresponding cumulative efflux over time. *p< 0.05 as compared to WT (unpaired Student’s $t$ test).
Figure 4.2- Homomeric Kir6.1-V65M (but not Kir6.1-V65L) expressed with SUR2A increases $K_{\text{ATP}}$ channel activity under Metabolic Inhibition (MI) and in the presence of pinacidil in intact cells. Cumulative $^{86}\text{Rb}^+$ efflux, as a function of time, was measured in GFP-transfected control cells (black symbols), and in cells transiently expressing SUR2A with Kir6.1-WT (white triangles), Kir6.1-V65M (blue squares) or Kir6.1-V65L (light grey circles). Experiments were done under basal conditions (A), in the presence of the K+ channel opener pinacidil (B), or metabolic inhibitors (MI) oligomycin and 2-deoxy-d-glucose (C). The data represents the means ± S.E.M. of 3 experiments. Flux data from each condition was fit with a double-exponential equation to obtain the rate constants for $K_{\text{ATP}}$-dependent efflux, $k_2$ (right), where lines show mean fitted relationships. Graphical representation of the determined $k_2$ rate constants are shown on the right of the corresponding cumulative efflux over time. * $p<0.05$ as compared to WT (unpaired Student’s $t$ test).
Figure 4.3- Homomeric Kir6.2-V64M–but not Kir6.2-V64L– expressed with SUR1 increases K_ATP channel activity in basal conditions and in the presence of Diazoxide in intact cells. The cumulative $^{86}\text{Rb}^+$ efflux, as a function of time, was measured in GFP-transfected control cells (black symbols), and in cells transiently expressing SUR1 with Kir6.2-WT (white triangles), Kir6.2-V64M (navy squares) or Kir6.2-V64L (dark grey circles). Experiments were done in basal conditions (A), in the presence of the K+ channel opener diazoxide (B), or metabolic inhibitors (MI) oligomycin and 2-deoxy-d-glucose (C). The data represents the means ± S.E.M. of 3-5 experiments. Flux data from each condition was fit with a double-exponential equation to obtain the rate constants for K_ATP-dependent efflux, $k_2$ (right), where lines show mean fitted relationships. Graphical representation of the determined $k_2$ rate constants are shown on the right of the corresponding cumulative efflux over time. * $p<0.05$ as compared to WT (unpaired Student’s $t$ test).
Figure 4.4- Homomeric Kir6.2-V64M–but not Kir6.2-V64L–with SUR2A increases $K_{ATP}$ channel activity in basal conditions and in the presence of pinacidil in intact cells. The cumulative $^{86}\text{Rb}^+$ efflux, as a function of time, was measured in GFP-transfected control cells (black symbols), and in cells transiently expressing SUR2A with Kir6.2-WT (white triangles), Kir6.2-V64M (navy squares) or Kir6.2-V64L (dark grey circles). Experiments were done in basal conditions (A), in the presence of the K+ channel opener pinacidil (B), or metabolic inhibitors (MI) oligomycin and 2-deoxy-d-glucose (C). The data represents the means ± S.E.M. of 3-6 experiments. Flux data from each condition was fit with a double-exponential equation to obtain the rate constants for $K_{ATP}$-dependent efflux, $k_2$ (right), where lines show mean fitted relationships. Graphical representation of the determined $k_2$ rate constants are shown on the right of the corresponding cumulative efflux over time. * p< 0.05 as compared to WT (unpaired Student’s t test).
These trends were also seen in the presence of diazoxide or pinacidil (Figure 4.3B and 4.4B). In MI Kir6.2 WT, Kir6.2-V64M and Kir6.2-V64L reached the same maximum with comparable efflux rates, whether paired with SUR1 or SUR2, indicating that these mutations do not alter the expression of $K_{\text{ATP}}$ channels (Figure 4.3C and 4.4C). Overall, compared to WT channels, the valine-to-methionine substitution in both Kir6.1 and Kir6.2 results in a gain of function in $K_{\text{ATP}}$ activity, but the valine-to-leucine mutation exhibits comparable channel activity (Figures 4.1-4.4).

Kir6.2-V64M overactivity results from decreased ATP sensitivity

The $^{86}\text{Rb}^+$ efflux experiments demonstrate that the behavior of valine-to-methionine and valine-to-leucine substitutions are consistent whether expressed in Kir6.1 or Kir6.2, but do not reveal underlying molecular mechanism(s). Unlike Kir6.2, Kir6.1-based channels quickly run down and require activatory nucleotides to maintain channel activity, making assessment of nucleotide sensitivity experimentally challenging (Babenko et al., 2000). To assess the details of increased channel activity, inside-out excised patch-clamp experiments on Kir6.2-WT-, Kir6.2-V64M- and V64L-based channels paired with SUR2A were carried out. Intrinsic sensitivity to ATP inhibition (in zero Mg$^{2+}$) was similar for WT ($K_i = 25 \ \mu\text{M}$) and Kir6.2-V64L ($K_i = 16 \ \mu\text{M}$) channels. However, $K_{\text{ATP}}$ channels expressing Kir6.2-V64M exhibited a significant right shift in the [ATP]-response ($K_i = 289 \ \mu\text{M}$) (Fig. 4.5B). A decreased sensitivity to intracellular ATP could account for the increased activity of $K_{\text{ATP}}$ channels under basal conditions in the intact cell (Figure 4.4A). PIP$_2$ potentiation experiments were carried out on wild type and Kir6.2-V64M-based channels to assess ATP-independent open state stability (Enkvetchakul and Nichols, 2003) (Figure 4.6B). As seen in Figure 4.6C, wild type channels have a relative open probability of
Figure 4.5- Gain of function in Kir6.2-V64M due to decreased ATP-sensitivity. Representative excised patch current recordings from COSm6 cells co-expressing SUR2A-WT with Kir6.2-WT, Kir6.2-V64M or Kir6.2-V64L (A). Membrane potential was held at -50mV, and currents were recorded continuously on-cell and in inside-out excised patches exposed to K\textsubscript{INT} in the absence or in the presence of 0.01, 0.1 or 1 mM ATP. Dose-response data (mean ± SEM 10 patches each) were fit to equation 1 to estimate the ATP concentration for half-maximal inhibition $K_i$: WT (24.7 μM), Kir6.2-V64M (16 μM), and Kir6.2-V64L (289.3 μM) (B).
Figure 4.6- Increased open state stability in Kir6.2-V64M-based channels underlies decreased ATP sensitivity. Representative $K_{ATP}$ current traces for SUR2A-WT expressed with Kir6.2-WT (A) or Kir6.2-V64M (B) following excision and in the presence of 10 mM ATP, 1mM ATP, or 5 µg/µL-PIP$_2$. The relative $P_o$ for individual patches from WT (white diamonds) and V64M (navy blue squares) was determined as a ratio of the maximum steady-state current upon excision, in the absence of nucleotides, over the maximum current measured in PIP$_2$. The average $P_o$ (black bars) is the mean of 8 individual patches for each construct ± SEM. The relative $P_o$ for WT channels is $0.73 \pm 0.08$ (WT) and $1.05 \pm 0.07$ for Kir6.2-V64M based channels. *$p<0.05$ as compared to WT (unpaired Student’s $t$ test).
0.73±0.08 where as Kir6.2-V64M channels have a significantly increased $P_o$ of 1.05±0.07, indicating that the valine-to-methionine substitutions also affect channel $P_o$.

4.3 Discussion

Cantu Syndrome, a complex multi-organ disease, was first described in 1982 (Cantu et al., 1982). Recently, several studies have demonstrated that the majority of CS patients have a mutation in $ABCC9$ (Harakalova et al., 2012; van Bon et al., 2012). In addition, two patients with clinically defined CS have been identified with coding mutations in $KCNJ8$ (Brownstein et al., 2013; Cooper et al., 2014), and two of these studies, together with the present study, confirm that the disease therefore results from gain of function in $K_{ATP}$ channel activity that is generated by the SUR2 and Kir6.1 subunits encoded by these genes.

*Effects of equivalent residue mutations on $K_{ATP}$ channel activity*

In a previously reported case of neonatal diabetes, a mutation at the equivalent position in Kir6.2 (V64L) was reported along with an additional mutation at F60Y. When these mutations were expressed separately and channel activity was assessed, Kir6.2-V64L was determined to have no effect on $K_{ATP}$ activity, and GOF in $K_{ATP}$ activity was solely a result of the increased $K_{ATP}$ channel activity from the F60Y mutation (Mannikko et al., 2009). Similarly, Kir6.2-V64A does not perturb $K_{ATP}$ activity (Li et al., 2013). In the present study I have confirmed this finding, and shown that mutation of the equivalent valine to leucine in either Kir6.1 or Kir6.2 is without effect on recombinant channel activity. Conversely, mutation to methionine in either Kir6.1 or Kir6.2 generates a net gain of function. The inability of either leucine or alanine substitutions at this position to alter $K_{ATP}$ activity might suggest that the bulkiness of the mutated residue at this position is relevant to if and how it alters $K_{ATP}$ channel activity.
Mechanism of increased $K_{\text{ATP}}$ channel activity through Kir6.1-V65M and Kir6.2-V64M-based channels

Given the experimental difficulty of recording Kir6.1/SUR2 currents (Cooper et al., 2014), Kir6.2-V64M and V64L were instead paired with SUR2A to determine nucleotide sensitivity of $K_{\text{ATP}}$ channels formed with each mutation. In excised patches, the ATP-sensitivity of Kir6.2-V64M based channels, but not Kir6.2-V64L, was significantly reduced, compared to Kir6.2-WT channels. This result is consistent with the overall gain of function in channel activity in Kir6.2-V64M and Kir6.1-V65M being a result of decreased ATP-sensitivity (Figure 4.5). As has previously been described, mutations in Kir6.2 can reduce ATP sensitivity by two distinct mechanisms: 1) by directly altering ATP binding and/or 2) by increasing ATP-independent open probability, thus indirectly reducing ATP sensitivity (Koster et al., 2008a). By measuring the relative PIP$_2$ activation of Kir6.2-V64M compared to Kir6.2-WT-based channels, we have shown that the $P_o$ is increased in the absence of ATP (Figure 4.6). This result is consistent with this mutation lying outside the ATP binding pocket, and thereby altering ATP sensitivity indirectly.
5.1 Introduction

The functional consequences of multiple uncharacterized CS-mutations remain unclear. In this paper, we have focused on determining the functional consequences of 3 documented human CS-associated \textit{ABCC9} mutations: human P432L, A478V and C1043Y. The mutations were engineered in the equivalent position in rat SUR2A (P429L, A475V and C1039Y) and each was co-expressed with mouse Kir6.2. Using macroscopic rubidium (\textsuperscript{86}Rb\textsuperscript{+}) efflux experiments, we show that \textit{K\textsubscript{ATP}} channels formed with P429L, A475V or C1039Y mutants enhance \textit{K\textsubscript{ATP}} activity compared to WT channels. We used inside-out patch-clamp electrophysiology to measure channel sensitivity to ATP-inhibition and MgADP-activation. For P429L and A475V mutants, sensitivity to ATP inhibition was comparable to WT channels, but activation by MgADP was significantly greater. C1039Y-dependent channels were significantly less sensitive to inhibition by ATP or by glibenclamide, but MgADP activation was comparable to WT. The results indicate that these three Cantu Syndrome mutations all lead to overactive \textit{K\textsubscript{ATP}} channels, but at least two mechanisms underlie the observed gain of function: decreased ATP inhibition and enhanced MgADP activation.

5.2 Methods

All rubidium efflux experiments were done by Monica Sala-Rabanal. Sequence alignment and homology model by Sun Joo Lee.
Homology Modeling

Models of human SUR2A (Figure 5.1) were built using Modeller v9.8 from the template of the multidrug ABC transporter Sav1866 (2ONJ, to model the ‘open’ conformation) and heterodimeric ABC transporter TM287-TM288 (3QF4, to model the ‘closed’ conformation). The TM0 domain and L0 loop (1-281) were omitted due to lack of sequence homology to any proteins of known structure. Two extended loops unique to SUR2A were also omitted: one connecting TM1 and NBD1 (614-672) and a second connecting NBD1 and TM2 (920-966). A multiple sequence alignment (MSA) was carried out using ClustalW2 between SUR2 and the two template sequences. Due to low sequence identity, other proteins in the human ABCC family (ABCC1, 2, 3, 4, 5, 6, 8 and 10) were included in the alignment for TM1 and NBD1. Highly conserved sequence identity enabled a reliable sequence alignment of TMD2 and NBD2 by MSA between SUR2 and SUR1, 2ONJ, and 3QF4.

5.3 Results

Homomorphic mutant channels are overactive in various metabolic conditions

Cantu syndrome-associated mutations have been found throughout the coding sequence (Figure 5.1). For the present study, we focused on two previously unexamined mutations (human P432L and C1043Y, corresponding to rat P429L and C1039Y, respectively), located in the TMD1 and TMD2 segments, and A478 (corresponding to rat A475V), also located in the TMD1 region. To examine $K_{ATP}$ channel activity in intact cells, we performed $^{86}$Rb$^+$ efflux experiments under four different conditions: basal; metabolic inhibition (MI); in the presence of pinacidil (PIN); and MI and pinacidil combined (MI + PIN). As shown in Figures 5.2 and 5.3, homomeric expression of
Figure 5.1- Cantu Syndrome mutations in SUR2 Alignment of SUR2 (ABCC9) with the multidrug ABC transporter Sav1866 (2ONJ) and heterodimeric ABC transporter TM287-TM288 (3QF4), upon which we built homology models. Key structural domains TMD1,2 and NBD1,2) are indicated, as well as predicted alpha helical (pink) and beta strand (green) segments (A). Homology models of ‘open’ (B) and ‘closed’ (C) conformations of the SUR2A protein, which are based on Staphylococcus aureus Sav1866, a bacterial homolog of the human ABC transporter Mdr1 and hetero-dimeric ABC transporter TM287-TM288 (TM287/288) from Thermotoga maritime, respectively. Published CS mutations from previous reports are shown in green (open) and light pink (closed).
Figure 5.2- Increased channel activity in intact cells expressing homomeric P429L, A475V or C1039Y-containing K_{ATP} channels. $^{86}\text{Rb}^+$ efflux, as a function of time, was measured in GFP-transfected control cells (dashed), and in cells transiently expressing reconstituted Kir6.2-based K_{ATP} channels with WT, P429L, A475V or C1039Y SUR2 subunits in homomeric configuration, in basal conditions (A), in the presence of metabolic inhibitors (MI) oligomycin and 2-deoxy-d-glucose (B), or in the K$^+$ channel opener pinacidil (C), or in the presence of both MI and pinacidil (D). The data represent means ± S.E.M. of 6-10 experiments. Flux data were fit with equation 1 (GFP) to obtain the rate constant $k_1$ or equation 2 to obtain the rate constants for K_{ATP}-dependent efflux, $k_2$ (Figure 5.3), where lines show mean fitted relationships.
Figure 5.3- K$_{ATP}$ conductance is increased in basal and stimulated conditions in intact cells expressing homomeric P429L and A475V-based K$_{ATP}$ channels. Rate constants for K$_{ATP}$-dependent $^{86}$Rb$^+$ efflux ($k_1$ in gray, proportional to non-specific K$^+$ conductance and $k_2$, proportional to K$_{ATP}$ specific K$^+$ conductance), were calculated from data shown in Figure 5.2. *p< 0.05 as compared to WT (unpaired Student’s t test).
SUR2A-P429L, A475V or C1039Y channels, results in significantly higher basal $^{86}\text{Rb}^+$ efflux rates compared to WT (Figure 5.2A and Figure 5.3A). P429L and A475V-based channels, but not those composed of C1039Y, also showed significantly higher rate of efflux compared to WT channels under MI conditions and in the presence of pinacidil (Figures 5.2B-C and 5.3B-C), consistent with the gain of function(GOF) observed in the basal condition. Maximal efflux rates (estimated using simultaneous exposure to MI and pinacidil) of P429L and A475V were comparable to SUR2A-WT, implying similar channel densities at the cell surface (Figure 5.2D and 5.3D). C1039Y showed significantly lower absolute fluxes in all stimulatory conditions (Figures 5.2B-D and 5.3B-D), implying a lower channel density at the membrane (Figure 5.4).

The ratio of $K_{\text{ATP}}$-dependent ($k_2$) rate constants in basal, MI and pinacidil to the maximal $K_{\text{ATP}}$-dependent rate constant ($k_2$ in MI+PIN) should reflect the relative activation of each channel in these specific conditions. In the basal condition, the ratio was higher for all three mutations, compared to WT, markedly so for C1039Y (Figure 5.4A). In pinacidil alone, while only P429L and A475V reach significance, the ratio of active channels in all three mutations are also increased (Figure 5.4C). Together, the results suggest that each mutant will result in higher channel activity under any stimulatory conditions, particularly for C1039Y.

$C1039Y$ ($hC1043Y$) overactivity results from decreased ATP-sensitivity via increased $P_o$

$^{86}\text{Rb}^+$ flux assays provide evidence for overactivity in CS mutants, but do not provide any indication of underlying molecular mechanisms. We assessed the details of channel properties in inside-out excised patch-clamp electrophysiology experiments. Intrinsic sensitivity to ATP inhibition (in zero Mg$^{2+}$), was similar for WT, P429L and A475V channels ($K_i = 7-9$ μM).
Figure 5.4- Relative $K_{\text{ATP}}$ conductance is markedly increased in basal and stimulated conditions in intact cells expressing homomeric C1039Y $K_{\text{ATP}}$ channels. The ratio of the rate constants for $K_{\text{ATP}}$-dependent $^{86}\text{Rb}^+$ efflux ($k_2$) in basal and pinacidil- or MI-stimulated conditions to the maximal activation (in MI+Pinacidil) is plotted for WT and mutant channels. *p< 0.05 as compared to WT (unpaired Student’s $t$ test).
However, $K_{\text{ATP}}$ channels expressing C1039Y exhibited a significant right shift in ATP-sensitivity ($K_i = 21.3 \ \mu\text{M}$) (Figure 5.5B). A diminished sensitivity to intracellular ATP may account, at least in part, for the increased activity of C1039Y-based $K_{\text{ATP}}$ channels in basal conditions in the intact cell (Figures. 5.2A and 5.3A). Consistent with reduced channel density, the maximal current in zero ATP was significantly lower in C1039Y channels than WT or the P429L and A475V mutant channels (Figure 5.5C).

In PIP$_2$ activation experiments, WT and C1039Y channels lost ATP sensitivity the more the patch was exposed to PIP$_2$. Simultaneously, the maximum currents for WT channels increased while the maximum current in C1039Y channels activity remained the same (Figure 5.6). The ratio of the maximum current in zero ATP over max current in PIP$_2$ for WT patches ($0.70 \pm 0.11$) was significantly lower than that of C1039Y channel patches ($1.26 \pm 0.21$), which implies increased intrinsic activity from C1039Y channels.

Overactivity in P429L (hP432L) and A475V (hA478V) results from increased MgADP activation

We investigated the current response to intracellular MgADP in the presence of 0.1 mM ATP (Figure 5.7A). MgADP-dependent activation was estimated as the ratio between the steady-state activated current in MgADP+ATP and the maximal current in zero ATP, immediately after excision (Figure 5.7B). In P429L, the relative current in MgADP was markedly higher than WT for P429L and A475V channels. Using this analysis, the current was also statistically higher for C1039Y channels. However, this analysis fails to account for the intrinsically lower sensitivity of C1039Y channels to ATP inhibition. An alternative estimation for the stimulatory action of Mg-
**Figure 5.5- ATP-sensitivity is decreased in C1039Y channels.** Representative excised patch clamp recordings from COSm6 cells co-expressing Kir6.2 and WT or CS mutant SUR2 subunits P429L, A475V or C1039Y (A). Membrane potential was held at -50mV, and negative currents (plotted as upward deflections) were recorded continuously on-cell and in inside-out excised patches exposed to K\textsubscript{INT} in the absence or in the presence of 0.01, 0.1 or 1 mM ATP; *arrowheads* mark the point of excision. Dose-response data (mean ± SEM from 8-11 patches) was fit with equation 3 to estimate the ATP concentration for half-maximal inhibition $K_i$: WT (9 µM), P429L (9 µM), A475V (7 µM), and C1039Y (21 µM) (B). The maximum patch current determined immediately following patch excision (mean ± SEM from 8-11 patches) (C).
Figure 5.6- C1039Y channels decreased ATP-sensitivity is due to increased $P_o$
Representative $K_{\text{ATP}}$ current traces following excision and in the presence of 1 mM ATP or 5
$\mu$g/$\mu$L-PIP$_2$ as indicated (A). Relative $P_o$ determined as a ratio of the maximum steady-state
current in the patch upon excision, in the absence of nucleotides, over the maximum current
measured in PIP$_2$. Individual patch data represented by symbols (n = 7-10); bars are the
means ± SEM respectively, where the relative $P_o$ for WT= 0.70 ± 0.11 and 1.26 ± 0.21 for
C1039Y (B). *$p< 0.05$ as compared to WT (unpaired Student’s $t$ test).
Figure 5.7- P429L and A475V show enhanced MgADP activation Representative $K_{ATP}$ current traces following excision and in the presence of nucleotides as indicated (A). Relative MgADP activation in individual patch data represented by symbols (n = 11-24); bars are the means ± SEM respectively (B). *p< 0.05 as compared to WT (unpaired Student’s t test). Insert shows the mean current measured in patches in the presence of 0.1 mM Mg-free ATP (from Figure 5.5B) subtracted from the mean value of steady-state current measured in patches in the presence of MgADP (from Figure 5.7A). Error bars represent the propagated error from both experiments half-time of activation by MgADP (C).
nucleotides is the ratio of current in MgADP+ATP to the current in Mg-free ATP alone (Figure 5.7B, inset). This analysis implies no difference in the MgADP activation of C1039Y and WT channels. In addition, while the MgADP activation for P429L and A475V channels is more rapid than WT, activation of C1039Y channel is even slower than WT (Figure 5.7C).

*Gain of function is reduced in heteromeric mutant channels*

All documented CS patients with mutations in *ABCC9* are heterozygous. To mimic the predicted heteromeric composition of channels in such patients we also expressed each mutation in a 1:1 ratio with SUR2A-WT plus Kir6.2-WT, and assessed channel activity by $^{86}\text{Rb}^+$ efflux experiments (Figures 5.8 and 5.9). In all conditions P429L, A475V and C1039Y heteromeric channels display no significant increases in the rates of $^{86}\text{Rb}^+$ efflux, compared to WT channels (Figures 5.8A and 5.9A). However, when normalized to the maximal efflux rates, channels with heteromeric expression of C1039Y are still considerably more active than WT. Additionally, in the heteromeric state, maximal C1039Y channel fluxes were markedly higher than in the homomeric state (Figure 5.9D). Taken together, these results imply a partial rescue of the surface expression of C1039Y subunits by WT subunits (Figure 5.7B-D and 5.8B-D).

*Altered response to glibenclamide in C1039Y channels*

Some NDM patients with GOF mutations in Kir6.2 or SUR1 have successfully switched from insulin therapy to $K_{\text{ATP}}$ channel inhibitors, such as glibenclamide (Zung et al., 2004). However, a correlation between increased channel activity and the diminished effectiveness of such drugs has been noted (Koster et al., 2005b). Therefore to test the effectiveness of glibenclamide on overactive P429L, A475V or C1039Y channels, expressed both homomerically and heteromerically, $^{86}\text{Rb}^+$ efflux experiments were carried out in the presence of MI+ 10 µM.
Figure 5.8- Channel activity in cells expressing heteromeric P429L, A475V or C1039Y-based K<sub>ATP</sub> channels. 86Rb<sup>+</sup> efflux, as a function of time, was measured in GFP-transfected control cells (dashed), and in cells transiently expressing reconstituted Kir6.2-based K<sub>ATP</sub> channels with WT or 1:1 mixtures of WT and P429L, A475V or C1039Y mutant SUR2 subunits, in basal conditions (A), in the presence of metabolic inhibitors (MI) oligomycin and 2-deoxy-d-glucose (B), or in the K<sup>+</sup> channel opener pinacidil (C), or in the presence of both MI and pinacidil (D). The data represent means ± S.E.M. of 6-10 experiments. Flux data were fit with equation 1 (GFP) to obtain the rate constant k<sub>1</sub> or equation 2 to obtain the rate constants for K<sub>ATP</sub>-dependent efflux, k<sub>2</sub> (**Figure 5.9**), where lines show mean fitted relationships.
Figure 5.9- \( \text{K}_{\text{ATP}} \) conductance normalized in the basal condition and in stimulated conditions for heteromeric P429L, A475V or C1039Y-based \( \text{K}_{\text{ATP}} \) channels. The rate constants for non-specific efflux (\( k_1 \) represented by gray area) and \( \text{K}_{\text{ATP}} \)-dependent \( ^{86}\text{Rb}^+ \) efflux (\( k_2 \)), proportional to \( \text{K}_{\text{ATP}} \) specific \( \text{K}^+ \) conductance, were estimated from data shown in Figure 5.8. *p< 0.05 as compared to WT (unpaired Student’s \( t \) test).
glibenclamide (Fig. 5.10). The time course of \(^{86}Rb^+\) effluxes shows an unusual behavior in that although initial fluxes are markedly lower than in MI without glibenclamide, the inhibition is not maintained through the time course of the assay. It is well understood that the inhibitory action of glibenclamide is a complex function of the metabolic conditions, and decreases under conditions of metabolic inhibition (Findlay, 1993; Koster et al., 1999). To account for this behavior, the efflux data in glibenclamide were again fit by equation 2, where negative values for \(k_2\) result in the rate of efflux actually increasing with time for A475V and P429L channels. From this analysis, the ratio of the \(k_2\) in MI+glibenclamide and \(k_2\) in MI alone approximates the relative glibenclamide sensitivity (Figure 5.10C). While the sensitivity of A475V and P429L channels was not different from WT, C1039Y channels are notably less sensitive. Qualitatively similar results were obtained with channels expressed in heteromeric mixture with WT subunits (Figures 5.9B,D). As discussed below, this is consistent with similar findings for SUR1 mutations that results in increased activity of the resultant K\(_{ATP}\) channels.

5.3 Discussion

Distinct mechanisms of K\(_{ATP}\) GOF in Cantu Syndrome ABCC9 mutations

Cantu Syndrome (CS) is a rare disease characterized by complex vascular and skeletal anomalies, the underlying cellular and molecular mechanisms of which we are only now beginning to understand (Nichols et al., 2013). The majority of genotyped CS patients are heterozygous for mutations in the genes encoding ABCC9 (SUR2) (Harakalova et al., 2012; van Bon et al., 2012) in most cases, and KCNJ8 (Kir6.1) (Brownstein et al., 2013; Cooper et al., 2014) in others. A select few of these mutations, to date, have been shown to form overactive K\(_{ATP}\) channels (Cooper et al., 2014; Harakalova et al., 2012), but most remain unexplored. Here, we characterized three CS-associated ABCC9 mutations, namely P429L (hP432L), A475V
Figure 5.10- Decreased sensitivity to glibenclamide inhibition in C1039Y channels. $^{86}$Rb$^+$ efflux, as a function of time, was measured in GFP-transfected control cells, and in cells transiently expressing $K_{ATP}$ channels composed of Kir6.2 plus WT or mutant SUR2 subunits (A), as well as in cells expressing 1:1 mixture of WT and mutant SUR2 subunits (B). Experiments were performed in MI+ glibenclamide. The data represent the means ± S.E.M. of 3-6 experiments. Data were fit with equation 2 to obtain rate constants for $K_{ATP}$-dependent efflux ($k_2$). $k_2$ in glibenclamide + MI was divided by $k_2$ in MI for each condition (C,D).
(hA478V) and C1039Y (hC1043Y), that localize to distinct regions of the SUR2 protein (Figure 5.1). Each mutation leads to $K_{\text{ATP}}$ channel gain of function (GOF) (Figures 5.2-5.7), consistent with previous findings on isolated CS-associated $ABCC9$ mutations (Harakalova et al., 2012).

In the only previous assessment of CS-associated $ABCC9$ mutations, Harakalova et al. measured channel sensitivity to ATP in the presence of Mg$^{2+}$. Mg-nucleotides have complex activatory/inhibitory effects on $K_{\text{ATP}}$ channels: MgATP can both inhibit the Kir6.X subunit and be hydrolyzed to activatory MgADP at the SUR subunits (Nichols, 2006). Therefore, such an analysis cannot separate mutant effects on ATP inhibition from those on Mg-nucleotide activation. By separately assessing channel activity in response to Mg-free ATP and to MgADP we show that CS-associated $ABCC9$ mutations lead to increased channel activity via different mechanisms, as has also been observed with NDM-associated $ABCC8$ mutations (de Wet et al., 2008; de Wet et al., 2007; Masia et al., 2007; Zhou et al., 2010). Specifically, we demonstrate two mechanisms of increased channel activity, where P429L and A475V channels results from increased MgADP activation and C1039Y channels results predominantly from decreased ATP sensitivity.

$K_{\text{ATP}}$ mutations in SUR1 have been shown to decrease ATP-sensitivity (Takagi et al., 2013; Tarasov et al., 2008). There are at least two mechanisms to decrease ATP sensitivity in $K_{\text{ATP}}$ channels including decreased ATP binding or increased open probability ($P_o$) (Denton and Jacobson, 2012). Considering the SUR2-C1039Y mutation is not likely to disrupt binding of ATP to the Kir6.X subunit we tested for changes in channel $P_o$. The simple gating model, $C_i \leftrightarrow C \leftrightarrow O$, $C_i$ represents closed channels inhibited by ATP, $C$ represents intrinsic closing and $O$ represents open channels. PIP$_2$ activates $K_{\text{ATP}}$ currents by increasing the intrinsic $P_o$, or in
reference to a simple gating model, PIP\(_2\) forces channels to stay in the open state, preventing transition from C to C\(_i\) by ATP binding (Shyng and Nichols, 1998). Therefore, the ability of PIP\(_2\) to decrease ATP sensitivity, but not increase the maximum currents from C1039Y channels imply the P\(_o\) of these channels is near 1 (relative P\(_o\) = 1.26 ± 0.21) which is significantly higher than the P\(_o\) for WT channels (relative P\(_o\) = 0.70 ± 0.11) (Figure 5.6), and thus ultimately results in decreased ATP sensitivity (Figure 5.5).

In addition to increasing channel activity, homomeric expression of C1039Y appears to decrease expression of channels in the membrane. A similar dual effect of GOF mutations in both Kir6.2 (Lin et al., 2013) and SUR1 (Zhou et al., 2010) has previously been shown. In these previous examples, decreased surface expression was demonstrated by western blot analysis. Significant rescue of channel activity in heteromeric C1039Y/WT channels is consistent with the patterns from such mutations (Figures 5.8 and 5.9), suggesting that in addition to reducing ATP-sensitivity of expressed channels, C1039Y also decreases K\(_{ATP}\) surface expression.

*Implications for tissue pathology in in Cantu Syndrome*

Kir6.1 GOF mutations have also been found in two Cantu Syndrome patients (Brownstein et al., 2013; Cooper et al., 2014), confirming Kir6.1/SUR2 as the key subunit combination generating the overactive channels. Given the experimental difficulty of recording Kir6.1/SUR2 currents (Cooper et al., 2014), we used Kir6.2+SUR2A channels in the present study. *ABCC9* encodes two splice variants: SUR2A and SUR2B, which vary only in the last 40 amino acids. All CS-associated SUR2 mutations identified to date are located within the core of the SUR2 protein, such that these mutations will be present in both SUR2A and SUR2B. SUR2A is predominant in skeletal muscle and heart, while SUR2B is expressed in the vasculature. Kir6.1/SUR2B may be
the more dramatically affected combination, since vascular phenotypes seem to predominate in CS patients (Nichols et al., 2013). Conversely, neither skeletal muscle weakness nor shortening of the QT interval on the ECG have been noted, as might be expected if Kir6.2/SUR2A were markedly activated.

The severity of disease varies between Cantu Syndrome patients such that not all reported features are exhibited by all patients. Direct comparison of the phenotypes of patients carrying the A475V and C1039Y equivalent mutations (Harakalova et al., 2012) revealed hypertrichosis, macrosomia, macrocephaly, coarse features, cardiac anomalies, and umbilical hernia to be common to both. However, pulmonary hypertension, cardiomegaly, osteopenia, hyperextensibility and lymphedema were only reported for the A475V equivalent patient. The reduced sensitivity to ATP inhibition, due to the C1039Y mutation, results in greater relative basal channel activity than the enhanced Mg-nucleotide activation, due to A475V. However, a less severe disease phenotype for C1039Y suggests that reduced expression of this mutation may also be a consequence in vivo, as has also been reported for SUR1 NDM mutations (Zhou et al., 2010).

*Implications for therapeutic intervention in Cantu Syndrome*

Finally, sulfonylureas, which inhibit K\textsubscript{ATP} channels via interaction with the SUR subunits, are effective treatments for NDM patients with \textit{ABCC8}-activating mutations (Babenko et al., 2006), and are promising potential therapies for Cantu Syndrome (Nichols, 2013 #7510). However, GOF mutations in Kir6.2 (Koster et al., 2005a) and SUR1 (Takagi et al., 2013) frequently also decrease sensitivity to sulfonylureas, which may result in a lack of sulfonylurea therapeutic efficacy in patients with these mutations. Assessment of $^{86}\text{Rb}^+$ efflux in glibenclamide
demonstrates that WT, P429L and A475V channels show complex time dependence of efflux (Figure 5.10). Each required a very negative $k_2$ rate constant, which is indicative of channel activation versus inactivation. We interpret this activation to be reflective of a loss of glibenclamide inhibition over time. Divergent from the others, the $k_2$ for C1039Y has a very small positive value, which is consistent with some channel inactivation. Similarly, $k_2$ values in MI+glibenclamide WT, P429L and A475V channel activity drops to less than 10% in MI alone while C1039Y activity drops to ~50%. Taken together this would indicate that the C1039Y channel is glibenclamide insensitive. However, in general, SUR2 sensitivity to sulfonylureas is much lower than SUR1-sensitivity (Dorschner et al., 1999). Therefore, given the further reduction in drug sensitivity of GOF mutants, the introduction of sulfonylureas to treat CS patients is likely to require high doses, which would result in undesired inhibition of SUR1-based $K_{ATP}$ channels. SUR1 inhibition can lead to hypoglycemic effects, which will need to be carefully considered. Such concerns may ultimately make necessary the development of Kir6.1 or SUR2 specific inhibitors to provide a safer and more effective treatment options for CS patients.
CHAPTER 6: DISCUSSION

GOF in $K_{ATP}$ channel underlies CS

In 2012, genetic analysis of 2 cohorts of CS patients demonstrated that the majority have mutations in $ABBC9$, which encodes the SUR2 protein (Harakalova et al., 2012; van Bon et al., 2012). Initial studies of a few CS-SUR2 mutations expressed with Kir6.2 demonstrated increased $K_{ATP}$ channel activity (Harakalova et al., 2012). My analysis of new, previously uncharacterized CS-associated SUR2 mutations confirms this effect. In 2013, two additional CS patients were reported (Brownstein et al., 2013; Cooper et al., 2014) who presented with the hallmark features of CS, but were negative for a SUR2 mutation. Instead, both carry a mutation in $KCNJ8$ (Kir6.1-V65M and C176S). I also demonstrate that each of these CS-Kir6.1 mutations (V64M and C176S) also increase $K_{ATP}$ channel activity. Therefore, having demonstrated that both SUR2 and Kir6.1 CS-mutations increase $K_{ATP}$ channel activity, I conclude that gain of function (GOF) in $K_{ATP}$ channel activity underlies CS. The previous data showing that multiple SUR2 mutations result in a GOF in channel activity was strong evidence to support increased $K_{ATP}$ channel activity underlying CS. However, the demonstration that CS-associated mutations in Kir6.1 also increase $K_{ATP}$ channel activity was crucial in confirming this, as there are reports of SUR2 involvement in $K_{ATP}$ channel independent activity (Aggarwal et al., 2010; Stoller et al., 2010).

Molecular basis of $K_{ATP}$ channel gain of function

Gain of Function (GOF) in channel activity can arise from increases in channel activity and/or from increased channel expression. The majority of Neonatal Diabetes Mellitus (NDM) patients carry mutations in Kir6.2 or SUR1 that increase $K_{ATP}$ channel activity (Babenko et al., 2006; Gloyn et al., 2004). Several mechanisms by which NDM mutations enhance $K_{ATP}$ channel
activity have been described (Denton and Jacobson, 2012). For example, Kir6.2 NDM mutations have been shown to enhance channel open probability ($P_o$) (Mannikko et al., 2009) or to decrease ATP binding (Proks et al., 2004). SUR1 NDM mutations have been shown to enhance $K_{ATP}$ channel activity by enhancing ATPase activity (de Wet et al., 2008), by allosteric changes that indirectly decrease ATP sensitivity (Babenko et al., 2006), or by increased MgADP activation (Masia et al., 2007). However, no molecular details of CS-causing Kir6.1 or SUR2 mutation mechanisms have previously been described.

In this project, I have sought to address this question. To do this, I examined nucleotide sensitivity for SUR2 mutations in inside out patch clamp experiments. For Kir6.1 mutations, nucleotide sensitivity was also assessed and, by estimating PIP$_2$ activation, I also determined relative $P_o$. CS-associated Kir6.1 and SUR2 GOF mutations result from several distinct mechanisms. I have, for example, demonstrated that SUR2 mutations can disrupt response to ATP or MgADP. The SUR2-C1039Y mutation results in GOF, by indirectly decreasing sensitivity to ATP inhibition without affecting MgADP activation. Conversely, both SUR-P429L and A475V increase channel activity by enhancing MgADP activation. A third mechanism, in both Kir6.1 mutations (Kir6.1-V65M and C176S), enhances $P_o$, thereby indirectly decreasing channel sensitivity to ATP inhibition. Additionally, because one of the SUR2 mutations (C1039Y) unexpectedly demonstrates decreased trafficking, surface expression may also confound disease presentation.

*Kir6.1/SUR2 expression and CS symptoms*

At the tissue level, the effects of NDM and CS mutations depend on the tissues where the channels are expressed, and on how sensitive each tissue is to $K_{ATP}$ channel activity. Kir6.1 is expressed ubiquitously and has been confirmed in the vasculature (Inagaki et al., 1995c). SUR2
can be alternatively spliced into two major isoforms: SUR2A and SUR2B (Isomoto et al., 1996). The splice variant SUR2A is expressed in the heart and skeletal muscle (Chutkow et al., 1996). In parallel to Kir6.1, the splice variant SUR2B is also expressed ubiquitously and confirmed in the vasculature (Chutkow et al., 1996; Inagaki et al., 1995c). Conversely, Kir6.2 and SUR1 are expressed primarily in pancreatic beta cells, brain, heart and skeletal muscle (Inagaki et al., 1995a).

There have been several reports of tissues expressing both Kir6.1 and Kir6.2 (Insuk et al., 2003; Teramoto et al., 2009). However, to date, there appears to be no overlap in symptoms of CS and NDM patients experience. CS patients generally present with cardiomegaly, hypertrichosis, macrocephaly, osteochondysplasia, and macrosomia (Grange 2006, van Bon 2012, and Harakalova 2012). Conversely, NDM patients experience hyperglycemia and hypoinsulinemia along with neurological complications in the most severe cases. This lack of overlap in symptoms implies that where expression of pore-forming subunits overlaps, the predominance of non-mutated pore-forming subunits might be protective.

Potential for CS genotype/phenotype correlation

NDM presents with varying severity, from Transient NDM (TNDM), to Permanent NDM (PNDM), to developmental delay, epilepsy and neonatal diabetes (DEND) (Naylor et al., 2011). The severity of the disease correlates with the molecular severity of the Kir6.2 or SUR2 mutations (Flanagan et al., 2009; Hattersley and Ashcroft, 2005). TNDM patients have hyperglycemia and hypoinsulinemia that is present at birth, but is not permanent. The Kir6.2 or SUR1 mutations associated with this form of NDM have weak shifts in ATP sensitivity or enhancement of MgADP activation (Gloyn et al., 2005; Proks et al., 2004). PNDM patients have
hyperglycemia and hypoinsulinemia that also appears within the first 6 months of life, but persists throughout life. Compared to TNDM mutations, the Kir6.2 and SUR1 mutations in this form of NDM typically have larger shifts in ATP sensitivity or enhancement of MgADP activation (Gloyn et al., 2004; Proks et al., 2004). Along with the diabetes, DEND patients can have developmental delay and epilepsy. Intermediate DEND (iDEND) is a form of DEND with milder developmental delay and no epilepsy. Kir6.2 and SUR1 mutations causing iDEND and DEND have the most severe shifts in ATP sensitivity or enhancement of MgADP activation compared with all other forms of this disease (Proks et al., 2004; Proks et al., 2006; Proks et al., 2005). Is there a range of symptoms in CS? CS patients generally present with cardiomegaly, hypertrichosis, macrocephaly, osteochondysplasia, and macrosomia (Grange 2006, van Bon 2012, and Harakalova 2012). There are also symptoms that are only found in small subsets of patients, including patent ductus arteriosus (PDA), pericardial effusions, and valvular abnormalities. This raises the question: do CS mutations correlate with symptomatic presentation?

In considering this question with just the patients with Kir6.1 or SUR2 mutations which I have functionally described (Table 6.1), the major common symptoms were present in all. The mutations carried by patients who have all the major symptoms with the addition of at least one of the rare symptoms included: Kir6.1-V65M, C176S or SUR2-A475V. Based on $^{86}$Rb$^+$ efflux experiments (Figures 3.1A and 3.2A, Figure 4.1, and Figures 5.2A and 5.3A) these mutations result in the largest fluxes in the basal and pinacidil stimulated conditions. Of the other SUR2 mutations considered, A475V has one of the largest increases in relative $^{86}$Rb$^+$ efflux under basal conditions. Both the CS-Kir6.1 patients carrying the Kir6.1-V65M and Kir6.1-C176S mutations
Table 6.1- Cantu Syndrome Mutations and Clinical Symptoms.

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The + symbols indicates clinical symptoms present in patients, - symbol indicates clinical symptoms that are absent in the patients. The unk designation indicates symptoms not test and/or reported in the patients. Common CS symptoms are indicated in black and symptoms present only in small subsets of CS patients are indicated in red.
have similar symptoms and the mutant channels displayed comparable rates of $^{86}\text{Rb}^+$ efflux in the basal condition (Figures 4.1 and 4.2). Taken together, potential genotype/phenotype correlations exist, but additional studies in a larger set of CS Kir6.1 and SUR2 mutations will need to be done to accompany the clinical findings reported.

**Potential for treating CS**

There is currently no specific treatment for CS. Sulfonylureas bind directly to the SUR subunits of $\text{K}_{\text{ATP}}$ channel to inhibit $\text{K}_{\text{ATP}}$ channel activity (Ashfield et al., 1999). With the understanding that the molecular basis of CS is GOF in $\text{K}_{\text{ATP}}$ channel activity, use of a sulfonylurea drugs, such as glyburide, a $\text{K}_{\text{ATP}}$ channel-specific inhibitor that is currently used to treat diabetes (Feldman, 1985), may be a treatment option. However, a potential detrimental side effect to using glyburide, or other sulfonylurea drugs, as a CS treatment option, is that $\text{K}_{\text{ATP}}$ channels containing SUR1 are typically $\sim 100$fold more sensitive to these drugs than $\text{K}_{\text{ATP}}$ channels containing SUR2 (Gribble et al., 1998). Even SUR2 mutations with reduced sulfonylurea sensitivity may be capable of being inhibited partially by these drugs, but perhaps require higher doses. However, in addition to the efficacy of these drugs to inhibit SUR2 based channels, they will also bind and inhibit SUR1-based channels from increasing insulin secretion, which can result in hypoglycemia. Thus, along with the potential benefits of administering sulfonylureas to CS patients, there would also be a risk of patients becoming hypoglycemic which, if left unmanaged, can be lethal.

An alternate option to sulfonylureas would be inhibitors that target $\text{K}_{\text{ATP}}$ channels composed of Kir6.1+SUR2A specifically. Currently, there is a reportedly Kir6.1-specific inhibitor, U-37883 or PNU-37883A. Electrophysiology experiments, in a heterologous expression system, demonstrate that PNU-37883A preferentially inhibits $\sim 80\%$ of Kir6.1 based
channels at 100µM, but also partially inhibits ~15% of Kir6.2-based channels (Surah-Narwal et al., 1999). While this Kir6.1 selective inhibitor is not be perfect, it may be a good starting point for the development of a specific CS treatment.

Is there a Kir6.1/SUR2 loss of function (LOF) disease?

Kir6.2 and SUR1 mutations which result in a loss of function (LOF) in $K_{ATP}$ channel activity underlie Persistent Hyperinsulinemic Hypoglycemia of Infancy (PHHI) also known as congenital hyperinsulinemia (Darendeliler et al., 2002). PHHI is characterized by increased plasma levels of insulin in hypoglycemic conditions along with decreased levels of ketone bodies and free fatty acids (Aguilar-Bryan and Bryan, 1999), i.e., the opposite of NDM. CS has now been identified as the result of Kir6.1/SUR2 GOF, but there is currently no known LOF disease caused by Kir6.1/SUR2 mutations. Sudden Infant Death Syndrome (SIDS), is characterized by the sudden and usually unexplained death of an infant (Dawes, 1968). Two Kir6.1 mutations (in-frame deletion E332del and a missense mutation V346I) identified in babies with SIDS results in LOF in $K_{ATP}$ channel activity, when expressed with SUR2 (Tester et al., 2011). Though there are no reported SIDS patients to date with SUR2 mutations, multiple SIDS babies with a Kir6.1-LOF mutation make SIDS a strong candidate Kir6.1/SUR2 LOF disease.

The tissues impacted by GOF in Kir6.1 and SUR2 most commonly include the heart, hair, vasculature and bone (Nichols et al., 2013). So what would be the result of LOF in those same tissues? Most CS patients have enlarged hearts (Grange et al., 2014) and, thus, in a disease linked with a LOF of $K_{ATP}$ channel activity, a small heart might be anticipated. The opposite of the failure of the ductus arteriosus to close, which is seen in CS, would be premature closure. When considering the hair of LOF patients, the converse of excessive hair growth, i.e., reduced or no hair would be potential symptoms. In the vasculature, CS patients have low blood pressure, and
the opposite of that, which may be present in LOF patients, would be hypertension. Finally, $K_{\text{ATP}}$ channels have been reported in mesenchymal cells, which differentiate along an osteogenic lineage to form bone. Considering these various symptoms in different target tissues, CS may therefore, provide insights into Kir6.1/SUR2 LOF disease(s)
REFERENCES


potassium channel ubiquitously expressed in rat tissues, including pancreatic islets, pituitary, skeletal muscle, and heart. J Biol Chem 270, 5691-5694.


