Physiological and Pharmacological Switches Combine to Uniquely Modulate the Most Common Cardiac Sodium Channel Mutant, E1784K

by
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Abstract

The SCN5a gene encodes the cardiac voltage-gated sodium channel (Na\textsubscript{v}1.5) mainly expressed in cardiac muscle cells. The inward sodium current (I\textsubscript{Na}) conducted by Na\textsubscript{v}1.5 triggers depolarization in the cardiac action potential. Mutations in SCN5a predominantly give rise to Long-QT syndrome 3 (LQT3), Brugada syndrome 1 (BrS1), and their overlapping phenotypes (mixed syndrome). The most common SCN5a mutation, expressed as E1784K in the Na\textsubscript{v}1.5 C-terminal domain (CTD), mainly displays LQT3 and sometimes mixed syndromes. E1784K causes mixed channel defects by decreasing the inward peak I\textsubscript{Na} and increasing late I\textsubscript{Na}, thought to underlie BrS1 and LQT3 pathogeneses, respectively. Very little is known, however, on how physiological and pharmacological switches modulate E1784K channel properties. These triggers may often govern phenotypes in SCN5a mutation carriers. The goal of my thesis is to study how exercise-related physiological triggers and pharmacological agents modulate E1784K ion channel properties. I used the whole-cell patch clamp technique to study elevated temperature, elevated cytosolic calcium, and their combined effects with ranolazine, on E1784K. Ranolazine is an antianginal drug with preferential selectivity for blocking late I\textsubscript{Na} versus peak I\textsubscript{Na}. My main results show that E1784K is uniquely altered by the triggers studied, compared to other Na\textsubscript{v}1.5 mutants: (1) Elevated temperature augments late I\textsubscript{Na} in E1784K. (2) Elevated cytosolic calcium, which correlates with exercise-ameliorated LQT3, effectively blocks late I\textsubscript{Na} in most Na\textsubscript{v}1.5 mutants. However, E1784K is resistant to the native calcium-induced block on late I\textsubscript{Na}. (3) When temperature and cytosolic calcium are combined, they decrease ranolazine efficacy to suppress late I\textsubscript{Na} in E1784K. The calcium-sensitivity in E1784K is clearly affected due to the mutant-induced instability in the CTD, which may cause a steric clash between the channel and ranolazine. To predict E1784K effects on arrhythmogenesis, I simulated a dynamic action potential model to account for the frequency-dependent elevations in cytosolic calcium. Alternans is observed at high heart rates in E1784K and is exacerbated by febrile temperatures and ranolazine. This work demonstrates the importance of personalized medicine since Na\textsubscript{v}1.5 mutants like E1784K display unique sensitivity to physiological triggers that potentially govern antiarrhythmic efficacy.

Keywords: Mixed Syndrome; Whole-cell patch clamp; Electrophysiology; Temperature; Cytosolic Calcium; Ranolazine; Exercise
Dedication

To the soul of my dearest, Rowis Abdelsayed, whose blessings support me, and to my parents, without whom this thesis would not have existed, and my life would have been very different.

The Lord makes firm the steps of the one who delights in him;
though he may stumble, he will not fall,
for the Lord upholds him with his hand.

(Psalm 37: 23-24)
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I would like to thank Dr. David Jones and Dr. Stan Sokolov who were the first to get me started on a research project at MCPG, in fall 2011. A year after, both Dave and Stan left the Ruben lab, leaving me behind with another colleague, Colin Peters. I could’ve never found a better teammate, mentor, and a friend than Colin, who was always a role model. Being a genius ginger and a sober researcher, Colin’s research standards were stupendous. This motivated me to constantly learn and discover new knowledge in cardiac electrophysiology. Shortly after enrolling in the M.Sc. program, I met Reza Ghovanloo, who become an inspiration and a very close brother. I will never forget the fun time we spent together at the Biophysical (2016) and Gordon Research (2017) conferences, which were both held at California. The inspiring conversations we had regarding the future, motivated us to advance forward. The warm connection linking the three of us together, ‘the Rubenites’, made the lab my second home. I cherish the nights we all spent together patching, laughing, and challenging our intellectual abilities. Thanks also to the future physicians, Alec Yu and Manpreet Ruprai, who were both talented undergrads and made great contributions to my work.

The only reason why the Ruben lab felt this cozy was because of its intrepid leader, Dr. Peter Ruben. Besides learning electrophysiology, patch-clamp, and other technical skills, I learned many virtues from Peter. It was in BPK 205 (fall 2011) when Peter advertised job opportunities in MCPG. I was courageous to ask Peter to join the lab after his final lecture. Ever since, Peter has greatly impacted, not only my scientific career, but my life, in general. Because of Peter’s genuine personality, I was able to deal with many tribulations along the way, which often threatened my continuity in the degree. Peter never lost hope. He continued to support and encourage me. If it weren’t for Peter, I really couldn’t have done this work. Thank you, Peter!

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Chapter 1. Genotype-Phenotype Correlations in Cardiac SCN5a Mutations

This chapter describes part of the work published in the journal *Channels*, “Voltage-gated sodium channels: pharmaceutical targets via anticonvulsants to treat epileptic syndromes”, with minor modifications and formatting changes to suit the thesis style.

1.1. Introduction

Cardiovascular disease (CD) is the most prevalent cause of morbidity and mortality worldwide (Myerburg & Junttila, 2012; Laslett *et al.*, 2012). In many cases CD results in cardiac arrest which, if not resuscitated within a few minutes, culminates in sudden cardiac death (SCD) (Modi & Krahn, 2011; Harris & Lysitsas, 2016). SCD accounts for approximately 50% of the mortality from cardiovascular disease and 15 – 20% of all deaths (Zipes & Wellens, 1998; Deo & Albert, 2012). The principle factor underlying cardiac arrest, and thus SCD, is malignant ventricular arrhythmia (Chugh *et al.*, 2008; Millar *et al.*, 2012; Harris & Lysitsas, 2016). Pathophysiological arrhythmias involve re-entering electrical impulses in the heart, which lead to ventricular tachycardia, often followed by ventricular fibrillation.

Arrhythmias are caused by genetic and/or environmental factors. Inherited arrhythmias usually arise from mutations in the main and/or auxiliary subunits of cardiac ion channels, known as ‘channelopathies’. Mutations in the SCN5a gene, located in chromosome 3p21, have been implicated in cardiac disease. This gene encodes the ion-conducting α-subunit of the cardiac voltage-gated sodium channel (VGSC), Na\(\text{V}_{1.5}\). Na\(\text{V}_{1.5}\) is mainly expressed in cardiac myocytes, and along with other Na\(\text{V}\) isoforms in cardiac conduction system tissues, like Purkinje fibers (Haufe *et al.*, 2005; Qu *et al.*, 2007; Zimmer *et al.*, 2014). The Na\(^+\)-selective Na\(\text{V}_{1.5}\) conducts a transient inward sodium current (peak I\(\text{Na}\)), underlying phase 0 (depolarization) in the cardiac action potential (CAP). Peak I\(\text{Na}\) is quickly curtailed by fast inactivation. Some fast-inactivated channels revert back into the open state, causing a late I\(\text{Na}\) current, which partly contributes to phase 2 (plateau) in the CAP.
Approximately 382 SCN5a mutations have been discovered (See NCBI ClinVar Database and inherited arrhythmias database - Fondazione Salvatore Maugeri, http://triad.fsm.it/cardmoc/). SCN5a mutations have variable penetrance, where 98% of diagnosed probands express either a pure or a mixed clinical arrhythmogenic phenotype (Figure 1-1). Disease phenotypes include Long-QT syndrome 3 (LQT3), Brugada Syndrome 1 (BrS1), Progressive familial heart block 1A (PFHB1A), Sick sinus syndrome 1 (SSS1), Familial paroxysmal ventricular fibrillation 1 (VF1), Sudden infant death syndrome (SIDS), Atrial standstill 1 (ATRST1), Dilated cardiomyopathy 1E (CMD1E), and Familial atrial fibrillation 10 (ATFB10). Often the first syndrome an SCN5a mutation carrier may express is lethal malignant ventricular arrhythmia or sudden death.

**Figure 1-1   Arrhythmia Syndromes Caused by SCN5a mutations**
Inherited SCN5a mutations can cause either pure or overlapping phenotypes. The pie chart includes both types.

The three most prevalent cardiac syndromes caused by SCN5a mutations are Brugada Syndrome 1 (BrS1), Long-QT syndrome 3 (LQT3), and their overlapping phenotype, known as mixed syndrome (Figure 1-1). Environmental/physiological and pharmacological triggers associated with exercise or provocative drug testing, respectively, are often required to determine the disease identity (Wu *et al.*, 2008; Serletis-Bizios *et al.*, 2009; Yuasa *et al.*, 2014).
Establishing a clear genotype-phenotype correlation in inherited \textit{SCN5a} conditions is often difficult. Not only do mutations in the \textit{SCN5a} gene give rise to multiple cardiac phenotypes but a single mutation can express several or overlapping phenotypes (i.e., variable expressivity). The ion channel sequence variation profile, known as "channotype", ultimately determines the biophysical attributes imposed on the channel by the mutation, resulting in gain-of-function, loss-of-function, or both (Klassen \textit{et al.}, 2011; Noebels, 2015). The interaction between channotype and physiological and/or pharmacological triggers may underlie the highly variable phenotypic expressivity in \textit{SCN5a} mutation carriers.

Over the past two decades, many discoveries have linked cardiac arrhythmia pathogenesis to underlying malfunctions in ion channels (Balser, 2002; Jones & Ruben, 2008; Pitt, 2009; Amin \textit{et al.}, 2010b; Catterall, 2012; Horvath & Bers, 2014; Ahern \textit{et al.}, 2016). This effort has elucidated the role played by ion channels as contributors, if not the primary cause behind arrhythmias and sudden cardiac death. Advances in understanding cardiac pathophysiology were mainly determined using classic electrophysiology techniques, which correlate channotype in \textit{SCN5a} mutations with their corresponding clinical phenotypes (Bezzina \textit{et al.}, 1999; Veldkamp \textit{et al.}, 2000a; Baroudi & Chahine, 2000; Viswanathan \textit{et al.}, 2001; Clancy & Rudy, 2002; Groenewegen \textit{et al.}, 2003; Keller \textit{et al.}, 2006). Nevertheless, the shortcomings in this approach are apparent from the phenotypic variability in \textit{SCN5a} mutation carriers.

1.2. Phenotypes: Clinical Diagnosis & Pathophysiology

1.2.1. Brugada Syndrome Type 1

Brugada syndrome (BrS) was first described by Josep and Pedro Brugada, in 1992, who diagnosed the disease as a right bundle branch block (RBBB) accompanied with ST-elevation in V₁-V₃ precordial leads (Brugada & Brugada, 1992). The type 1 Brugada ECG pattern is diagnostic compared to the other two ECG types (\textbf{Figure 1-2}). Brugada pattern type 1 is characterized by a coved ST-segment elevation (≥ 2 mm) followed by a T-wave inversion in the right precordial leads, V₁-V₃ (Antzelevitch, 2001a; Francis & Antzelevitch, 2003; Castro Hevia \textit{et al.}, 2006; Antzelevitch & Yan, 2010; Tomita \textit{et al.}, 2012). Type 2 and 3 patterns are characterized by an elevated J-wave (≥ 2 mm) followed by a positive T-wave (\textbf{Figure 1-2}) (Francis & Antzelevitch, 2003;
Asymptomatic BrS patients with types 2 or 3 phenotypes may express type 1 with provocative drug tests (Barajas-Martinez et al., 2008; Doetzer et al., 2011; Obeyesekere et al., 2011; Barra et al., 2013; Zhou et al., 2013, 2013; Yu et al., 2013). BrS is often associated with conduction abnormalities that prolong the PR interval and QRS duration (Amin et al., 2009; Tomita et al., 2012; Aizawa et al., 2013).

Approximately 15 – 30 % of patients with congenital BrS carry an SCN5a mutation displaying an autosomal dominant mode of inheritance with incomplete penetrance (Antzelevitch, 2007; Modi & Krahn, 2011). BrS1 mutants cause loss-of-function in Na\textsubscript{v}1.5 by decreasing sodium channel expression and/or conduction. The resultant decrease in the depolarizing current increases the risk for arrhythmias as envisaged by the depolarizing and repolarizing hypotheses. The depolarization hypothesis suggests that the decrease in conduction velocity to the right ventricular outflow tract (RVOT) creates spatial heterogeneity in the right ventricle (RV) (Meregalli et al., 2005; Wilde et al., 2010). The repolarization hypothesis indicates that high K\textsubscript{v}4.3 expression (passing transient outward K\textsuperscript{+} current, I\textsubscript{Kto}) abrogates the diminished depolarizing wave in the RV epicardium, causing a loss in the action potential dome (Yan & Antzelevitch, 1999; Antzelevitch, 2001; Di Diego, 2002; Wilde et al., 2010). The transmural heterogeneity in RV creates a substrate for phase 2 re-entry (Figure 1-2), increasing the susceptibility for premature extrasystoles (Antzelevitch et al., 2005; Di Diego et al., 2005). The two mechanisms are not mutually exclusive, and both may contribute to BrS pathophysiology (Sieira et al., 2016).
Figure 1-2   Brugada Syndrome Electrophysiological and Electrocardiographic features

Electrical heterogeneity exists between the three myocardial cells. The depolarization wave proceeds from the endocardium to the midmyocardium to the epicardium as shown in panel a. This is followed by repolarization in the opposite order. In the normal ECG, T peak and T end complements epicardial and midmyocardial repolarization, respectively. The difference between T peak – T end measures Transmural Dispersion of Repolarization (TDR). Panel b shows an exacerbated epicardial notch in BrS. This produces a saddleback ST-elevation with a positive T-wave. This is observed in type 2 BrS patterns while type 3 may have both saddle-back or coved type ST-elevation. Panel c shows the diagnostic type 1 BrS ECG pattern. In this case, there is a prolonged epicardial action potential giving rise to a negative T-wave. Panel d shows a loss in the epicardial action potential dome, which may lead to phase 2 reentry. The epicardial tissue recovers from the refractory period quickly conducting another action potential, thereby exacerbating the ST-elevation and the negative T-wave in panel e. Reproduced from Antzelevitch (2001).

1.2.2. Long-QT Syndrome Type 3

Long-QT syndrome (LQTS) was first described in the mid-20th century (Jervell & Lange-Nielsen, 1957). Inherited LQTS is mainly autosomal dominant (Romano-Ward Syndrome) compared to the rare autosomal recessive mode (Jervell and Lange-Nielsen Syndrome), which is accompanied with deafness (Noh et al., 1995; Shimizu &
Antzelevitch, 1998, 2000). Congenital LQTS arises from 17 genes (LQT1 – 17), where the three most prevalent are \( \text{KCNQ1} \) (\( \text{KV7.1, LQT1} \)), \( \text{HERG} \) (\( \text{KV11.1, LQT2} \)), and \( \text{SCN5a} \) (\( \text{NaV1.5, LQT3} \)) (Schwartz et al., 1995, 2001; Takenaka et al., 2003). Despite its rarity, LQT3 is the most lethal inherited LQTS (Antzelevitch et al., 1996; Shimizu & Antzelevitch, 1998; Yan & Antzelevitch, 1998; Shimizu & Antzelevitch, 1999).

LQTS has a prolonged QT interval with a diagnostic QTc \( \geq 500 \text{ ms} \) (Antzelevitch & Oliva, 2006; Priori et al., 2013). The cellular mechanism underlying LQTS involves either a reduction in the repolarizing current or an increase in the depolarizing current, mediated by \( \text{I}_k/\text{I}_{ks} \) or late \( \text{I}_{\text{Na}} \), respectively (Antzelevitch et al., 1996; Sicouri et al., 2007; Antzelevitch et al., 2014). Both mechanisms delay cardiac repolarization, especially in the mid-myocardium. The augmented depolarizing current during phase 2 in the CAP can generate early after-depolarizations (EADs) or drive the sodium-calcium exchanger (NCX) in reverse mode, causing intracellular calcium and sodium overload, leading to delayed after-depolarizations (DADs) (Akar, 2002; Shimizu et al., 2005; Gaur et al., 2009; Antzelevitch & Burashnikov, 2011).

1.2.3. Common Pathophysiology in BrS1 and LQT3

A shared mechanism between BrS and LQTS is exacerbated transmural dispersion of repolarization (TDR), the primary substrate precipitating arrhythmias (Castro Hevia et al., 2006; Antzelevitch et al., 2007). TDR is measured as the peak – end difference in the T-wave. The \( \text{T}_{\text{peak}} \) and \( \text{T}_{\text{end}} \) correlate with epicardial and mid-myocardial action potential termination, respectively (Figure 1-2) (Antzelevitch, 2001b). The right epicardial action potential dome loss in BrS, as in the repolarization hypothesis, increases TDR (Lukas & Antzelevitch, 1996; Dumaine et al., 1999; Antzelevitch & Patocskai, 2015). In LQTS, the relatively prolonged APD in mid-myocardium compared to the endocardium and epicardium, exacerbates the TDR (Zygmunt et al., 2001). Increased TDR enlarges the vulnerable window during which premature extrasystoles may occur, leading to ventricular tachycardia/fibrillation.

1.3. Phenotype Variability in BrS1 and LQT3

Phenotypes resulting from \( \text{SCN5a} \) mutations can vary with sex, age, ethnicity, circadian rhythms, and physiological state. All these factors modify \( \text{SCN5a} \) transcription
and translation. The exercise stress test is a common diagnostic tool used to unmask latent arrhythmias in patients with SCN5a mutations. During exercise, heightened sympathetic tone elevates heart rate and contractility. Various intracellular cascades are activated in cardiomyocytes that alter \( \text{Na}_\text{v} \)1.5 transcription, translation, and gating. Environmental factors, like hypoxia, acidemia (either metabolic or respiratory), or elevations in body temperature, accompany exercise.

### 1.3.1. Environmental/Physiological triggers in BrS

BrS patients may not express diagnostic phenotypes; however, they may still die from SCD. Many BrS patients require an external trigger to precipitate their latent cardiac disease (Fish & Antzelevitch, 2004a; Minoura et al., 2012). Various triggers like exercise, acidemia, fever, and sodium channel blockers unmask BrS (Wakita et al., 2004; Ozeke et al., 2005; Peng et al., 2005; García-Borbolla et al., 2007; Kukla et al., 2007; Grimster et al., 2008; Amin et al., 2009; Jayasuriya & Whitman, 2011; Kumar et al., 2013; Masrur et al., 2015). A large clinical group created the website, www.brugadadrugs.org, to identify high risk BrS provocative drugs. Environmental and pharmacological triggers modify \( I_{\text{Na}} \) (Di Diego et al., 2005; Minoura et al., 2012). Other factors may modulate the extent to which external triggers unmask BrS. For instance, elevated testosterone levels in males upregulates \( \text{K}_\text{v}4.3 \) expression in the RV epicardium, thereby increasing their risk for BrS (Shimizu et al., 2007). Thus, BrS is predominant in males, with an 8:1 (male:female) ratio, especially in Southeastern Asian populations (Wilde, 2002; Antzelevitch et al., 2002; Ackerman et al., 2004; Antzelevitch et al., 2005; Antzelevitch, 2005). These factors often account for complete versus incomplete penetrance in carriers of BrS1 mutations.

Exercise elevates the J-point in BrS1 patients (Amin et al., 2009). Some reports show exercise-induced J-point elevation during peak exercise while others during recovery from exercise (Figure 1-3) (García-Borbolla et al., 2007; Grimster et al., 2008; Amin et al., 2009; Makimoto et al., 2010; Walker et al., 2010; Jayasuriya & Whitman, 2011; Masrur et al., 2015). This conflicting observation may be partly explained by the fact that there are controversies regarding effects of sympathetic versus parasympathetic tone in BrS. Increased sympathetic output is thought to ameliorate the syndrome by increasing the depolarizing current through \( \text{I}_{\text{Na}} \) and \( \text{I}_{\text{Ca}} \) (Makimoto et al., 2010; Antzelevitch & Patocskai, 2015). Thus, medications for BrS include quinidine
(blocks $I_{Kt0}$), β adrenergic agents, anticholinergic agents, and implantable cardioverter defibrillators (ICDs) (Antzelevitch & Fish, 2006). However, the elevated sympathetic tone during exercise may increase $NaV1.5$ use-dependence, precipitating BrS1 (Bezzina et al., 1999; Veldkamp et al., 2000a). Decreased $NaV1.5$ availability leads to conduction blocks diagnosed as prolonged QRS durations in BrS1 patients (Amin et al., 2009). When challenged with flecainide, BrS1 patients also manifest a QTc prolongation during exercise (Amin et al., 2009). The reduced $I_{Na}$ accompanying BrS1, indirectly accentuates phase 1 notch in the CAP, mediated by $I_{to}$. Thus, the augmented outward repolarizing current, temporarily delays $I_{CaL}$ activation, thereby prolonging phase 2 and 3 in CAP (Krishnan & Antzelevitch, 1993).

Most BrS patients suffer from arrhythmias during sleep, such as in sudden infant death syndrome (Skinner, 2005; Makielski, 2006; Huang et al., 2009; Qiu et al., 2009; Andreasen et al., 2013). Although sleep is a restful state, it shares some physiological properties with exercise; the rapid-eye movement (REM) phase is accompanied with elevated heart rate (HR) and blood pressure (BP) (Somers et al., 1993). Whether this physiological change is sufficient to cause arrhythmias remains unknown. The relative changes in HR and BP during sleep may mimic exercise effects. High vagal input during non-REM sleep may trigger BrS in patients (Trinder et al., 2001). Additionally, sleep is accompanied with decreased body temperature and sometimes increased blood acidosis due to sleep apnea, which may precipitate arrhythmias (Fish & Antzelevitch, 2004b). As with exercise, sleep physiology is complicated as physiological fluctuations occur in REM and non-REM sleep.

Even with no genetic mutations, environmental factors may induce Brugada phenocopy in non-athletes and athletes (Kovacic & Kuchar, 2004), primarily due to a reduction in the depolarizing current carried by peak $I_{Na}$ (Di Diego et al., 2005). The combined interaction between environmental and genetic factors may synergistically heighten arrhythmia lethality in patients with SCN5a mutations.
Figure 1-3  Brugada Syndrome response to exercise
The J-point size is plotted against the exercise stage. Control subjects have no significant variation in their J-points compared to both BrS patients with and without SCN5a mutations. Both patient types have elevated J-points at peak exercise, which is maintained during recovery. Reproduced from Amin et al. (2009).

1.3.2. Environmental/Physiological triggers in LQTS

Unmasking congenital LQTS depends on the genotype. The common trigger for cardiac events in LQT1 patients is exercise, especially swimming (Shimizu & Antzelevitch, 1998). Most cardiac events in LQT2 patients are provoked by emotional arousals while only a few occur with rest/sleep (Schwartz et al., 1995; Shimizu & Antzelevitch, 2000; Paavonen et al., 2001; Takenaka et al., 2003). The latter trigger, which accompanies decreased heart rate, is very common for precipitating LQT3 (Figure 1-4) (Schwartz et al., 1995, 2001). Only a few LQT3 patients express their syndromes during exercise or emotion (Shimizu & Antzelevitch, 2000; Aziz et al., 2011). Exercise is a general therapy for LQT3 mutant carriers. Recommended therapies for LQT3 include β₁ blockers (most common in LQT1), Naᵥ blockers like Mexiletine or Flecaainide, or ICDs (especially in LQT3) (Haverkamp, 2000; Bennett et al., 2014). The LQT3 mutant signature, however, determines the patients’ response to exercise
(Veldkamp et al., 2000a; Chen et al., 2015). Most LQT3 patients ameliorated by exercise were screened positive for the ΔKPQ mutant (Schwartz et al., 1995).

**Figure 1-4**  Trigger Effects on Inherited LQTS
Reproduced from Schwartz et al. (2001).

1.4. Why Variable Phenotypic Penetrance?

BrS1 and LQT3 phenotypes can be correlated with the channotype-induced biophysical defects. The phenotypic variability observed in SCN5a mutation carriers can be ascribed to the various stages of modification in Na\textsubscript{v}1.5 life-cycle: SCN5a transcription, post-transcription by RNA processing, translation of mRNA transcript, post-translational modification, and protein degradation (Liu et al., 2014). Transcription and translation modifiers ultimately downregulate or upregulate Na\textsubscript{v}1.5 expression and trafficking, which is modulated by channotype. These factors vary with age, sex, and physiological state.

Various protein kinases, such as mitogen-activated protein kinases (MAPKs), affect Na\textsubscript{v}1.5 translation, folding, and trafficking (Muslin, 2008). Protein kinase C (PKC) and Protein kinase A (PKA) have antagonistic effects on channel trafficking (Muslin, 2008; Hallaq et al., 2012). Unfortunately, few reports describe how SCN5a mutations impact transcription and translation. Sodium channel research was mainly directed at understanding how channotype affects gating. This reductionist approach for studying channotype is inadequate and may not fully explain the phenotypic heterogeneity observed in SCN5a mutation carriers. Na\textsubscript{v}1.5 is co-expressed and modified by various...
other subunits and molecules, so channel modification sites may be altered with channotype.

1.4.1. Na\textsubscript{V}1.5 and Cardiac Disease

The voltage-gated sodium channel (Na\textsubscript{V}) exists in humans as nine different isoforms (Na\textsubscript{V}1.1-1.9). The isoforms commonly expressed in the central nervous system include Na\textsubscript{V}1.1, Na\textsubscript{V}1.2, Na\textsubscript{V}1.3, and Na\textsubscript{V}1.6. Dorsal root ganglion cells in the peripheral nervous system mainly express Na\textsubscript{V}1.7, Na\textsubscript{V}1.8, and Na\textsubscript{V}1.9. Na\textsubscript{V}1.4 is predominant in skeletal muscle and is minimally expressed along with the predominant cardiac-specific isoform, Na\textsubscript{V}1.5, in the heart (Qu et al., 2007; Zimmer et al., 2014). TTX-sensitive Na\textsubscript{Vs} (Na\textsubscript{V}1.1-1.4, 1.6, and 1.7) are not prevalent in mammalian hearts as confirmed by normal ECG and action potential recordings in hearts perfused with a low TTX concentration (Santarelli et al., 2007; Zimmer et al., 2014). However, the TTX-resistant Na\textsubscript{V}1.8 (encoded by SCN10a) is expressed in the heart and SCN10a mutations cause BrS18 (Zimmer et al., 2014; Fukuyama et al., 2016).

Na\textsubscript{V}1.5 functions to depolarize cardiomyocytes. The inward peak $I_{\text{Na}}$ is altered in cardiac disease. Genetic factors, such as those causing BrS1, mainly affect peak $I_{\text{Na}}$ by modulating channel gating (Deschênes et al., 2000; Di Diego, 2002; Tester et al., 2005; Antzelevitch, 2007; Kapplinger et al., 2010; Wilde et al., 2010). Na\textsubscript{V}1.5 mainly transitions between a closed-state (deactivated), activated, and fast- or slow-inactivated states in a voltage-dependent manner. Gating defects caused by BrS1 mutants decrease Na\textsubscript{V}1.5 open probability via a voltage-dependent depolarization in activation, or hyperpolarization in fast or slow inactivation, or enhanced onset or decelerated recovery from inactivation (Wang et al., 2000; Baroudi et al., 2002; Wang, 2004; Huang et al., 2006; Keller et al., 2006; Poelzing et al., 2006; Tan et al., 2006; Casini et al., 2007; Jones & Ruben, 2008; Makita et al., 2008b; Lizotte et al., 2009; Gui et al., 2010). BrS1 mutants may also perturb Na\textsubscript{V}1.5 trafficking and expression, thereby suppressing peak $I_{\text{Na}}$.

LQT3 is mainly associated with exacerbated late $I_{\text{Na}}$. This non-inactivating $I_{\text{Na}}$ serves as the pathological substrate underlying various cardiac disease states, associated with myocardial ischemia/infarction and heart failure (Schwartz & Wolf, 1978; Antzelevitch et al., 1996; Yan & Antzelevitch, 1998; Shimizu & Antzelevitch, 1999;
The increased late $I_{Na}$ in LQT3 is caused by late re-opening of NaV1.5 due to destabilized fast inactivation. Following fast inactivation, a few NaV1.5 channels recover from fast inactivation, bursting back into the open state, thereby enhancing the depolarizing current during phase 2 in CAP (Quandt, 1987; Maltsev & Undrovinas, 2006; Maltsev et al., 2009). The augmented late $I_{Na}$ in LQT3 delays cardiac repolarization. Late $I_{Na}$ is larger in the midmyocardium compared to endocardium and epicardium due to decreased $K_{V7.1}$ ($I_{Ks}$) expression and increased $I_{NCX}$, thereby reducing the repolarizing current causing the late depolarizing current to prevail (Liu et al., 1993; Antzelevitch et al., 1996; Schwartz et al., 2000; Akar, 2002).

1.4.2. Channel Function and Phenotype Correlations

Approximately 14% of SCN5a mutations have been characterized using the voltage-clamp technique. Channotype positively correlates with phenotype in the SCN5a variants characterized (Figure 1-5). A positive correlation involves LQT3 mutants expressing NaV1.5 gain-of-function, BrS1 mutants expressing NaV1.5 loss-of-function, and the mixed phenotype (BrS1 & LQT3) expressing both channel defects. Intriguingly, a mixed correlation exists between channotype and phenotype, which is as prevalent as the positive correlation in BrS1 and LQT3 (Figure 1-5). This mixed correlation involves either a pure phenotype accompanied with mixed channel function, or vice versa. Very few cases show a negative correlation (Figure 1-5).
1.4.3. Triggers determining channel behaviour and phenotype in some SCN5a mutations

The most common SCN5a mutation expresses as the E1784K mutant in the NaV1.5 C-terminal Domain (CTD) (Kaplinger et al., 2010; Sumitomo, 2014; Takahashi et al., 2014; Veltmann et al., 2016). This mutation is common in the Okinawa islands in Japan, where more than 80% of the genotype-positive children express LQTS (Nakajima et al., 2011; Takahashi et al., 2014). Although commonly associated with LQTS, E1784K cohorts express other phenotypes including sinus node dysfunction (SND), LQT3/SND, LQT3/BrS1, and LQT3/BrS1/SND (Wei et al., 1999; Deschênes et al., 2000; Makita et al., 2008a). Electrophysiology data confirms that E1784K displays mixed channel behavior (Wei et al., 1999; Makita et al., 2008a; Sumitomo, 2014; Peters et al., 2016; Veltmann et al., 2016; Abdelsayed et al., 2017). Very little is known, however, on how arrhythmogenic triggers alter E1784K channel behavior, which could be the reason for the differential phenotypic variability in cohorts (Makita et al., 2008a).

The founder Netherlands SCN5a mutation expressed in NaV1.5 CTD as the 1795insD mutant, displays LQT3 at rest and BrS1 during peak exercise (Veldkamp et al., 2000a; Clancy & Rudy, 2002; Veldkamp et al., 2003; Remme et al., 2006). Biophysical characterization reveals reduced and augmented peak and late I_{Na}, respectively, in 1795insD (Bezzina et al., 1999; Rivolta, 2001; Clancy & Rudy, 2002;
Veldkamp et al., 2003; Beaufort-Krol et al., 2005; Remme et al., 2006). These biophysical defects are modified by stimulation frequency, where high rates further reduce peak $I_{Na}$ and low rates increase late $I_{Na}$ percentage compared to peak $I_{Na}$ (Veldkamp et al., 2000a; Clancy & Rudy, 2002; Veldkamp et al., 2003).

Beneficial effects of exercise on LQT3 patients were validated using data from patients screened positive for $\Delta$KPQ, the first discovered LQT3 mutant (Schwartz et al., 1995). Generalizing exercise as a therapeutic for the LQT3 patient population can be misleading since Na\textsubscript{v}1.5 mutants have unique responses to exercise. The exercise-ameliorated LQT3 in $\Delta$KPQ carriers was correlated with reduced late $I_{Na}$ caused by elevated cytosolic calcium, high stimulation frequency, and $\beta_1$ adrenergic activation (Chandra et al., 1998, 1999; Fredj et al., 2006a; Potet et al., 2015). These are amongst a multitude of factors that are upregulated in cardiomyocytes during exercise. Various protein kinases are activated during exercise and pathophysiological states (Steenbergen et al., 1987; Belardinelli, 2006a; Pitt, 2009; Horvath & Bers, 2014). PKA and PKC underlie the epinephrine-induced QT\textsubscript{c} prolongation and sinus node dysfunction in V2016M carriers (Na\textsubscript{v}1.5 CTD mutant) (Chen et al., 2015).

It is notable that many CTD mutants are linked to mixed syndromes and have variable sensitivity to exercise-related factors.

### 1.5. Cardiac Voltage-Gated Sodium Channels (VGSC) Structure & Function Overview

The heteromeric voltage-gated sodium channel is composed of four Domains (DI-DIV) consisting of six transmembrane segments (S1-S6). The voltage-sensing domain (VSD) and the pore-forming domain (PFD) is encompassed by S1-S4 and S5-S6, respectively, in each homologous Domain. The four Domains are connected by intracellular linkers, which modulate channel gating (Bennett, 1999, 2001, 2004). The PFDs and VSDs form a functional channel, which can be modified by “auxiliary” $\beta$-subunits (McCormick et al., 1998; Bennett, 1999; Lee et al., 2014; Yan et al., 2017b).
1.5.1. Activation

Channel activation is controlled by DI, DII, and partially DIII voltage sensor movements (Figure 1-6) (Cha et al., 1999). Changes in the membrane electric field are sensed by the peripheral VSDs and relayed by the S4-S5 linkers to the central PFDs (Yang & Horn, 1995; Payandeh et al., 2011; Zhang et al., 2012; Catterall et al., 2012; Shen et al., 2017; Yan et al., 2017b). The S4s contain conserved arginine and lysine residues, which form salt bridges with residues in S2 and S3 segments (DeCaen et al., 2009; Yarov-Yarovoy et al., 2012). Upon membrane depolarization, a rearrangement occurs in the ionic bonds, and the S4s are outwardly displaced, as envisaged by the sliding-helix model (Yang & Horn, 1995; DeCaen et al., 2009; Kubota et al., 2017). The voltage sensor transition to the depolarized state is rapid (microsecond scale) and causes a conformational change in the PFD (Payandeh et al., 2011; Zhang et al., 2012). The S5 and S6 segments are thought to slide laterally, dilating the channel's inner vestibule. The PFD rearrangement results in ionic conduction passing a transient inward sodium current, rushed into the cell by a steep electrochemical driving force. Sodium ions move into the outer vestibule, where Na⁺ is stripped from its hydration shell. This is followed by Na⁺ passing through a selectivity filter formed by amino acid residues, DEKA, in each of the homologous Domains (i.e. D in Domain I, E in Domain II, K in Domain III, and A in Domain IV) (Payandeh et al., 2011; Zhang et al., 2012; Shen et al., 2017). Na⁺ is then rehydrated in the central cavity and the inner vestibule (Payandeh et al., 2011).

1.5.2. Fast inactivation

A few milliseconds following activation, the inward sodium current is curtailed by fast inactivation. The DIV S4 movement is correlated with fast inactivation onset and is suggested to be its rate-determining step (Armstrong & Bezanilla, 1977; Bezanilla & Armstrong, 1977; Kühn & Greeff, 1999; Groome et al., 2007; Capes et al., 2013). Near resting potential, the DIV S4 activates in a small population of channels giving rise to closed-state fast inactivated state (Figure 1-6) (Groome et al., 2011); therefore, only half-maximal channel availability exists at resting potential (Starkus et al., 1993). The outward helical movement in DIV S4 causes hydrophobic residues in the DIII-DIV linker, the IFMT (1485-1488) motif, to bind to residues in the inner vestibule and S4-S5 linkers in DIII and DIV (Richmond et al., 1997; Ulbricht, 2005; Groome et al., 2007). The IFM-
QQQ mutant completely abolishes fast inactivation (Featherstone et al., 1996). Positively charged residues in the DIII-DIV linker form electrostatic interactions with their counter charges in the inner vestibule, thereby stabilizing fast inactivation (Ulbricht, 2005; Groome et al., 2007).

1.5.3. Persistent Current

Not all channels are fast inactivated; rather, some channels recover into the open-state and conduct a small residual sodium current, referred to as the non-inactivated, persistent, or late $I_{Na}$ (Quandt, 1987; Maltsev & Undrovinas, 2006). Late $I_{Na}$ partially underlies phase 2 stabilization in the CAP (Maltsev et al., 2009).

1.5.4. Slow inactivation

With maintained or repetitive depolarizations, Na$_V$ channels recover from fast inactivation and transition into a distinct mode of inactivation, known as slow inactivation. Slow inactivation is mechanistically and pharmacologically distinct from fast inactivation (Featherstone et al., 1996; Richmond et al., 1997, 1998; Vilin et al., 2001; Goldin, 2003; Ulbricht, 2005). Slow inactivation occurs over several seconds and is thought to involve conformational changes in the selectivity filter, voltage sensors, and lateral pores known as fenestrations (Ruben et al., 1992; Vilin et al., 2001; Webb, 2009; Payandeh et al., 2011, 2012, Silva & Goldstein, 2013a, 2013b). During slow inactivation, the voltage sensors adapt to an activated locked-up state in which the pore collapses (Ruben et al., 1992). There is an inverse relationship between fast and slow inactivation in Na$_V$. More channels slow inactivate when fast inactivation is abolished with the IFM-QQQ mutant. Thus, charge immobilization in DIV S4, which impedes full recovery from fast inactivation, limits the extent of slow inactivation (Featherstone et al., 1996; Nuss et al., 1996; Richmond et al., 1997).

1.5.5. Deactivation

The membrane must sufficiently repolarize for channels to recover from fast and slow inactivation and then deactivate. Upon repolarizing, the voltage-sensors move back into their deactivated states and the channel PFD is re-orientated into the ‘activation-ready’ conformation. Deactivation can be measured by very short depolarization pulses
followed by abrupt hyperpolarization (Hodgkin & Huxley, 1952; Hodgkin et al., 1952; Groome et al., 2003). This method allows for a large inward sodium current surge, capturing the ‘tail $I_{Na}$’ carried by channels which are not fast-inactivated.

![Figure 1-6 Schematic Diagram of Sodium Channel Gating](image)

**Figure 1-6 Schematic Diagram of Sodium Channel Gating**
The Markov scheme shows the series of voltage-dependent steps required for sodium channel activation. All closed states are in equilibrium with inactivation, giving rise to closed-state inactivation. The forward and reverse rates are represented as $\alpha$ and $\beta$, respectively. Reproduced from (Irvine et al., 1999).

### 1.6. Extrinsic and Intrinsic Na$_V$1.5 Modifiers

#### 1.6.1. β subunits

The Na$_V$1.5 is co-expressed in cardiomyocytes with one or two "accessory" β-subunits encoded by the $SCN1B$ (β1), $SCN2B$ (β2), $SCN3B$ (β3), and $SCN4B$ (β4) genes (McCormick et al., 1998; Malhotra et al., 2001; Gilchrist et al., 2013). β2 and β4 form covalent disulfide links with the DIV S5-S6 extracellular linker, whereas β1 and β3 form non-covalent interactions with the α-subunit (Qu et al., 1995; Chen et al., 2012; Gilchrist et al., 2013). β-subunits usually increase sodium channel expression and alter inactivation kinetics in an isoform-dependent or mutant-dependent fashion (Brackenbury & Isom, 2008; Aman et al., 2009; Patino & Isom, 2010). The biophysical defects caused by the BrS1 mutant, T1620M, are aggravated by β1 co-expression (Makita et al., 2000). Mutations in $SCN1B$, $SCN2B$, $SCN3B$ cause BrS5, BrS7, and BrS17, respectively. $SCN4B$ mutations cause LQT10, such as with β4-L179F which destabilizes fast inactivation (Medeiros-Domingo et al., 2007).
1.6.2. C-terminal Domain

The C-terminal domain (CTD) in Na\textsubscript{v}1.5 plays a crucial role in governing fast inactivation. Both intra-CTD and inter-CTD interactions are necessary for proper channel function (Tan \textit{et al.}, 2002; Deschenes, 2002; Cormier \textit{et al.}, 2002; Van Petegem \textit{et al.}, 2012). Chimera studies, interchanging Na\textsubscript{v}1.2 and Na\textsubscript{v}1.5 CTDs, show that Na\textsubscript{v}1.5 CTD does not stabilize or enhance onset in fast inactivation compared to Na\textsubscript{v}1.2 (Mantegazza \textit{et al.}, 2001). The proximal half of the Na\textsubscript{v}1.5 CTD, however, is important in regulating inactivation compared to the distal half (Mantegazza \textit{et al.}, 2001). Mutants in Na\textsubscript{v}1.5 CTD proximal half perturb the normal CTD control on fast inactivation, potentially inducing Na\textsubscript{v}1.2 fast inactivation characterized by a hyperpolarized voltage-dependence and accelerated onset kinetics (Mantegazza \textit{et al.}, 2001; Tan \textit{et al.}, 2002; Cormier \textit{et al.}, 2002; Mori \textit{et al.}, 2003).

Fluorescence resonance energy transfer (FRET) has been used to elucidate Na\textsubscript{v}1.5 intra-CTD interactions (Glaaser \textit{et al.}, 2012). The CTD contains a pair of EF-like hand domains (\(\alpha_1-\alpha_4\)) composed of a helix-loop-helix, approximately 120 residues upstream from an IQ domain (\(\alpha_6\)), which binds calmodulin (Tan \textit{et al.}, 2002; Wingo \textit{et al.}, 2004; Glaaser \textit{et al.}, 2006; Chagot \textit{et al.}, 2009; Van Petegem \textit{et al.}, 2012; Bagnéris \textit{et al.}, 2013). The IQ motif interacts with aromatic residues in \(\alpha_1\) (F1791 and Y1795) and with residues in \(\alpha_1-\alpha_2\) and \(\alpha_2-\alpha_3\) linkers via its N-terminus (Chagot \textit{et al.}, 2009; Glaaser \textit{et al.}, 2012; Gabelli \textit{et al.}, 2014). The S1904L mutant, located four residues upstream from the IQ motif, causes a steric clash with the EF-like hand domain (Bankston \textit{et al.}, 2007). Disrupting the intra-CTD interactions involves decoupling CTD from inter-connections with the DIII-DIV linker, as with S1904L, which increases late I\textsubscript{Na} (Glaaser \textit{et al.}, 2006; Wang \textit{et al.}, 2014). Intra-CTD interactions are also crucial in Na\textsubscript{v}1.5 heterodimers where the IQ motif of one channel interacts with the EF-like hand domain of the neighboring channel (Gabelli \textit{et al.}, 2014).

Interconnections between the CTD and the DIII-DIV linker in Na\textsubscript{v}1.5 are modulated by the calcium-calmodulin complex under a calcium signal (Potet \textit{et al.}, 2009; Sarhan \textit{et al.}, 2009). During fast inactivation, the CTD fastens like a latch on the DIII-DIV linker via electrostatic and hydrophobic interactions, stabilizing fast inactivation (Motoike, 2004; Sarhan \textit{et al.}, 2009; Wang \textit{et al.}, 2012; Van Petegem \textit{et al.}, 2012; Bagnéris \textit{et al.}, 2013; Pitt & Lee, 2016; Yan \textit{et al.}, 2017a). A proline-rich region (PIPRP, 1509-1513) in
the DIII-DIV linker partially constitutes the intermolecular interactions with the CTD (Motoike, 2004). SCN5a mutations expressed in the CTD usually halt this mode of regulation, thereby destabilizing inactivation and augmenting late $i_{Na}$ (An et al., 1998; Wei et al., 1999; Bezzina et al., 1999; Wang et al., 2007).

### 1.6.3. Calcium-calmodulin complex

Unlike in Na$_V$1.4, which contains an additional aspartate in the CTD EF-like hand domain, Na$_V$1.5 does not chelate calcium (Herzog et al., 2003; Kim et al., 2004; Shah et al., 2006; Pitt, 2007; Chagot et al., 2009; Van Petegem et al., 2012; Ben-Johny et al., 2014; Pitt & Lee, 2016). Calmodulin initially binds to the IQ domain via its C-lobe but reverts and weakly binds via its N-lobe with a calcium signal (Chagot et al., 2009; Miloushev et al., 2009; Sarhan et al., 2012). The calmodulin C-lobe is then free to bind to other channel regions, primarily the DIII-DIV linker $\alpha$-helix formed by residues 1498-1501 (Potet et al., 2009; Sarhan et al., 2012). The tripartite complex formed by calmodulin destabilizes and stabilizes the fast inactivation particle at resting and depolarized potentials, respectively (Sarhan et al., 2012; Potet et al., 2015). Thus, calcium-dependent facilitation (CDF) dominates at resting potentials, thereby sustaining rapid heart rhythms, and calcium-dependent inhibition (CDI) at depolarized potentials, which reduces EADs and DADs (Shah et al., 2006; Sarhan et al., 2012). There are conflicting opinions on the role of calcium-calmodulin in modulating Na$_V$1.5. The controversies arise from the differences in experimental conditions between the studies. Calcium-dependent facilitation in Na$_V$1.5 is not observed under dynamic calcium influx measured using photo-uncaging or with calcium spillover from neighboring calcium channels (Ben-Johny et al., 2014). Some studies suggest that Na$_V$1.5 functions like Ca$_V$1.2, refuting a tripartite complex formation and localizing CDF and CDI solely to the CTD (Pitt & Lee, 2016).

Both arrhythmia and epilepsy-associated mutations expressed in Na$_V$1.5 and Na$_V$1.2 CTDs, weaken non-calcified calmodulin affinity, thereby destabilizing fast inactivation (Miloushev et al., 2009; Yan et al., 2017a). Calmodulin over-expression rescues biophysical defects in Na$_V$1.5 CTD mutants that perturb calmodulin’s binding affinity except in the EF-like hand mutant, D1790G (Yan et al., 2017a).
1.6.4. Ca$^{2+}$/calmodulin-dependent protein kinase II (Ca$^{2+}$-CaMKII)

Besides regulating Na$\text{v}$1.5, calcium-calmodulin activates protein kinase II (Ca$^{2+}$-CaMKII) which antagonizes CDF and CDI (Wagner et al., 2006; Pitt, 2007; Scheuer, 2011; Herren et al., 2013). This adaptive ‘fail-safe’ mechanism induced by Ca$^{2+}$-CaMKII may be useful with inherited SCN5a mutations, especially those expressed in the CTD, and thus disrupt normal calcium-calmodulin regulation of Na$\text{v}$1.5.

CaMKIIδc is the main isoform expressed in the heart and phosphorylates Na$\text{v}$1.5 at S516, S571 and T594 (Wagner et al., 2006; Maltsev et al., 2009; Herren et al., 2013; Marionneau & Abriel, 2015). Over-expression of CaMKIIδc hyperpolarizes the voltage-dependence of fast inactivation, enhances intermediate/slow inactivation, slows recovery from inactivation, and increased late $I_{\text{Na}}$ (Wagner et al., 2006; Livshitz & Rudy, 2007; Hund et al., 2008; Herren et al., 2013). The biophysical shifts caused by Ca$^{2+}$-CaMKII may augment mixed syndrome pathogenesis due to inherited and/or acquired cardiac disease (Herren et al., 2013).

1.6.5. Protein Kinases A & C

Protein kinase A (PKA) and protein kinase C (PKC) have opposing effects on Na$\text{v}$1.5 conductance (Qu et al., 1996; Chen et al., 2006; Marionneau & Abriel, 2015). Substances known to increase PKC activity include 2-dioctanoyl-rac-glycerol (DOG), 1-oleoyl-2-acetyl-sn-glycerol (OAG), and phorbol 12-myristate 13-acetate (PMA). PKC phosphorylates S1505 (in rodent Na$\text{v}$1.5, S1503 in human Na$\text{v}$1.5) in the DIII-DIV linker, which stabilizes closed-state fast inactivation (Qu et al., 1996; Chen et al., 2006). An alanine scan to the consensus PKC binding residues abolishes the PKC-induced reduction in peak $I_{\text{Na}}$ caused by voltage shifts in closed-state fast inactivation (Qu et al., 1996). PKC effects on open-state fast inactivation and late $I_{\text{Na}}$ remain unknown.

The cAMP-dependent PKA phosphorylates S525 and S528 in the DI-DII linker (Murphy et al., 1996; Frohnwieser et al., 1997; Tateyama et al., 2003). In vitro, in silico, and proteomic analyses suggest the presence of additional PKA phosphorylation sites in Na$\text{v}$1.5 (Marionneau & Abriel, 2015). However, the extent to which these sites are phosphorylated is unknown. PKA increases Na$\text{v}$1.5 trafficking and hyperpolarizes the voltage-dependence of activation and fast inactivation (Murphy et al., 1996; Frohnwieser
et al., 1997; Tateyama et al., 2003; Chen et al., 2006; Scheuer, 2011). The D1790G mutant has a unique response to PKA compared Y1795C and Y1795H (Tateyama et al., 2003). Late \( I_N \) in D1790G is markedly increased by PKA (Tateyama et al., 2003). Although this charge-neutralizing mutant is located in the CTD, it enhances PKA activity which binds to S36 and S525 residues, suggesting that the CTD may have direct or indirect interactions with the N-terminus and/or the DI-DII linker. A crosstalk mechanism may exist between \( \text{Na}_V \text{1.5} \) inter-linkers, and the N- and C-termini, thereby modulating PKA activity. The R526H mutant, however, found adjacent to the PKA phosphorylation site, inhibits PKA and exacerbates loss-of-function in \( \text{Na}_V \text{1.5} \) (Marionneau & Abriel, 2015).

1.6.6. Temperature

Temperature plays an important role in regulating channel behavior and kinetics (Collins & Rojas, 1982; Matteson & Armstrong, 1982; Smeets et al., 1986). A major caveat in the electrophysiology studies is the temperature at which patch-clamp recordings are performed. Most experiments are conducted at room temperature (22 °C). Patch-clamp recordings become unstable at elevated temperatures. Reports of \( Q_{10} \) coefficients for the different \( \text{Na}_V \text{1.5} \) states are limited, and extrapolating biophysical data using a uniform \( Q_{10} \) coefficient cannot be generalized since \( \text{Na}_V \text{1.5} \) variants are differentially altered by temperature (Mohammadi et al., 2003; Mok et al., 2003; Keller et al., 2006; Trip et al., 2007; Egri et al., 2012; Holzherr et al., 2014).

Governed by anabolism and catabolism, body temperature is usually around 37 °C. Body temperature varies with sex, age, time of the day, and the physiological state. During sleep, body temperature generally decreases (~ 36 °C) but may increase during REM sleep. Elevations in body temperature accompany exercise, reaching as high as 40 °C. Body temperature may drop below 35 °C or rise to 45 °C under extreme hypothermia or hyperthermia, respectively. Hyperthermia may serve as an arrhythmogenic trigger, unmasking BrS1 (Wakita et al., 2004; Peng et al., 2005; Makaryus et al., 2009).

One case study reported a 54 year-old man displaying BrS1 patterns during a fever (41 °C) which recovered during the afebrile state (Mok et al., 2003). The man carried the H681P \( \text{Na}_V \text{1.5} \) mutant. This mutant stabilizes activation and inactivation
states leading to a decrease in window current, reducing the net $I_{Na}$ (Mok et al., 2003). H681P was not characterized at elevated temperature, however.

Elevated temperatures depolarized the voltage-dependence of activation in the BrS1 mutant, T1620M, located in DIV S3-S4 linker (Dumaine et al., 1999). Decreased Na\textsubscript{V}1.5 availability explains the decelerated cardiac conduction velocity resulting in right bundle branch block and H-R interval prolongation in T1620M carriers (Dumaine et al., 1999). Similarly, hyperthermia destabilizes the voltage-dependence of activation and enhances fast inactivation recovery kinetics in the F1344S mutant. The former biophysical shift overrides the latter thereby unmasking BrS1 in F1344S carriers (Keller et al., 2006).

Hyperthermia-induced LQTS was mainly reported in LQT1 and LQT2 patients (Amin et al., 2008; Burashnikov et al., 2008; Amin et al., 2010a; Elbey et al., 2012; Nakajima et al., 2015). Less is known about LQT3 patients’ response to hyperthermia. The LQT3 mutant, ΔKPQ, was functionally characterized at elevated temperature and was not thermosensitive (Nagatomo et al., 1998). Nevertheless, no clinical data correlate with this biophysical finding.

1.6.7. Temperature and Calcium Interaction

Hypothermia elevates cytosolic calcium by mainly increasing RyR open probability (more leaky), thereby increasing heart contracture in hibernators (Hannon, 1958; Johansson, 1996; Nolan, 2003; Shutt & Howlett, 2008; El-Sayed et al., 2012; Polderman, 2013; Nielsen et al., 2013). Hypothermia slows Ca\textsubscript{V}1.2 kinetics and the turnover rate of other transporters such as SERCA and NCX, and reduces troponin C and calmodulin’s binding affinities to calcium (Pelzmann et al., 1998; da Silva et al., 2002; Fu et al., 2005).

Shifts in cytosolic calcium caused by hyperthermia have not been studied extensively as hypothermia. Elevated temperature increases L-type $I_{Ca}$ amplitude and Ca\textsubscript{V}1.2 kinetics in cardiomyocytes, and also increase NCX’s and SERCA’s turnover rates (Elias et al., 2001; Ferrier et al., 2003). However, it is difficult to determine cytosolic calcium levels from the large amplitude and short lasting $I_{Ca}$ transients. A hyperthermia stimulus to human colon cancer cells initially reduces cytosolic calcium but is followed by
a marked rise in cytosolic calcium with maintained stimulation (Mikkelsen et al., 1991). Other confounding variables may contribute to the rise in cytosolic calcium, falsely attributed to elevated temperature in physiological settings (Marks, 2003). β₁ adrenergic agonists indirectly modulate cytosolic calcium. PKA activation increases L-type I_{Ca} influx and the open-probability of RyR (Marks, 2003). PKA phosphorylates Troponin I, thereby reducing Troponin C’s affinity for cytosolic calcium, causing a faster attenuation to the calcium spark (Peña & Wolska, 2004). PKA also phosphorylates phospholamban, increasing calcium uptake into the sarcoplasmic reticulum (Song et al., 2001). Thus, the overall net increase in cytosolic calcium accompanying exercise is complex and governed by many factors modulating the inflow and outflow of cytosolic calcium.

1.7. Antiarrhythmic Drugs and VGSCs

Voltage gated sodium channels are a target for many antiarrhythmics, anticonvulsants, and local anesthetics. Various toxins and pharmacological modulators bind to different channel modes (Hille, 1977; Tikhonov & Zhorov, 2017). The modulated receptor hypothesis relates the functional state of VGSC with pharmacodynamics (Ragsdale et al., 1996; Li et al., 1999; Denac et al., 2000). Although the hypothesis was originally based on local anesthetics, it is applicable to antiarrhythmics and postulates that Na\textsubscript{V} blockers bind to the activated/inactivated states versus the deactivated state.

The Food and Drug Administration (FDA) approved 12 class I a-c antiarrhythmics (Table 1-1). Other antiarrhythmics function as β₁ blockers (slow heart rate, class II), potassium channel blockers (class III), calcium channel blockers (class IV), and ones with variable pharmacological mechanisms (class V). The yellow-highlighted class I antiarrhythmics in Table 1-1 are no longer prescribed in the clinic due to their dangerous effects.
Table 1-1  Class I antiarrhythmics

<table>
<thead>
<tr>
<th>Class Name</th>
<th>Generic Name</th>
<th>Brand Name</th>
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</thead>
<tbody>
<tr>
<td>Class Ia</td>
<td>Quinidine</td>
<td>Cardioquin</td>
</tr>
<tr>
<td></td>
<td>Ajmaline</td>
<td>Gilurytmal</td>
</tr>
<tr>
<td></td>
<td>Procainamide</td>
<td>Pronestyl</td>
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<tr>
<td></td>
<td>Disopyramide</td>
<td>Norpace</td>
</tr>
<tr>
<td>Class Ib</td>
<td>Lidocaine</td>
<td>Anestacaine</td>
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<tr>
<td></td>
<td>Phenytoin</td>
<td>Dilantin</td>
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<td></td>
<td>Mexiletine</td>
<td>Mexitil</td>
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<tr>
<td></td>
<td>Tocainide</td>
<td>Tonocard</td>
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<tr>
<td>Class Ic</td>
<td>Encaainide</td>
<td>Enkaid</td>
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<tr>
<td></td>
<td>Flecaainide</td>
<td>Tambocor</td>
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<td></td>
<td>Propafenone</td>
<td>Rythmol</td>
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<td></td>
<td>Moricizine</td>
<td>Ethmozine</td>
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1.7.1. Antiarrhythmics mode of Action

Most class I antiarrhythmics share common molecular determinants with other local anesthetics and anticonvulsants (Ragsdale et al., 1996; Ragsdale & Avoli, 1998; Clare et al., 2000; Czapiński et al., 2005; Sheets et al., 2010; Lipkind & Fozzard, 2010; O’Reilly et al., 2012; Lee et al., 2012). Residues F1760 and Y1767 in the inner vestibule of Na\textsubscript{v}1.5 form the bindings sites for class I antiarrhythmics (Ragsdale et al., 1996; Fredj et al., 2006b; Lipkind & Fozzard, 2010; Huang et al., 2011; O’Reilly et al., 2012). The location of these residues indicates that these drugs adopt an intracellular binding mode in Na\textsubscript{v}1.5. In the open-state conformation, the C-termini of S6 in DI to DIV form a wide opening, which exposes the two receptor sites in the pore. In this high affinity conformational state, small class I antiarrhythmics and anesthetics like Lidocaine and Phenytoin, form interactions with F1760 and Y1767 aromatic rings, thereby blocking the pore (Fredj et al., 2006b; Sheets et al., 2010; Lipkind & Fozzard, 2010; O’Reilly et al., 2012). Phenytoin forms a non-polar aromatic-aromatic interaction with the Y1767 residue, while the other amide containing branch (hydantoin ring) forms a polar bond between the electron-donating π electron in F1760 aromatic residue (Lipkind & Fozzard, 2010). Phenytoin partially interacts with residues near the IFM motif, thereby stabilizing the fast-inactivated state. F1760 and Y1767 form thermodynamically favourable interactions with the IFM motif and also stabilize fast inactivation (Ulbricht, 2005).

Lidocaine has similar effects as Phenytoin. Alanine scans to the receptors sites reduce Lidocaine binding affinity to Na\textsubscript{v}1.5, which reduces the drug’s voltage-dependent and frequency-dependent block (Li et al., 1999).
Minimal research has been devoted to drug effects on channel trafficking or expression. The M1766L is the only Na\textsubscript{v}1.5 mutant where the expression defect is rescued by Mexiletine (Valdivia et al., 2002).

### 1.7.2. Arrhythmias provoked by Antiarrhythmic Drugs

Antiarrhythmics do not always ameliorate arrhythmias, rather they may be clinically used to provoke, and hence test for positive SCN5a mutation carriers (Haverkamp, 2000; Fenichel et al., 2004; Fish & Antzelevitch, 2004a). E1784K carriers express BrS more preferentially when administered with Ajmaline compared to Flecainide or Pilsicainide (Makita et al., 2008a). Flecainide has proven to be therapeutic in certain SCN5a mutations; however, it induces a large tonic peak I\textsubscript{Na} block in certain Na\textsubscript{v}1.5 mutants, posing a pro-arrhythmic threat for BrS1 and mixed syndrome mutants (Viswanathan et al., 2001; Moss et al., 2005). Arrhythmias may occur with enhanced peak I\textsubscript{Na} tonic-block, inducing channel loss-of-function. Flecainide is therapeutic compared to lidocaine in treating LQT3-induced phenotypes in D1790G by increasing channel use-dependence (Benhorin et al., 2000; Abriel et al., 2000; Zhu et al., 2006). Similarly, lidocaine is effective in treating LQT3, but augments channel use-dependence in V232I+L1308F, which unmasks BrS (Balser et al., 1996; Sheets & Hanck, 2007; Barajas-Martinez et al., 2008).

Acquired BrS and LQTS are usually caused by class I antiarrhythmics and class III antiarrhythmics, respectively (Day et al., 1990; Shimizu & Antzelevitch, 1999; Fenichel et al., 2004). Quinidine is implicated in acquired LQTS due to its high potency for blocking HERG (Paul et al., 2002). To selectively target LQT3 in SCN5a mutations and prevent the occurrence of BrS, the pharmaceutical industry has developed LQT3-specific drugs: ranolazine and new compounds like aryl sulfonamides selectively block late I\textsubscript{Na} compared to peak I\textsubscript{Na} (Antzelevitch, 2004; Antzelevitch et al., 2004; Belardinelli, 2006b; Ahuja et al., 2015).

### 1.7.3. Ranolazine

Ranolazine is used to treat angina pectoris, cardiac instability, arrhythmias, and reduced contractility due to improper sodium balance within myocytes caused by augmented late I\textsubscript{Na} (Antzelevitch et al., 2004; Belardinelli, 2006a). Sodium overload
reverses NCX function causing calcium overload, thereby increasing the risk for electrical and mechanical abnormalities. Ranolazine preferentially blocks late $I_{Na}$, thereby reducing intracellular sodium and calcium overload.

A Cysteine substitution to the second receptor site in Na\textsubscript{v}1.5, Y1767, abolishes the inhibitory effects of Quinidine, Mexiletine, and Flecainide on late $I_{Na}$ (Huang et al., 2011). Ranolazine, however, is not affected by Y1767C and effectively suppresses late $I_{Na}$, suggesting that ranolazine adapts a different binding mode compared to classic antiarrhythmics (Ahern et al., 2008; Huang et al., 2011). Replacing the large aromatic group in Y1767 with a smaller cysteine side chain may reduce the steric hindrance experienced by ranolazine while accessing its binding site at the inner pore (Huang et al., 2011; Kaczmarowski & Corry, 2014). Ranolazine binding mode is not limited, however, to the inner pore; the fenestrations are an accessory route for large and bulky Na\textsubscript{v} blockers (Kaczmarowski & Corry, 2014). Thus, it is plausible for ranolazine to regulate Na\textsubscript{v} inactivation at the fenestrations or at the interphase between the inner vestibule drug binding sites and the fenestrations.

In addition to its inhibitory effects on Na\textsubscript{v}1.5, ranolazine also blocks neuronal Na\textsubscript{v} isoforms. Ranolazine reduces pathological late $I_{Na}$ caused by epilepsy-associated Na\textsubscript{v}1.1 mutants (Kahlig et al., 2010). In most Na\textsubscript{v} isoforms, ranolazine stabilizes the slow inactivated state by accelerating onset and decelerating recovery kinetics of slow inactivation (Peters et al., 2013). Acidosis accentuates these drug effects in both Na\textsubscript{v}1.2 and Na\textsubscript{v}1.5, which is correlated with attenuated late $I_{Na}$ (Sokolov et al., 2013; Peters et al., 2013) Ranolazine, may thus adopt a different binding mode from that of the receptor modular hypothesis. Ranolazine is structurally similar to Lidocaine. Movement of DIII and DIV S4s stabilizes Lidocaine binding (Sheets & Hanck, 2007). Thus, ranolazine may stabilize slow inactivation by interacting with DIII or DIV segments and not necessarily bind to the inner vestibule.

1.7.4. Na\textsubscript{v} targets exhibiting novel modes of action

Lacosamide is a novel anticonvulsant that effectively treats partial seizures. Lacosamide, like ranolazine, stabilizes the slow-inactivated state compared to other anticonvulsants that primarily affect fast inactivation. By increasing channel use-dependence, Lacosamide is able to reduce long-lasting neuronal repetitive firing spikes
elicited by prolonged depolarizations (Errington et al., 2007). Phenytoin, Carbamazepine, and Lamotrigine exert their action over substantially shorter time scale (Ragsdale et al., 1996; Errington et al., 2007; Yang et al., 2010).

As opposed to drugs binding to the inner vestibule or through the fenestrations, recent drug design attempts to target Na\textsubscript{v} extracellularly. Ionic compounds, such as aryl sulfonamides like GX-936, form salt bridges with the fourth arginine (R4) residue in DIV S4 (Ahuja et al., 2015). This ionic bond stabilizes the activated fast inactivation segment (DIV S4), thereby reducing late I\textsubscript{Na} (Sheets & Hanck, 2007; Ahuja et al., 2015). Although a breakthrough, further modifications to aryl sulfonamide compounds is required to achieve higher Na\textsubscript{v}-isoform selectivity.

1.8. Thesis Significance

The most common SCN5a mutation, expressed as the E1784K mutant, is very well characterized. Functional data support a correlation between channotype and phenotype in E1784K. However, the high phenotype heterogeneity in E1784K carriers must be further elucidated. The cardiac conduction abnormalities manifesting as BrS1 or heart block accompanied by prolonged QT intervals in E1784K carriers may ultimately trickle down to the effects of external triggers on channotype. E1784K distorts Na\textsubscript{v}1.5 CTD integrity. Despite E1784’s presence prior to the EF-like hand domain, it forms electrostatic interactions with downstream residues in the IQ domain, establishing an intact CTD (Chagot et al., 2009; Yan et al., 2017a). The charge reversal mutant distorts the normal salt bridges required for CTD maintenance, thereby eliminating the adaptive roles adopted by several CTD-interacting molecules like calmodulin, in rescuing biophysical defects (Yan et al., 2017a). Various CTD mutants disrupt calmodulin’s inherent role in limiting pathogenic late I\textsubscript{Na} either by directly or indirectly affecting the molecule’s binding affinity for the CTD. Furthermore, both the dependent and independent calcium-calmodulin interactions between the CTD and the DIII-DIV linker may be affected in E1784K. I hypothesize that exercise-related factors like elevated temperature and cytosolic calcium affect channotype expressivity in E1784K. Temperature enhances channel kinetics, thereby augmenting E1784K biophysical defects. Cytosolic calcium, although is known to be therapeutic for LQT3 mutants, is not effective in ameliorating E1784K since the mutant deprives Na\textsubscript{v}1.5 from calcium-calmodulin regulation. The disintegration in intra-CTD interactions caused by E1784K
may decouple the CTD and the DIII-DIV linker, further reducing calcium-calmodulin regulation. I hypothesize that ranolazine is an effective therapy for E1784K since it preferentially blocks augmented late $I_{\text{Na}}$.

1.9. Thesis Objectives

My thesis objectives are to study the effect of (1) elevated temperature, (2) elevated cytosolic calcium, and (3) their combined triggering effects on E1784K when screened against ranolazine. An in-depth biophysical characterization of wild type and mutant channel gating is done using the whole-cell patch clamp technique. Chinese Hamster Ovary (CHOK1) or Human Embryonic Kidney (HEK293) cells, with varying calmodulin levels, are used to control for temperature and cytosolic calcium effects, separately. Temperature effects are studied on E1784K along with a reference mixed syndrome mutant, R1193Q, expressed in the DII-DIII linker. Cytosolic calcium effects are studied on E1784K and other DIII-DIV linker and CTD mutants like $\Delta$KPQ, 1795insD, and Q1909R. These mutants are chosen specifically for comparative reasons since their position may uniquely modify calcium-calmodulin regulation in Na$_v$1.5. Ranolazine is screened against E1784K at elevated temperature and cytosolic calcium. *In-silico* cardiac action potentials are simulated using modified ten Tüsscher (2006) and O’Hara-Rudy (2011) models. A dynamic simulation is included using the latter model in objectives (2) and (3) to account for the positive-staircase phenomenon of cytosolic calcium. Thus, I am able to account for modifications in sodium currents caused by cytosolic calcium. Molecular docking is used to understand ranolazine binding to Na$_v$1.5. Ranolazine was auto-docked against the Na$_v$1.5 homology model based on the new American cockroach Na$_v$Pas structure.
Chapter 2. Differential Thermosensitivity in Mixed Syndrome Cardiac Sodium Channel Mutants

This chapter describes the work published in (Abdelsayed et al., 2015) with minor modifications and formatting changes to suit the thesis style.

2.1. Abstract

Introduction: Cardiac arrhythmias are often associated with mutations in SCN5a, the gene that encodes the cardiac paralog of the voltage-gated sodium channel, Na\textsubscript{v}1.5. The Na\textsubscript{v}1.5 mutants R1193Q and E1784K give rise to both Long QT and Brugada syndromes. Various environmental factors, including temperature, may unmask arrhythmia. We sought to determine whether temperature might be an arrhythmogenic trigger in these two mixed syndrome mutants.

Methods: Whole-cell patch-clamp was used to measure the biophysical properties of Na\textsubscript{v}1.5 WT, E1784K and R1193Q mutants. Recordings were performed using Chinese Hamster Ovary (CHOK1) cells transiently transfected with the Na\textsubscript{v}1.5 α subunit (wild type, E1784K, or R1193Q), β1 subunit, and eGFP. The channels’ voltage-dependent and kinetic properties were measured at three different temperatures: 10 ºC, 22 ºC, and 34 ºC.

Results: The E1784K mutant is more thermosensitive than either WT or R1193Q channels. When temperature is elevated from 22 ºC to 34 ºC, there is a greater increase in late I\textsubscript{Na} and use-dependent inactivation in E1784K than WT or R1193Q. However, when temperature is lowered to 10 ºC, the two mutants show a decrease in channel availability. Action potential modeling using Q\textsubscript{10} fit values, extrapolated to physiological and febrile temperatures, show a larger transmural voltage gradient in E1784K compared to R1193Q and WT with hyperthermia.

Conclusions: The E1784K mutant is more thermosensitive than WT or R1193Q channels. This enhanced thermosensitivity may be a mechanism for arrhythmogenesis in patients with E1784K sodium channels.
2.2. Introduction

Voltage-gated sodium channels are responsible for the upstroke of action potentials in most cardiac, skeletal, and neuronal tissue. In this study, we focus on Na\textsubscript{v}1.5, the sodium channel most prevalent in the cardiac conduction system and cardiomyocytes (Remme et al., 2009). Mutations in SCN5a, the gene encoding Na\textsubscript{v}1.5, give rise to a large spectrum of potentially arrhythmogenic disorders including Brugada and Long QT-3 syndromes.

Long QT syndrome 3 (LQT3) is due to gain-of-function mutants of Na\textsubscript{v}1.5, in which the S4 voltage sensor, the DIII-DIV linker segment, and the C-terminus are most commonly affected (Glaaser et al., 2012). These mutants hinder fast inactivation and lead to an increase in late I\textsubscript{Na} which disrupts the normal balance between outward and inward currents that maintain the plateau phase of the cardiac action potential (AP). An increase in late I\textsubscript{Na} delays cardiomyocyte repolarization and prolongs the cardiac action potential. In contrast, loss-of-function Na\textsubscript{v}1.5 mutants that diminish peak I\textsubscript{Na} can cause Brugada syndrome 1 (BrS1). Many of the mechanisms underlying BrS1 involve stabilizing inactivated states and/or destabilizing the activated state (Grant et al., 2002). Due to heterogeneous channel expression in the heart tissue, BrS1 preferentially affects the right epicardial AP. Relatively high levels of I\textsubscript{K\textsubscript{to}} (transient outward potassium current), found in the right ventricular epicardium, can cause the loss of the AP plateau in myocytes with decreased I\textsubscript{Na}. This loss generates a transmural voltage gradient causing ST segment elevation, visible in the right precordial leads. This phenomenon is also known as “phase 2 reentry” (Chen et al., 1998; Antzelevitch, 2005). There are three different manifestations of this phenomenon in the ECG: Type I is characterized by a J-point elevation, a coved ST-segment, and an inverted T wave in V\textsubscript{1}-V\textsubscript{3}; Type II shows a saddleback-type ST-segment elevation; Type III shows an ST-segment elevation accompanied by a J-point elevation. Types II and III are not diagnostic of BrS (Antzelevitch, 2005).

BrS1 and LQT3 have been reported to precipitate ventricular tachycardia and ventricular fibrillation leading to sudden death in men and women, and sudden infant death syndrome in young children (Priori et al., 2000; García-Borbolla et al., 2007; Makaryus et al., 2009). BrS1 may be unmasked by decreases in plasma pH, changes in body core temperatures, and pharmacological agents that block sodium channels (Mok...
et al., 2003; Makaryus et al., 2009; Doetzer et al., 2011). Elevated temperatures exacerbate the decrease in channel function in the BrS1 mutant T1620M by causing a larger destabilization of the activated state (Dumaine et al., 1999). Additionally, there is an enhanced onset into inactivation at elevated temperatures (Dumaine et al., 1999). In another thermosensitive-mutant, F1344S, the conductance is further destabilized at elevated temperatures (Keller et al., 2006).

Interestingly, some Na\textsubscript{v}1.5 mutants, including R1193Q and E1784K, can cause both LQT3 and BrS1 and are referred to as mixed syndromes (Bezzina et al., 1999; Veldkamp et al., 2000a; Grant et al., 2002). Molecular genetic screening revealed that a heterozygous mutation, in which a guanine was replaced with an adenine in the 3578\textsuperscript{th} position in exon 20 of SCN5a, results in the R1193Q mutant (Huang et al., 2006). R1193Q is located in the intracellular DII-DIII linker region. This mutant stabilizes the fast-inactivated state (Wang, 2004; Huang et al., 2006). Enhanced inactivation may lead to a decrease in sodium currents, consistent with the phenotype of BrS1. However, the mutant increases late I\textsubscript{Na}, associated with LQT3 (Sun et al., 2008). A guanine to an adenine mutation in the 5349\textsuperscript{th} position of SCN5a results in the E1784K mutant in the C-terminus of Na\textsubscript{v}1.5 (Splawski et al., 2000; Tester et al., 2005). E1784K tends to stabilize steady-state inactivation but also increases late I\textsubscript{Na} (Wei et al., 1999; Makita et al., 2008a).

Our study focused on the effects of temperature on wild-type (WT), R1193Q, and E1784K channels. Our results suggest differential thermosensitivity in the E1784K and R1193Q mutants. E1784K is most strongly affected by temperature changes. Action potential simulations suggest that increasing temperature may attenuate the epicardial AP dome in cardiomyocytes expressing the E1784K mutant. Temperature increases may therefore be arrhythmogenic in E1784K.

2.3. Methods

2.3.1. Ethical approval

The research was approved by Biohazards review 251-2012 issued by the office of the Environmental Health and Safety at Simon Fraser University, Burnaby, BC, Canada.
2.3.2. Cell Culture

Chinese hamster ovary (CHOK1) cells (Sigma-Aldrich) were grown at pH 7.4 in filter sterile F12 (Ham) nutrient medium (Life Technologies, NY, USA), supplemented with 5% FBS and maintained in a humidified environment at 37°C with 5% CO2. Twenty-four hours prior to electrophysiology experiments, cells were transfected with cDNA for the sodium channel α and β subunits as well as green fluorescent protein (eGFP). Eight hours after transfection, cells were dissociated with 0.25% trypsin-EDTA (Life Technologies, NY, USA) and then plated on sterile cover slips. These time intervals were used to control for channel expression.

2.3.3. Transfection

Transfection followed the procedures suggested by Qiagen. Briefly, 1 µg of the Na\textsubscript{v}1.5 α subunit, 0.5 µg of the sodium channel β1 subunit, and 1 µg of eGFP were allowed to incubate with 15 µl of polyfect transfection reagent (Qiagen) and 147 µl of unsupplemented medium for 10 minutes. The cDNA mixture was then allowed to incubate with the CHOK1 cells for 8 hours before plating on coverslips. Na\textsubscript{v}1.5 mutations were generously provided by Dr. Charles Antzelevitch (E1784K) and Dr. Mohamed Chahine (R1193Q).

2.3.4. Electrophysiology

Whole-cell recordings were performed in extracellular solution containing (mM): 140 NaCl, 4 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, and 10 HEPES (pH 7.4). Solutions were titrated with CsOH to pH 7.4. Pipettes were fabricated with a P-1000 puller using borosilicate glass (Sutter Instruments, CA, USA), dipped in dental wax to reduce capacitance, then thermally polished to a resistance of 1.0-1.5 MΩ. Low resistance electrodes were used to minimize series resistance between pipette and intracellular solution resulting in typical access resistances of 3.5 MΩ or less, thereby minimizing voltage measurement error. Pipettes were filled with intracellular solution, containing (mM): 130 CsF, 10 NaCl, 10 HEPES, and 10 EGTA titrated to pH 7.4.

All recordings were made using an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) digitized at 20 kHz using an ITC-16 interface (HEKA
Data were acquired and low-pass-filtered (5 kHz) using PatchMaster/FitMaster software (HEKA Elektronik, Lambrecht, Germany) running on an Apple iMac (Apple Computer, Cupertino, CA). Leak subtraction was performed online using a P/4 procedure. Bath solution temperature was controlled using a Peltier device driven by a TC-10 Temperature Controller (Dagan, Minneapolis, MN). Bath temperature was maintained at 10 °C, 22 °C or 34 °C. Experiments were not performed at physiological temperatures because of the inherent instability of cells at temperatures above 34 °C. Using a Q10 relationship we extrapolated data to physiological temperatures (described below). After a Giga-ohm seal-resistance was achieved, the whole-cell configuration was attained. Currents were then allowed to stabilize such that currents measured by successive trains of five 10 ms depolarizations at 1 Hz to 0 mV were similar. Run-down was assessed by comparing peak current amplitudes before and after each protocol. With the exception of use-dependent inactivation protocols only protocols with less than 5% run-down were used. Use-dependent protocols showing more than 5% rundown were corrected post hoc. The holding potential between protocols was -110 mV. We recorded $I_{Na}$ from cells that expressed currents no greater than –5 nA. Cells with larger currents were not used since they gave rise to voltage-error issues. The average voltage error calculated for all cells used in this study is 6.15 mV obtained from a total of 114 cells (Table 2-1). There are no differences between the voltage-errors in the different conditions ($p>0.05$).

Table 2-1    Temperature Voltage Error

<table>
<thead>
<tr>
<th>Condition</th>
<th>Voltage Error</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-10</td>
<td>6.59 ± 0.95</td>
<td>14</td>
</tr>
<tr>
<td>WT-22</td>
<td>5.15 ± 0.95</td>
<td>11</td>
</tr>
<tr>
<td>WT-34</td>
<td>6.49 ± 1.08</td>
<td>9</td>
</tr>
<tr>
<td>RQ-10</td>
<td>6.95 ± 1.12</td>
<td>14</td>
</tr>
<tr>
<td>RQ-22</td>
<td>6.58 ± 1.37</td>
<td>6</td>
</tr>
<tr>
<td>RQ-34</td>
<td>7.10 ± 0.86</td>
<td>15</td>
</tr>
<tr>
<td>EK-10</td>
<td>6.23 ± 1.47</td>
<td>10</td>
</tr>
<tr>
<td>EK-22</td>
<td>6.75 ± 1.08</td>
<td>14</td>
</tr>
<tr>
<td>EK-34</td>
<td>8.73 ± 0.81</td>
<td>21</td>
</tr>
</tbody>
</table>
2.3.5. Analysis

Analysis and graphing were done using FitMaster software (HEKA Elektronik, Lambrecht, Germany) and Igor Pro (Wavemetrics, Lake Oswego, OR, USA) with statistical information derived using JMP statistical software. All data acquisition and analysis programs were run on an Apple iMac (Apple Computer, Cupertino, CA). Statistical significance was accepted at p < 0.05 using a two-factor completely randomized design (CRD) ANOVA test followed by a post-hoc Tukey test. We used a post hoc Student’s t test to analyze temperature effect on late current (22 °C and 34 °C) and steady-state slow inactivation (10 °C and 22 °C). Statistical analysis was performed on the averages between three variables: channel variant (WT, R1193Q, E1784K), temperature (10 °C, 22 °C, 34 °C), and channel variant × temperature. The latter represented the statistical significance of a change in channel variants as a result of temperature changes. All values reported are given as means ± standard error of means.

2.3.6. Voltage Protocols

2.3.6.1 Activation

To determine the voltage dependence of activation, we measured the peak current amplitude at test pulse potentials ranging from -100 mV to +80 mV in increments of +10 mV for 19 ms. Prior to the test pulse, channels were allowed to recover from fast inactivation at -130 mV for 197 ms. Channel conductance was calculated from peak INa.

1. \( G_{Na} = \frac{I_{Na}}{V - E_{rev}} \)

where \( G_{Na} \) is sodium channel conductance, \( I_{Na} \) is peak sodium current in response to the command potential \( V \), and \( E_{rev} \) is the reversal potential. Calculated values for conductance were fit with the Boltzmann function:

2. \( \frac{G}{G_{max}} = \frac{1}{1 + \exp[-ze_0(V_m - V_{1/2})/kT]} \)

where \( \frac{G}{G_{max}} \) is the normalized conductance amplitude, \( V_m \) is the command potential, \( z \) is the apparent valence, \( e_0 \) is the elementary charge, \( V_{1/2} \) is the midpoint voltage, \( k \) is the Boltzmann constant, and \( T \) is temperature in °K.
2.3.6.2 Steady-State Fast Inactivation (SSFI)

The voltage-dependence of SSFI was measured by preconditioning the channels to a hyperpolarizing potential of -130 mV and then eliciting prepulse potentials that range from -130 or -150 to +10 mV in increments of 10 mV for 200 ms. Channel availability was assessed by a test pulse to 0 mV. Different hyperpolarizing prepulse potentials (-130 mV or -150 mV) were used to obtain a plateau for the SSFI curve. Normalized current amplitude as a function of voltage was fit using the Boltzmann function:

\[ \frac{I}{I_{\text{max}}} = \frac{1}{1 + \exp(\frac{-ze_0 (V_m - V_{1/2})}{kT})} \]

where \( \frac{I}{I_{\text{max}}} \) is the normalized current amplitude, \( z \) is apparent valence, \( e_0 \) is the elementary charge, \( V_m \) is the prepulse potential, \( V_{1/2} \) is the midpoint voltage of SSFI, \( k \) is the Boltzmann constant, and \( T \) is temperature in °K.

2.3.6.3 Fast Inactivation Onset

Time constants for open-state fast inactivation were derived by fitting a single exponential function to the decay of current obtained from the activation protocol. To measure onset into fast inactivation, channels were preconditioned at -130 mV prior to a prepulse at -50 mV, -70 mV, or -90 mV for 0 – 0.256 s. Current amplitude was measured during a test pulse to 0 mV for 20 ms. Normalized current amplitudes as a function of time were fit using a single exponential equation:

\[ I = I_{ss} + \alpha \exp(-\frac{(t-t_0)}{\tau}) \]

where \( I \) is the current amplitude, \( I_{ss} \) is the plateau amplitude, \( \alpha \) is the amplitude at time 0 for time constant \( \tau \), and \( t_0 \) is initial time. Rates of recovery from and onset into fast inactivation were calculated from the reciprocal of the time constants (\( \tau \)) obtained from the single-exponential fits.

2.3.6.4 Fast Inactivation Recovery

Channels were fast-inactivated during a 200 ms depolarizing step to 0 mV. Recovery was measured during a 19 ms test pulse to 0 mV following 0 – 1.024 s conditioning pulses at -130 mV, -110 mV, or -90 mV. Time constants of fast inactivation recovery as a function of time were fit using a single exponential equation, as above.
2.3.6.5  

**Late Current**

Late current was measured between 45 – 50 ms during a 50 ms depolarizing pulse to 0 mV from a holding potential of –130 mV. An average of 60 pulses was used to increase the signal-to-noise ratio.

2.3.6.6  

**Use-Dependent Inactivation (1 Hz and 3 Hz)**

Channels accumulated into a use-dependent inactivated state during either a series of 300 380 ms depolarizing pulses to 0 mV followed by a 615 ms – 90 mV recovery pulse at a frequency 1 Hz, or 500 220 ms depolarizing pulses to 0 mV followed by a 110 ms – 90 mV recovery pulse at a frequency 3 Hz. Normalized current amplitude as a function of time was fit with a double exponential.

\[
5. \quad I = I_{ss} + \alpha_1 e^{(-t/\tau_1)} + \alpha_2 e^{(-t/\tau_2)}
\]

where \( I \) is the current amplitude, \( I_{ss} \) is the plateau amplitude, \( \alpha_1 \) and \( \alpha_2 \) are the amplitudes at time 0 for time constants \( \tau_1 \) and \( \tau_2 \), and \( t \) is time.

2.3.6.7  

**Slow Inactivation Onset**

To measure onset into slow inactivation, channels were preconditioned at -130 mV for 30 s prior to a prepulse at 0 mV for 0 – 64 s. A test pulse to 0 mV followed a -130 mV recovery pulse from fast inactivation for 20 ms. Normalized current amplitudes as a function of time were fit using a double exponential equation.

2.3.6.8  

**Steady-State Slow Inactivation (SSSI)**

The voltage-dependence of SSSI was measured by preconditioning the channels to a hyperpolarizing potential of -150 mV for 30 s and then eliciting prepulse potentials that range from -150 to -10 mV in increments of 20 mV for 60 s. Channel availability was assessed by a test pulse to 0 mV following a -130 mV recovery pulse from fast inactivation at 20 ms. Normalized current amplitude as a function of voltage was fit using a modified Boltzmann function:

\[
6. \quad I/I_{max} = (l_1 - l_2)/(1 + \exp(-ze_0 (V_M - V_{1/2})/kT) + l_2
\]

where \( l_1 \) and \( l_2 \) are maximum and minimum values of fit. The other symbols are as previously stated.
2.3.6.9 Slow Inactivation Recovery

To measure recovery from slow inactivation, channels were preconditioned at -130 mV for 30 s prior to a prepulse at 0 mV for 60 s, followed by series of test pulses to 0 mV for 20 ms between increasing incremental recovery durations at -130 mV for 0 – 32s. Normalized current amplitudes as a function of time were fit using a double exponential equation with the plateau equal to 1.00.

2.3.6.10 Q10 Coefficients

To determine an appropriate fit for the kinetic or thermodynamic parameters plotted as a function temperature, we used the Q10 formula:

7. \[ Q_{10} = \left(\frac{R_2}{R_1}\right)^{10/(T_2-T_1)} \]

where \( R \) is the rate and \( T \) is temperature (1 and 2 refer to initial and secondary, respectively). Rate was calculated by the inverse of the \( \tau \) value. \( Q_{10} \) fits for steady-state midpoints and slopes were calculated by replacing the Rs with \( V_{1/2} \) and \( z \) values. Fits for \( y_0 \) were calculated based of the \( 1/y_0 \) to yield optimal \( Q_{10} \) values. The best fit was used with three points obtained at 10 ºC, 22 ºC and 34 ºC to increase the accuracy of the \( Q_{10} \) fit. The fit was extrapolated to physiological and febrile temperatures.

2.3.7. Action Potential Modeling

Cardiac action potential modeling was based on a modified ten Tüsscher 2006 model, previously described (ten Tüsscher et al., 2004; ten Tüsscher, 2006; Jones et al., 2011). All action potentials were programmed and run in the Python language. The \( I_{\text{to}} \) formulation and maximal conductance were modified as suggested by Dumaine et al. 1999 (Dumaine et al., 1999). The epicardial L-type calcium current was decreased by 50% to obtain a more realistic action potential length (Dumaine et al., 1999; Xia et al., 2006). Simulations were run at 1 Hz. The 34 ºC data were incorporated into the model to account for hypothermic conditions. Predicted channel kinetics and steady state properties at 37 ºC (normothermia) and 41 ºC (hyperthermia) temperatures were obtained from the \( Q_{10} \) extrapolations. Our model only accounted for current density, activation, steady-state fast inactivation, fast inactivation kinetics, and late \( I_{\text{Na}} \). The slow inactivation parameters were excluded from the model since we did not have a full slow inactivation kinetics profile. As described earlier, it is challenging to patch at elevated
temperatures because of a loss of patch stability. Temperature shifts affect the kinetics of other channels that contribute to the maintenance of the cardiac action potential, thus changes in temperature will have other effects on cardiac action potential morphology, for which we are unable to account. We used the ten Tüsscher model to determine the effects of temperature on cardiac action potential based only on shifts occurring in Na\textsubscript{v}1.5. We modeled epicardial action potentials with increasing I\textsubscript{K,ls} to simulate changes across the heart wall and between the right and left ventricles.

2.4. Results

2.4.1. Activation

We measured current density from the ratio of peak current amplitude to the cell membrane capacitance (pA/pF). Representative raw current traces are shown for the different channel variants at the three temperatures studied (Figure 2-1). Peak I\textsubscript{Na} density was higher in WT and E1784K when temperature was elevated from 22 ºC to 34 ºC (p<0.05, Table 2-2). The Q\textsubscript{10} values are consistent with temperature insensitivity in R1193Q compared to WT and E1784K (Table 2-2).

We measured activation rate by fitting the rise phase of I\textsubscript{Na} at 0 mV, from the beginning of the test pulse to the half peak I\textsubscript{Na}. Both mutants, E1784K and R1193Q, were slower to reach half peak I\textsubscript{Na} than WT channels at 10 ºC (p<0.0001, Table 2-2). Time to half peak I\textsubscript{Na} was reduced (p<0.0001) in E1784K (550.2 μs ± 52.4 μs) and R1193Q (525.4 μs ± 60.0 μs) compared to WT (270.3 μs ± 47.0 μs) when temperature was elevated from 10 ºC to 22 ºC (Figure 2-1 and Table 2-2). With further elevations in temperature (22 ºC to 34 ºC) time to half peak I\textsubscript{Na} was further reduced (p>0.05) in E1784K (124.4 μs ± 49.0 μs) compared to smaller changes in WT and R1193Q (Figure 2-1 and Table 2-2). Table 2-2 includes Q\textsubscript{10} fit values which are consistent with overall trends in which E1784K exhibits higher thermosensitivity compared to WT and R1193Q.
Figure 2-1  Temperature effects on current density and activation kinetics
Current recordings of the channel variants are shown at three temperatures. Panel A shows peak current density for the different channel variants as a function of temperature. Panel B shows the time to half peak of maximal $I_{Na}$ measured at 0 mV.

Table 2-2  Temperature Peak Current Density and Time to Half peak $I_{Na}$

<table>
<thead>
<tr>
<th>Condition</th>
<th>Peak $I_{Na}$ Density (pA/pF)</th>
<th>N</th>
<th>Time to half peak $I_{Na}$ (µs)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-10</td>
<td>328.11 ± 40.61</td>
<td>16</td>
<td>530 ± 56 *2</td>
<td>16</td>
</tr>
<tr>
<td>WT-22</td>
<td>399.70 ± 65.83</td>
<td>11</td>
<td>260 ± 26</td>
<td>11</td>
</tr>
<tr>
<td>WT-34</td>
<td>848.80 ± 132.77 *1</td>
<td>9</td>
<td>230 ± 42</td>
<td>10</td>
</tr>
<tr>
<td>WT-Q10</td>
<td></td>
<td></td>
<td>1.61</td>
<td></td>
</tr>
<tr>
<td>RQ-10</td>
<td>351.96 ± 56.84</td>
<td>15</td>
<td>810 ± 24 *2</td>
<td>8</td>
</tr>
<tr>
<td>RQ-22</td>
<td>360.60 ± 48.20</td>
<td>8</td>
<td>280 ± 50</td>
<td>8</td>
</tr>
<tr>
<td>RQ-34</td>
<td>543.18 ± 76.47</td>
<td>17</td>
<td>320 ± 9.0</td>
<td>14</td>
</tr>
<tr>
<td>RQ-Q10</td>
<td>1.22</td>
<td></td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>EK-10</td>
<td>255.93 ± 40.43</td>
<td>11</td>
<td>830 ± 35 *2</td>
<td>11</td>
</tr>
<tr>
<td>EK-22</td>
<td>394.22 ± 60.13</td>
<td>16</td>
<td>280 ± 16</td>
<td>10</td>
</tr>
<tr>
<td>EK-34</td>
<td>853.23 ± 91.86 *1</td>
<td>22</td>
<td>160 ± 9.2</td>
<td>15</td>
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<tr>
<td>EK-Q10</td>
<td>1.75</td>
<td></td>
<td>1.8</td>
<td></td>
</tr>
</tbody>
</table>

*p<0.05 vs. 22 ºC of same channel variant, *2 p<0.0001 vs. 22 ºC and 34 ºC of same channel variant
We show conductance as a function of membrane potential in Figure 2-2. The conductance midpoint (GV-V1/2) was depolarized (p<0.0001) in E1784K compared to WT and R1193Q (Figure 2-2 and Table 2-3). GV-V1/2 in both E1784K and R1193Q was hyperpolarized (p<0.0001) by approximately 10 mV when temperature was increased from 10 °C to 22 °C compared to WT channels. Further elevations in temperature did not significantly shift GV-V1/2 in the mutants. The Q_{10} fit values are reported in Table 2-3 and are consistent with a slightly higher Q_{10} value for E1784K compared to R1193Q and WT.

The conductance slope (GV-z) was significantly different between the channel variants in which E1784K had a lower slope value compared to WT and R1193Q (p<0.01, Figure 2-2). GV-z increased when temperature was elevated (p<0.0001, Table 2-3). The changes in slope due to temperature were not significantly different between the channel variants (p>0.05). The Q_{10} fit values reported in Table 2-3 are relatively equal between the channel variants.
Figure 2-2  Temperature effects on activation and steady-state fast inactivation
Panels A-C show the voltage-dependence of activation and fast inactivation as normalized conductance and normalized current, respectively, plotted against membrane potential. The insets in panel C show pulse protocols used to measure GV and SSFI.
Table 2-3  Temperature Conductance

<table>
<thead>
<tr>
<th>Condition</th>
<th>GV-V\textsubscript{1/2}</th>
<th>GV-z</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-10</td>
<td>-30.56 ± 1.99</td>
<td>2.33 ± 0.11</td>
<td>14</td>
</tr>
<tr>
<td>WT-22</td>
<td>-31.22 ± 3.12</td>
<td>2.88 ± 0.20</td>
<td>9</td>
</tr>
<tr>
<td>WT-34</td>
<td>-40.22 ± 2.39</td>
<td>3.97 ± 0.33</td>
<td>10</td>
</tr>
<tr>
<td>WT-Q\textsubscript{10}</td>
<td>1.13</td>
<td>1.26</td>
<td></td>
</tr>
<tr>
<td>RQ-10</td>
<td>-25.82 ± 2.14</td>
<td>2.05 ± 0.09</td>
<td>15</td>
</tr>
<tr>
<td>RQ-22</td>
<td>-38.12 ± 1.58</td>
<td>3.34 ± 0.32</td>
<td>7</td>
</tr>
<tr>
<td>RQ-34</td>
<td>-36.56 ± 1.69</td>
<td>3.70 ± 0.15</td>
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</tr>
<tr>
<td>RQ-Q\textsubscript{10}</td>
<td>1.13</td>
<td>1.24</td>
<td></td>
</tr>
<tr>
<td>EK-10</td>
<td>-17.22 ± 1.82</td>
<td>1.76 ± 0.07</td>
<td>11</td>
</tr>
<tr>
<td>EK-22</td>
<td>-25.85 ± 2.09</td>
<td>2.40 ± 0.13</td>
<td>16</td>
</tr>
<tr>
<td>EK-34</td>
<td>-32.46 ± 1.79</td>
<td>3.28 ± 0.23</td>
<td>23</td>
</tr>
<tr>
<td>EK-Q\textsubscript{10}</td>
<td>1.28</td>
<td>1.30</td>
<td></td>
</tr>
</tbody>
</table>

*p<0.0001 vs. 22 ºC and 34 ºC of same channel variant

2.4.2. Fast Inactivation

Normalized currents obtained from the SSFI protocols are plotted as a function of membrane potential (Figure 2-2). The E1784K SSFI-V\textsubscript{1/2} was hyperpolarized (p<0.0001) compared to WT and R1193Q at all temperatures measured (Figure 2-2 and Table 2-4). At 34 ºC, SSFI-V\textsubscript{1/2} was hyperpolarized (p<0.01) compared to 22 ºC and 10 ºC (Figure 2-2 and Table 2-4). The differences in SSFI-V\textsubscript{1/2} due to temperature were not significantly different between the channel variants (p>0.05, Figure 2-2). The Q\textsubscript{10} values reported in Table 2-4 are almost identical between the different channel variants.

The E1784K mutant had a lower (p<0.0001) steady-state fast inactivation slope (SSFI-z) than R1193Q and WT (Table 2-4). The WT SSFI-z was increased (p<0.0001) when temperature was elevated from 10 ºC to 22 ºC (Table 2-4). In R1193Q, the SSFI-z was increased (p<0.0001) between 10 ºC and 34 ºC (Table 2-4). Q\textsubscript{10} fit values are relatively consistent between the different channel variants reported in Table 2-4.
Time constants measured from single exponential fits to the recovery from fast inactivation at -130 mV are shown in Figure 2-3. Insets in Figure 2-3 show time constants plotted against the membrane potential. The values of the time constants (ms) are reported in Table 2-5 and Table 2-6, for time constants between -130 mV to -70 mV, and -50 mV to +10 mV, respectively. E1784K had enhanced fast inactivation kinetics between -90 mV to +10 mV compared to R1193Q and WT (p<0.01, Figure 2-3). Fast inactivation kinetics of all channel variants between -130 mV to -90 mV, and -30 mV to -10 mV had a significantly larger (p<0.01) time constant (decelerated kinetics) at 10 °C compared to 22 °C and 34 °C, which were not significantly different (Table 2-5 and Table 2-6). At -70 mV, both WT and R1193Q fast inactivation time constants decrease significantly from 10 °C to 34 °C (p<0.01) compared to a minor (p>0.05) change in E1784K. At -50 mV, WT and E1784K channels had reduced time constants when temperature increased from 10 °C to 34 °C compared to R1193Q which decreased from 10 °C to 22 °C (p<0.01). The Q10 fit values for the different channel variants are reported in Table 2-5 and Table 2-6.
Figure 2-3  Temperature effects on fast inactivation kinetics
Panels A-B show the recovery from fast inactivation at -130 mV as normalized currents versus recovery time duration. The insets show the fast inactivation single-exponential time constants ($\tau$, ms) plotted against the membrane potential. Panels C shows fast inactivation $Q_{10}$s for all three channel variants plotted against the membrane potential.
Table 2-5 Temperature -130 mV and -70 mV Fast inactivation time constants (ms)

<table>
<thead>
<tr>
<th>Condition</th>
<th>-130 mV τ</th>
<th>n</th>
<th>-110 mV τ</th>
<th>n</th>
<th>-90 mV τ</th>
<th>n</th>
<th>-70 mV τ</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-10</td>
<td>18.0 ± 1.8</td>
<td>5</td>
<td>36.4 ± 5.2</td>
<td>5</td>
<td>176.0 ± 52.5</td>
<td>4</td>
<td>55.0 ± 10.0</td>
<td>4</td>
</tr>
<tr>
<td>WT-22</td>
<td>5.5 ± 1.3</td>
<td>6</td>
<td>12.4 ± 2.6</td>
<td>5</td>
<td>46.5 ± 8.3</td>
<td>4</td>
<td>57.2 ± 3.1</td>
<td>5</td>
</tr>
<tr>
<td>WT-34</td>
<td>3.0 ± 0.5</td>
<td>5</td>
<td>6.2 ± 0.8</td>
<td>5</td>
<td>19.9 ± 2.5</td>
<td>5</td>
<td>8.2 ± 2.1</td>
<td>5</td>
</tr>
<tr>
<td>WT-Q10</td>
<td>1.86</td>
<td>5</td>
<td>1.92</td>
<td>4</td>
<td>2.18</td>
<td>4</td>
<td>4.03</td>
<td>5</td>
</tr>
<tr>
<td>RQ-10</td>
<td>15.4 ± 1.8</td>
<td>7</td>
<td>54.4 ± 6.8</td>
<td>4</td>
<td>103.2 ± 19.2</td>
<td>4</td>
<td>55.5 ± 6.2</td>
<td>4</td>
</tr>
<tr>
<td>RQ-22</td>
<td>4.4 ± 0.2</td>
<td>5</td>
<td>14.7 ± 4.4</td>
<td>5</td>
<td>57.2 ± 6.2</td>
<td>5</td>
<td>38.5 ± 10.5</td>
<td>5</td>
</tr>
<tr>
<td>RQ-34</td>
<td>3.4 ± 0.6</td>
<td>5</td>
<td>9.6 ± 2.2</td>
<td>5</td>
<td>23.7 ± 4.2</td>
<td>4</td>
<td>12.6 ± 3.0</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 2-6 Temperature -50 mV and +10 mV Fast inactivation time constants (ms)

<table>
<thead>
<tr>
<th>Condition</th>
<th>-50 mV</th>
<th>n</th>
<th>-30 mV</th>
<th>n</th>
<th>-10 mV</th>
<th>n</th>
<th>+10 mV</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-10</td>
<td>24.40 ± 4.86</td>
<td>4</td>
<td>7.39 ± 0.48</td>
<td>9</td>
<td>4.96 ± 0.29</td>
<td>9</td>
<td>4.34 ± 0.29</td>
<td>9</td>
</tr>
<tr>
<td>WT-22</td>
<td>20.10 ± 5.11</td>
<td>5</td>
<td>1.98 ± 0.29</td>
<td>8</td>
<td>0.96 ± 0.10</td>
<td>8</td>
<td>0.67 ± 0.09</td>
<td>8</td>
</tr>
<tr>
<td>WT-34</td>
<td>2.09 ± 0.59</td>
<td>5</td>
<td>0.88 ± 0.16</td>
<td>8</td>
<td>0.61 ± 0.13</td>
<td>8</td>
<td>0.45 ± 0.09</td>
<td>8</td>
</tr>
<tr>
<td>WT-Q10</td>
<td>5.80</td>
<td>5</td>
<td>1.71</td>
<td>1.96</td>
<td>1.71</td>
<td>1.96</td>
<td>1.71</td>
<td>1.96</td>
</tr>
<tr>
<td>RQ-10</td>
<td>23.60 ± 3.69</td>
<td>4</td>
<td>7.08 ± 0.42</td>
<td>10</td>
<td>4.49 ± 0.35</td>
<td>10</td>
<td>3.76 ± 0.24</td>
<td>10</td>
</tr>
<tr>
<td>RQ-22</td>
<td>3.34 ± 0.54</td>
<td>6</td>
<td>1.29 ± 0.10</td>
<td>6</td>
<td>0.73 ± 0.03</td>
<td>6</td>
<td>0.53 ± 0.04</td>
<td>6</td>
</tr>
<tr>
<td>RQ-34</td>
<td>1.80 ± 0.27</td>
<td>11</td>
<td>0.75 ± 0.12</td>
<td>11</td>
<td>0.43 ± 0.07</td>
<td>11</td>
<td>0.35 ± 0.04</td>
<td>11</td>
</tr>
<tr>
<td>RQ-Q10</td>
<td>2.02</td>
<td>10</td>
<td>1.89</td>
<td>1.90</td>
<td>1.89</td>
<td>1.90</td>
<td>1.89</td>
<td>1.90</td>
</tr>
<tr>
<td>EK-10</td>
<td>8.59 ± 0.71</td>
<td>6</td>
<td>4.42 ± 0.36</td>
<td>8</td>
<td>3.47 ± 0.26</td>
<td>8</td>
<td>3.20 ± 0.21</td>
<td>8</td>
</tr>
<tr>
<td>EK-22</td>
<td>1.73 ± 0.23</td>
<td>10</td>
<td>0.90 ± 0.12</td>
<td>10</td>
<td>0.58 ± 0.06</td>
<td>10</td>
<td>0.49 ± 0.05</td>
<td>10</td>
</tr>
<tr>
<td>EK-34</td>
<td>0.91 ± 0.18</td>
<td>10</td>
<td>0.45 ± 0.07</td>
<td>10</td>
<td>0.36 ± 0.05</td>
<td>10</td>
<td>0.31 ± 0.05</td>
<td>10</td>
</tr>
<tr>
<td>EK-Q10</td>
<td>1.99</td>
<td>10</td>
<td>2.03</td>
<td>1.84</td>
<td>2.03</td>
<td>1.84</td>
<td>2.03</td>
<td>1.84</td>
</tr>
</tbody>
</table>

2.4.3. Late Sodium Current

We show representative normalized current traces of late $I_{Na}$ for the channel variants only at 22 °C and 34 °C in Figure 2-4. Figure 2-4 includes bar graphs of late $I_{Na}$ percent along with the Q10 values. We eliminated 10 °C late current traces since currents at 10 °C took a longer time to reach plateau. The late $I_{Na}$ percentage was larger in E1784K compared to WT and R1193Q ($p<0.0001$, Table 2-7). This effect is only present at 34 °C compared to 22 °C ($p<0.0001$, Figure 2-4 and Table 2-7). Late $I_{Na}$ percent was increased by 2.67 % ± 0.46% in E1784K compared to a minor change in WT and R1193Q when temperature was elevated from 22 °C to 34 °C (Figure 2-4 and Table 2-7). Late $I_{Na}$ density was also increased in E1784K in a temperature-dependent fashion.
(Table 2-7). The Q_{10} fit values reported in Table 2-7 are consistent with a higher thermosensitivity in E1784K compared to WT and R1193Q. At febrile temperatures (41 °C) E1784K has 9.24% late current.

**Figure 2-4** Temperature effects on late I_{Na}
Panels A-B show normalized current plotted as a function of time with insets that focus on narrower current window to show late I_{Na}. The pulse protocol inset is shown in Panel A. Panel C shows the late I_{Na} percent as a function of channel variant and temperature. Panel D shows the Q_{10} values for late I_{Na} of all the channel variants.

<table>
<thead>
<tr>
<th>Table 2-7</th>
<th>Temperature Late I_{Na}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condition</td>
<td>Late (%)</td>
</tr>
<tr>
<td>WT-22</td>
<td>0.74 ± 0.18</td>
</tr>
<tr>
<td>WT-34</td>
<td>1.67 ± 0.39</td>
</tr>
<tr>
<td>WT-Q_{10}</td>
<td>1.98</td>
</tr>
<tr>
<td>RQ-22</td>
<td>0.64 ± 0.14</td>
</tr>
<tr>
<td>RQ-34</td>
<td>1.28 ± 0.29</td>
</tr>
<tr>
<td>RQ-Q_{10}</td>
<td>1.78</td>
</tr>
<tr>
<td>EK-22</td>
<td>1.53 ± 0.30</td>
</tr>
<tr>
<td>EK-34</td>
<td>4.20 ± 0.52 *[1]</td>
</tr>
<tr>
<td>EK-Q_{10}</td>
<td>2.32</td>
</tr>
</tbody>
</table>

*1 p<0.05 vs. 22 °C of same conditions

**2.4.4. Use-dependent Inactivation**

Normalized currents from use-dependent inactivation (1 Hz) are plotted against time in Figure 2-5. The decay of current was best fit by a double exponential equation.
The $\tau_1$ was unaffected by the channel variant or temperature, separately (Figure 2-5 and Table 2-8); however, there was an interaction effect on $\tau_1$ ($p<0.05$). When temperature increased from 10 °C to 34 °C, $\tau_1$ decreased in E1784K by 8.21 s ± 2.47 s compared to WT and R1193Q (Figure 2-5 and Table 2-8). The $\tau_2$ value significantly decreased in E1784K ($p<0.05$) by 82.8 s ± 26.5 s when temperature was increased from 10 °C to 34 °C (Figure 2-5 and Table 2-8). The UDI plateau ($y_0$) decreased by 0.21 ± 0.07 in E1784K when temperature was increased from 10 °C to 34 °C ($p<0.05$) as opposed to minor shifts in WT and R1193Q (Figure 2-5 and Table 2-8). The reported $Q_{10}$ fit values in Table 2-8 are consistent with a markedly heightened thermosensitivity in E1784K $\tau_1$, $\tau_2$ and $y_0$ compared to R1193Q and WT.

To indirectly measure elevated heart rate effects on channel function, we elicited use-dependent inactivation at 3 Hz. Normalized currents from 3 Hz use-dependent inactivation protocols are plotted against time in Figure 2-5. The $\tau_1$ was not significantly affected by channel variants or temperature, separately. However, when temperature was elevated from 10 °C to 34 °C, $\tau_1$ increased and decreased ($p<0.01$) in WT and E1784K, respectively compared to a non-significant change in R1193Q (Figure 2-5 and Table 2-8). Similar shifts were observed in $\tau_2$ ($p<0.01$) when temperature was elevated from 10 °C to 34 °C. The UDI (3 Hz) $y_0$ was not significantly affected by channel variant, temperature, and the interaction between both factors ($p>0.05$, Figure 2-5).
Figure 2-5  Temperature effects on $I_{Na}$ use-dependence at 1Hz and 3Hz
Panels AI-AIII show normalized current plotted as a function of time measured at 1Hz. The inset in panel AIII includes the pulse protocols used to measure UDI 1 Hz. Panel AIV shows the UDI 1Hz $Q_{10}$ of both the fast and slow rates as a function of channel variants. Panels BI-BIII show normalized current plotted as a function of time measured at 3Hz. The inset in panel BIII includes the pulse protocols used to measure UDI 3 Hz. BIV shows the UDI 3Hz $Q_{10}$ of both the fast and slow rates as a function of channel variants.
<table>
<thead>
<tr>
<th>Condition</th>
<th>1Hz-(y_0)</th>
<th>1Hz-(\tau_1) (s)</th>
<th>1Hz-(\tau_2) (s)</th>
<th>N</th>
<th>3Hz-(y_0)</th>
<th>3Hz-(\tau_1) (s)</th>
<th>3Hz-(\tau_2) (s)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-10</td>
<td>0.44 ± 0.07</td>
<td>4.52 ± 1.31</td>
<td>96.33 ± 8.77</td>
<td>4</td>
<td>0.45 ± 0.07</td>
<td>0.55 ± 0.39&lt;sup&gt;2&lt;/sup&gt;</td>
<td>11.30 ± 4.86</td>
<td>6</td>
</tr>
<tr>
<td>WT-22</td>
<td>0.62 ± 0.07</td>
<td>7.01 ± 0.74</td>
<td>105.30 ± 12.92</td>
<td>5</td>
<td>0.55 ± 0.07</td>
<td>2.91 ± 0.43</td>
<td>49.24 ± 7.04</td>
<td>6</td>
</tr>
<tr>
<td>WT-34</td>
<td>0.52 ± 0.05</td>
<td>7.59 ± 2.76</td>
<td>66.83 ± 18.32</td>
<td>5</td>
<td>0.47 ± 0.05</td>
<td>3.52 ± 0.99</td>
<td>69.22 ± 21.03&lt;sup&gt;3&lt;/sup&gt;</td>
<td>6</td>
</tr>
<tr>
<td>WT-Q&lt;sub&gt;10&lt;/sub&gt;</td>
<td>1.06</td>
<td>0.97</td>
<td>1.44</td>
<td></td>
<td></td>
<td>1.06</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>RQ-10</td>
<td>0.52 ± 0.07</td>
<td>8.27 ± 2.22</td>
<td>115.16 ± 28.17</td>
<td>6</td>
<td>0.54 ± 0.06</td>
<td>1.33 ± 0.21</td>
<td>31.77 ± 4.65</td>
<td>6</td>
</tr>
<tr>
<td>RQ-22</td>
<td>0.45 ± 0.05</td>
<td>5.16 ± 0.87</td>
<td>112.23 ± 9.27</td>
<td>6</td>
<td>0.53 ± 0.07</td>
<td>2.22 ± 0.42</td>
<td>50.54 ± 9.43</td>
<td>6</td>
</tr>
<tr>
<td>RQ-34</td>
<td>0.58 ± 0.05</td>
<td>5.41 ± 1.26</td>
<td>75.96 ± 22.01</td>
<td>7</td>
<td>0.63 ± 0.06</td>
<td>2.42 ± 0.67</td>
<td>25.61 ± 4.63</td>
<td>6</td>
</tr>
<tr>
<td>RQ-Q&lt;sub&gt;10&lt;/sub&gt;</td>
<td>1.05</td>
<td>1.07</td>
<td>2.00</td>
<td></td>
<td></td>
<td>1.07</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>EK-10</td>
<td>0.58 ± 0.03&lt;sup&gt;1&lt;/sup&gt;</td>
<td>10.66 ± 2.82&lt;sup&gt;1&lt;/sup&gt;</td>
<td>125.85 ± 23.32&lt;sup&gt;1&lt;/sup&gt;</td>
<td>5</td>
<td>0.62 ± 0.04</td>
<td>4.82 ± 1.53&lt;sup&gt;2&lt;/sup&gt;</td>
<td>68.37 ± 17.76</td>
<td>5</td>
</tr>
<tr>
<td>EK-22</td>
<td>0.51 ± 0.03</td>
<td>7.98 ± 2.17</td>
<td>116.01 ± 15.02</td>
<td>4</td>
<td>0.56 ± 0.03</td>
<td>2.45 ± 0.29</td>
<td>81.27 ± 11.88</td>
<td>7</td>
</tr>
<tr>
<td>EK-34</td>
<td>0.37 ± 0.07</td>
<td>2.44 ± 0.70</td>
<td>43.05 ± 10.37</td>
<td>6</td>
<td>0.51 ± 0.05</td>
<td>1.28 ± 0.28</td>
<td>27.23 ± 7.74&lt;sup&gt;3&lt;/sup&gt;</td>
<td>6</td>
</tr>
<tr>
<td>EK-Q&lt;sub&gt;10&lt;/sub&gt;</td>
<td>0.84</td>
<td>5.18</td>
<td>2.28</td>
<td></td>
<td>0.92</td>
<td>1.54</td>
<td>1.77</td>
<td></td>
</tr>
</tbody>
</table>

*<sup>1</sup>p<0.05 vs EK 34 °C, <sup>2</sup>p<0.01 vs. 34 °C of same channel variant, <sup>3</sup>p<0.01 vs. 22 °C of same channel variant
2.4.5. Slow Inactivation

Slow inactivation onset was measured with a double-pulse protocol and fit with a double exponential curve. The $\tau_2$ and $y_0$ values were not affected by channel variant, temperature or both interactions ($p>0.05$). The $\tau_1$ value was affected significantly by temperature ($p<0.01$), where the slow inactivation onset kinetics was enhanced at 10 °C compared to 22 °C and 34 °C in all channel variants (Table 2-9).

### Table 2-9 Temperature Slow Inactivation Onset

<table>
<thead>
<tr>
<th>Condition</th>
<th>$\text{Slonset-y}_0$</th>
<th>$\text{Slonset-}\tau_1$ (s)</th>
<th>$\text{Slonset-}\tau_2$ (s)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-10</td>
<td>0.29 ± 0.04</td>
<td>0.16 ± 0.04$^\dagger$</td>
<td>21.27 ± 3.73</td>
<td>6</td>
</tr>
<tr>
<td>WT-22</td>
<td>0.25 ± 0.05</td>
<td>3.89 ± 1.28</td>
<td>23.83 ± 8.57</td>
<td>4</td>
</tr>
<tr>
<td>WT-34</td>
<td>0.32 ± 0.05</td>
<td>2.49 ± 0.84</td>
<td>19.31 ± 6.30</td>
<td>5</td>
</tr>
<tr>
<td>RQ-10</td>
<td>0.31 ± 0.04</td>
<td>0.12 ± 0.04$^\dagger$</td>
<td>17.29 ± 3.63</td>
<td>7</td>
</tr>
<tr>
<td>RQ-22</td>
<td>0.29 ± 0.05</td>
<td>2.14 ± 1.00</td>
<td>14.97 ± 3.96</td>
<td>6</td>
</tr>
<tr>
<td>RQ-34</td>
<td>0.21 ± 0.03</td>
<td>1.68 ± 0.35</td>
<td>17.61 ± 2.33</td>
<td>5</td>
</tr>
<tr>
<td>EK-10</td>
<td>0.31 ± 0.06</td>
<td>1.33 ± 1.07$^\dagger$</td>
<td>21.95 ± 4.29</td>
<td>6</td>
</tr>
<tr>
<td>EK-22</td>
<td>0.23 ± 0.05</td>
<td>4.02 ± 1.73</td>
<td>13.71 ± 2.38</td>
<td>5</td>
</tr>
<tr>
<td>EK-34</td>
<td>0.23 ± 0.04</td>
<td>2.99 ± 0.84</td>
<td>11.78 ± 4.63</td>
<td>6</td>
</tr>
</tbody>
</table>

$^\dagger p<0.01$ vs 22 °C and 34 °C of same channel variant

SSSI measurement and ANOVA analysis were only limited to the channel variant (3 levels) and the temperature factor with only two levels (10 °C and 22 °C). The 34 °C data were excluded from the analysis since we were not able to record a full steady-state slow inactivation for E1784K at 34 °C. The SSSI midpoint (SSSI-V1/2) for E1784K (34 °C) was hyperpolarized. To obtain a full plateau the prepulse potential was reduced to potentials like -170 mV. With cellular instability at patch temperatures of 34 °C, cells did not survive this protocol. The SSSI-V1/2 in all channel variants were unaffected by temperature ($p>0.05$, Table 2-10). The SSSI slope and plateau were unaffected by channel variant, temperature, or their interaction ($p>0.05$).
Table 2-10  Temperature Steady-State Slow Inactivation

<table>
<thead>
<tr>
<th>Condition</th>
<th>SSSI-V_{1/2}</th>
<th>SSSI-z</th>
<th>SSSI-y0</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-10</td>
<td>-105.35 ± 3.18</td>
<td>-2.70 ± 0.07</td>
<td>0.27 ± 0.04</td>
<td>8</td>
</tr>
<tr>
<td>WT-22</td>
<td>-95.27 ± 4.04</td>
<td>-2.27 ± 0.57</td>
<td>0.26 ± 0.04</td>
<td>4</td>
</tr>
<tr>
<td>WT-34</td>
<td>-85.76 ± 5.90</td>
<td>-1.97 ± 0.58</td>
<td>0.40 ± 0.06</td>
<td>4</td>
</tr>
<tr>
<td>RQ-10</td>
<td>-100.86 ± 5.21</td>
<td>-2.89 ± 0.64</td>
<td>0.34 ± 0.04</td>
<td>7</td>
</tr>
<tr>
<td>RQ-22</td>
<td>-94.76 ± 6.57</td>
<td>-1.85 ± 0.24</td>
<td>0.30 ± 0.02</td>
<td>5</td>
</tr>
<tr>
<td>RQ-34</td>
<td>-89.03 ± 7.45</td>
<td>-1.95 ± 0.28</td>
<td>0.32 ± 0.06</td>
<td>4</td>
</tr>
<tr>
<td>EK-10</td>
<td>-110.02 ± 1.90</td>
<td>-2.27 ± 0.22</td>
<td>0.26 ± 0.04</td>
<td>6</td>
</tr>
<tr>
<td>EK-22</td>
<td>-104.39 ± 7.21</td>
<td>-1.89 ± 0.29</td>
<td>0.32 ± 0.03</td>
<td>9</td>
</tr>
<tr>
<td>EK-34</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

The \( \tau_1 \) values of slow inactivation recovery were not affected by channel variant, temperature, or both interactions (p> 0.05). The \( \tau_2 \) value was decreased at 10 ºC compared to 22 ºC and 34 ºC (p<0.01).

Table 2-11  Temperature Slow Inactivation Recovery

<table>
<thead>
<tr>
<th>Condition</th>
<th>Slrec-( \tau_1 ) (s)</th>
<th>Slrec-( \tau_2 ) (s)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-10</td>
<td>0.05 ± 0.02</td>
<td>3.30 ± 0.69</td>
<td>5</td>
</tr>
<tr>
<td>WT-22</td>
<td>0.04 ± 0.02</td>
<td>1.37 ± 0.41</td>
<td>5</td>
</tr>
<tr>
<td>WT-34</td>
<td>0.02 ± 0.00</td>
<td>0.85 ± 0.06</td>
<td>4</td>
</tr>
<tr>
<td>RQ-10</td>
<td>0.05 ± 0.02</td>
<td>2.93 ± 0.93</td>
<td>4</td>
</tr>
<tr>
<td>RQ-22</td>
<td>0.03 ± 0.02</td>
<td>1.11 ± 0.19</td>
<td>5</td>
</tr>
<tr>
<td>RQ-34</td>
<td>0.04 ± 0.01</td>
<td>2.19 ± 0.43</td>
<td>5</td>
</tr>
<tr>
<td>EK-10</td>
<td>0.02 ± 0.01</td>
<td>2.36 ± 0.35</td>
<td>3</td>
</tr>
<tr>
<td>EK-22</td>
<td>0.04 ± 0.02</td>
<td>1.73 ± 0.43</td>
<td>5</td>
</tr>
<tr>
<td>EK-34</td>
<td>0.04 ± 0.01</td>
<td>0.94 ± 0.29</td>
<td>6</td>
</tr>
</tbody>
</table>

\(^*1\) p<0.01 vs 22 ºC and 34 ºC of same channel variant

2.4.6. Action Potential Simulations

We used a modified the ten Tüsscher model to simulate epicardial and endocardial action potentials. We focused specific attention on the transmural voltage gradient between both walls of the heart. Figure 2-6 shows the 1999th and 2000th simulated APs for the different channel variants at three different physiological temperatures (34 ºC, 37 ºC, 41 ºC). The transient outward potassium current conductance was varied in the epicardium of the heart (0.400 pA/pF to 0.700 pA/pF) since its expression is heterogeneous. Across all the temperatures, the E1784K channel had a less negative resting membrane potential (RMP) compared to R1193Q and WT. The E1784K mutant cardiomyocytes showed a decrease in the initial depolarization. The decrease in E1784K AP plateau is not constant across all the temperatures. At 34 ºC the
loss of AP plateau in E1784K alternates with the second AP at 0.700 pA/pF of \(I_{Kto}\) current relative to the lower 0.400 pA/pF (Figure 2-6). At 37 °C there is a loss of AP plateau which is exacerbated at 41 °C.

![Figure 2-6](Figure 2-6: Temperature effects on cardiac action potentials at varying \(I_{Kto}\) densities)

**2.5. Discussion**

Febrile temperatures are known to unmask Brugada Syndrome (Meggiolaro *et al.*, 2013; Salinski & Worrilow, 2014). We characterized the temperature sensitivity of two mixed syndrome mutants, E1784K and R1193Q in the cardiac sodium channel \(Na_v 1.5\). Long lasting recordings at physiological temperatures are difficult to obtain due to membrane instability. Thus, channel behavior was assessed at three different, albeit non-physiological, temperatures to allow data to be extrapolated to physiologically-relevant temperatures. We found the E1784K mutant to be more temperature-sensitive than WT \(Na_v 1.5\) or R1193Q in ways that suggest temperature could be an arrhythmogenic trigger.

Previous studies on the E1784K and R1193Q mutants have shown either no effect or a decrease in channel current density (Wei *et al.*, 1999; Huang *et al.*, 2006; Makita *et al.*, 2008a). These inconsistencies may be due to differences in the expression
systems used in different studies or to the variability in current amplitudes in transiently
transfected heterologous expression systems. One previous study quantifying E1784K
expression using fluorescence showed no difference in cell surface expression between
E1784K and WT (Makita et al., 2008a). Our results using CHOK1 cells also suggest that
the E1784K mutant does not affect channel expression compared to WT.
Arrhythmogenesis in the E1784K mutant may thus be related to changes in channel
gating rather than expression. Current density in E1784K was, however, acutely
sensitive to temperature. This result should be interpreted with caution due to the
variability in currents as mentioned above. In contrast to E1784K, we show no change
in WT conductance midpoints even with a greater than 10 °C change in temperature,
consistent with previous studies (Nagatomo et al., 1998).

The greater temperature sensitivity of E1784K is reflected in the AP model, in
which E1784K mutant decreases the rise of the initial upstroke of AP and also
attenuates the epicardial AP plateau. This effect is exacerbated with elevated
temperatures and with larger $I_{K,10}$. This is not the case with R1193Q as expected from the
relatively lower $Q_{10}$ values for both current density and time to half peak $I_{Na}$. The
increase in late current in E1784K has the greatest temperature sensitivity, suggesting
temperature has the potential to be arrhythmogenic in this mixed syndrome mutant.
Although the data suggest the AP should be prolonged at greater temperature,
consistent with LQT3, the predominate effect in our AP model was a loss-of-function due
to decreased epicardial E1784K sodium currents. Decreased sodium current in E1784K
could lead to a failure to activate L-type Ca$^{2+}$ channels and a subsequent loss of the
epicardial AP plateau, causing a transmural voltage-gradient between endocardium and
epicardium typical of BrS1. In addition, greater use-dependent inactivation in the
E1784K mutant, which was not accounted for within the model, is predicted to further
exacerbate loss-of-function in $I_{Na}$, in vivo. Our in vitro data show that with higher heart
rates the stabilization in use-dependent inactivation in E1784K compared to WT is no
longer apparent. Thus, higher frequencies may partially ameliorate the biophysical
defects associated with E1784K.

At high stimulation frequencies, we observed a fast rate of entry into use-
dependent inactivation in WT below 22 °C and above 34 °C, yielding a U-shaped
temperature dependence. This is consistent with the slow inactivation onset kinetics
measured. Previous literature shows that both fast inactivation (with prepulses of at least
500 ms) and slow inactivation are stabilized in Nav1.5 and Nav1.4 channels as temperature decreases, as observed in different heterologous expression systems (Murray et al., 1990; Ruff, 1999; Carle et al., 2009). Murray et al. (1990) suggests this effect may be due to disturbances in lipid-channel interactions or metabolic disturbances affecting charge transfer across the membrane (Murray et al., 1990). Although biophysically curious, the U-shaped temperature dependence of WT channels does not appear in the range of physiological temperatures extrapolated in this study.

The effects of E1784K on activation, fast inactivation and use-dependent inactivation confirm previous studies that highlight the role of the C-terminus in sodium channel gating (Shah et al., 2006; Sarhan et al., 2012). With a Ca²⁺ signal, the C-terminus interacts with the DIII-DIV linker through the actions of calmodulin. This interaction was reported to shift the inactivation curve and decrease late currents with increased [Ca²⁺] (Shah et al., 2006; Sarhan et al., 2012). We limited our study, however, to the apo-Ca²⁺ condition, using intracellular EGTA to chelate [Ca²⁺]. Other studies reported a direct interaction between the C-terminus and the DIII-DIV linker under apo-Ca²⁺ conditions (Cormier et al., 2002; Motoike, 2004). In those studies, a series of residues following the IFM motif, PIPR (non-alpha helical structure), was thought to interact with the C-terminus. In this interpretation, the C-terminus does not affect the extent of inactivation but rather ensures that the IFM motif latch is in place upon occluding the pore. The 1885stop mutation (truncation of helix VI of C-terminus) in Nav1.5 causes a large increase in late current compared to 1921stop (intact C-terminus including helix VI) (Motoike, 2004). Helix VI, the IQ motif, plays an essential role in maintaining inactivation, preventing increases in late I<sub>Na</sub>. The E1784K mutant is in a region prior to helix I in the C-terminus (EF-hand domain). The E1784K charge reversal mutation may disrupt the interaction between helix VI and helices I-IV, thus altering the mechanism by which helix VI modulates inactivation via the DIII-DIV linker, which could explain the large increase in late I<sub>Na</sub>. Chimera studies on the DI-DII and DII-DIII linkers, as well as the C-terminus, have shown that these regions modulate channel activation and account for many isoform-specific differences (Bennett, 1999, 2001; Choi et al., 2004). Slow inactivation is stabilized when fast inactivation is removed (Featherstone et al., 1996; Richmond et al., 1998). Late current and use-dependent inactivation increases are largest in the E1784K mutant when temperature is elevated to 34 °C, consistent with the previously reported inverse relationship between fast and slow inactivation.
In conclusion, we show that the E1784K mutant shows enhanced thermosensitivity compared to WT channels and another mixed syndrome mutant, R1193Q. Heightened thermosensitivity in E1784K may play a role in arrhythmogenesis during a fever or intense exercise by prolonging the cardiac action potential or causing loss-of function.

2.6. Acknowledgments

The authors thank Dr. David Jones and Dr. Stanislav Sokolov for their contribution and their support.
Chapter 3. Differential Calcium Sensitivity in Na\textsubscript{v}1.5 Mixed Syndrome Mutants

This chapter describes the work published in (Abdelsayed et al., 2017) with minor modifications and formatting changes to suit the thesis style.

3.1. Abstracts

Introduction: Inherited arrhythmias may arise from mutations in the SCN5a gene, which encodes the cardiac voltage-gated sodium channel, Na\textsubscript{v}1.5. Mutants in Na\textsubscript{v}1.5 result in Brugada Syndrome (BrS1), Long-QT Syndrome (LQT3), or mixed syndromes (an overlap of BrS1/LQT3). Exercise is a potential arrhythmogenic trigger in mixed syndromes. We sought to determine the effects of elevated cytosolic calcium, common during exercise, in mixed syndrome Na\textsubscript{v}1.5 mutants.

Methods: We used whole-cell patch-clamp to assess the biophysical properties of Na\textsubscript{v}1.5 wild-type (WT), ∆KPQ, E1784K, 1795insD, and Q1909R mutants in Human Embryonic Kidney (HEK293) cells transiently transfected with the Na\textsubscript{v}1.5 α subunit (WT or mutants), β1 subunit, and eGFP. Voltage-dependence and kinetics were measured at approximately 0 nM, 500 nM, and 2500 nM cytosolic calcium levels. In silico, action potential (AP) model simulations were performed using a modified O’Hara-Rudy model.

Results: Elevated cytosolic calcium attenuates the late sodium current in ∆KPQ, 1795insD, and Q1909R but not in E1784K. Elevated cytosolic calcium restores steady-state slow inactivation (SSSI) to the WT-form in Q1909R, but depolarized SSSI in E1784K. Our AP simulations showed a frequency-dependent reduction of action potential duration (APD) in ∆KPQ, 1795insD, and Q1909R carriers. In E1784K, APD is relatively prolonged at both low and high heart rates, resulting in a sodium overload.

Conclusions: Cellular perturbations during exercise may affect BrS1/LQT3 patients differently depending on their individual genetic signature. Thus, exercise may be therapeutic or may be an arrhythmogenic trigger in some patients with SCN5a mutations.
3.2. Introduction

Inherited arrhythmias may arise from mutations in the SCN5a gene, which encodes the cardiac voltage-gated sodium channel (NaV1.5) (Jones et al., 2011; Priori et al., 2013). These mutations result in gain-of-function (Long QT Syndrome, LQT3) or loss-of-function (Brugada Syndrome, BrS1) in NaV1.5. Although BrS1 and LQT3 are clinically distinct, a subset of mutations simultaneously trigger both syndromes (BrS1/LQT3, Mixed Syndromes) Rivolta et al., 2001; Clancy & Rudy, 2002; Makita et al., 2008).

BrS1 and LQT3 patients have differential expressivities depending on the physiological triggers, such as exercise (Shimizu & Antzelevitch, 2000; Veldkamp et al., 2000b; Schwartz et al., 2001; Masrur et al., 2015). BrS1 patients manifest the diagnostic ST-elevation during both exercise and recovery from exercise (Amin et al., 2009; Masrur et al., 2015). In contrast, LQT phenotype manifestation during exercise is genotype-dependent (Schwartz et al., 2001). Whereas LQT1 patients have cardiac events during exercise, LQT3 is mainly associated with arrhythmia during sleep (Schwartz et al., 1995; Shimizu & Antzelevitch, 2000). Although sleep is primarily a restful physiological state, the REM phase includes exercise-like physiological properties (Somers et al., 1993). A small proportion of LQT3 patients have lethal cardiac events during exercise (Schwartz et al., 1995).

Studies showing exercise-induced QTc shortening in LQT3 patients have focused on cases positive for the ∆KPQ mutation (Schwartz et al., 1995; Chandra et al., 1998). In ∆KPQ patients, the exercise-induced QTc shortening was linked to reductions in late sodium current (late INa) caused by elevated cytosolic calcium (Potet et al., 2015). To assume that the LQT3 phenotype is rescued by exercise is highly controversial since SCN5a mutation responses to exercise-induced triggers vary (Abdelsayed et al., 2015; Chen et al., 2015). For example, the C-terminal mutant, E1784K, has greater channel availability with elevated temperature and stimulation frequencies (Abdelsayed et al., 2015), both of which are associated with exercise. E1784K carriers generally have a high phenotypic gain-of-function (LQT3) expressivity, as in the Okinawa islands, where the mutation is most prevalent, whereas a minority of patients display sinus node dysfunction, BrS1, or mixed syndrome phenotypes (Makita et al., 2008a; Takahashi et al., 2014). Another C-terminal mutant, V2016M, expresses protein kinase-induced gain-
and loss-of-function translating to epinephrine-induced LQT3 and sinus node dysfunction (Chen et al., 2015). Unfortunately, little is known regarding the response of SCN5a mutations to physiological factors accompanying exercise. To better understand the variability in response to exercise among SCN5a-mutation patients, it is critical to study Na\textsubscript{v}1.5 mutants with cellular perturbations that mimic exercise.

With elevated heart rates, a rise in sympathetic tone triggers multiple cellular cascades which elevate cytosolic calcium (Song et al., 2001; Baartscheer et al., 2003). The Na\textsubscript{v}1.5 C-terminus contains a pair of EF-hand domains and an IQ motif (Kim et al., 2004). Calcium effects are mediated via calmodulin binding to the IQ motif (Chagot et al., 2009; Miloushev et al., 2009; Van Petegem et al., 2012). The calcium-calmodulin complex interacts with the DIII-DIV linker, destabilizing inactivation, and increasing Na\textsubscript{v}1.5 channel availability during elevated heart rates (Shah et al., 2006). The presence of intact intra- and inter-C-terminal interactions are essential for calcium binding (Shah et al., 2006). We hypothesized that mixed syndrome mutants located in intracellular linkers or the C-terminus may either strengthen or weaken the calcium-calmodulin modulation of Na\textsubscript{v}1.5. Thus, elevated cytosolic calcium may alleviate or exacerbate gain/loss-of-function properties in mutants, and consequently rescuing or unmasking the phenotype. The BrS1/LQT3 mutants we studied include the DIII-DIV linker mutant, \( \Delta \)KPQ, and C-terminal mutants, E1784K, 1795insD, and Q1909R (Figure 3-1). All these mutants are located in calcium-sensitive regions known to modulate Na\textsubscript{v}1.5 fast and slow inactivation.

**Figure 3-1** Mutants characterized in Na\textsubscript{v}1.5
3.3. Methods

3.3.1. Ethical approval

The research was approved by Biohazards review 251-2012 issued by the office of the Environmental Health and Safety at Simon Fraser University, Burnaby, BC, Canada.

3.3.2. Cell Culture and Transfection

Human Embryonic Kidneys (HEK293) cells were grown at pH 7.4 in a DMEM (1×) nutrient medium (Life Technologies, NY, USA), supplemented with 10 % FBS and maintained in a humidified environment at 37 °C with 5 % CO₂. To preserve calcium-calmodulin effects on Naᵥ1.5, HEK293 cells were selected for this study since they contain a relatively high [calmodulin]free level compared to other cell lines (Black et al., 2004).

Transfection followed the procedures suggested by Qiagen. Briefly, 1.5 µg of the Naᵥ1.5 α subunit, 0.75 µg of the sodium channel β₁ subunit, and 1.5 µg of eGFP were allowed to incubate with 15 µl of polyfect transfection reagent (Qiagen) and 146 µl of unsupplemented medium for 10 minutes. The cDNA mixture was then allowed to incubate with the HEK293 cells for 8 hours before plating on coverslips.

3.3.3. Electrophysiology

Whole-cell patch clamp recordings were performed in extracellular solution containing (mM): 96 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, and 10 HEPES (pH 7.4). Solutions were titrated with CsOH. Pipettes were fabricated with a P-1000 puller using borosilicate glass (Sutter Instruments, CA, USA), dipped in dental wax to reduce capacitance, then thermally polished to a resistance of 1.0-1.5 MΩ. Voltage measurement error were minimized by using low resistance electrodes to limit series resistance between pipette and intracellular solution to 3.5 MΩ or less. Pipettes were filled with intracellular solution. For minimal cytosolic calcium levels, reported below as 0 nM, pipettes contained (mM): 130 CsF, 9.6 NaCl, 10 HEPES, and 10 EGTA titrated to pH 7.4. To mimic average and peak systolic cytosolic calcium we calculated, using the Ca-EGTA Calculator TS v1.3 –
Maxchelator, the amount of CaCl$_2$ added to bring cytosolic calcium to 500 nM and 2500 nM: 8.04 mM and 9.5 mM CaCl$_2$, respectively (Steenbergen et al., 1987; Kirschenlohr et al., 2000).

All recordings were made using an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) digitized at 20 kHz using an ITC-16 interface (HEKA Elektronik, Lambrecht, Germany). Data were acquired and low-pass-filtered (5 kHz) using PatchMaster/FitMaster software (HEKA Elektronik, Lambrecht, Germany) running on an Apple iMac (Apple Computer, Cupertino, CA). Leak subtraction was performed online using a P/4 procedure. Recordings were performed at room temperature (22 ºC). After a giga ohm resistance seal was achieved, the whole-cell configuration was attained. The holding potential between protocols was -110 mV. We recorded $I_{\text{Na}}$ from cells that expressed currents no greater than –5 nA. The average voltage error calculated for all cells used in this study (n=362) is 5.16 mV ± 0.24 mV obtained (Table 3-1). There are no differences between the voltage-errors in the different conditions (p>0.05).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Voltage Error</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - 0 nM</td>
<td>4.45 ± 0.42</td>
<td>26</td>
</tr>
<tr>
<td>WT - 500 nM</td>
<td>5.10 ± 0.81</td>
<td>14</td>
</tr>
<tr>
<td>WT - 2500 nM</td>
<td>7.17 ± 1.21</td>
<td>13</td>
</tr>
<tr>
<td>△KPQ - 0 nM</td>
<td>7.18 ± 0.82</td>
<td>33</td>
</tr>
<tr>
<td>△KPQ - 500 nM</td>
<td>5.98 ± 0.83</td>
<td>24</td>
</tr>
<tr>
<td>△KPQ - 2500 nM</td>
<td>7.67 ± 0.99</td>
<td>12</td>
</tr>
<tr>
<td>EK - 0 nM</td>
<td>6.63 ± 0.63</td>
<td>33</td>
</tr>
<tr>
<td>EK - 500 nM</td>
<td>4.49 ± 0.55</td>
<td>31</td>
</tr>
<tr>
<td>EK - 2500 nM</td>
<td>5.98 ± 0.56</td>
<td>20</td>
</tr>
<tr>
<td>1795insD - 0 nM</td>
<td>2.56 ± 0.32</td>
<td>28</td>
</tr>
<tr>
<td>1795insD - 500 nM</td>
<td>2.97 ± 0.51</td>
<td>21</td>
</tr>
<tr>
<td>1795insD - 2500 nM</td>
<td>2.98 ± 0.29</td>
<td>23</td>
</tr>
<tr>
<td>QR - 0 nM</td>
<td>5.35 ± 0.46</td>
<td>36</td>
</tr>
<tr>
<td>QR - 500 nM</td>
<td>4.33 ± 0.83</td>
<td>27</td>
</tr>
<tr>
<td>QR - 2500 nM</td>
<td>5.02 ± 0.69</td>
<td>21</td>
</tr>
</tbody>
</table>

3.3.4. TTX-subtraction experiments

To confirm the mutant-induced increases in late $I_{\text{Na}}$, we performed TTX-subtraction experiments at 0 nM cytosolic calcium, where the late $I_{\text{Na}}$ in mutant channels
was the largest. The concentration of TTX used was 40 µM in extracellular solution. The 40 µM TTX current trace was subtracted from the control (non-TTX) current trace in all conditions, to calculate the TTX-sensitive late $I_{Na}$.

### 3.3.5. Analysis and Statistics

Analysis and graphing were done using FitMaster software (HEKA Elektronik, Lambrecht, Germany) and Igor Pro (Wavemetrics, Lake Oswego, OR, USA) with statistical information derived using JMP statistical software. Statistical significance was accepted at $p < 0.05$ using a two-factor completely randomized design (CRD) ANOVA test followed by a post-hoc Tukey test. Statistical results were obtained for the channel variant, calcium, and channel variant $\times$ calcium factors. We report the statistical results for the mutants and the interaction between mutants and calcium. All values reported are given as means ± standard error of means.

### 3.3.6. Voltage Protocols

#### 3.3.6.1 Peak Sodium Current Density ($I_{Na}$)

We measured current density from the ratio of peak current amplitude in pA to the cell membrane capacitance in pF.

#### 3.3.6.2 Maximal Peak and Late Sodium Conductance Density ($G_{Na}$)

Maximal conductance density relates to membrane channel trafficking and expression. We could not directly measure maximal peak or late conductance density, rather we indirectly calculated it from the conductance at 0 mV, measured by a test pulse described under $Conductance (GV)$:

1. $G_{Na} = g_{Na}/C_M$

where $G_{Na}$ is the conductance at 0 mV, $g_{Na}$ is the conductance at 0 mV (calculated using equation 2) and $C_M$ is the membrane capacitance in pF.

#### 3.3.6.3 Conductance ($GV$)

To determine the voltage dependence of activation, we measured the peak current amplitude at test pulse potentials ranging from -100 mV to +80 mV in increments
of +10 mV for 19 ms. Prior to the test pulse, channels were allowed to recover from fast inactivation at -130 mV for 197 ms. Channel conductance was calculated from peak $I_{Na}$,

$$2. \quad g_{Na} = \frac{I_{Na}}{V - E_{rev}}$$

where $g_{Na}$ is sodium channel conductance, $I_{Na}$ is peak sodium current in response to the command potential $V$, and $E_{rev}$ is the reversal potential. Calculated values for conductance were fit with the Boltzmann function:

$$3. \quad \frac{G}{G_{max}} = \frac{1}{1 + \exp[-ze_0(V_m - V_{1/2})/kT]}$$

where $G/G_{max}$ is the normalized conductance amplitude, $V_m$ is the command potential, $z$ is the apparent valence, $e_0$ is the elementary charge, $V_{1/2}$ is the midpoint voltage, $k$ is the Boltzmann constant, and $T$ is temperature in °K.

### 3.3.6.4 Steady-State Fast Inactivation (SSFI)

The voltage-dependence of SSFI was measured by preconditioning the channels to a hyperpolarizing potential of -130 mV and then eliciting prepulses between -150 mV and +10 mV in increments of 10 mV for 500 ms. Channel availability was assessed during a test pulse to 0 mV. Different hyperpolarizing prepulse potentials (-130 mV or -150 mV) were used to obtain a plateau for the SSFI curve. Normalized current amplitude as a function of voltage was fit using the Boltzmann function:

$$4. \quad \frac{I}{I_{max}} = \frac{1}{1 + \exp(-ze_0 (V_m - V_{1/2})/kT)}$$

where $I/I_{max}$ is the normalized current amplitude, $z$ is apparent valence, $e_0$ is the elementary charge, $V_m$ is the prepulse potential, $V_{1/2}$ is the midpoint voltage of SSFI, $k$ is the Boltzmann constant, and $T$ is temperature in °K.

### 3.3.6.5 Fast Inactivation Onset

Time constants for open-state fast inactivation were derived by fitting a double exponential function to the decay of current obtained from the activation protocol. To measure closed-state fast inactivation onset, channels were preconditioned at -130 mV prior to a prepulse at -50 mV, -70 mV, or -90 mV for 0 – 0.256 s. Current amplitude was measured during a test pulse to 0 mV for 20 ms. Normalized current amplitudes as a function of time were fit using a double exponential equation:
5. \[ I = I_{ss} + \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2) \]

where \( I \) is current amplitude, \( I_{ss} \) is the plateau amplitude, \( \alpha_1 \) and \( \alpha_2 \) are the amplitudes at time 0 for time constants \( \tau_1 \) and \( \tau_2 \), and \( t \) is time.

### 3.3.6.6 Fast Inactivation Recovery

Channels were fast-inactivated during a 500 ms depolarizing step to 0 mV. Recovery was measured during a 19 ms test pulse to 0 mV following 0 – 1.024 s conditioning pulses at -130 mV, -110 mV, or -90 mV. Time constants of fast inactivation recovery as a function of time were fit using a double exponential equation, as above.

### 3.3.6.7 Late \( I_{Na} \) Current

Late \( I_{Na} \) current was measured between 450 – 500 ms during a 500 ms depolarizing pulse to 0 mV from a holding potential of -130 mV. An average of 30 pulses was used to increase the signal-to-noise ratio.

### 3.3.6.8 Steady-State Slow Inactivation (SSSI)

The voltage-dependence of SSSI was measured by preconditioning the channels to -150 mV for 30 s and then eliciting prepulse potentials that range from -150 to -10 mV in increments of 20 mV for 60 s. Channel availability was assessed during a test pulse to 0 mV following a -130 mV recovery pulse from fast inactivation at 20 ms. Normalized current amplitude as a function of voltage was fit using a modified Boltzmann function:

\[
6. \quad \frac{I}{I_{max}} = \frac{(I_1 - I_2)}{(1 + \exp(-z e_0 (V_M - V_{1/2})/kT)) + I_2}
\]

where \( I_1 \) and \( I_2 \) are maximum and minimum values of fit. The other symbols are as previously stated.

### 3.3.6.9 Slow Inactivation Onset

To measure onset into slow inactivation, channels were preconditioned at -130 mV for 30 s prior to a prepulse at 0 mV for 0 – 64 s. A test pulse to 0 mV followed a -130 mV fast inactivation recovery pulse for 20 ms. Normalized current amplitude as a function of time was fit with a double exponential.
3.3.6.10 **Slow Inactivation Recovery**

To measure recovery from slow inactivation, channels were preconditioned at -130 mV for 30 s prior to a prepulse at 0 mV for 60 s, followed by series of test pulses to 0 mV for 20 ms between increasing incremental recovery durations at -130 mV for 0 – 32s. Normalized current amplitudes as a function of time were fit using a double exponential equation with the plateau equal to 1.00.

3.3.6.11 **Weighted Slow Inactivation Time Constants**

The weighted slow inactivation time constant at each voltage was calculated using the double $\tau$ values obtained from equation 5:

7. $\tau_{\text{slow-weighted}} = ((\alpha_1 \times \tau_1) + (\alpha_2 \times \tau_2))/(\alpha_1 + \alpha_2)$

3.3.6.12 **Slow Inactivation $\alpha_j$ and $\beta_j$ Rates**

The recovery from slow inactivation (forward, $\alpha_j$) and the onset into slow inactivation (reverse, $\beta_j$) rates were calculated using Hodgkin-Huxley formulations:

8. $\alpha_j = j_\infty/\tau_j$
9. $\beta_j = (1-j_\infty)/\tau_j$

where $j_\infty$ is channel availability obtained from equation 6 and $\tau_j$ is the weighted slow inactivation time constant obtained from equation 7, at a given voltage. The slow inactivation $\alpha_j$ rates versus voltage were fit using a single exponential curve and beta rates were fit with a line with a slope of 0.

3.3.7. **Myocardial Action Potential (AP) Modeling**

Action potentials were simulated using a modified version of the O’Hara-Rudy (O’Rd) model at 37 ºC programmed in Python (O’Hara et al., 2011). The sodium data were extrapolated to physiological temperatures using previously reported Q10 values for WT, $\triangle$KoQ, and E1784K variants (Nagatomo et al., 1998; Abdelsayed et al., 2015). The thermosensitivity of 1795insD and Q1909R mutants was never reported; thus, we used the WT temperature-dependence for these mutants. The maximal $G_{Na}$ density was 75 mS/µF across all channel variants in the O’Rd model. We modified the gating $I_{Na}$
parameters data in accordance with our biophysical data for the various channel variants. The GV, SSFI, and SSSI midpoints and slopes of the channel variants extrapolated to 37 °C were normalized to the original O'Rd parameters. The normalized midpoint and slope values for GV and SSFI were then incorporated into the Boltzmann curve (equation 4) and SSSI into the modified Boltzmann curve (equation 6). The original O'Rd SSSI (j∞) curve was equated to the SSFI curve (h∞), which had no plateau. Our experimental SSSI plateaus were subtracted from the original O'Rd value of 0, to compensate for this caveat. The phosphorylated steady-state fast inactivation midpoints in all channel variants were equally hyperpolarized by 6.2 mV. Late INa density was normalized to the original O'Rd value and multiplied by the percentage of late to peak INa calculated above. The relationship between fast inactivation time constants versus voltage was calculated using an inverse double exponential distribution at 37 °C. The O'Rd slow inactivation time constant equation was used for all the channel variants since it encompassed a comprehensive range of voltages.

To model the calcium-dependence of our INa data, we fit the extrapolated biophysical parameters to 37 °C with a Hill equation:

\[ Z = Y_0 + \frac{(Y_M - Y_0)}{1 + (X_{1/2}/X)^b} \]

where Z is the biophysical parameter of interest, Y0 is the minimum value, YM is the maximum value, X1/2 is the midpoint of the curve, X is the intracellular cytosolic calcium, b is the rate.

Subspace calcium was not accounted for due to the lack of experimental data. Thus, the modified O'Rd model is a dynamic simulation of the calcium-induced shifts which are observed with increasing intracellular calcium levels as a function of pacing frequency, comprising the positive staircase phenomenon.

Simulations were run on endocardial and epicardial ventricular myocytes using a 0.5 ms stimulus pulse with an amplitude of -80 µA/µF. The stimulus protocol was designed accordingly to step up the frequency gradually from 0.5 Hz to 2.5 Hz. The stimulus protocol had a cycle length of 2000 ms for the first 75 APs, 1000 ms for the following 75 APs, 667 ms for the following 75 APs, 500 ms for the following 75 APs, 400 ms for the following 100 APs.
Analysis of APs only included those that fully recovered and were restored to baseline. Action potential duration (APD) was measured at 30 %, 60 %, and 90 % of repolarization by multiplying these percentages by the resting membrane potential (RMP) value prior to the current stimulus pulse. The \( \text{APD}_x \) (\( X=30, 60, 90 \)) values were plotted versus the diastolic interval (DI = BCL \( - \) \( \text{APD}_x \)), where BCL is the basic cycle length, creating electrical restitution curves. The curves were fitted with a double-exponential equation 5. Action potential velocity was calculated from initiation of the current stimulus to the maximal peak.

### 3.4. Results

#### 3.4.1. Peak current amplitude and conductance density

Raw \( I_{Na} \) traces are shown in Figure 3-2 for all the channel variants at 0 nM and 2500 nM. The current density of 1795insD was smaller (\( p<0.0001, \) Table 3-2) compared to WT and the rest of the mutants. Elevations in cytosolic calcium had no effect on current density in any of the channel variants (\( p>0.05, \) Figure 3-2 and Table 3-2). Peak conductance density was also reduced in 1795insD compared to WT and the mutants (\( p<0.0001, \) Figure 3-2 and Table 3-2). In \( \Delta \)KPQ, peak conductance density was larger (\( p<0.01, \) Table 3-2) by 4.59 pA/pF ± 1.06 pA/pF with 500 nM to 2500 nM elevations in cytosolic calcium. No other mutants were affected by elevations in cytosolic calcium (\( p>0.05, \) Table 3-2).
Figure 3-2  Wild type and Mutant Currents and Conductance
Raw current traces for the wild-type and mutant channels at 0 nM and 2500 nM cytosolic calcium. Panels A and B show bar graphs of peak $I_{Na}$ current density and peak $I_{Na}$ conductance density, respectively, as a function of the three cytosolic calcium levels. Panel B includes an asterisk indicating a significant rise in $\Delta KPQ$ conductance density with 500 nM to 2500 nM elevations in cytosolic calcium. Panels C shows normalized conductance versus membrane potential for the mutant effect at 0 nM cytosolic calcium.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Peak I$_{Na}$ Density (pA/pF)</th>
<th>N</th>
<th>Peak GV Density (nS/pF)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - 0 nM</td>
<td>352.99 ± 62.86</td>
<td>21</td>
<td>4.66 ± 0.57</td>
<td>19</td>
</tr>
<tr>
<td>WT - 500 nM</td>
<td>351.68 ± 44.06</td>
<td>13</td>
<td>6.01 ± 0.75</td>
<td>13</td>
</tr>
<tr>
<td>WT - 2500 nM</td>
<td>542.91 ± 124.29</td>
<td>8</td>
<td>7.58 ± 1.47</td>
<td>7</td>
</tr>
<tr>
<td>∆KPQ - 0 nM</td>
<td>358.88 ± 44.00</td>
<td>28</td>
<td>5.83 ± 0.71</td>
<td>27</td>
</tr>
<tr>
<td>∆KPQ - 500 nM</td>
<td>308.51 ± 37.56</td>
<td>20</td>
<td>4.88 ± 0.53</td>
<td>19</td>
</tr>
<tr>
<td>∆KPQ - 2500 nM</td>
<td>554.2 ± 81.95</td>
<td>11</td>
<td>9.47 ± 1.40 *1</td>
<td>11</td>
</tr>
<tr>
<td>EK - 0 nM</td>
<td>399.15 ± 40.85</td>
<td>23</td>
<td>6.44 ± 0.61</td>
<td>22</td>
</tr>
<tr>
<td>EK - 500 nM</td>
<td>333.65 ± 35.27</td>
<td>26</td>
<td>4.70 ± 0.46</td>
<td>29</td>
</tr>
<tr>
<td>EK - 2500 nM</td>
<td>419.13 ± 45.16</td>
<td>13</td>
<td>7.16 ± 0.77</td>
<td>13</td>
</tr>
<tr>
<td>1795insD - 0 nM</td>
<td>166.90 ± 27.14</td>
<td>17</td>
<td>2.85 ± 0.46</td>
<td>17</td>
</tr>
<tr>
<td>1795insD - 500 nM</td>
<td>191.35 ± 24.73</td>
<td>19</td>
<td>3.27 ± 0.42</td>
<td>19</td>
</tr>
<tr>
<td>1795insD - 2500 nM</td>
<td>211.93 ± 39.87</td>
<td>12</td>
<td>3.62 ± 0.68</td>
<td>12</td>
</tr>
<tr>
<td>QR - 0 nM</td>
<td>346.55 ± 61.56</td>
<td>25</td>
<td>4.62 ± 0.57</td>
<td>23</td>
</tr>
<tr>
<td>QR - 500 nM</td>
<td>306.78 ± 36.87</td>
<td>19</td>
<td>5.00 ± 0.64</td>
<td>20</td>
</tr>
<tr>
<td>QR - 2500 nM</td>
<td>253.19 ± 34.58</td>
<td>15</td>
<td>4.33 ± 0.59</td>
<td>15</td>
</tr>
</tbody>
</table>

*1 p<0.01 versus ∆KPQ (0 nM and 500 nM)
3.4.2. Activation voltage-dependence

Normalized conductance is plotted as a function of membrane potential in Figure 3-2. Activation midpoint (GV-V$_{1/2}$) was depolarized in all mutants compared to WT (p<0.0001, Table 3-3). The mutants depolarized GV-V$_{1/2}$ in an increasing order compared to WT: Q1909R, 1795insD, E1784K, and ΔKPQ, respectively. In all channel variants, GV-V$_{1/2}$ was not affected by elevations in cytosolic calcium (p>0.05, Table 3-3). Activation slope (GV-z) was reduced in the same order as the mutant-induced GV-V$_{1/2}$ shifts (p<0.0001, Table 3-3). Elevations in cytosolic calcium did not affect GV-z in any channel variant (p>0.05, Table 3-3).

<table>
<thead>
<tr>
<th>Condition</th>
<th>GV-V$_{1/2}$ (mV)</th>
<th>GV-z</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - 0 nM</td>
<td>-44.42 ± 1.20</td>
<td>4.87 ± 0.25</td>
<td>19</td>
</tr>
<tr>
<td>WT - 500 nM</td>
<td>-41.45 ± 1.66</td>
<td>3.79 ± 0.24</td>
<td>13</td>
</tr>
<tr>
<td>WT - 2500 nM</td>
<td>-46.95 ± 1.88</td>
<td>4.89 ± 0.38</td>
<td>8</td>
</tr>
<tr>
<td>ΔKPQ - 0 nM</td>
<td>-34.98 ± 1.30</td>
<td>2.56 ± 0.10</td>
<td>30</td>
</tr>
<tr>
<td>ΔKPQ - 500 nM</td>
<td>-34.94 ± 1.69</td>
<td>2.89 ± 0.13</td>
<td>16</td>
</tr>
<tr>
<td>ΔKPQ - 2500 nM</td>
<td>-38.46 ± 1.09</td>
<td>3.54 ± 0.16</td>
<td>11</td>
</tr>
<tr>
<td>EK - 0 nM</td>
<td>-39.03 ± 1.21</td>
<td>3.28 ± 0.13</td>
<td>23</td>
</tr>
<tr>
<td>EK - 500 nM</td>
<td>-38.20 ± 1.52</td>
<td>3.29 ± 0.16</td>
<td>30</td>
</tr>
<tr>
<td>EK -2500 nM</td>
<td>-38.38 ± 1.11</td>
<td>3.71 ± 0.22</td>
<td>13</td>
</tr>
<tr>
<td>1795insD - 0 nM</td>
<td>-41.51 ± 1.08</td>
<td>3.72 ± 0.21</td>
<td>17</td>
</tr>
<tr>
<td>1795insD - 500 nM</td>
<td>-39.43 ± 1.02</td>
<td>3.81 ± 0.22</td>
<td>19</td>
</tr>
<tr>
<td>1795insD - 2500 nM</td>
<td>-39.02 ± 1.38</td>
<td>3.29 ± 0.17</td>
<td>12</td>
</tr>
<tr>
<td>QR - 0 nM</td>
<td>-41.89 ± 1.20</td>
<td>4.14 ± 0.18</td>
<td>25</td>
</tr>
<tr>
<td>QR - 500 nM</td>
<td>-42.96 ± 1.12</td>
<td>4.03 ± 0.23</td>
<td>22</td>
</tr>
<tr>
<td>QR - 2500 nM</td>
<td>-43.18 ± 1.63</td>
<td>4.38 ± 0.16</td>
<td>16</td>
</tr>
</tbody>
</table>

3.4.3. Fast and intermediate inactivation voltage-dependence

Normalized current versus membrane potential is shown in Figure 3-3. The steady-state fast inactivation midpoint (SSFI-V$_{1/2}$) was hyperpolarized by ΔKPQ, E1784K, and 1795insD compared to WT (p<0.0001, Table 3-4). The SSFI-V$_{1/2}$ of ΔKPQ was depolarized (p<0.01) by elevations in cytosolic calcium compared to the other channel variants (p>0.05, Figure 3-3 and Table 3-4). When cytosolic calcium was elevated from 0 nM to 500 nM, the SSFI-V$_{1/2}$ of ΔKPQ depolarized by 8.98 mV ± 2.35 mV (p<0.01, Table 3-4). Additional elevations in cytosolic calcium to 2500 nM did not further depolarize the SSFI-V$_{1/2}$ of ΔKPQ (p>0.05, Figure 3-3 and Table 3-4). The steady-state fast inactivation slope (SSFI-z) was reduced in E1784K, 1795insD, and
Q1909R compared to WT (p=0.0005, Table 3-4). Elevations in cytosolic calcium had no effects on SSFI-z in any channel variant (p>0.05, Table 3-4). Steady-state intermediate inactivation (SSII) was measured using a similar protocol to SSFI, except with 1000 ms prepulse durations (Table 3-4). The SSII-V1/2 of E1784K and 1795insD are hyperpolarized compared to WT (p<0.0001, Table 3-4). Both mutants also reduced SSII-z (p<0.0001, Table 3-4) compared to WT. Elevations in cytosolic calcium had no effect on both SSII-V1/2 and SSII-z in any channel variant (p>0.05, Table 3-4).
Figure 3-3  Effects of cytosolic calcium on steady-state fast inactivation

Panels I shows the mutant effect at 0 nM cytosolic calcium and contains a voltage pulse inset. Panels A-D show the calcium effect on each individual mutant compared to WT. Normalized current versus membrane potential was fit with a Boltzmann fit.
### Table 3-4 Calcium Steady-State Fast and Intermediate Inactivation

<table>
<thead>
<tr>
<th>Condition</th>
<th>SSFI-V(_{1/2}) (mV)</th>
<th>SSFI-(z)</th>
<th>N</th>
<th>SSII-V(_{1/2}) (mV)</th>
<th>SSII-(z)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - 0 nM</td>
<td>-87.95 ± 2.06</td>
<td>-3.84 ± 0.27</td>
<td>7</td>
<td>-86.17 ± 2.21</td>
<td>-4.24 ± 0.11</td>
<td>9</td>
</tr>
<tr>
<td>WT - 500 nM</td>
<td>-89.73 ± 0.79</td>
<td>-3.91 ± 0.23</td>
<td>8</td>
<td>-90.97 ± 2.99</td>
<td>-3.87 ± 0.20</td>
<td>6</td>
</tr>
<tr>
<td>WT - 2500 nM</td>
<td>-88.24 ± 0.89</td>
<td>-3.92 ± 0.17</td>
<td>8</td>
<td>-90.56 ± 0.68</td>
<td>-3.90 ± 0.14</td>
<td>8</td>
</tr>
<tr>
<td>ΔKPQ - 0 nM</td>
<td>-102.15 ± 2.07 (^*)</td>
<td>-3.71 ± 0.12</td>
<td>17</td>
<td>-97.19 ± 2.68</td>
<td>-3.70 ± 0.19</td>
<td>7</td>
</tr>
<tr>
<td>ΔKPQ - 500 nM</td>
<td>-93.17 ± 1.06</td>
<td>-3.58 ± 0.18</td>
<td>13</td>
<td>-95.12 ± 2.64</td>
<td>-4.02 ± 0.13</td>
<td>9</td>
</tr>
<tr>
<td>ΔKPQ - 2500 nM</td>
<td>-99.83 ± 0.76</td>
<td>-3.91 ± 0.08</td>
<td>8</td>
<td>-90.98 ± 0.80</td>
<td>-3.86 ± 0.09</td>
<td>8</td>
</tr>
<tr>
<td>EK - 0 nM</td>
<td>-97.62 ± 1.84</td>
<td>-3.38 ± 0.07</td>
<td>7</td>
<td>-99.33 ± 1.34</td>
<td>-3.08 ± 0.11</td>
<td>8</td>
</tr>
<tr>
<td>EK - 500 nM</td>
<td>-97.82 ± 2.98</td>
<td>-3.32 ± 0.11</td>
<td>8</td>
<td>-100.73 ± 2.98</td>
<td>-3.27 ± 0.13</td>
<td>8</td>
</tr>
<tr>
<td>EK -2500 nM</td>
<td>-99.06 ± 2.82</td>
<td>-3.25 ± 0.13</td>
<td>8</td>
<td>-100.70 ± 1.97</td>
<td>-3.10 ± 0.10</td>
<td>8</td>
</tr>
<tr>
<td>1795insD - 0 nM</td>
<td>-99.03 ± 1.95</td>
<td>-3.71 ± 0.11</td>
<td>7</td>
<td>-100.64 ± 1.78</td>
<td>-3.78 ± 0.10</td>
<td>8</td>
</tr>
<tr>
<td>1795insD - 500 nM</td>
<td>-95.70 ± 2.03</td>
<td>-3.37 ± 0.21</td>
<td>13</td>
<td>-100.53 ± 2.45</td>
<td>-3.55 ± 0.16</td>
<td>12</td>
</tr>
<tr>
<td>1795insD - 2500 nM</td>
<td>-101.01 ± 2.37</td>
<td>-3.22 ± 0.16</td>
<td>13</td>
<td>-102.81 ± 2.24</td>
<td>-3.18 ± 0.15</td>
<td>12</td>
</tr>
<tr>
<td>QR - 0 nM</td>
<td>-86.28 ± 2.16</td>
<td>-3.28 ± 0.17</td>
<td>7</td>
<td>-90.53 ± 2.37</td>
<td>-3.53 ± 0.17</td>
<td>8</td>
</tr>
<tr>
<td>QR - 500 nM</td>
<td>-89.30 ± 1.89</td>
<td>-3.46 ± 0.16</td>
<td>7</td>
<td>-88.00 ± 2.34</td>
<td>-3.98 ± 0.37</td>
<td>7</td>
</tr>
<tr>
<td>QR - 2500 nM</td>
<td>-89.78 ± 2.51</td>
<td>-3.56 ± 0.15</td>
<td>9</td>
<td>-90.18 ± 2.33</td>
<td>-3.49 ± 0.10</td>
<td>7</td>
</tr>
</tbody>
</table>

\(^*\) \(p<0.01\) versus ΔKPQ (500 nM and 2500 nM)

### 3.4.4. Fast inactivation recovery and onset kinetics

Double-pulse protocols were used to measure onset (\(\tau_{on}\)) and recovery (\(\tau_{off}\)) kinetics of fast inactivation. The time constant (\(\tau\)) obtained from the fits to the recovery and onset curves equals the inverse of the sum of both the forward (recovery) and reverse (onset) rates. At voltages hyperpolarized relative to SSFI-V\(_{1/2}\), recovery from fast inactivation predominates. At voltages depolarized relative to SSFI-V\(_{1/2}\), onset into fast inactivation predominates. At SSFI-V\(_{1/2}\), both onset and recovery are in equilibrium.

Fast inactivation time constants as a function of voltage are reported in **Table 3-5** and **Table 3-6**. At -10 mV, the slow component (\(\tau_2\)) of the fast inactivation onset in ΔKPQ decreased when cytosolic calcium was elevated from 0 nM to 500 nM. 1795insD fast inactivation time constant (\(\tau_2\)) is increased at -30 mV when cytosolic calcium was elevated from 500 nM to 2500 nM. Q1909R fast inactivation kinetics were decelerated at both open-state and closed-state voltages with elevations in cytosolic calcium (**Table 3-5** and **Table 3-6**).
Table 3-5  Calcium -130 mV to -70 mV Fast Inactivation Time Constants (ms)

<table>
<thead>
<tr>
<th>Condition</th>
<th>-130 $\tau_1$</th>
<th>-130 $\tau_2$</th>
<th>n</th>
<th>-110 $\tau_1$</th>
<th>-110 $\tau_2$</th>
<th>n</th>
<th>-90 $\tau_1$</th>
<th>-90 $\tau_2$</th>
<th>n</th>
<th>-70 $\tau_1$</th>
<th>-70 $\tau_2$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT – 0 nM</td>
<td>3.94 ± 0.58</td>
<td>36.03</td>
<td>7</td>
<td>13.92 ± 2.04</td>
<td>83.60</td>
<td>5</td>
<td>70.37 ± 11.48</td>
<td>91.75 ± 5.50</td>
<td>10</td>
<td>43.21 ± 10.94</td>
<td>155.03 ± 27.72</td>
<td>10</td>
</tr>
<tr>
<td>WT – 500 nM</td>
<td>4.33 ± 0.49</td>
<td>89.52</td>
<td>7</td>
<td>12.50 ± 0.45</td>
<td>73.01</td>
<td>5</td>
<td>56.26 ± 7.92</td>
<td>177.29 ± 37.91</td>
<td>9</td>
<td>20.70 ± 4.16</td>
<td>91.38 ± 17.83</td>
<td>9</td>
</tr>
<tr>
<td>WT – 2500 nM</td>
<td>5.28 ± 0.44</td>
<td>24.52</td>
<td>7</td>
<td>17.25 ± 0.86</td>
<td>14.30</td>
<td>7</td>
<td>75.67 ± 5.82</td>
<td>94.14 ± 8.63</td>
<td>7</td>
<td>17.93 ± 1.54</td>
<td>82.97 ± 15.41</td>
<td>7</td>
</tr>
<tr>
<td>$\Delta$KPQ – 0 nM</td>
<td>3.10 ± 0.26</td>
<td>6.61</td>
<td>7</td>
<td>7.96 ± 0.71</td>
<td>17.35</td>
<td>7</td>
<td>8.47 ± 1.05</td>
<td>29.45 ± 8.37</td>
<td>8</td>
<td>3.80 ± 0.65</td>
<td>24.97 ± 12.37</td>
<td>6</td>
</tr>
<tr>
<td>$\Delta$KPQ – 500 nM</td>
<td>3.00 ± 0.25</td>
<td>13.39</td>
<td>7</td>
<td>7.16 ± 0.45</td>
<td>16.23</td>
<td>8</td>
<td>8.67 ± 1.04</td>
<td>29.21 ± 10.68</td>
<td>8</td>
<td>3.44 ± 0.41</td>
<td>10.51 ± 6.88</td>
<td>8</td>
</tr>
<tr>
<td>$\Delta$KPQ – 2500 nM</td>
<td>3.33 ± 0.23</td>
<td>12.93</td>
<td>7</td>
<td>8.37 ± 0.71</td>
<td>36.71</td>
<td>7</td>
<td>10.02 ± 0.88</td>
<td>52.00 ± 19.54</td>
<td>6</td>
<td>6.41 ± 0.38</td>
<td>28.55 ± 15.48</td>
<td>6</td>
</tr>
<tr>
<td>EK – 0 nM</td>
<td>3.04 ± 0.30</td>
<td>70.23</td>
<td>7</td>
<td>7.48 ± 0.47</td>
<td>23.74</td>
<td>5</td>
<td>9.17 ± 1.02</td>
<td>31.89 ± 7.28</td>
<td>7</td>
<td>3.76 ± 0.48</td>
<td>16.85 ± 5.46</td>
<td>7</td>
</tr>
<tr>
<td>EK – 500 nM</td>
<td>3.25 ± 0.53</td>
<td>24.57</td>
<td>7</td>
<td>7.89 ± 1.25</td>
<td>58.34</td>
<td>6</td>
<td>7.45 ± 1.06</td>
<td>20.34 ± 5.41</td>
<td>7</td>
<td>3.60 ± 0.82</td>
<td>20.19 ± 7.54</td>
<td>7</td>
</tr>
<tr>
<td>EK – 2500 nM</td>
<td>5.06 ± 0.86</td>
<td>56.86</td>
<td>7</td>
<td>12.90 ± 1.88</td>
<td>60.28 ± 17.81</td>
<td>7</td>
<td>11.02 ± 2.06</td>
<td>61.37 ± 22.90</td>
<td>7</td>
<td>3.81 ± 0.56</td>
<td>13.38 ± 2.92</td>
<td>7</td>
</tr>
<tr>
<td>179insD – 0 nM</td>
<td>8.60 ± 1.23</td>
<td>32.04</td>
<td>7</td>
<td>24.90 ± 3.65</td>
<td>84.10</td>
<td>6</td>
<td>21.87 ± 5.41</td>
<td>79.80 ± 12.43</td>
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<td>10.44 ± 2.10</td>
<td>29.19 ± 5.89</td>
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<tr>
<td>179insD – 500 nM</td>
<td>6.37 ± 1.00</td>
<td>156.73</td>
<td>12</td>
<td>19.95 ± 3.54</td>
<td>39.38</td>
<td>7</td>
<td>16.37 ± 2.67</td>
<td>78.86 ± 9.55</td>
<td>15</td>
<td>10.95 ± 1.98</td>
<td>37.08 ± 5.40</td>
<td>14</td>
</tr>
<tr>
<td>179insD – 2500 nM</td>
<td>6.44 ± 0.44</td>
<td>29.90</td>
<td>10</td>
<td>17.68 ± 2.29</td>
<td>46.22</td>
<td>8</td>
<td>37.03 ± 15.64</td>
<td>91.95 ± 21.14</td>
<td>6</td>
<td>3.54 ± 1.08</td>
<td>22.99 ± 5.19</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>9.92 ± 2.10</td>
<td>145.74 ± 26.44</td>
<td>5</td>
<td>58.39 ± 12.43</td>
<td>80.10 ± 14.21</td>
<td>6</td>
<td>26.72 ± 6.32</td>
<td>89.13 ± 11.00</td>
<td>5</td>
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</tr>
<tr>
<td>QR – 0 nM</td>
<td>3.43 ± 0.40</td>
<td>68.023 ± 11.15</td>
<td>6</td>
<td>9.92 ± 2.10</td>
<td>145.74 ± 26.44</td>
<td>5</td>
<td>58.39 ± 12.43</td>
<td>80.10 ± 14.21</td>
<td>6</td>
<td>26.72 ± 6.32</td>
<td>89.13 ± 11.00</td>
<td>5</td>
</tr>
<tr>
<td>QR – 500 nM</td>
<td>4.72 ± 0.84</td>
<td>116.14 ± 31.40</td>
<td>7</td>
<td>10.86 ± 2.23</td>
<td>227.29 ± 39.76</td>
<td>10</td>
<td>51.69 ± 19.72</td>
<td>48.62 ± 6.98</td>
<td>9</td>
<td>18.96 ± 5.03</td>
<td>74.54 ± 10.70</td>
<td>9</td>
</tr>
<tr>
<td>QR – 2500 nM</td>
<td>5.85 ± 0.59</td>
<td>128.70 ± 28.24</td>
<td>6</td>
<td>20.74 ± 1.82</td>
<td>160.05 ± 45.20</td>
<td>6</td>
<td>83.22 ± 35.21</td>
<td>136.73 ± 35.41</td>
<td>7</td>
<td>33.25 ± 10.01</td>
<td>102.47 ± 27.95</td>
<td>8</td>
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</tbody>
</table>

*p<0.001 versus 0 nM and 500 nM Q1909R, *2 p<0.05 versus 0 nM WT
<table>
<thead>
<tr>
<th>Condition</th>
<th>-50 $\tau_1$</th>
<th>-50 $\tau_2$</th>
<th>n</th>
<th>-30 $\tau_1$</th>
<th>-30 $\tau_2$</th>
<th>n</th>
<th>-10 $\tau_1$</th>
<th>-10 $\tau_2$</th>
<th>n</th>
<th>+10 $\tau_1$</th>
<th>+10 $\tau_2$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT – 0 nM</td>
<td>6.32 ± 1.00</td>
<td>14.05 ± 6.12</td>
<td>10</td>
<td>0.88 ± 0.07</td>
<td>3.23 ± 0.23</td>
<td>21</td>
<td>0.50 ± 0.02</td>
<td>2.56 ± 0.44</td>
<td>21</td>
<td>0.39 ± 0.02</td>
<td>4.19 ± 1.80</td>
<td>18</td>
</tr>
<tr>
<td>WT – 500 nM</td>
<td>3.09 ± 0.2</td>
<td>3.45 ± 0.47</td>
<td>5</td>
<td>0.93 ± 0.10</td>
<td>3.36 ± 0.35</td>
<td>16</td>
<td>0.55 ± 0.05</td>
<td>3.25 ± 0.36</td>
<td>15</td>
<td>0.39 ± 0.05</td>
<td>1.97 ± 0.37</td>
<td>14</td>
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<tr>
<td>WT – 2500 nM</td>
<td>4.41 ± 0.47</td>
<td>4.51 ± 0.58</td>
<td>7</td>
<td>0.93 ± 0.10</td>
<td>6.35 ± 2.85</td>
<td>8</td>
<td>0.58 ± 0.03</td>
<td>1.99 ± 0.46</td>
<td>8</td>
<td>0.44 ± 0.02</td>
<td>2.68 ± 0.75</td>
<td>8</td>
</tr>
<tr>
<td>∆KPQ –0 nM</td>
<td>1.41 ± 0.22</td>
<td>1.42 ± 0.22</td>
<td>6</td>
<td>0.48 ± 0.02</td>
<td>3.68 ± 0.62</td>
<td>34</td>
<td>0.45 ± 0.02 *3</td>
<td>5.46 ± 1.35</td>
<td>33</td>
<td>0.46 ± 0.02</td>
<td>4.95 ± 1.98</td>
<td>32</td>
</tr>
<tr>
<td>∆KPQ –500 nM</td>
<td>1.00 ± 0.09</td>
<td>2.16 ± 0.85</td>
<td>8</td>
<td>0.58 ± 0.05</td>
<td>3.38 ± 0.68</td>
<td>19</td>
<td>0.50 ± 0.03</td>
<td>3.99 ± 1.01</td>
<td>19</td>
<td>0.45 ± 0.02</td>
<td>3.89 ± 0.70</td>
<td>14</td>
</tr>
<tr>
<td>∆KPQ –2500 nM</td>
<td>1.47 ± 0.30</td>
<td>1.93 ± 0.24</td>
<td>6</td>
<td>0.61 ± 0.02</td>
<td>5.00 ± 0.90</td>
<td>11</td>
<td>0.58 ± 0.02</td>
<td>4.34 ± 0.80</td>
<td>11</td>
<td>0.59 ± 0.03 *5</td>
<td>3.15 ± 0.71</td>
<td>11</td>
</tr>
<tr>
<td>EK – 0 nM</td>
<td>0.75 ± 0.30</td>
<td>1.55 ± 0.35</td>
<td>5</td>
<td>0.46 ± 0.03</td>
<td>6.62 ± 3.34</td>
<td>22</td>
<td>0.33 ± 0.02</td>
<td>4.17 ± 1.24</td>
<td>22</td>
<td>0.27 ± 0.02</td>
<td>2.31 ± 0.75</td>
<td>21</td>
</tr>
<tr>
<td>EK – 500 nM</td>
<td>1.09 ± 0.24</td>
<td>1.21 ± 0.15</td>
<td>7</td>
<td>0.50 ± 0.03</td>
<td>2.13 ± 0.48</td>
<td>29</td>
<td>0.34 ± 0.01</td>
<td>3.29 ± 1.43</td>
<td>29</td>
<td>0.26 ± 0.02</td>
<td>2.01 ± 0.67</td>
<td>27</td>
</tr>
<tr>
<td>EK – 2500 nM</td>
<td>1.59 ± 0.19</td>
<td>1.79 ± 0.22</td>
<td>7</td>
<td>0.53 ± 0.03</td>
<td>3.80 ± 1.38</td>
<td>13</td>
<td>0.40 ± 0.01</td>
<td>2.13 ± 0.34</td>
<td>13</td>
<td>0.34 ± 0.01</td>
<td>1.46 ± 0.26</td>
<td>14</td>
</tr>
<tr>
<td>1795insD –0 nM</td>
<td>3.53 ± 0.37</td>
<td>3.54 ± 0.40</td>
<td>6</td>
<td>0.81 ± 0.03</td>
<td>4.62 ± 0.85</td>
<td>15</td>
<td>0.46 ± 0.02</td>
<td>3.15 ± 0.67</td>
<td>15</td>
<td>0.31 ± 0.02</td>
<td>2.49 ± 0.84</td>
<td>15</td>
</tr>
<tr>
<td>1795insD –500 nM</td>
<td>4.74 ± 0.65</td>
<td>4.69 ± 0.62</td>
<td>5</td>
<td>0.90 ± 0.06</td>
<td>9.18 ± 4.16</td>
<td>19</td>
<td>0.51 ± 0.03</td>
<td>5.50 ± 1.91</td>
<td>18</td>
<td>0.41 ± 0.02</td>
<td>3.93 ± 1.19</td>
<td>15</td>
</tr>
<tr>
<td>1795insD –2500 nM</td>
<td>4.22 ± 0.38</td>
<td>4.23 ± 0.40</td>
<td>6</td>
<td>0.96 ± 0.10</td>
<td>18.72 ± 5.98 *2</td>
<td>13</td>
<td>0.56 ± 0.03</td>
<td>4.63 ± 1.15</td>
<td>12</td>
<td>0.41 ± 0.02</td>
<td>5.99 ± 1.48</td>
<td>11</td>
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</tr>
<tr>
<td><strong>QR – 0 nM</strong></td>
<td>5.90 ±</td>
<td>0.89</td>
<td>21.73 ±</td>
<td>4.74</td>
<td>0.99 ±</td>
<td>0.08</td>
<td>9.12 ±</td>
<td>2.81</td>
<td>0.60 ±</td>
<td>0.02</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>0.64</td>
<td></td>
<td>15.49 ±</td>
<td>3.06</td>
<td>2.48 ±</td>
<td>0.39</td>
<td>4.2 ±</td>
<td>0.60</td>
<td>2.44 ±</td>
<td>0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>QR – 500 nM</strong></td>
<td>5.37 ±</td>
<td>1.24</td>
<td>20.44 ±</td>
<td>4.78</td>
<td>0.88 ±</td>
<td>0.07</td>
<td>4.63 ±</td>
<td>0.54</td>
<td>0.53 ±</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.67</td>
<td></td>
<td>18.07 ±</td>
<td>3.04</td>
<td>2.36 ±</td>
<td>0.40</td>
<td>4.11 ±</td>
<td>0.60</td>
<td>2.30 ±</td>
<td>0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>QR – 2500 nM</strong></td>
<td>7.86 ±</td>
<td>0.74</td>
<td>34.01 ±</td>
<td>9.82</td>
<td>1.33 ±</td>
<td>0.13 *</td>
<td>7.18 ±</td>
<td>1.55</td>
<td>0.75 ±</td>
<td>0.04 *</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.66</td>
<td></td>
<td>29.21 ±</td>
<td>6.02</td>
<td>2.21 ±</td>
<td>0.34</td>
<td>4.08 ±</td>
<td>0.70</td>
<td>2.16 ±</td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*1 p<0.05 vs. 0 nM and 500 nM Q1909R, *2 p<0.05 vs. 0 nM 1795insD, *3 p<0.05 vs. 500 nM and 2500 nM ΔKPQ, *4 p<0.05 vs. 0 nM and 500 nM Q1909R, *5 p<0.05 vs 0 nM and 500 nM ΔKPQ, *6 p<0.05 vs 0 nM and 500 nM Q1909R
3.4.5. Late current amplitude and conductance density

Late $I_{Na}$ conductance density was measured similarly to peak conductance density. Elevations in cytosolic calcium had no effect on late conductance density in any of the channel variants ($p>0.05$, Table 3-7).

Raw $I_{Na}$ current records emphasizing late $I_{Na}$ are shown in Figure 3-4: Al-DI. Traces of TTX-sensitive late $I_{Na}$ in all mutants compared to WT are shown in Figure 3-4: All-DII. The current traces were normalized to peak $I_{Na}$. Late $I_{Na}$ was larger in the mutants compared to WT ($p<0.0001$, Table 3-7). Late $I_{Na}$ was lower by 1.58 % ± 0.49 % in ∆KPQ and by 1.68 % ± 0.47 % in 1795insD when cytosolic calcium was elevated from 0 nM to 2500 nM ($p<0.0001$, Figure 3-4 and Table 3-7). In Q1909R, late $I_{Na}$ was lower by 2.11 % ± 0.44 % when cytosolic calcium was elevated from 0 nM to 500 nM ($p<0.0001$, Figure 3-4 and Table 3-7). Further elevations in cytosolic calcium did not affect late $I_{Na}$ of Q1909R ($p>0.05$, Table 3-7). Late $I_{Na}$ of E1784K was not affected by elevations in cytosolic calcium ($p>0.05$, Table 3-7).
Figure 3-4  Effects of cytosolic calcium on late \( I_{\text{Na}} \)
Panels AI-DI show the effects of cytosolic calcium on the mutants compared to WT. The voltage pulse used to measure late \( I_{\text{Na}} \) is shown in panel AI. Panels All-II show TTX-subtracted late \( I_{\text{Na}} \) for all channel variants at 0 nM cytosolic calcium. Panel E shows a bar graph of late \( I_{\text{Na}} \) as a percentage of peak \( I_{\text{Na}} \) versus the three cytosolic calcium concentrations.

Table 3-7  Calcium Late \( I_{\text{Na}} \)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Late GV Density (nS/pF)</th>
<th>N</th>
<th>Late Percent (%)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - 0 nM</td>
<td>0.07 ± 0.02</td>
<td>16</td>
<td>0.40 ± 0.08</td>
<td>9</td>
</tr>
<tr>
<td>WT - 500 nM</td>
<td>0.08 ± 0.01</td>
<td>5</td>
<td>0.94 ± 0.12</td>
<td>6</td>
</tr>
<tr>
<td>WT - 2500 nM</td>
<td>0.05 ± 0.02</td>
<td>8</td>
<td>0.62 ± 0.13</td>
<td>6</td>
</tr>
<tr>
<td>( \Delta )KPQ - 0 nM</td>
<td>0.12 ± 0.02</td>
<td>13</td>
<td>1.94 ± 0.35 ( ^*1 )</td>
<td>9</td>
</tr>
<tr>
<td>( \Delta )KPQ - 500 nM</td>
<td>0.18 ± 0.05</td>
<td>9</td>
<td>1.48 ± 0.36</td>
<td>5</td>
</tr>
<tr>
<td>( \Delta )KPQ - 2500 nM</td>
<td>0.12 ± 0.04</td>
<td>10</td>
<td>0.36 ± 0.13</td>
<td>6</td>
</tr>
<tr>
<td>EK - 0 nM</td>
<td>0.07 ± 0.02</td>
<td>11</td>
<td>0.95 ± 0.15</td>
<td>11</td>
</tr>
<tr>
<td>EK - 500 nM</td>
<td>0.13 ± 0.02</td>
<td>19</td>
<td>2.11 ± 0.32</td>
<td>14</td>
</tr>
<tr>
<td>EK - 2500 nM</td>
<td>0.09 ± 0.01</td>
<td>15</td>
<td>1.71 ± 0.34</td>
<td>14</td>
</tr>
<tr>
<td>1795insD - 0 nM</td>
<td>0.07 ± 0.02</td>
<td>11</td>
<td>3.42 ± 0.37 ( ^*1 )</td>
<td>7</td>
</tr>
<tr>
<td>1795insD - 500 nM</td>
<td>0.12 ± 0.02</td>
<td>6</td>
<td>2.72 ± 0.48</td>
<td>5</td>
</tr>
<tr>
<td>1795insD - 2500 nM</td>
<td>0.07 ± 0.02</td>
<td>13</td>
<td>1.74 ± 0.18</td>
<td>9</td>
</tr>
<tr>
<td>QR - 0 nM</td>
<td>0.11 ± 0.02</td>
<td>14</td>
<td>2.94 ± 0.48 ( ^*2 )</td>
<td>10</td>
</tr>
<tr>
<td>QR - 500 nM</td>
<td>0.04 ± 0.02</td>
<td>5</td>
<td>0.82 ± 0.34</td>
<td>8</td>
</tr>
<tr>
<td>QR - 2500 nM</td>
<td>0.08 ± 0.01</td>
<td>7</td>
<td>1.01 ± 0.24</td>
<td>6</td>
</tr>
</tbody>
</table>

\( ^*1 \) p<0.0001 versus same mutant (2500 nM), \( ^*2 \) p<0.0001 versus Q1909R (500 nM and 2500 nM)

3.4.6. Slow inactivation voltage-dependence

Normalized current versus membrane potential is shown in Figure 3-5. The steady-state slow inactivation midpoint (SSSI-V\(_{1/2}\)) was not different in any of the channel variants (p>0.05, Table 3-8). The SSSI-V\(_{1/2}\) of E1784K was depolarized by 23.9 mV ± 6.61 mV when cytosolic calcium was elevated from 500 nM to 2500 nM (p<0.01, Figure 3-5 and Table 3-8). The steady-state slow inactivation slope (SSSI-z) was not affected by the mutants (p>0.05, Table 3-8). SSSI-z of 1795insD was larger by 0.85 ± 0.19 with 0 nM to 2500 nM elevations in cytosolic calcium (p<0.05, Table 3-8). The steady-state slow inactivation plateau (SSSI-y\(_{0}\)) was higher in E1784K by 20.0 % ± 4.9 % when cytosolic calcium was elevated from 0 nM to 500 nM (p<0.01, Figure 3-5 and Table 3-8). Further elevations in cytosolic calcium did not have an effect on SSSI-y\(_{0}\) in E1784K. The SSSI-y\(_{0}\) of Q1909R was higher by 18.0% ± 0.05% with 0 nM to 500 nM elevations in cytosolic calcium (p<0.01, Figure 3-5 and Table 3-8).
Figure 3-5  Effects of cytosolic calcium on steady-state slow inactivation
Panels I-II show the mutant effects at 0 nM and 2500 nM cytosolic calcium. Panel II shows a voltage pulse inset. Panels A-D show the calcium effect on each individual mutant compared to WT. Normalized current versus membrane potential was fit with a modified Boltzmann fit.
Table 3-8  Calcium Steady-State Slow Inactivation

<table>
<thead>
<tr>
<th>Condition</th>
<th>SSSI-V1/2 (mV)</th>
<th>SSSI-z</th>
<th>SSSI-y0</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - 0 nM</td>
<td>-69.26 ± 2.43</td>
<td>-1.33 ± 0.06</td>
<td>0.27 ± 0.04</td>
<td>7</td>
</tr>
<tr>
<td>WT - 500 nM</td>
<td>-69.99 ± 2.67</td>
<td>-1.56 ± 0.16</td>
<td>0.35 ± 0.01</td>
<td>3</td>
</tr>
<tr>
<td>WT - 2500 nM</td>
<td>-68.86 ± 2.47</td>
<td>-1.33 ± 0.14</td>
<td>0.34 ± 0.03</td>
<td>8</td>
</tr>
<tr>
<td>ΔKPQ - 0 nM</td>
<td>-66.16 ± 2.18</td>
<td>-1.50 ± 0.20</td>
<td>0.32 ± 0.03</td>
<td>5</td>
</tr>
<tr>
<td>ΔKPQ - 500 nM</td>
<td>-74.75 ± 8.18</td>
<td>-1.63 ± 0.19</td>
<td>0.37 ± 0.03</td>
<td>6</td>
</tr>
<tr>
<td>ΔKPQ - 2500 nM</td>
<td>-73.17 ± 2.05</td>
<td>-1.57 ± 0.16</td>
<td>0.48 ± 0.03</td>
<td>6</td>
</tr>
<tr>
<td>EK - 0 nM</td>
<td>-73.44 ± 2.41</td>
<td>-1.22 ± 0.10</td>
<td>0.21 ± 0.02</td>
<td>6</td>
</tr>
<tr>
<td>EK - 500 nM</td>
<td>-80.60 ± 5.18</td>
<td>-1.41 ± 0.24</td>
<td>0.41 ± 0.03</td>
<td>5</td>
</tr>
<tr>
<td>EK - 2500 nM</td>
<td>-56.66 ± 4.48</td>
<td>-1.02 ± 0.06</td>
<td>0.43 ± 0.04</td>
<td>4</td>
</tr>
<tr>
<td>1795insD - 0 nM</td>
<td>-84.92 ± 3.88</td>
<td>-1.86 ± 0.21</td>
<td>0.28 ± 0.03</td>
<td>8</td>
</tr>
<tr>
<td>1795insD - 500 nM</td>
<td>-75.10 ± 6.65</td>
<td>-1.25 ± 0.21</td>
<td>0.30 ± 0.02</td>
<td>6</td>
</tr>
<tr>
<td>1795insD - 2500 nM</td>
<td>-72.72 ± 3.58</td>
<td>-1.00 ± 0.06</td>
<td>0.26 ± 0.05</td>
<td>8</td>
</tr>
<tr>
<td>QR - 0 nM</td>
<td>-76.67 ± 2.82</td>
<td>-1.51 ± 0.14</td>
<td>0.20 ± 0.02</td>
<td>7</td>
</tr>
<tr>
<td>QR - 500 nM</td>
<td>-73.10 ± 1.55</td>
<td>-1.40 ± 0.10</td>
<td>0.38 ± 0.02</td>
<td>5</td>
</tr>
<tr>
<td>QR - 2500 nM</td>
<td>-77.47 ± 2.66</td>
<td>-1.27 ± 0.08</td>
<td>0.33 ± 0.02</td>
<td>7</td>
</tr>
</tbody>
</table>

*1 p<0.01 (V1/2) and p<0.05 (y0) versus E1784K (0 nM and 500 nM), *2 p<0.05 versus 1795insD (0 nM), *3 p<0.05 versus Q1909R (500 nM and 2500 nM)

3.4.7 Slow inactivation recovery and onset kinetics

The weighted slow inactivation time constants are plotted as a function of the membrane potential in Figure 3-6. Slow inactivation recovery (α) rates are plotted against voltage in Figure 3-6 and slow inactivation onset (β) rates in Figure 3-6. Mutant effects on τ are reported in Table 3-9 and Table 3-10. At -130 mV and -110 mV, recovery kinetics of Q1909R are accelerated (p<0.0001, Table 3-9) when cytosolic calcium was elevated from 0 nM to 500 nM. Slow inactivation recovery kinetics were also accelerated in E1784K, at -110 mV (p<0.0001, Table 3-9). The other channel variants had no significant changes in recovery. At voltages greater than -70 mV, slow inactivation kinetics were not affected by elevations in cytosolic calcium in any channel variant (Table 3-9 and Table 3-10).
Figure 3-6  Effects of cytosolic calcium on slow inactivation kinetics

Panels I-II show the mutant effects on the weighted slow inactivation time constant at 0 nM and 2500 nM cytosolic calcium. Panels A-D show the calcium effects on the slow inactivation $\alpha$-rate for the mutants compared to WT. The bar graph below shows the slow inactivation $\beta$-rate as a function of calcium and channel variant. Voltage pulse protocols of recovery and onset were not shown for clarity. Please refer to Methods.
### Table 3-9 Calcium -130 mV to -70 mV Slow Inactivation Time Constant (s)

<table>
<thead>
<tr>
<th>Condition</th>
<th>-130 mV ( \tau ) weight</th>
<th>n</th>
<th>-110 mV ( \tau ) weight</th>
<th>n</th>
<th>-70 mV ( \tau ) weight</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - 0 nM</td>
<td>0.43 ± 0.06</td>
<td>6</td>
<td>1.22 ± 0.19</td>
<td>5</td>
<td>8.03 ± 1.69</td>
<td>8</td>
</tr>
<tr>
<td>WT - 500 nM</td>
<td>0.40 ± 0.07</td>
<td>5</td>
<td>1.41 ± 0.12</td>
<td>5</td>
<td>9.25 ± 0.99</td>
<td>5</td>
</tr>
<tr>
<td>WT - 2500 nM</td>
<td>0.70 ± 0.05</td>
<td>7</td>
<td>2.06 ± 0.15</td>
<td>7</td>
<td>6.32 ± 2.67</td>
<td>5</td>
</tr>
<tr>
<td>( \Delta KPQ ) - 0 nM</td>
<td>0.97 ± 0.12</td>
<td>7</td>
<td>1.64 ± 0.29</td>
<td>6</td>
<td>9.31 ± 2.56</td>
<td>4</td>
</tr>
<tr>
<td>( \Delta KPQ ) - 500 nM</td>
<td>0.58 ± 0.04</td>
<td>7</td>
<td>0.98 ± 0.08</td>
<td>6</td>
<td>6.41 ± 0.96</td>
<td>4</td>
</tr>
<tr>
<td>( \Delta KPQ ) - 2500 nM</td>
<td>0.54 ± 0.07</td>
<td>5</td>
<td>1.25 ± 0.18</td>
<td>6</td>
<td>11.4 ± 1.35</td>
<td>5</td>
</tr>
<tr>
<td>WT - 0 nM</td>
<td>1.15 ± 0.27</td>
<td>5</td>
<td>3.50 ± 0.89*1</td>
<td>6</td>
<td>14.3 ± 2.23</td>
<td>5</td>
</tr>
<tr>
<td>WT - 500 nM</td>
<td>0.95 ± 0.24</td>
<td>6</td>
<td>1.37 ± 0.23</td>
<td>6</td>
<td>18.9 ± 4.31</td>
<td>5</td>
</tr>
<tr>
<td>WT - 2500 nM</td>
<td>0.60 ± 0.09</td>
<td>8</td>
<td>1.00 ± 0.12</td>
<td>5</td>
<td>16.7 ± 2.22</td>
<td>4</td>
</tr>
<tr>
<td>( \Delta KPQ ) - 0 nM</td>
<td>1.30 ± 0.42</td>
<td>3</td>
<td>1.50 ± 0.37</td>
<td>2</td>
<td>11.8 ± 0.69</td>
<td>5</td>
</tr>
<tr>
<td>( \Delta KPQ ) - 500 nM</td>
<td>1.78 ± 0.28</td>
<td>5</td>
<td>2.92 ± 0.55</td>
<td>4</td>
<td>11.2 ± 0.53</td>
<td>4</td>
</tr>
<tr>
<td>( \Delta KPQ ) - 2500 nM</td>
<td>0.83 ± 0.17</td>
<td>5</td>
<td>1.40 ± 0.16</td>
<td>4</td>
<td>13.4 ± 0.94</td>
<td>4</td>
</tr>
<tr>
<td>QR - 0 nM</td>
<td>2.49 ± 0.56*1</td>
<td>5</td>
<td>5.72 ± 0.84*1</td>
<td>4</td>
<td>12.6 ± 0.94</td>
<td>5</td>
</tr>
<tr>
<td>QR - 500 nM</td>
<td>0.68 ± 0.08</td>
<td>6</td>
<td>1.36 ± 0.25</td>
<td>6</td>
<td>6.98 ± 1.29</td>
<td>6</td>
</tr>
<tr>
<td>QR - 2500 nM</td>
<td>0.68 ± 0.10</td>
<td>5</td>
<td>1.76 ± 0.36</td>
<td>5</td>
<td>5.86 ± 0.38</td>
<td>5</td>
</tr>
</tbody>
</table>

*1 p<0.0001 versus same mutant (500 nM and 2500 nM)

### Table 3-10 Calcium -50 mV to -10 mV Slow Inactivation Time Constant (s)

<table>
<thead>
<tr>
<th>Condition</th>
<th>-50 mV ( \tau ) weight</th>
<th>n</th>
<th>-30 mV ( \tau ) weight</th>
<th>n</th>
<th>-10 mV ( \tau ) weight</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - 0 nM</td>
<td>9.38 ± 1.00</td>
<td>5</td>
<td>6.32 ± 0.27</td>
<td>4</td>
<td>7.01 ± 1.15</td>
<td>5</td>
</tr>
<tr>
<td>WT - 500 nM</td>
<td>10.9 ± 0.83</td>
<td>5</td>
<td>7.17 ± 0.30</td>
<td>6</td>
<td>5.91 ± 0.65</td>
<td>6</td>
</tr>
<tr>
<td>WT - 2500 nM</td>
<td>5.62 ± 1.83</td>
<td>5</td>
<td>5.88 ± 1.77</td>
<td>5</td>
<td>5.27 ± 1.19</td>
<td>4</td>
</tr>
<tr>
<td>( \Delta KPQ ) - 0 nM</td>
<td>9.15 ± 1.90</td>
<td>6</td>
<td>8.48 ± 1.59</td>
<td>5</td>
<td>5.86 ± 1.44</td>
<td>5</td>
</tr>
<tr>
<td>( \Delta KPQ ) - 500 nM</td>
<td>14.6 ± 1.77</td>
<td>5</td>
<td>10.4 ± 1.39</td>
<td>4</td>
<td>10.7 ± 1.76</td>
<td>5</td>
</tr>
<tr>
<td>( \Delta KPQ ) - 2500 nM</td>
<td>12.3 ± 1.13</td>
<td>5</td>
<td>12.1 ± 0.56</td>
<td>5</td>
<td>10.0 ± 0.72</td>
<td>5</td>
</tr>
<tr>
<td>EK - 0 nM</td>
<td>9.12 ± 0.98</td>
<td>5</td>
<td>7.10 ± 0.39</td>
<td>5</td>
<td>5.27 ± 1.02</td>
<td>4</td>
</tr>
<tr>
<td>EK -2500 nM</td>
<td>15.5 ± 2.81</td>
<td>5</td>
<td>9.36 ± 1.06</td>
<td>5</td>
<td>9.23 ± 1.07</td>
<td>5</td>
</tr>
<tr>
<td>1795insD - 0 nM</td>
<td>14.5 ± 1.48</td>
<td>5</td>
<td>9.76 ± 1.83</td>
<td>5</td>
<td>10.2 ± 1.58</td>
<td>4</td>
</tr>
<tr>
<td>1795insD - 500 nM</td>
<td>8.05 ± 1.72</td>
<td>6</td>
<td>5.96 ± 1.30</td>
<td>5</td>
<td>6.48 ± 1.35</td>
<td>5</td>
</tr>
<tr>
<td>1795insD - 2500 nM</td>
<td>7.82 ± 1.93</td>
<td>5</td>
<td>5.56 ± 1.59</td>
<td>5</td>
<td>6.48 ± 1.52</td>
<td>5</td>
</tr>
<tr>
<td>QR - 0 nM</td>
<td>7.17 ± 0.43</td>
<td>5</td>
<td>6.69 ± 0.49</td>
<td>5</td>
<td>8.68 ± 0.76</td>
<td>5</td>
</tr>
<tr>
<td>QR - 500 nM</td>
<td>7.56 ± 0.88</td>
<td>5</td>
<td>5.17 ± 0.30</td>
<td>5</td>
<td>5.48 ± 0.78</td>
<td>5</td>
</tr>
<tr>
<td>QR - 2500 nM</td>
<td>7.47 ± 1.35</td>
<td>6</td>
<td>7.02 ± 0.44</td>
<td>5</td>
<td>5.67 ± 0.97</td>
<td>5</td>
</tr>
</tbody>
</table>

### 3.4.8. Ventricular action potential simulations

With increasing pacing frequency stimulations, intracellular calcium naturally increases inside the cell, known as the positive stair-case phenomenon. To simulate dynamic properties of the experimentally observed calcium-induced \( I_{Na} \) shifts, we used a modified version of the O’Rd model to generate action potentials in the endocardial and epicardial cells. The \( I_{Na} \) gating parameters were held uniform across all the three cell types.

83
types. The last action potential (AP) simulated at each frequency is shown in Figure 3-7 for all the channel variants at bradycardia, normal, and tachycardia heart rates. In E1784K, AP is lost with every other beat at elevated heart rates. Thus, both the final two E1784K APs at each frequency are shown in Figure 3-7.

**Figure 3-7  Ventricular action potential simulations**
The AP simulations are shown for endocardial and epicardial cells as a function of frequencies (0.5 Hz, 1.5 Hz, and 2.5 Hz).
Action potential upstroke velocity and action potential duration at 30 %, 60 %, and 90 % of repolarization were measured and are shown in Figure 3-8. Action potential upstroke velocity was substantially reduced in ΔKPQ, E1784K, and 1795insD compared to WT and Q1909R (Figure 3-8: A-B). With decreasing BCLs, particularly below 600 ms, the Q1909R upstroke velocities are reduced. AP upstroke velocity in the remaining mutants seemed to be insensitive to any BCL shortening.

Action potential durations (APD$_X$, X=30,60,90) are plotted versus the diastolic interval in Figure 3-8: C to create electrical restitution curves (ERCs). The plateaus of the ERC curves were increased with increasing APD$_X$. The greatest change in ERC as function of repolarization time was evident in E1784K compared to other variants, which displays prolonged APD at reduced diastolic intervals. In addition, the ERC slope is greatest in E1784K (APD$_{90}$), suggesting heightened arrhythmogenicity.

The shifts observed in APD as a function of heart rate in all the mutants can be ascribed to the positive-staircase rise in cytosolic calcium. When the calcium-dependent gating in $I_{Na}$ was disabled (results not shown), APD$_{90}$ was less sensitive to higher pacing frequencies, suggesting a calcium-mediated effect on AP morphology in the mutants studied.

To further investigate the frequency-dependent shortening of APD, we calculated the difference in APD$_{90}$ between successive frequencies shown in Figure 3-8: D. In the endocardial cell, the APD$_{90}$ shortening displays a relatively U-type frequency dependence. E1784K exacerbates the U-type frequency dependence by prolonging greatly (Figure 3-8: D). This result suggests a frequency-dependent APD prolongation in E1784K.
Figure 3-8  Action potential velocity and electrical restitution curves (ERC)
Panels A and B show the action potential velocity as a function of BCL. Panel C shows the ERC curve in the endocardium and Panel D shows the difference (Δ) in APD90 as a function of frequency.

Each of the mutants characterized in this study increases the late I_{Na} compared to WT. Increases in intracellular sodium, [Na], known as sodium overload, causes the sodium-calcium exchanger (NCX) to function in reverse-mode inducing a calcium-overload, which underlies cardiac diastolic dysfunction. The rise in intracellular calcium ([Ca^{2+}]) during the action potential time course is shown in Figure 3-9: A-D. All mutants affect [Ca^{2+}], similar to WT, except in E1784K, which has relatively suppressed [Ca^{2+}] during the AP plateau and elevated [Ca^{2+}] during the refractory period (Figure 3-9: A-D inset). The [Na]^{3+}:[Ca^{2+}] ratio was calculated for all channel variants at all frequencies and plotted against the APD90 for the endocardial (Figure 3-9: E). The [Na]^{3+}:[Ca^{2+}] ratio decreased in WT, ∆KPQ, 1795insD, and Q1909R as a function of frequency. E1784K displays differential sensitivity to frequency-induced [Na]^{3+}:[Ca^{2+}] shifts. In E1784K, the perturbations in [Na]^{3+}:[Ca^{2+}] follow a helter-skelter manner resulting in a sodium overload at bradycardia and near tachycardia frequencies. Analysis of sodium-calcium levels at frequencies higher than 2.0 Hz in E1784K was difficult due to presence of alternans. Elevated [Na]^{3+}:[Ca^{2+}] were sufficient to induce NCX reverse mode in the presence of alternans in E1784K (results not shown).
Figures 3-9  Sodium-calcium overload
Panels A-D show the intracellular calcium in all the channel variants at 0.5 Hz, 1.5 Hz, and 2.5 Hz simulated in endocardial cells. Panel E shows the intracellular sodium-calcium ratio as function of APD90 in the endocardium.

3.5. Discussion

Elevated cytosolic calcium, induced by sympathetic stimulation during exercise, may serve as a potential arrhythmogenic trigger in patients with SCN5a mutations. The goal of our study was to explore the response of mixed syndrome mutants to elevated
cytosolic calcium. The mutants studied here have differential cytosolic calcium sensitivities. We found biophysical defects becoming either ameliorated or exacerbated by elevated cytosolic calcium in a mutant-dependent fashion.

Elevated cytosolic calcium clearly attenuates late $I_{Na}$ in ∆KPQ, 1795insD, and Q1909R. Since late $I_{Na}$ is a key pathophysiological substrate in LQT3, elevated cytosolic calcium rescues the gain-of-function phenotype in those Na\(_v\)1.5 mutants. In contrast, the therapeutic effect of cytosolic calcium is hampered in E1784K, suggesting carriers may express LQT3 properties under elevated heart rates. Although our experiments were performed at room temperature, we predict our results, and their phenotypic sequelae, may be exacerbated at higher temperatures since E1784K is also highly thermosensitive (Abdelsayed et al., 2015). Our action potential modelling confirms that the most arrhythmogenic mutant is E1784K and predicts a large dispersion of repolarization across the ventricular wall. E1784K was the only mutant which was predicted to cause a $[Na^+]_i$-overload under both bradycardia and tachycardia by our model. The other mutants characterized showed decreases in $[Na^+]_i$ with elevated heart rates. The model also predicts $[Na^+]_i$-overload, exacerbating E1784K arrhythmogenicity by driving NCX to run in reverse mode, further increasing cytosolic calcium. Increased cytosolic sodium and calcium results in electrical and mechanical cardiac abnormalities (Antzelevitch et al., 2014).

### 3.5.1. Biophysical Implications

The DIII-DIV linker and C-terminus are crucial for both fast and slow inactivation in sodium channels. Mutations in these regions perturb their normal interaction both under diastolic calcium levels and upon a calcium signal, causing a defect in Na\(_v\)1.5 inactivation. The C-terminus contains indirect binding sites for calcium (Mori et al., 2003; Biswas et al., 2009), including a pair of EF-hand domains composed of a helix-loop-helix, from Glu1773 to Asp1852 (comprising helices 1-4, H1-H4) Wingo et al., 2004; Shah et al., 2006). Approximately 120 residues downstream, an IQ domain binds to calmodulin (Tan et al., 2002; Cormier et al., 2002; Wingo et al., 2004). The influence of calcium on inactivation has been studied using electrophysiology and isothermal titration calorimetry (ITC) experiments (Shah et al., 2006; Sarhan et al., 2012). Under low calcium conditions, calmodulin binds via the C-lobe to the IQ domain of the C-terminus (Chagot et al., 2009). When cytosolic calcium levels rise, the calmodulin N-lobe binds to
the IQ domain and the C-lobe binds to the DIII-DIV linker, as in the tripartite complex (Shah et al., 2006; Biswas et al., 2009; Gaudioso et al., 2011; Sarhan et al., 2012).

Previous studies report intra C-terminal interactions, observed by fluorescence resonance energy transfer. The IQ motif (H6) in the C-terminus interacts with the aromatic residues of H1 (F1791 and Y1795) and the N-terminal residues of H6 interact with residues in H1-H2 and H2-H3 linkers (Glaaser et al., 2006; Chagot et al., 2009). Proximity of the EF-hand domain to the IQ motif is required for proper inactivation (Glaaser et al., 2012). The intra C-terminal interaction associates with the DIII-DIV linker, modulating inactivation (Bankston et al., 2007).

Studies supporting the tripartite complex show that residues 1498-1501 in the DIII-DIV linker form an alpha helix that interacts with the C-lobe of calmodulin, whereas the rest of the residues within the linker (1502-1522) are disordered (Sarhan et al., 2012). Deletion of residues 1505-1507 in ∆KPQ normally increases late $I_{Na}$ and stabilizes steady-state fast inactivation at RMP (Chandra et al., 1998). Stabilized inactivation may occur due to a tighter association between the truncated DIII-DIV linker and its binding site in the inner vestibule of NaV1.5. Shortening the DIII-DIV linker in ∆KPQ may increase the affinity for calmodulin binding upon a calcium signal. This further destabilizes fast inactivation and increases channel availability as evident in the calcium-induced depolarized shift in SSFI midpoint.

We predict the charge-reversal in E1784K disrupts crucial electrostatic interactions linking the IQ motif to the EF-hand domain, resulting in an abnormally structured C-terminus. The positive lysine may form electrostatic interactions with downstream negative charges in H1, altering the IQ-EF-hand interaction, and resulting in a loosely bound IQ. Consequently, the IQ-calmodulin complex may be more mobile and unable to modulate fast inactivation via the DIII-DIV linker, as IQ-calmodulin requires anchorage to H1 (Pitt & Lee, 2016). Since the IQ-EF-hand interaction is destabilized, calcium affinity might decrease (Shah et al., 2006; Chagot et al., 2009). As a result, elevations in cytosolic calcium may be unable to attenuate increases in late $I_{Na}$ in E1784K. The fast inactivation mechanism elicited by the DIII-DIV linker is thought to function in synchrony with the C-terminus, affecting slow inactivation (Motoike, 2004). A charge-reversal mutant may uncouple both structures and thus affect the interaction between fast and slow inactivation. Consequently, the increase in E1784K late $I_{Na}$ with
elevations in cytosolic calcium accompany destabilized slow inactivation at depolarized potentials (Featherstone et al., 1998; Richmond et al., 1998).

The 1795insD mutant in the EF-hand domain (H1) adds a negative charge to the region. Chagot et al. (2009) show that Y1795 is a key residue, establishing hydrophobic interactions with the IQ motif. Adding a negative charge may stabilize the EF-hand domain and preserve the intactness of the C-terminal interactions yielding greater sensitivity by Na\textsubscript{v}1.5 to calcium-calmodulin effects. The Q1909R mutation is in the initial segment of the IQ motif, the region that calmodulin associates with via its N-terminus during a calcium signal and via its C-lobe during diastolic calcium levels. In the IQ motif, Arginine-3 and Arginine-6 are bound by hydrogen bonds to different calmodulin residues. Thus, the introduction of an additional Arginine residue in Q1909R should enhance calmodulin binding. Fast inactivation $\tau_{\text{onset}}$ in both 1795insD and Q1909R was decelerated at depolarized potentials in a calcium-dependent fashion, agreeing with past data showing calcium-induced deceleration of fast inactivation $\tau_{\text{onset}}$ in WT Na\textsubscript{v}1.5 (Biswas et al., 2009).

3.5.2. Physiological Implications

Accentuated gain-of-function in E1784K by elevated cytosolic calcium increases [Na\textsuperscript{+}]-overload. The O'Rd model simulations suggested that prolonged APDs are ameliorated by elevated pacing in $\Delta$KPQ, 1795insD, and Q1909R. E1784K APD is relatively prolonged, even during tachycardia. Furthermore, in all mutants except E1784K, the [Na\textsuperscript{+}]:[Ca\textsuperscript{2+}] ratio is adjusted or minimized. In E1784K, a discordant curve develops, in which the sodium overload is observed at different heart rates. Sodium overload induces multiple cellular cascades including the reverse mode in NCX, a factor known to shorten APD at elevated heart rates (Faber & Rudy, 2000; Moreau et al., 2013). Reverse mode NCX causes a rise in intracellular calcium, which threatens diastolic stability resulting in diastolic dysfunction. In addition to those mechanical abnormalities, calcium overload disturbs the electrical stability of myocardial tissue by inducing DADs, increasing the likelihood of lethal cardiac events including ventricular tachycardia/fibrillation.
3.5.3. Clinical Implications

The effect of exercise on LQT3 is an unsettled controversial matter. Schwartz et al. (1995) reported a marked reduction in QTc during exercise in LQT3 patients. This effect is lost during recovery from exercise. Most of the patients in that study carried the ΔKPQ mutation. A reduced QTc in ΔKPQ may be explained by frequency- or calcium-dependent attenuation of late \( I_{Na} \). Nevertheless, a relatively minor percentage of LQT3 patients develop cardiac events during exercise. Questions remain as to whether sleep or rest without arousal (i.e. low heart rates) fully explains arrhythmogenesis in LQT3, since the REM phase of sleep is accompanied by high sympathetic tone. Thus, both sleep and exercise share some common physiological properties and may share a mechanism of arrhythmogenesis in some LQT3 cases. These commonalities raise questions about the utility of β-blockers as potential therapeutics for all LQT3 patients, regardless of the causative mutation. The variance seen in BrS1/LQT3 response to exercise depends, at least in part, on the exact \( SCN5a \) mutation. Thus, genetic screening is a critical diagnostic tool to determine whether exercise may be therapeutic or an arrhythmogenic trigger in patients with \( SCN5a \) mutations.

3.6. Conclusion

Greater calcium sensitivity in E1784K is reflected by the AP model, in which E1784K prolongs the APD. Compared to the other mutants, E1784K results in the largest transmural voltage gradient. Our results may lead to further refined treatments for BrS1/LQT3. Antiarrhythmics, potent for LQT3, should be tested in mixed syndrome cases since the propensity to be phenotypically LQT3 and/or BrS1 may be affected by triggers such as exercise. Cellular cascades, such as activation of the \( \text{Ca}^{2+}/\text{CaMKII} \) and other protein kinases, may determine the mutant-specific biophysical effects of arrhythmogenic mutations (Herren et al., 2013). This study is likely biased to the effects of calcium on predominantly phosphorylated \( \text{Nav}1.5 \) due to the use of internal fluoride, which inhibits phosphatases (Proud, 1994). Future assays should measure phosphorylation-induced shifts on gating and channel expression to further elucidate how physiological changes impact the arrhythmogenicity of these mutants. Our results support the case for more detailed biophysical analysis of all mutants underlying sudden cardiac death.
3.7. Acknowledgments

The authors thank Dr. David Jones, Dr. Sam Goodchild, Mr. Colin Peters, and Mr. Mohammed-Reza Ghovanloo for their valuable input and support.
Chapter 4. The efficacy of ranolazine on E1784K is altered by temperature and calcium

The content of this chapter has been submitted for publication in the journal of Scientific Reports.

4.1. Abstract

E1784K is the most common mixed syndrome SCN5a mutation underpinning both Brugada syndrome type 1 (BrS1) and Long-QT syndrome type 3 (LQT3). The charge reversal mutant enhances the late sodium current (INa) passed by the cardiac voltage-gated sodium channel (NaV1.5), delaying cardiac repolarization. Exercise-induced triggers, like elevated temperature and cytosolic calcium, exacerbate E1784K late INa. In this study, we tested the effects of ranolazine, the late INa blocker, on voltage-dependent and kinetic properties of E1784K at elevated temperature and cytosolic calcium. We used whole-cell patch clamp to measure INa from wild type and E1784K channels expressed in HEK293 cells. At elevated temperature, ranolazine attenuated gain-of-function in E1784K by decreasing late INa, hyperpolarizing steady-state fast inactivation, and increasing channel use-dependent inactivation. Both elevated temperature and cytosolic calcium hampered the capacity of ranolazine to suppress E1784K late INa. In-silico action potential (AP) simulations were done using a modified O’Hara-Rudy (O’Rd) cardiac model. Simulations showed that ranolazine failed to shorten AP duration, an effect augmented at febrile temperatures. The channel/drug interaction is clearly affected by external triggers, as reported previously with ischemia. Determining drug efficacy under various physiological states in SCN5a cohorts is crucial for accurate management of arrhythmias.

4.2. Introduction

The alpha subunit of the cardiac voltage-gated sodium channel, NaV1.5, is encoded by the SCN5a gene. Mutations in this gene usually cause long-QT syndrome type 3 (LQT3), Brugada syndrome type 1 (BrS1), or both (mixed syndromes) (Bezzina et al., 1999; Shimizu & Antzelevitch, 2000; Rivolta, 2001; Antzelevitch et al., 2005;
Kapplinger et al., 2010). These clinical conditions are elicited by expression of gating dysfunctions in Na\textsubscript{v}1.5 (Dumaine et al., 1996; Baroudi & Chahine, 2000; Baroudi et al., 2002; Makita et al., 2008b; Sun et al., 2011). Gain- and loss-of-function mutations can modify the inward sodium current (I\textsubscript{Na}). Gain-of-function (GoF) in Na\textsubscript{v}1.5 arises from loss in channel fast inactivation, thereby increasing the non-inactivating, late I\textsubscript{Na}, underlying LQT3 (Dumaine et al., 1996, 1996; Chandra et al., 1998; Wei et al., 1999; Groenewegen et al., 2003; Bankston et al., 2007; Kwon et al., 2012; Moreau et al., 2013). Loss-of-function (LoF) mainly arises from decreased peak I\textsubscript{Na} resulting in BrS1 (Bezzina et al., 1999; Veldkamp et al., 2000a; Wang et al., 2000; Baroudi & Chahine, 2000; Mok et al., 2003; Hwang et al., 2005). Interestingly, both GoF and LoF defects can occur simultaneously in a number of mutants (Wei et al., 1999; Veldkamp et al., 2000a; Baroudi & Chahine, 2000; Huang et al., 2006; Makita et al., 2008a; Postema et al., 2009; Chen et al., 2015).

A guanine to an adenine substitution at position 5349 in SCN5a expresses the charge reversal mutant, E1784K, in the Na\textsubscript{v}1.5 C-terminal domain (CTD) (Splawski et al., 2000; Tester et al., 2005). E1784K is the most common mixed syndrome mutant, particularly prevalent in the Okinawa Islands in Japan, where carriers mainly express diagnostic LQT3 (Takahashi et al., 2014). Clinical studies reveal differential phenotypic expressivity in E1784K cohorts (Wei et al., 1999; Deschênes et al., 2000; Makita et al., 2008a; Postema et al., 2009; Sumitomo, 2014).

E1784 is located directly upstream of the acidic globular EF-like hand domain (\(\alpha_1-\alpha_4\)). The residue contributes to the electrostatic interactions formed between the acidic domain and the downstream basic IQ domain (\(\alpha_6\). Figure 4-1 compares WT to E1784K structure) (Mantegazza et al., 2001; Cormier et al., 2002; Kim et al., 2004; Glaaser et al., 2006; Chagot et al., 2009). The proximal CTD, in which E1784K resides, has the largest effects on kinetics and steady-state inactivation (Mantegazza et al., 2001; Motoike, 2004; Glaaser et al., 2006, 2012). The charge reversal mutant, E1784K, is thought to disturb the integrity of the CTD, causing the \(\alpha_6\) to become more mobile (Figure 4-1) (Abdelsayed et al., 2017). A disturbance to \(\alpha_6\) integrity has been correlated with elevations in late I\textsubscript{Na} and enhanced slow inactivation (Mori et al., 2003; Bankston et al., 2007; Glaaser et al., 2012; Van Petegem et al., 2012; Yan et al., 2017a), which are key biophysical attributes in E1784K (Wei et al., 1999; Deschênes et al., 2000; Makita et al., 2008a).
Figure 4-1  Sodium Channel Schematic Diagram
Domain III and Domain IV along with their inter-linker and the CTD are important in regulating channel function and calcium sensitivity. The DIII-DIV linker has the fast inactivation particle (IFM motif). DIV-S6 contains the putative drug-binding residue, F1760, which is key for binding ranolazine. The enlarged CTD contains six α-helices which aggregate to form an intact domain for calmodulin binding. Calmodulin binds to the IQ-domain (α6) under low cytosolic calcium conditions (structure adapted from Chagot et al., 2009 and Gabelli et al., 2014). We speculate that a disarrangement occurs in the CTD upon mutagenesis to E1784K, which may affect calmodulin interaction with other channel sites, such as the DIII-DIV linker.

Recent studies characterized the effects of exercise-induced triggers on E1784K. These triggers include acidosis, elevated temperatures and cytosolic calcium. Acidosis and elevated temperatures augment late $I_{Na}$ and decrease peak $I_{Na}$ in E1784K (Abdelsayed et al., 2015; Peters et al., 2016, 2017). Use-dependence in E1784K is reduced with high stimulation frequencies at elevated temperatures (Abdelsayed et al., 2015). Compared to other mutants, E1784K tends to hamper the native potency of cytosolic calcium to block late $I_{Na}$ in Na\textsubscript{v}1.5 (Potet et al., 2015; Abdelsayed et al., 2017). Elevated cytosolic calcium augments channel availability in E1784K by depolarizing the voltage-dependence of slow inactivation (Abdelsayed et al., 2017). Dynamic in silico
action potential (AP) simulations in cardiac cells show E1784K-induced alternans at sinus rhythm and with tachycardia (Abdelsayed et al., 2015, 2017).

We hypothesize that ranolazine, which preferentially blocks late $I_{Na}$, is suitable for ameliorating the thermal and calcium-induced defects in E1784K. Although prescribed as an anti-anginal drug for diastolic dysfunction treatment (Antzelevitch, 2004; Antzelevitch et al., 2004; Sossalla et al., 2008), Ranolazine has antiarrhythmic efficacy proven to be useful in treating SCN5a inherited conditions (Moss et al., 2008; Rajamani et al., 2009; Huang et al., 2011; Sokolov et al., 2013). Ranolazine efficacy is enhanced with SCN5a mutations or channel triggers, such as acidosis, which augment late $I_{Na}$ (Belardinelli, 2006a; Fredj et al., 2006b; Huang et al., 2011; Sokolov et al., 2013; Peters et al., 2013). We predicted that the channel mutation-trigger interaction may alter drug efficacy. Our goal is to study the effects of ranolazine on E1784K under conditions of elevated temperature and cytosolic calcium levels.

### 4.3. Methods

#### 4.3.1. Homology Modelling and Auto-Docking

Homology modeling was performed using the Swiss-Model server (https://swissmodel.expasy.org) (Bordoli et al., 2008). The newly cryo-EM solved American cockroach voltage-gated sodium channel (Na$\nu$Pas) structure (3.8-Å resolution) was used as a template against the Na$\nu$1.5 sequence. Modeling was done according to the protocol established by Bordoli et al., (2008). Sequence alignment was done according to Uniprot Align (http://www.uniprot.org/align/) for SCN5A_HUMAN (Na$\nu$1.5) and SCNA1_PERAM (Na$\nu$Pas).

Ranolazine was virtually docked using AutoDock4 against the Na$\nu$1.5 homology model built on Na$\nu$Pas (Na$\nu$1.5-Na$\nu$Pas) (Morris et al., 2009). PyMOL-pdb viewer was used for optimization and visualization of the auto-docking results.
4.3.2. Ethical approval

The research was approved by Biohazards review 251-2012 issued by the office of the Environmental Health and Safety at Simon Fraser University, Burnaby, BC, Canada.

4.3.3. Cell Culture

HEK293 cells were grown at pH 7.4 in a DMEM (1×) nutrient medium (Life Technologies, NY, USA), supplemented with 10 % FBS and maintained in a humidified environment at 37 °C with 5 % CO₂. The α subunits (WT or E1784K) were co-transfected with the β1 subunit and green fluorescent protein, eGFP (1.50 µg: 0.75 µg: 1.50 µg, respectively). The cDNA mixture was then allowed to incubate with the HEK293 cells before plating on coverslips. The HEK293 cells were selected for this study since they contain a relatively elevated [calmodulin]free level compared to other cell lines, thereby controlling for calcium-calmodulin effects on NaV1.5 (Black et al., 2004).

4.3.4. Electrophysiology

Whole-cell patch clamp recordings were performed in extracellular solution containing (mM): 96 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, and 10 HEPES (pH 7.4). Solutions were titrated with CsOH to pH 7.4. Pipettes were fabricated with a P-1000 puller using borosilicate glass (Sutter Instruments, CA, USA), dipped in dental wax to reduce capacitance, then thermally polished to a resistance of 1.0-1.5 MΩ. Low resistance electrodes were used to minimize series resistance between pipette and intracellular solution resulting in typical access resistances of 3.5 MΩ or less, thereby minimizing voltage measurement error. Pipettes were filled with intracellular solution. For minimal cytosolic calcium levels, pipettes contained (mM): 130 CsF, 9.6 NaCl, 10 HEPES, and 10 EGTA titrated to pH 7.4. The intracellular pipette solution was manipulated to mimic peak systolic cytosolic calcium (Steenbergen et al., 1987; Kirschenlohr et al., 2000). To do so, we calculated, using the Ca-EGTA Calculator v1.3, the amount of CaCl₂ (in mM) added to bring cytosolic calcium to 2500nM at both 22 °C and 34 °C: 9.53 and 9.60, respectively.
All recordings were made using an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) digitized at 20 kHz using an ITC-16 interface (HEKA Elektronik, Lambrecht, Germany). Data were acquired and low-pass-filtered (5 kHz) using PatchMaster/FitMaster software (HEKA Elektronik, Lambrecht, Germany) running on an Apple iMac (Apple Computer, Cupertino, CA). Leak subtraction was performed online using a P/4 procedure. Bath solution temperature was controlled using a Peltier device driven by a TC-10 Temperature Controller (Dagan, Minneapolis, MN). Bath temperature was maintained at 22 °C or 34 °C. Experiments were not performed at physiological temperatures because of the inherent instability of cells at temperatures above 34 °C. Using a Q10 relationship, which were confirmed with Arrhenius calculations, we extrapolated data to physiological temperatures (described below). After a giga-ohm seal resistance was achieved, the whole-cell configuration was attained. The holding potential between protocols was -110 mV. We recorded $I_{Na}$ from cells that expressed currents no greater than –5 nA. The average voltage error calculated for all cells used in this study (n = 250) is 6.06 mV ± 0.40 mV obtained (Table 4-1). There are no differences between the voltage-errors in the different conditions (p>0.05).
### Table 4-1 Ranolazine Voltage Error

<table>
<thead>
<tr>
<th>Condition</th>
<th>Voltage Error (mV)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - 22 °C - 0 nM Ca²⁺ - 0 Ran</td>
<td>3.52 ± 0.97</td>
<td>9</td>
</tr>
<tr>
<td>WT - 22 °C - 0 nM Ca²⁺ - 10 Ran</td>
<td>7.83 ± 1.47</td>
<td>8</td>
</tr>
<tr>
<td>WT - 22 °C - 0 nM Ca²⁺ - 100 Ran</td>
<td>5.70 ± 2.05</td>
<td>6</td>
</tr>
<tr>
<td>WT - 22 °C - 2500 nM Ca²⁺ - 0 Ran</td>
<td>4.89 ± 1.29</td>
<td>9</td>
</tr>
<tr>
<td>WT - 22 °C - 2500 nM Ca²⁺ - 10 Ran</td>
<td>6.54 ± 1.30</td>
<td>11</td>
</tr>
<tr>
<td>WT - 22 °C - 2500 nM Ca²⁺ - 100 Ran</td>
<td>3.12 ± 0.88</td>
<td>10</td>
</tr>
<tr>
<td>WT - 34 °C - 0 nM Ca²⁺ - 0 Ran</td>
<td>8.35 ± 1.56</td>
<td>11</td>
</tr>
<tr>
<td>WT - 34 °C - 0 nM Ca²⁺ - 10 Ran</td>
<td>9.08 ± 1.32</td>
<td>9</td>
</tr>
<tr>
<td>WT - 34 °C - 0 nM Ca²⁺ - 100 Ran</td>
<td>7.18 ± 1.16</td>
<td>19</td>
</tr>
<tr>
<td>WT - 34 °C - 2500 nM Ca²⁺ - 0 Ran</td>
<td>8.64 ± 0.98</td>
<td>7</td>
</tr>
<tr>
<td>WT - 34 °C - 2500 nM Ca²⁺ - 10 Ran</td>
<td>8.53 ± 1.79</td>
<td>9</td>
</tr>
<tr>
<td>WT - 34 °C - 2500 nM Ca²⁺ - 100 Ran</td>
<td>8.14 ± 1.57</td>
<td>6</td>
</tr>
<tr>
<td>EK - 22 °C - 0 nM Ca²⁺ - 0 Ran</td>
<td>4.41 ± 0.65</td>
<td>19</td>
</tr>
<tr>
<td>EK - 22 °C - 0 nM Ca²⁺ - 10 Ran</td>
<td>5.99 ± 1.22</td>
<td>10</td>
</tr>
<tr>
<td>EK - 22 °C - 0 nM Ca²⁺ - 100 Ran</td>
<td>4.28 ± 0.72</td>
<td>14</td>
</tr>
<tr>
<td>EK - 22 °C - 2500 nM Ca²⁺ - 0 Ran</td>
<td>3.01 ± 0.71</td>
<td>6</td>
</tr>
<tr>
<td>EK - 22 °C - 2500 nM Ca²⁺ - 10 Ran</td>
<td>4.49 ± 1.04</td>
<td>9</td>
</tr>
<tr>
<td>EK - 22 °C - 2500 nM Ca²⁺ - 100 Ran</td>
<td>4.63 ± 1.21</td>
<td>10</td>
</tr>
<tr>
<td>EK - 34 °C - 0 nM Ca²⁺ - 0 Ran</td>
<td>6.50 ± 1.09</td>
<td>19</td>
</tr>
<tr>
<td>EK - 34 °C - 0 nM Ca²⁺ - 10 Ran</td>
<td>6.74 ± 1.73</td>
<td>10</td>
</tr>
<tr>
<td>EK - 34 °C - 0 nM Ca²⁺ - 100 Ran</td>
<td>5.52 ± 0.98</td>
<td>12</td>
</tr>
<tr>
<td>EK - 34 °C - 2500 nM Ca²⁺ - 0 Ran</td>
<td>5.63 ± 1.32</td>
<td>8</td>
</tr>
<tr>
<td>EK - 34 °C - 2500 nM Ca²⁺ - 10 Ran</td>
<td>8.95 ± 1.97</td>
<td>8</td>
</tr>
<tr>
<td>EK - 34 °C - 2500 nM Ca²⁺ - 100 Ran</td>
<td>3.67 ± 0.69</td>
<td>11</td>
</tr>
</tbody>
</table>

#### 4.3.5. Drug Preparation

Ranolazine was obtained from Gilead Sciences (Foster City, CA) in powder form, diluted to 100 mM stock in 0.1 M HCl, aliquoted at 10 mM and stored at −20°C. Working concentrations of 10 μM (therapeutic concentration) or 100 μM (non-therapeutic) were freshly prepared in bath solution. pH was readjusted before performing electrophysiological experiments. Due to the large number of experimental conditions and the challenges of maintaining whole-cell recordings at elevated temperature, we performed unmatched pair experiments.

#### 4.3.6. Analysis and Statistics

Analysis and graphing were done using FitMaster software (HEKA Elektronik, Lambrecht, Germany) and Igor Pro (Wavemetrics, Lake Oswego, OR, USA) with statistical information derived using JMP statistical software. Statistical significance was
accepted at p < 0.05 using a four-factor completely randomized design (CRD) ANOVA test followed by a post-hoc Tukey test. Our statistical model was a full factorial in which all the factors were allowed to interact together yielding multiple effect tests: Ranolazine, Channel Variant, Ranolazine \times Channel Variant, Temperature, Ranolazine \times Temperature, Channel Variant \times Temperature, Ranolazine \times Channel Variant \times Temperature, Calcium, Ranolazine \times Calcium, Channel Variant \times Calcium, Ranolazine \times Channel Variant \times Calcium, Temperature \times Calcium, Channel Variant \times Temperature \times Calcium, Ranolazine \times Channel Variant \times Temperature \times Calcium. All values reported in the results sections are given as means \pm standard error of means.

4.3.7. Voltage Protocols

4.3.7.1 Current Density

We measured current density from the ratio of current amplitude to the cell membrane capacitance (pA/pF).

4.3.7.2 Conductance Density

Channel conductance was calculated from peak $I_{Na}$ using ohm’s law at 0 mV.

$$G_{Na} = \frac{I_{Na}}{V - E_{rev}}$$

where $G_{Na}$ is sodium channel conductance, $I_{Na}$ is peak sodium current in response to the command potential $V=0$ mV, and $E_{rev}$ is the reversal potential. We measured conductance density from the ratio of conductance to the cell membrane capacitance (nS/pF).

4.3.7.3 Activation (GV)

To determine the voltage dependence of activation, we measured the peak current amplitude at test pulse potentials ranging from -100 mV to +80 mV in increments of +10 mV for 19 ms. Prior to the test pulse, channels were allowed to recover from fast inactivation at -130 mV for 197 ms. Channel conductance was calculated from peak $I_{Na}$ using Formula (1). Calculated values for conductance were normalized to the maximal conductance and fit with the Boltzmann function:
2. \[ \frac{G}{G_{\text{max}}} = \frac{1}{1 + \exp(-ze_0[V_m - V_{1/2}]/kT)} \]

where \( \frac{G}{G_{\text{max}}} \) is the normalized conductance amplitude, \( V_m \) is the command potential, \( z \) is the apparent valence, \( e_0 \) is the elementary charge, \( V_{1/2} \) is the midpoint voltage, \( k \) is the Boltzmann constant, and \( T \) is temperature in °K.

**4.3.7.4 Steady-State Fast Inactivation (SSFI)**

The voltage-dependence of SSFI was measured by preconditioning the channels to a hyperpolarizing potential of -130 mV and then eliciting prepulses from -130 or -150 to +10 mV in increments of 10 mV for 500 ms. Channel availability was assessed during a test pulse to 0 mV. Normalized current amplitude as a function of voltage was fit using the Boltzmann function:

3. \[ \frac{I}{I_{\text{max}}} = \frac{1}{1 + \exp(-ze_0 (V_{m} - V_{1/2})/kT)} \]

where \( \frac{I}{I_{\text{max}}} \) is the normalized current amplitude, \( z \) is apparent valence, \( e_0 \) is the elementary charge, \( V_m \) is the prepulse potential, \( V_{1/2} \) is the midpoint voltage of SSFI, \( k \) is the Boltzmann constant, and \( T \) is temperature in °K.

**4.3.7.5 Fast Inactivation Onset**

Time constants for open-state fast inactivation were derived by fitting a single exponential function to the decay of current obtained from the activation protocol.

4. \[ I = I_{ss} + \alpha \exp(-(t-t_0)/\tau) \]

where \( I \) is current amplitude, \( I_{ss} \) is the plateau amplitude, \( \alpha \) is the amplitude at time 0 for time constant \( \tau \), and \( t \) is time.

**4.3.7.6 Late \( I_{\text{Na}} \) Current**

Late \( I_{\text{Na}} \) was measured between 40 – 50 ms during a 50 ms depolarizing pulse to 0 mV from a holding potential of -130 mV. An average of 10 pulses was used to increase the signal-to-noise ratio.

**4.3.7.7 Use-Dependent Inactivation (UDI, 1 Hz and 3 Hz)**

Channels accumulated into a use-dependent inactivated state during either a series of 300 380 ms depolarizing pulses to 0 mV followed by a 615 ms – 110 mV
recovery pulse at a frequency 1 Hz, or 500 220 ms depolarizing pulses to 0 mV followed by a 110 ms – 110 mV recovery pulse at a frequency 3 Hz. Normalized current amplitude as a function of time was fit with a double exponential.

5. \[ I = I_{ss} + \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2) \]

where \( I \) is current amplitude, \( I_{ss} \) is the plateau amplitude, \( \alpha_1 \) and \( \alpha_2 \) are the amplitudes at time 0 for time constants \( \tau_1 \) and \( \tau_2 \), and \( t \) is time.

### 4.3.7.8 \( Q_{10} \) Coefficients

The temperature coefficient for kinetic and thermodynamic parameters plotted as a function temperature was calculated in Igor:

6. \[ Q_{10} = \left( \frac{R_2}{R_1} \right)^{10/(T_2-T_1)} \]

where \( R \) is the rate and \( T \) is temperature (1 and 2 are the two temperatures measured). Rate was calculated by the inverse of the \( \tau \) value. \( Q_{10} \) fits for steady-state midpoints and slopes were calculated by replacing the \( R_X \) with \( V_{1/2} \) and \( z \) values. Fits for \( y_0 \) were calculated based of the \( 1/y_0 \) to yield optimal \( Q_{10} \) values. The fit was extrapolated to physiological (37 °C) and febrile (41 °C) temperatures.

### 4.3.7.9 Arrhenius Calculations

The Arrhenius linear relationship for the natural exponent of kinetic or thermodynamic parameters as a function of inverse temperature was calculated in Igor:

7. \[ \ln(k) = \ln(A) - \frac{(E_a/R)}{(1/T)} \]

where \( k \) is the rate constant, steady-state midpoint, or slope, \( A \) is the pre-exponential factor, \( E_a \) is the activation energy, \( R \) is the universal gas constant, and \( T \) is temperature in °K.

### 4.3.8. Myocardial Action Potential (AP) Modeling

### 4.3.8.1 Simulations

Action potentials were simulated using a modified version of the O’Hara-Rudy (O’Rd) model at 37 °C and 41 °C programmed in Matlab (O’Hara et al., 2011). The
sodium data were extrapolated to physiological and febrile temperatures Q10 values for WT and E1784K at 0 µM and 10 µM ranolazine. The maximal GNa density was 150 mS/µF in all conditions simulated. We modified the gating INa parameters data in accordance with our biophysical data for the various conditions. The GV and SSFI midpoints and slopes were extrapolated to 37 ºC and 41 ºC and normalized to the original O’Rd parameters. The phosphorylated steady-state fast inactivation midpoints in all channel variants were equally hyperpolarized by 6.2 mV. Late INa density was normalized to the original O’Rd value and multiplied by the percentage of late to peak INa calculated above.

To model the calcium-dependence of our late INa data, we fit the biophysical parameters extrapolated to 37 ºC and 41 ºC with a Hill equation:

8. \[ Z = Y_0 + \frac{(Y_M - Y_0)}{(1 + (X_{1/2}/X)^b)} \]

where Z is the biophysical parameter of interest, Y0 is the minimum value, YM is the maximum value, X1/2 is the midpoint of the curve, X is the intracellular cytosolic calcium, b is the rate.

Subspace calcium was not accounted for due to the lack of experimental data. Thus, the modified O’Rd model is a dynamic simulation of the calcium-induced shifts which are observed with increasing intracellular calcium levels as a function of pacing frequency, comprising the positive staircase phenomenon (Kort et al., 1985; Song et al., 2001).

Simulations at febrile temperature (41 ºC) included modifications to the major ionic currents, IKto (Yang & Zheng, 2014), ICaL (ten Tüsscher, 2003; Radzicki et al., 2013), IKr (Mauerhöfer & Bauer, 2016), and IKs (G. et al., 2001), in the O’Rd model based on previously published Q10 values.

Simulations were run on endocardial, midmyocardial, and epicardial ventricular myocytes using a 0.5 ms stimulus pulse with an amplitude of -80 µA/µF. The stimulus protocol was designed to step up the frequency gradually from 0.5 Hz to 2.5 Hz, with 1000 beats per frequency step to ensure attainment of steady-state.
### 4.3.8.2 Analysis

Analysis of APs only included those that fully recovered and were restored to baseline. Action potential duration (APD) was measured at 90% of repolarization by multiplying the resting membrane potential (RMP) value, prior to the current stimulus pulse, by 0.9. The APD\textsubscript{90} of the final two beats in the frequency step were plotted versus the diastolic interval (DI = BCL – APD\textsubscript{90}), where BCL is the basic cycle length, creating electrical restitution curves.

### 4.4. Results

#### 4.4.1. Ranolazine binds to Na\textsubscript{v}1.5 inner vestibule

The Na\textsubscript{v}1.5 homology model based on Na\textsubscript{v}Pas (Na\textsubscript{v}1.5-Na\textsubscript{v}Pas) is shown in Figure 4-2. The side view of the channel shows the four domains and their putative voltage and pore-forming segments (including the p-helices, extracellular and intracellular linkers). Na\textsubscript{v}Pas shares about 32% sequence identity with Na\textsubscript{v}1.5. The aligned the DIII-DIV linker and the CTD are shown in Figure 4-2. Ranolazine was auto-docked against Na\textsubscript{v}1.5-Na\textsubscript{v}Pas using AutoDock4. The highest affinity (-7.7 kcal/mol) binding mode is enlarged in Figure 4-2. The compound formed polar and Van der Waals interaction with various residues located in all four domains: S401, V405, C896, N927, F1418, S1458, L1462, N1463, I1466, F1760, V1764, I1768. The aromatic residue, F1760, is outlined in Figure 4-2 as it is a key putative binding site for many anti-arrhythmics, local anesthetics, and anticonvulsants (Fredj et al., 2006b; Lipkind & Fozzard, 2010). F1760 orientation with respect to ranolazine confirms its critical role in drug binding.
Figure 4-2  Ranolazine docked to Naᵥ1.5-NaᵥPas
The side view of Naᵥ1.5-NaᵥPas homology model is shown docked to ranolazine. The enlarged inset shows the cartoon structure of the drug binding to the central domains of the channel. The aromatic F1760 residue is outlined. Below the inset is a 3D-structure of ranolazine (Nitrogen is blue, Oxygen is red, Carbon is green, and Hydrogen is grey). Conserved residues in the DIII-DIV linker and the CTD between Naᵥ1.5 and NaᵥPas are indicated by a red asterisk.

4.4.2. Ranolazine does not affect conductance

Raw current traces in Figure 4-3 show the effects of 0 µM and 100 µM ranolazine on WT and E1784K at 0 nM and 2500 nM cytosolic calcium (only 34 ºC shown). E1784K reduced (p<0.0001) the peak current and conductance density compared to WT. Elevated temperature (34 ºC) increased (p<0.0001) peak current and conductance density in WT but not in E1784K. Ranolazine had no effect (p>0.05) on peak current or conductance density (Table 4-2 and Figure 4-3: B).
Figure 4-3  Ranolazine effects on channel conductance.

Top traces show raw $I_{Na}$ traces recorded at 34 °C. Panel A shows normalized conductance plotted against the test potential (pulse protocol shown in inset) at 34 °C. Panel B shows the peak conductance density bar graph versus all conditions at both 22 °C and 34 °C.
Table 4-2  Ranolazine Peak Current and Conductance Densities

<table>
<thead>
<tr>
<th>Condition</th>
<th>Peak I Density (pA/pF)</th>
<th>N</th>
<th>Peak GV Density (pS/pF)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - 22 ºC - 0 nM Ca(^{2+}) - 0 Ran</td>
<td>148.97 ± 35.10</td>
<td>7</td>
<td>2348.13 ± 584.87</td>
<td>7</td>
</tr>
<tr>
<td>WT - 22 ºC - 0 nM Ca(^{2+}) - 10 Ran</td>
<td>241.31 ± 41.80</td>
<td>5</td>
<td>3708.29 ± 580.79</td>
<td>5</td>
</tr>
<tr>
<td>WT - 22 ºC - 0 nM Ca(^{2+}) - 100 Ran</td>
<td>184.16 ± 40.16</td>
<td>5</td>
<td>3030.10 ± 640.76</td>
<td>5</td>
</tr>
<tr>
<td>WT - 22 ºC - 2500 nM Ca(^{2+}) - 0 Ran</td>
<td>302.91 ± 59.07</td>
<td>9</td>
<td>4714.99 ± 971.64</td>
<td>9</td>
</tr>
<tr>
<td>WT - 22 ºC - 2500 nM Ca(^{2+}) - 10 Ran</td>
<td>250.88 ± 45.34</td>
<td>7</td>
<td>4599.97 ± 788.18</td>
<td>7</td>
</tr>
<tr>
<td>WT - 22 ºC - 2500 nM Ca(^{2+}) - 100 Ran</td>
<td>128.74 ± 34.69</td>
<td>9</td>
<td>2088.79 ± 517.47</td>
<td>9</td>
</tr>
<tr>
<td>WT - 34 ºC - 0 nM Ca(^{2+}) - 0 Ran</td>
<td>354.74 ± 83.12</td>
<td>7</td>
<td>6067.75 ± 1197.96</td>
<td>6</td>
</tr>
<tr>
<td>WT - 34 ºC - 0 nM Ca(^{2+}) - 10 Ran</td>
<td>472.24 ± 55.47</td>
<td>5</td>
<td>7370.72 ± 654.58</td>
<td>7</td>
</tr>
<tr>
<td>WT - 34 ºC - 0 nM Ca(^{2+}) - 100 Ran</td>
<td>369.56 ± 81.13</td>
<td>8</td>
<td>5321.49 ± 1074.25</td>
<td>8</td>
</tr>
<tr>
<td>WT - 34 ºC - 2500 nM Ca(^{2+}) - 0 Ran</td>
<td>535.63 ± 70.44</td>
<td>6</td>
<td>7979.56 ± 433.11</td>
<td>5</td>
</tr>
<tr>
<td>WT - 34 ºC - 2500 nM Ca(^{2+}) - 10 Ran</td>
<td>447.46 ± 106.69</td>
<td>6</td>
<td>5945.51 ± 1253.7</td>
<td>6</td>
</tr>
<tr>
<td>WT - 34 ºC - 2500 nM Ca(^{2+}) - 100 Ran</td>
<td>599.72 ± 113.81</td>
<td>6</td>
<td>6935.36 ± 1048.34</td>
<td>5</td>
</tr>
<tr>
<td>EK - 22 ºC - 0 nM Ca(^{2+}) - 0 Ran</td>
<td>214.56 ± 50.18</td>
<td>7</td>
<td>3762.09 ± 835.36</td>
<td>7</td>
</tr>
<tr>
<td>EK - 22 ºC - 0 nM Ca(^{2+}) - 10 Ran</td>
<td>265.65 ± 51.36</td>
<td>8</td>
<td>4534.15 ± 651.10</td>
<td>10</td>
</tr>
<tr>
<td>EK - 22 ºC - 0 nM Ca(^{2+}) - 100 Ran</td>
<td>240.86 ± 24.43</td>
<td>7</td>
<td>3576.84 ± 301.37</td>
<td>7</td>
</tr>
<tr>
<td>EK - 22 ºC - 2500 nM Ca(^{2+}) - 0 Ran</td>
<td>170.41 ± 31.44</td>
<td>5</td>
<td>3660.43 ± 1006.25</td>
<td>6</td>
</tr>
<tr>
<td>EK - 22 ºC - 2500 nM Ca(^{2+}) - 10 Ran</td>
<td>250.36 ± 33.96</td>
<td>6</td>
<td>4562.87 ± 453.93</td>
<td>8</td>
</tr>
<tr>
<td>EK - 22 ºC - 2500 nM Ca(^{2+}) - 100 Ran</td>
<td>183.02 ± 44.18</td>
<td>5</td>
<td>2886.72 ± 498.80</td>
<td>5</td>
</tr>
<tr>
<td>EK - 34 ºC - 0 nM Ca(^{2+}) - 0 Ran</td>
<td>257.99 ± 34.07</td>
<td>8</td>
<td>3613.63 ± 524.71</td>
<td>9</td>
</tr>
<tr>
<td>EK - 34 ºC - 0 nM Ca(^{2+}) - 10 Ran</td>
<td>322.26 ± 47.45</td>
<td>6</td>
<td>5422.63 ± 833.24</td>
<td>8</td>
</tr>
<tr>
<td>EK - 34 ºC - 0 nM Ca(^{2+}) - 100 Ran</td>
<td>278.25 ± 67.75</td>
<td>8</td>
<td>4834.84 ± 766.20</td>
<td>7</td>
</tr>
<tr>
<td>EK - 34 ºC - 2500 nM Ca(^{2+}) - 0 Ran</td>
<td>354.25 ± 64.03</td>
<td>8</td>
<td>5587.74 ± 831.96</td>
<td>7</td>
</tr>
<tr>
<td>EK - 34 ºC - 2500 nM Ca(^{2+}) - 10 Ran</td>
<td>227.98 ± 41.82</td>
<td>5</td>
<td>3154.90 ± 685.41</td>
<td>5</td>
</tr>
<tr>
<td>EK - 34 ºC - 2500 nM Ca(^{2+}) - 100 Ran</td>
<td>212.41 ± 28.73</td>
<td>9</td>
<td>2959.51 ± 471.73</td>
<td>9</td>
</tr>
</tbody>
</table>

Figure 4-3: A shows normalized conductance plotted against the test potential at 34 ºC. E1784K (p<0.0001) and elevated temperature (p=0.0003) depolarized the conductance midpoint (GV-V_{1/2}). The conductance slope (GV-z) was reduced (p<0.0001) in E1784K compared to WT and increased (p<0.0001) when temperature was elevated in both channel variants. The interaction between channel variant and temperature had no effect on GV-V_{1/2} and GV-z. Normalized conductance was unchanged in all conditions with ranolazine (Table 4-3).
<table>
<thead>
<tr>
<th>Condition</th>
<th>GV-V_{1/2} (mV)</th>
<th>GV-z</th>
<th>N</th>
<th>SSFI-V_{1/2} (mV)</th>
<th>SSFI-z</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - 22 ºC - 0 nM Ca^{2+} - 0 Ran</td>
<td>-40.45 ± 1.24</td>
<td>3.76 ± 0.33</td>
<td>7</td>
<td>-88.62 ± 1.99</td>
<td>-3.93 ± 0.26</td>
<td>7</td>
</tr>
<tr>
<td>WT - 22 ºC - 0 nM Ca^{2+} - 10 Ran</td>
<td>-45.59 ± 1.09</td>
<td>5.37 ± 0.29</td>
<td>6</td>
<td>-86.51 ± 1.16</td>
<td>-3.95 ± 0.16</td>
<td>7</td>
</tr>
<tr>
<td>WT - 22 ºC - 0 nM Ca^{2+} - 100 Ran</td>
<td>-44.13 ± 3.07</td>
<td>4.84 ± 0.56</td>
<td>5</td>
<td>-88.45 ± 3.18</td>
<td>-2.87 ± 0.11</td>
<td>6</td>
</tr>
<tr>
<td>WT - 22 ºC - 2500 nM Ca^{2+} - 0 Ran</td>
<td>-42.22 ± 1.16</td>
<td>4.08 ± 0.25</td>
<td>9</td>
<td>-91.17 ± 1.72</td>
<td>-3.68 ± 0.15</td>
<td>9</td>
</tr>
<tr>
<td>WT - 22 ºC - 2500 nM Ca^{2+} - 10 Ran</td>
<td>-44.40 ± 2.22</td>
<td>3.87 ± 0.28</td>
<td>7</td>
<td>-91.44 ± 1.63</td>
<td>-3.93 ± 0.18</td>
<td>7</td>
</tr>
<tr>
<td>WT - 22 ºC - 2500 nM Ca^{2+} - 100 Ran</td>
<td>-44.76 ± 2.08</td>
<td>4.38 ± 0.36</td>
<td>7</td>
<td>-90.9 ± 1.64</td>
<td>-2.20 ± 0.13</td>
<td>7</td>
</tr>
<tr>
<td>WT - 34 ºC - 0 nM Ca^{2+} - 0 Ran</td>
<td>-43.44 ± 2.06</td>
<td>6.00 ± 0.45</td>
<td>6</td>
<td>-80.31 ± 1.17</td>
<td>-4.72 ± 0.18</td>
<td>6</td>
</tr>
<tr>
<td>WT - 34 ºC - 0 nM Ca^{2+} - 10 Ran</td>
<td>-37.93 ± 1.97</td>
<td>4.95 ± 0.28</td>
<td>7</td>
<td>-82.51 ± 2.26</td>
<td>-4.00 ± 0.14</td>
<td>8</td>
</tr>
<tr>
<td>WT - 34 ºC - 0 nM Ca^{2+} - 100 Ran</td>
<td>-37.58 ± 2.03</td>
<td>4.43 ± 0.33</td>
<td>7</td>
<td>-87.30 ± 2.73</td>
<td>-2.82 ± 0.16</td>
<td>7</td>
</tr>
<tr>
<td>WT - 34 ºC - 2500 nM Ca^{2+} - 0 Ran</td>
<td>-40.62 ± 1.44</td>
<td>5.96 ± 0.35</td>
<td>6</td>
<td>-80.25 ± 2.30</td>
<td>-4.24 ± 0.08</td>
<td>7</td>
</tr>
<tr>
<td>WT - 34 ºC - 2500 nM Ca^{2+} - 10 Ran</td>
<td>-38.03 ± 3.69</td>
<td>5.15 ± 0.40</td>
<td>6</td>
<td>-83.18 ± 3.95</td>
<td>-4.00 ± 0.13</td>
<td>6</td>
</tr>
<tr>
<td>WT - 34 ºC - 2500 nM Ca^{2+} - 100 Ran</td>
<td>-34.69 ± 0.98</td>
<td>6.30 ± 0.48</td>
<td>6</td>
<td>-90.43 ± 2.80</td>
<td>-3.18 ± 0.2</td>
<td>6</td>
</tr>
<tr>
<td>EK - 22 ºC - 0 nM Ca^{2+} - 0 Ran</td>
<td>-35.13 ± 2.70</td>
<td>2.99 ± 0.27</td>
<td>7</td>
<td>-100.46 ± 1.51</td>
<td>-2.97 ± 0.08</td>
<td>8</td>
</tr>
<tr>
<td>EK - 22 ºC - 0 nM Ca^{2+} - 10 Ran</td>
<td>-35.83 ± 1.65</td>
<td>3.14 ± 0.27</td>
<td>10</td>
<td>-99.37 ± 1.61</td>
<td>-2.88 ± 0.08</td>
<td>10</td>
</tr>
<tr>
<td>EK - 22 ºC - 0 nM Ca^{2+} - 100 Ran</td>
<td>-36.16 ± 1.87</td>
<td>3.95 ± 0.17</td>
<td>7</td>
<td>-103.83 ± 2.42</td>
<td>-1.77 ± 0.06</td>
<td>8</td>
</tr>
<tr>
<td>EK - 22 ºC - 2500 nM Ca^{2+} - 0 Ran</td>
<td>-39.33 ± 2.02</td>
<td>3.09 ± 0.16</td>
<td>5</td>
<td>-101.30 ± 3.14</td>
<td>-3.07 ± 0.21</td>
<td>6</td>
</tr>
<tr>
<td>EK - 22 ºC - 2500 nM Ca^{2+} - 10 Ran</td>
<td>-34.88 ± 1.57</td>
<td>2.83 ± 0.15</td>
<td>8</td>
<td>-100.76 ± 1.83</td>
<td>-3.11 ± 0.13</td>
<td>8</td>
</tr>
<tr>
<td>EK - 22 ºC - 2500 nM Ca^{2+} - 100 Ran</td>
<td>-33.72 ± 1.52</td>
<td>3.02 ± 0.35</td>
<td>5</td>
<td>-106.45 ± 2.29</td>
<td>-2.12 ± 0.08</td>
<td>5</td>
</tr>
<tr>
<td>EK - 34 ºC - 0 nM Ca^{2+} - 0 Ran</td>
<td>-30.22 ± 1.27</td>
<td>3.68 ± 0.28</td>
<td>9</td>
<td>-91.02 ± 2.79</td>
<td>-3.18 ± 0.14</td>
<td>9</td>
</tr>
<tr>
<td>EK - 34 ºC - 0 nM Ca^{2+} - 10 Ran</td>
<td>-36.80 ± 1.20</td>
<td>4.31 ± 0.38</td>
<td>9</td>
<td>-94.20 ± 3.73</td>
<td>-3.14 ± 0.13</td>
<td>9</td>
</tr>
<tr>
<td>EK - 34 ºC - 0 nM Ca^{2+} - 100 Ran</td>
<td>-35.53 ± 1.18</td>
<td>4.11 ± 0.22</td>
<td>8</td>
<td>-105.48 ± 1.64</td>
<td>-2.07 ± 0.07</td>
<td>8</td>
</tr>
<tr>
<td>EK - 34 ºC - 2500 nM Ca^{2+} - 0 Ran</td>
<td>-34.74 ± 1.48</td>
<td>4.06 ± 0.41</td>
<td>8</td>
<td>-96.78 ± 4.70</td>
<td>-3.26 ± 0.12</td>
<td>8</td>
</tr>
<tr>
<td>EK - 34 ºC - 2500 nM Ca^{2+} - 10 Ran</td>
<td>-31.25 ± 2.04</td>
<td>3.92 ± 0.35</td>
<td>7</td>
<td>-95.01 ± 3.23</td>
<td>-2.94 ± 0.15</td>
<td>7</td>
</tr>
<tr>
<td>EK - 34 ºC - 2500 nM Ca^{2+} - 100 Ran</td>
<td>-32.33 ± 1.64</td>
<td>3.39 ± 0.15</td>
<td>9</td>
<td>-105.73 ± 2.44</td>
<td>-2.00 ± 0.06</td>
<td>10</td>
</tr>
</tbody>
</table>

*1 p<0.01 and *2 p<0.05 vs 0 µM and 10 µM Ran of same condition
4.4.3. E1784K availability is decreased in ranolazine

Normalized current is plotted against membrane potential in Figure 4-4. E1784K hyperpolarized (p<0.0001) the SSFI midpoint (SSFI-V1/2) compared to WT. Elevated temperature depolarized (p<0.0001) SSFI-V1/2 in both WT and E1784K. At 34 °C and 0 nM cytosolic calcium, SSFI-V1/2 in E1784K was hyperpolarized (p<0.0001) in 100 µM ranolazine compared to WT (Figure 4-4: C and Table 4-3). This effect was not significant at 2500 nM cytosolic calcium. Analogous to the shifts on GV-z, SSFI-z was decreased in E1784K and increased with elevated temperature (p<0.0001). The slope was reduced in all conditions when ranolazine was increased from 10 µM to 100 µM (p<0.05, Table 4-3).

![Figure 4-4 Ranolazine effects on steady-state fast inactivation.](image)

Panels A-B (22 ºC) and C (34 ºC) shows steady-state fast inactivation as normalized current plotted against the prepulse potential (pulse protocol shown in C inset).

4.4.4. Fast inactivation onset kinetics are not altered with ranolazine

Fast inactivation onset kinetics at depolarized potentials (>50 mV) were measured from τ_{on} of the mono-exponential fits. E1784K fast inactivation onset kinetics were accelerated regardless of temperature (Figure 4-5: A-B). Onset kinetics were accelerated (decreased τ_{on}) with elevated temperature in WT compared to E1784K (p<0.01). WT and E1784K onset kinetics were decelerated (increased τ_{on}, p<0.05) in ranolazine as a function of voltage and cytosolic calcium at 22 ºC (values reported in Table 4-4). These drug effects on τ_{on} were not significant at elevated temperature.
Ranolazine effects on fast inactivation onset time constants. Panels A-B show the single-exponential time constants plotted against voltage. The pulse protocol is identical to that used to measure channel conductance (refer to Methods 4.3.7.3). Panel C includes Q_{10} coefficient values for all conditions between -50 mV to +10 mV. Elevated cytosolic calcium seems to modulate ranolazine effects on WT Q_{10}, as elevated cytosolic calcium heightened thermosensitivity in drug at -50 mV. E1784K thermosensitivity is dampened in elevated cytosolic at -50 mV.
Table 4-4  Ranolazine Fast Inactivation Onset Kinetics

<table>
<thead>
<tr>
<th>Condition</th>
<th>-50 mV $\tau$ (ms)</th>
<th>-30 mV $\tau$ (ms)</th>
<th>-10 mV $\tau$ (ms)</th>
<th>+10 mV $\tau$ (ms)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - 22 °C - 0 nM Ca$^{2+}$ - 0 Ran</td>
<td>3.47 ± 0.22</td>
<td>1.32 ± 0.11</td>
<td>0.69 ± 0.07</td>
<td>0.48 ± 0.04</td>
<td>7</td>
</tr>
<tr>
<td>WT - 22 °C - 0 nM Ca$^{2+}$ - 10 Ran</td>
<td>3.28 ± 0.45</td>
<td>1.13 ± 0.06</td>
<td>0.61 ± 0.04</td>
<td>0.44 ± 0.03</td>
<td>5</td>
</tr>
<tr>
<td>WT - 22 °C - 0 nM Ca$^{2+}$ - 100 Ran</td>
<td>5.72 ± 1.67 $^*$</td>
<td>1.61 ± 0.20 $^*$</td>
<td>0.74 ± 0.06</td>
<td>0.46 ± 0.03</td>
<td>5</td>
</tr>
<tr>
<td>WT - 22 °C - 2500 nM Ca$^{2+}$ - 0 Ran</td>
<td>3.59 ± 0.49</td>
<td>1.34 ± 0.12</td>
<td>0.69 ± 0.03</td>
<td>0.47 ± 0.01</td>
<td>8</td>
</tr>
<tr>
<td>WT - 22 °C - 2500 nM Ca$^{2+}$ - 10 Ran</td>
<td>3.03 ± 0.13</td>
<td>1.09 ± 0.05</td>
<td>0.58 ± 0.01</td>
<td>0.42 ± 0.01</td>
<td>6</td>
</tr>
<tr>
<td>WT - 22 °C - 2500 nM Ca$^{2+}$ - 100 Ran</td>
<td>3.02 ± 0.28</td>
<td>1.30 ± 0.09</td>
<td>0.71 ± 0.05</td>
<td>0.49 ± 0.04</td>
<td>9</td>
</tr>
<tr>
<td>WT - 34 °C - 0 nM Ca$^{2+}$ - 0 Ran</td>
<td>2.28 ± 0.52</td>
<td>0.66 ± 0.07</td>
<td>0.34 ± 0.02</td>
<td>0.23 ± 0.01</td>
<td>5</td>
</tr>
<tr>
<td>WT - 34 °C - 0 nM Ca$^{2+}$ - 10 Ran</td>
<td>2.82 ± 0.46</td>
<td>0.70 ± 0.08</td>
<td>0.35 ± 0.04</td>
<td>0.23 ± 0.02</td>
<td>7</td>
</tr>
<tr>
<td>WT - 34 °C - 0 nM Ca$^{2+}$ - 100 Ran</td>
<td>3.22 ± 0.45</td>
<td>0.78 ± 0.12</td>
<td>0.38 ± 0.06</td>
<td>0.22 ± 0.02</td>
<td>5</td>
</tr>
<tr>
<td>WT - 34 °C - 2500 nM Ca$^{2+}$ - 0 Ran</td>
<td>2.43 ± 0.45</td>
<td>0.48 ± 0.04</td>
<td>0.27 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>6</td>
</tr>
<tr>
<td>WT - 34 °C - 2500 nM Ca$^{2+}$ - 10 Ran</td>
<td>1.53 ± 0.24</td>
<td>0.60 ± 0.10</td>
<td>0.29 ± 0.03</td>
<td>0.20 ± 0.02</td>
<td>4</td>
</tr>
<tr>
<td>WT - 34 °C - 2500 nM Ca$^{2+}$ - 100 Ran</td>
<td>2.36 ± 0.32</td>
<td>0.69 ± 0.08</td>
<td>0.38 ± 0.04</td>
<td>0.25 ± 0.02</td>
<td>6</td>
</tr>
<tr>
<td>EK - 22 °C - 0 nM Ca$^{2+}$ - 0 Ran</td>
<td>1.35 ± 0.14</td>
<td>0.57 ± 0.03</td>
<td>0.40 ± 0.02</td>
<td>0.33 ± 0.02</td>
<td>7</td>
</tr>
<tr>
<td>EK - 22 °C - 0 nM Ca$^{2+}$ - 10 Ran</td>
<td>1.46 ± 0.17</td>
<td>0.54 ± 0.03</td>
<td>0.36 ± 0.02</td>
<td>0.30 ± 0.02</td>
<td>10</td>
</tr>
<tr>
<td>EK - 22 °C - 0 nM Ca$^{2+}$ - 100 Ran</td>
<td>1.65 ± 0.16</td>
<td>0.61 ± 0.03</td>
<td>0.37 ± 0.01</td>
<td>0.29 ± 0.01</td>
<td>6</td>
</tr>
<tr>
<td>EK - 22 °C - 2500 nM Ca$^{2+}$ - 0 Ran</td>
<td>0.71 ± 0.09</td>
<td>0.44 ± 0.02</td>
<td>0.32 ± 0.01</td>
<td>0.26 ± 0.01</td>
<td>6</td>
</tr>
<tr>
<td>EK - 22 °C - 2500 nM Ca$^{2+}$ - 10 Ran</td>
<td>1.23 ± 0.09</td>
<td>0.54 ± 0.03</td>
<td>0.37 ± 0.01</td>
<td>0.30 ± 0.01</td>
<td>7</td>
</tr>
<tr>
<td>EK - 22 °C - 2500 nM Ca$^{2+}$ - 100 Ran</td>
<td>1.65 ± 0.13</td>
<td>0.81 ± 0.09</td>
<td>0.54 ± 0.06 $^*$</td>
<td>0.40 ± 0.03 $^*$</td>
<td>5</td>
</tr>
<tr>
<td>EK - 34 °C - 0 nM Ca$^{2+}$ - 0 Ran</td>
<td>0.89 ± 0.12</td>
<td>0.34 ± 0.02</td>
<td>0.23 ± 0.02</td>
<td>0.18 ± 0.01</td>
<td>9</td>
</tr>
<tr>
<td>EK - 34 °C - 0 nM Ca$^{2+}$ - 10 Ran</td>
<td>0.88 ± 0.11</td>
<td>0.30 ± 0.02</td>
<td>0.21 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>9</td>
</tr>
<tr>
<td>EK - 34 °C - 0 nM Ca$^{2+}$ - 100 Ran</td>
<td>1.41 ± 0.34</td>
<td>0.47 ± 0.09</td>
<td>0.34 ± 0.07</td>
<td>0.24 ± 0.05</td>
<td>8</td>
</tr>
<tr>
<td>EK - 34 °C - 2500 nM Ca$^{2+}$ - 0 Ran</td>
<td>0.62 ± 0.11</td>
<td>0.27 ± 0.02</td>
<td>0.18 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>9</td>
</tr>
<tr>
<td>EK - 34 °C - 2500 nM Ca$^{2+}$ - 10 Ran</td>
<td>1.08 ± 0.07</td>
<td>0.35 ± 0.05</td>
<td>0.22 ± 0.02</td>
<td>0.17 ± 0.01</td>
<td>6</td>
</tr>
<tr>
<td>EK - 34 °C - 2500 nM Ca$^{2+}$ - 100 Ran</td>
<td>0.88 ± 0.11</td>
<td>0.37 ± 0.02</td>
<td>0.21 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>8</td>
</tr>
</tbody>
</table>

*p<0.05 vs 0 µM Ran of same condition, $^*$p<0.05 vs 10 µM Ran of same condition, $^*$p<0.01 and $^*$p<0.05 vs 0 µM Ran of same condition

**Figure 4-5: C** shows the $Q_{10}$ values at 0 µM and 10 µM ranolazine for all conditions. We observed high variability in the temperature coefficient at -50 mV compared to other voltages. At -50 mV, both ranolazine and cytosolic calcium mutually affect thermosensitivity in WT: $Q_{10}$ decreased at 0 nM cytosolic calcium and increased at 2500 nM cytosolic calcium in 10 µM ranolazine. At more depolarized voltages than -50 mV, subtle alterations occurred in WT $Q_{10}$ (**Figure 4-5: C**). E1784K $Q_{10}$ was not sensitive to ranolazine. However, E1784K thermosensitivity was dampened in cytosolic calcium at -50 mV compared to other voltages and to WT.
4.4.5. Ranolazine does not suppress thermosensitive late $I_{Na}$ in E1784K with elevated cytosolic calcium

Representative normalized late $I_{Na}$ current traces are shown in Figure 4-6: A-B at 0 µM and 100 µM ranolazine (only 34 °C shown). Late $I_{Na}$ percent and density are shown in Figure 4-6: C-D as bar graphs. Late $I_{Na}$ percent and density in E1784K increased (p<0.01) by 11-fold and 7-fold, respectively, with elevated temperature at 0 nM cytosolic calcium (Figure 4-6: D and Table 4-5). This increase in late $I_{Na}$ was almost fully attenuated in 10 µM ranolazine. Late $I_{Na}$ percent decreased in elevated cytosolic calcium (p<0.01) but there was no effect on late $I_{Na}$ density in E1784K. Late $I_{Na}$ percent and density in E1784K were not suppressed with ranolazine at 2500 nM cytosolic calcium (Figure 4-6: D and Table 4-5).
Figure 4-6  Ranolazine effects on late $I_{Na}$.
Panels A-B show normalized current traces, with emphasis on the non-inactivating, late $I_{Na}$, at 34 °C. The normalized late $I_{Na}$ in E1784K is enhanced drastically by elevated temperature. Cytosolic calcium suppresses the E1784K normalized late $I_{Na}$ (addressed in discussions). Ranolazine suppresses late $I_{Na}$ only at 0 nM compared to 2500 nM cytosolic calcium. Panels C-D show the late $I_{Na}$ percent and density bar graphs for all conditions at 22 °C and 34 °C, respectively.
Table 4-5  Ranolazine Late I_{Na}

<table>
<thead>
<tr>
<th>Condition</th>
<th>Late Density (pA/pF)</th>
<th>N</th>
<th>Late Percent (%)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - 22 °C - 0 nM Ca^{2+} - 0 Ran</td>
<td>1.95 ± 0.34</td>
<td>9</td>
<td>1.71 ± 0.54</td>
<td>9</td>
</tr>
<tr>
<td>WT - 22 °C - 0 nM Ca^{2+} - 10 Ran</td>
<td>1.35 ± 0.44</td>
<td>8</td>
<td>0.35 ± 0.12</td>
<td>8</td>
</tr>
<tr>
<td>WT - 22 °C - 0 nM Ca^{2+} - 100 Ran</td>
<td>1.55 ± 0.79</td>
<td>6</td>
<td>0.45 ± 0.20</td>
<td>6</td>
</tr>
<tr>
<td>WT - 22 °C - 2500 nM Ca^{2+} - 0 Ran</td>
<td>8.83 ± 1.33</td>
<td>8</td>
<td>2.41 ± 0.72</td>
<td>6</td>
</tr>
<tr>
<td>WT - 22 °C - 2500 nM Ca^{2+} - 10 Ran</td>
<td>3.78 ± 1.64</td>
<td>8</td>
<td>0.80 ± 0.48</td>
<td>7</td>
</tr>
<tr>
<td>WT - 22 °C - 2500 nM Ca^{2+} - 100 Ran</td>
<td>1.01 ± 0.38</td>
<td>7</td>
<td>0.30 ± 0.12</td>
<td>6</td>
</tr>
<tr>
<td>WT - 34 °C - 0 nM Ca^{2+} - 0 Ran</td>
<td>5.04 ± 1.42</td>
<td>7</td>
<td>1.57 ± 0.52</td>
<td>9</td>
</tr>
<tr>
<td>WT - 34 °C - 0 nM Ca^{2+} - 10 Ran</td>
<td>7.21 ± 1.61</td>
<td>8</td>
<td>1.54 ± 0.54</td>
<td>8</td>
</tr>
<tr>
<td>WT - 34 °C - 0 nM Ca^{2+} - 100 Ran</td>
<td>7.66 ± 2.47</td>
<td>9</td>
<td>1.67 ± 0.42</td>
<td>9</td>
</tr>
<tr>
<td>WT - 34 °C - 2500 nM Ca^{2+} - 0 Ran</td>
<td>10.57 ± 3.2</td>
<td>7</td>
<td>2.71 ± 0.90</td>
<td>9</td>
</tr>
<tr>
<td>WT - 34 °C - 2500 nM Ca^{2+} - 10 Ran</td>
<td>6.95 ± 1.97</td>
<td>8</td>
<td>1.09 ± 0.26</td>
<td>8</td>
</tr>
<tr>
<td>WT - 34 °C - 2500 nM Ca^{2+} - 100 Ran</td>
<td>5.11 ± 1.67</td>
<td>4</td>
<td>0.98 ± 0.25</td>
<td>5</td>
</tr>
<tr>
<td>EK - 22 °C - 0 nM Ca^{2+} - 0 Ran</td>
<td>2.51 ± 0.63</td>
<td>9</td>
<td>0.85 ± 0.20</td>
<td>9</td>
</tr>
<tr>
<td>EK - 22 °C - 0 nM Ca^{2+} - 10 Ran</td>
<td>2.86 ± 0.88</td>
<td>9</td>
<td>0.68 ± 0.21</td>
<td>8</td>
</tr>
<tr>
<td>EK - 22 °C - 0 nM Ca^{2+} - 100 Ran</td>
<td>3.01 ± 1.08</td>
<td>10</td>
<td>1.02 ± 0.39</td>
<td>8</td>
</tr>
<tr>
<td>EK - 22 °C - 2500 nM Ca^{2+} - 0 Ran</td>
<td>9.02 ± 2.31</td>
<td>5</td>
<td>2.64 ± 0.81</td>
<td>4</td>
</tr>
<tr>
<td>EK - 22 °C - 2500 nM Ca^{2+} - 10 Ran</td>
<td>3.68 ± 0.51</td>
<td>9</td>
<td>1.65 ± 0.30</td>
<td>9</td>
</tr>
<tr>
<td>EK - 22 °C - 2500 nM Ca^{2+} - 100 Ran</td>
<td>1.78 ± 0.65</td>
<td>8</td>
<td>0.61 ± 0.30</td>
<td>6</td>
</tr>
<tr>
<td>EK - 34 °C - 0 nM Ca^{2+} - 0 Ran</td>
<td>17.47 ± 1.54*</td>
<td>6</td>
<td>8.97 ± 1.02*</td>
<td>6</td>
</tr>
<tr>
<td>EK - 34 °C - 0 nM Ca^{2+} - 10 Ran</td>
<td>3.84 ± 1.39</td>
<td>4</td>
<td>0.86 ± 0.04</td>
<td>4</td>
</tr>
<tr>
<td>EK - 34 °C - 0 nM Ca^{2+} - 100 Ran</td>
<td>3.09 ± 0.96</td>
<td>6</td>
<td>0.89 ± 0.22</td>
<td>6</td>
</tr>
<tr>
<td>EK - 34 °C - 2500 nM Ca^{2+} - 0 Ran</td>
<td>11.01 ± 2.12</td>
<td>7</td>
<td>2.00 ± 0.34</td>
<td>9</td>
</tr>
<tr>
<td>EK - 34 °C - 2500 nM Ca^{2+} - 10 Ran</td>
<td>10.05 ± 3.08</td>
<td>5</td>
<td>3.28 ± 0.93</td>
<td>6</td>
</tr>
<tr>
<td>EK - 34 °C - 2500 nM Ca^{2+} - 100 Ran</td>
<td>15.73 ± 6.24</td>
<td>5</td>
<td>3.76 ± 1.10</td>
<td>4</td>
</tr>
</tbody>
</table>

*p<0.01 vs 10 µM and 100 µM Ran of same condition

4.4.6. Ranolazine does not enhance UDI in E1784K with elevated cytosolic calcium

Sustained or repetitive depolarizations induce slow inactivation in NaV1.5, which was indirectly measured by the use-dependent inactivation (UDI) protocols described in the methods. Use-dependence was measured at 1 Hz and 3 Hz, mimicking resting heart rate (60 bpm) and tachycardia (180 bpm), respectively. Normalized current plotted against time for UDI measured at 1 Hz and 3 Hz are shown in Figure 4-7 (only 34 °C shown).

UDI plateau (y₀) was greater (p=0.0430) at elevated temperature at both 1 Hz and 3 Hz, but the shift was larger in E1784K at 1Hz (Table 4-6). y₀ decreased to different levels in ranolazine (reported in Table 4-6). At high UDI frequencies, E1784K y₀ decreased in ranolazine at 34 °C compared to WT (Figure 4-7 shows only 34 °C, Table
Our statistical results suggest that the drug effects on UDI (3 Hz) in E1784K are limited in elevated cytosolic calcium (Table 4-6).

UDI onset kinetics were accelerated in E1784K and elevated temperature (p<0.05), measured by $\tau_1$, at 1 Hz and 3 Hz. Onset kinetics decelerated in elevated cytosolic calcium (p<0.0001) at 1 Hz predominately in WT compared to E1784K. WT and E1784K $\tau_1$ was decreased (p<0.0001) in ranolazine at 2500 nM cytosolic calcium and 34 ºC (Table 4-6). $\tau_1$ at 3 Hz was unaffected in ranolazine. $\tau_2$ was unaffected at 1 Hz and 3 Hz in all experimental conditions.

Figure 4-7 Ranolazine effects on use-dependence. Panels AI-II and Panels BI-II show normalized current versus time measuring UDI at 1 Hz and 3 Hz, respectively. Insets of pulse protocols are excluded for visual clarity (refer to methods). All three drug concentrations are included in the figures to show 10 µM ranolazine effect on UDI (3 Hz) in E1784K at 0 nM compared to 2500 nM (refer to Table 4-6 for values).
### Table 4-6  Ranolazine Use-Dependence 1Hz & 3Hz

<table>
<thead>
<tr>
<th>Condition</th>
<th>1 Hz - y0</th>
<th>1 Hz - τ1 (s)</th>
<th>1 Hz - τ2 (s)</th>
<th>N</th>
<th>3 Hz - y0</th>
<th>3 Hz - τ1 (s)</th>
<th>3 Hz - τ2 (s)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - 22 °C - 0 nM Ca²⁺ - 0 Ran</td>
<td>0.84 ± 0.03</td>
<td>3.13 ± 0.88</td>
<td>47.88 ± 9.44</td>
<td>6</td>
<td>0.63 ± 0.04</td>
<td>2.03 ± 0.41</td>
<td>40.53 ± 5.42</td>
<td>7</td>
</tr>
<tr>
<td>WT - 22 °C - 0 nM Ca²⁺ - 10 Ran</td>
<td>0.77 ± 0.01</td>
<td>2.96 ± 0.52</td>
<td>18.55 ± 4.50</td>
<td>7</td>
<td>0.52 ± 0.02</td>
<td>3.39 ± 0.62</td>
<td>23.00 ± 7.54</td>
<td>7</td>
</tr>
<tr>
<td>WT - 22 °C - 0 nM Ca²⁺ - 100 Ran</td>
<td>0.44 ± 0.02</td>
<td>2.19 ± 0.44</td>
<td>9.70 ± 3.31</td>
<td>5</td>
<td>0.15 ± 0.02</td>
<td>0.92 ± 0.18</td>
<td>7.02 ± 4.44</td>
<td>4</td>
</tr>
<tr>
<td>WT - 22 °C - 2500 nM Ca²⁺ - 0 Ran</td>
<td>0.81 ± 0.02</td>
<td>8.22 ± 2.84</td>
<td>114.32 ± 22.42</td>
<td>7</td>
<td>0.66 ± 0.03</td>
<td>1.83 ± 0.23</td>
<td>36.4 ± 7.98</td>
<td>7</td>
</tr>
<tr>
<td>WT - 22 °C - 2500 nM Ca²⁺ - 10 Ran</td>
<td>0.78 ± 0.01</td>
<td>6.93 ± 0.94</td>
<td>31.93 ± 10.85</td>
<td>6</td>
<td>0.52 ± 0.02</td>
<td>1.19 ± 0.19</td>
<td>8.57 ± 0.42</td>
<td>5</td>
</tr>
<tr>
<td>WT - 22 °C - 2500 nM Ca²⁺ - 100 Ran</td>
<td>0.35 ± 0.03</td>
<td>1.67 ± 0.12</td>
<td>34.32 ± 22.87</td>
<td>7</td>
<td>0.20 ± 0.03</td>
<td>0.69 ± 0.32</td>
<td>14.56 ± 13.09</td>
<td>3</td>
</tr>
<tr>
<td>WT - 34 °C - 0 nM Ca²⁺ - 0 Ran</td>
<td>0.92 ± 0.01</td>
<td>4.77 ± 3.14</td>
<td>39.42 ± 20.36</td>
<td>4</td>
<td>0.84 ± 0.03</td>
<td>2.75 ± 1.00</td>
<td>17.73 ± 9.63</td>
<td>5</td>
</tr>
<tr>
<td>WT - 34 °C - 0 nM Ca²⁺ - 10 Ran</td>
<td>0.76 ± 0.05</td>
<td>5.41 ± 3.35</td>
<td>62.60 ± 20.25</td>
<td>5</td>
<td>0.65 ± 0.06</td>
<td>0.98 ± 0.10</td>
<td>16.65 ± 6.29</td>
<td>6</td>
</tr>
<tr>
<td>WT - 34 °C - 0 nM Ca²⁺ - 100 Ran</td>
<td>0.60 ± 0.06</td>
<td>0.66 ± 0.10</td>
<td>43.51 ± 18.93</td>
<td>8</td>
<td>0.33 ± 0.05</td>
<td>0.39 ± 0.08</td>
<td>13.99 ± 8.03</td>
<td>8</td>
</tr>
<tr>
<td>WT - 34 °C - 2500 nM Ca²⁺ - 0 Ran</td>
<td>0.88 ± 0.03</td>
<td>30.10 ± 6.41</td>
<td>59.96 ± 37.08</td>
<td>4</td>
<td>0.79 ± 0.04</td>
<td>1.46 ± 0.17</td>
<td>38.85 ± 12.19</td>
<td>8</td>
</tr>
<tr>
<td>WT - 34 °C - 2500 nM Ca²⁺ - 10 Ran</td>
<td>0.82 ± 0.04</td>
<td>2.83 ± 1.02</td>
<td>67.05 ± 23.05</td>
<td>5</td>
<td>0.60 ± 0.03</td>
<td>1.02 ± 0.16</td>
<td>17.28 ± 6.89</td>
<td>7</td>
</tr>
<tr>
<td>WT - 34 °C - 2500 nM Ca²⁺ - 100 Ran</td>
<td>0.64 ± 0.02</td>
<td>0.44 ± 0.16</td>
<td>8.44 ± 2.77</td>
<td>5</td>
<td>0.27 ± 0.03</td>
<td>0.41 ± 0.06</td>
<td>17.80 ± 9.98</td>
<td>5</td>
</tr>
<tr>
<td>EK - 22 °C - 0 nM Ca²⁺ - 0 Ran</td>
<td>0.69 ± 0.06</td>
<td>3.37 ± 0.64</td>
<td>69.20 ± 25.09</td>
<td>6</td>
<td>0.79 ± 0.01</td>
<td>3.08 ± 1.61</td>
<td>32.65 ± 20.62</td>
<td>4</td>
</tr>
<tr>
<td>EK - 22 °C - 0 nM Ca²⁺ - 10 Ran</td>
<td>0.57 ± 0.05</td>
<td>3.25 ± 0.63</td>
<td>64.71 ± 37.46</td>
<td>7</td>
<td>0.47 ± 0.02</td>
<td>2.40 ± 0.37</td>
<td>16.28 ± 3.57</td>
<td>6</td>
</tr>
<tr>
<td>EK - 22 °C - 0 nM Ca²⁺ - 100 Ran</td>
<td>0.27 ± 0.05</td>
<td>1.87 ± 0.25</td>
<td>74.43 ± 28.56</td>
<td>8</td>
<td>0.14 ± 0.03</td>
<td>1.00 ± 0.34</td>
<td>1.29 ± 0.19</td>
<td>4</td>
</tr>
<tr>
<td>EK - 22 °C - 2500 nM Ca²⁺ - 0 Ran</td>
<td>0.73 ± 0.02</td>
<td>1.99 ± 1.02</td>
<td>31.37 ± 6.74</td>
<td>4</td>
<td>0.69 ± 0.06</td>
<td>3.59 ± 1.84</td>
<td>8.20 ± 3.73</td>
<td>4</td>
</tr>
<tr>
<td>EK - 22 °C - 2500 nM Ca²⁺ - 10 Ran</td>
<td>0.59 ± 0.06</td>
<td>7.17 ± 1.35</td>
<td>75.52 ± 20.26</td>
<td>5</td>
<td>0.47 ± 0.04</td>
<td>3.38 ± 0.43</td>
<td>13.21 ± 1.51</td>
<td>5</td>
</tr>
<tr>
<td>EK - 22 °C - 2500 nM Ca²⁺ - 100 Ran</td>
<td>0.24 ± 0.04</td>
<td>2.68 ± 1.13</td>
<td>70.86 ± 29.29</td>
<td>3</td>
<td>0.20 ± 0.06</td>
<td>0.82 ± 0.29</td>
<td>1.94 ± 0.31</td>
<td>3</td>
</tr>
<tr>
<td>EK - 34 °C - 0 nM Ca²⁺ - 0 Ran</td>
<td>0.82 ± 0.04</td>
<td>6.76 ± 1.43</td>
<td>68.66 ± 25.39</td>
<td>8</td>
<td>0.77 ± 0.03</td>
<td>2.66 ± 0.45</td>
<td>49.11 ± 13.27</td>
<td>9</td>
</tr>
<tr>
<td>EK - 34 °C - 0 nM Ca²⁺ - 10 Ran</td>
<td>0.77 ± 0.06</td>
<td>2.40 ± 0.55</td>
<td>78.22 ± 35.32</td>
<td>7</td>
<td>0.55 ± 0.03</td>
<td>1.63 ± 0.32</td>
<td>27.16 ± 15.43</td>
<td>5</td>
</tr>
<tr>
<td>EK - 34 °C - 0 nM Ca²⁺ - 100 Ran</td>
<td>0.47 ± 0.04</td>
<td>0.77 ± 0.07</td>
<td>19.07 ± 9.49</td>
<td>6</td>
<td>0.21 ± 0.05</td>
<td>0.40 ± 0.04</td>
<td>24.08 ± 18.78</td>
<td>5</td>
</tr>
<tr>
<td>EK - 34 °C - 2500 nM Ca²⁺ - 0 Ran</td>
<td>0.87 ± 0.03</td>
<td>15.05 ± 3.35</td>
<td>65.60 ± 19.19</td>
<td>5</td>
<td>0.74 ± 0.08</td>
<td>2.50 ± 0.65</td>
<td>30.82 ± 2.43</td>
<td>6</td>
</tr>
<tr>
<td>EK - 34 °C - 2500 nM Ca²⁺ - 10 Ran</td>
<td>0.77 ± 0.04</td>
<td>1.77 ± 0.48</td>
<td>48.78 ± 22.76</td>
<td>5</td>
<td>0.63 ± 0.03</td>
<td>1.13 ± 0.12</td>
<td>6.41 ± 3.13</td>
<td>6</td>
</tr>
<tr>
<td>EK - 34 °C - 2500 nM Ca²⁺ - 100 Ran</td>
<td>0.46 ± 0.05</td>
<td>1.21 ± 0.20</td>
<td>38.24 ± 19.24</td>
<td>5</td>
<td>0.26 ± 0.03</td>
<td>0.54 ± 0.14</td>
<td>6.89 ± 4.62</td>
<td>5</td>
</tr>
</tbody>
</table>

* p<0.0001 vs 0 µM and 10 µM Ran of same condition, * p<0.0001 vs 0 µM Ran of same condition, * p<0.0001 vs 10 µM and 100 µM Ran of same condition, * p<0.0001 vs 0 µM and 100 µM Ran of same condition,
4.4.7. E1784K-induced alternans is exacerbated with ranolazine

Biophysical data were extrapolated to physiological (37 °C) and febrile (41 °C) temperatures using a Q10 coefficient. Extrapolations were independently confirmed using Arrhenius relationships. Action potential (AP) traces are shown in Figure 4-8 for simulations conducted in endocardial cells at three frequencies: 0.5 Hz (bradycardia), 1.5 Hz (sinus rhythm), and 2.5 Hz (tachycardia). At febrile temperature, simulated APs show accelerated depolarizations and repolarizations in WT compared to E1784K.

![Figure 4-8](image.png)

Figure 4-8  Endocardial action potential simulations.
AP simulations are plotted against time (inset is shown in bottom right corner) at 37 °C and 41 °C. The last two AP beats were plotted in E1784K to show alternans-induction. Simulations only included therapeutic concentrations of ranolazine (10 µM).

Electrical restitution curves (ERCs) at 90 % repolarization were constructed from plotting APD₉₀ against the diastolic interval as shown in Figure 4-9: A-B in endocardial cells. The last two beats were included in the ERCs to exemplify bifurcation and alternans-induction at critical diastolic intervals. The APD₉₀ for WT follows a similar trend to previously published ERCs, typifying a relatively stable APD rate dependence (Nolasco & Dahlen, 1968; Franz, 2003; Jordan & Christini, 2004). E1784K has a higher APD₉₀ in all cardiac cells, especially the mid-myocardium, compared to WT (not shown, Figure 4-9). At 37 °C, the mutant causes bifurcation in APD₉₀, mainly in epicardial cells, indicative of alternans (not shown); however, endocardial cells also experience alternans at febrile temperature in addition to epicardial cells (Figure 4-9: B). Upon drug perfusion, bifurcations were observed at higher diastolic intervals in E1784K (Figure 4-9).
drug-induced bifurcations in ERCs were augmented at febrile temperature in all cardiac cells.

A linear relationship is established between the last two AP beats at each frequency step (shown in Figure 4-9: C-D), with no alternans. Divergence from linearity is indicative of alternans occurrence. At both 37 ºC and 41 ºC, WT cells had no alternans, even upon 10 µM ranolazine perfusion, showing linearity with a slope = 1 (Figure 4-9: C-D). In drug-free conditions, E1784K had a linear relationship at high BCLs (low frequencies), but deviated from linearity beginning at intermediate BCLs; distortion in linearity is observed at lower frequencies in epicardial cells. This relationship in E1784K is augmented with febrile temperature. The prolonged APD₉₀ in E1784K were shortened with ranolazine at very low frequencies, and alternans were quickly induced even during bradycardia, an effect exacerbated by febrile temperature.

Figure 4-9 Cardiac electrical restitution properties
Panel A-B shows the endocardial ERC curves at 37 ºC and 41 ºC. Panels C shows plots of the last two AP beats to determine alternans-induction in the three myocardial cells at 37 ºC and 41 ºC.

4.5. Discussion

Our goal was to determine whether ranolazine reduced channel dysfunction in E1784K under the triggering conditions of elevated temperature and cytosolic calcium. Ranolazine did not attenuate gain-of-function in E1784K when temperature and cytosolic
calcium were elevated. Ranolazine has minimal effects on conductance in Na\textsubscript{v}1.5 (Huang et al., 2011; Sokolov et al., 2013). The drug follows the modulated receptor hypothesis, targeting the open/inactivated states at depolarized potentials, thereby suppressing late I\textsubscript{Na} (Ragsdale et al., 1996). Physiological events, such as acidaemia, enhance ranolazine antiarrhythmic effect by augmenting late I\textsubscript{Na}, thus providing the drug with a larger open-state channel substrate to target (Jones et al., 2011; Vilin et al., 2012; Sokolov et al., 2013). In addition to physiological modulators, SCN5a mutations often alter voltage-dependence of the channel, which modify drug effects on Na\textsubscript{v}1.5. To date, ranolazine has been screened against only ∆KPQ (Fredj et al., 2006b; Moss et al., 2008), Y1767C (Huang et al., 2011), R1623Q (Rajamani et al., 2009), and D1790G (Chorin et al., 2016). Our study is the first to show the combined external triggers and SCN5a mutation effects on ranolazine.

Ranolazine efficacy was enhanced at elevated temperature. Similar to acidosis effects, elevated temperature increases the late open probability in E1784K (Belardinelli, 2006a; Sokolov et al., 2013). Late I\textsubscript{Na} percent and density increased by 11- and 7-fold, respectively, with elevated temperature. We previously reported a 3.54-fold increase in late I\textsubscript{Na} percent when temperature was elevated from 22 ºC to 34 ºC (Abdelsayed et al., 2015); however, we used CHOK1 cells to study E1784K thermosensitivity. The temperature coefficient (Q\textsubscript{10}) partly depends on lipid-channel interactions in the membrane, which differ between heterologous expression systems (Murray et al., 1990; Ruff, 1999). The HEK293 lipid bilayer is less viscous than CHOK1 cells as observed in our whole-cell recordings, which may justify the heightened Q\textsubscript{10} in the late I\textsubscript{Na} measurements. At elevated stimulation frequencies and temperature, therapeutic ranolazine decreased channel availability by increasing channel use-dependence in E1784K.

Although ranolazine efficacy appears to be increased by temperature, its efficacy appears to be dampened by the combination of both elevated temperature and cytosolic calcium. At 34 ºC, E1784K late I\textsubscript{Na} percent was depressed with elevated cytosolic calcium, but there was no effect on late I\textsubscript{Na} density. These opposing changes in late I\textsubscript{Na} percent and density may be attributed to the increased peak I\textsubscript{Na} density with elevated cytosolic calcium at 34 ºC. Although not significant, the shift contributes to late I\textsubscript{Na} percent calculation (Table 4-2). Late I\textsubscript{Na} in E1784K was not attenuated by ranolazine at elevated temperature and cytosolic calcium.
4.5.1. Drug Binding in Mutant-Trigger Context

Ranolazine action on Na\textsubscript{v}1.5 is commonly associated with the modulated receptor hypothesis (Hille, 1977). Sokolov et al. (2013) argued that the drug follows a modified form of binding, as their pharmacological analysis suggested drug-binding at hyperpolarized voltages (Sokolov et al., 2013). Ranolazine exhibits partial preference to the closed state in Na\textsubscript{v}1.5, as suggested by the hyperpolarizing shift in SSFI observed with ranolazine. The shift mainly decreases channel availability near resting potential in cardiomyocytes. Other classic antiarrhythmics, anticonvulsants, and local anesthetics have similar effects, yet follow the modulated receptor hypothesis (Ragsdale et al., 1996; Tikhonov & Zhorov, 2017). Sokolov et al. (2013) also report that the drug-induced block in late I\textsubscript{Na} occurs by a slow-mode recovery in slow inactivation, which is exacerbated at low pH (Sokolov et al., 2013). This effect was mildly observed in this study. However, it is difficult to correlate the drug effects in E1784K UDI to late I\textsubscript{Na}, as the former had minor but significant shifts.

Ranolazine shares a very similar structure with the class 1b antiarrhythmic drug lidocaine, which, like other sodium blockers, preferentially binds to F1760 and, to a lesser extent, Y1767 in DIVS6 (Fredj et al., 2006b; Huang et al., 2011). Ranolazine has high lipophilicity and can only bind to its receptor sites by traversing the phospholipid membrane and entering the central cavity through the inner vestibule. However, lateral pores, known as fenestrations, are alternative routes for large compounds like ranolazine to access the central cavity. The fenestrations in the Na\textsubscript{v}Pas-Na\textsubscript{v}1.5 model were unavailable for drug binding in auto-docking due to their constricted sizes (Shen et al., 2017). It would be interesting to determine whether E1784K alters fenestration size in Na\textsubscript{v}1.5, modifying drug entry via the fenestrations.

The interaction between ranolazine and extracellular channel regions is unknown, but is unlikely due to the drug’s lipophilicity; a crystal structure of the channel/drug interaction would elucidate the drug-induced modifications in gating. The newly discovered aryl sulfonamide antagonists preferentially stabilize Na\textsubscript{v}1.7 DIVS4 activation thereby stabilizing the fast inactivated state and suppressing late I\textsubscript{Na} (Ahuja et al., 2015). Ranolazine may be structurally modified to include other moieties, like anionic aryl sulfonamides, for further optimizing its selectivity for targeting late versus peak I\textsubscript{Na}. 
4.5.2. E1784K-induced Structural Rearrangements in Na\textsubscript{v}1.5 and its Impact on ranolazine

We speculate E1784K affects fast inactivation via two possible mechanisms in Na\textsubscript{v}1.5, thereby altering channel/drug interactions. Figure 4-1 shows the channel structures discussed, as follows.

(1) E1784K hyperpolarizes the voltage-dependence of SSFI, thus stabilizing the interaction between the channel and the fast inactivation particle (Dumaine et al., 1996; Chandra et al., 1998; Wei et al., 1999; Deschênes et al., 2000; Makita et al., 2008a). Fast inactivation onset is correlated with DIVS4 activation, whereas channel recovery is rate limited by charge immobilization of DIVS4 (Kühn & Greeff, 1999; Capes et al., 2013). The charge reversal mutant, E1784K, may enhance the transition of DIVS4 between closed and open states, as suggested by the Peters-Ruben model (Peters et al., 2017). We postulate that this effect may be due to an electrostatic repulsion between the CTD mutant and conserved positive residues in DIVS4, given their close proximity (Shen et al., 2017). This repulsion could make the DIVS4 in E1784K more mobile, which might explain the accelerated fast inactivation onset and recovery kinetics (Makita et al., 2008a; Abdelsayed et al., 2015, 2017; Peters et al., 2017). Fast inactivation kinetics in E1784K are not enhanced by temperature. Thus, it does not seem justifiable to attribute the thermosensitive late \(I_{\text{Na}}\) in E1784K to increased recovery kinetics, which are not thermosensitive. Rather a rearrangement may occur in DIVS4, conforming the voltage sensor to a state in which conductance in E1784K is higher (Peters et al., 2017).

(2) E1784K alters the structure of the CTD by disrupting the native hydrophobic and electrostatic interactions holding the EF-like hand domain (\(\alpha_1-\alpha_4\)) tight with the IQ motif (\(\alpha_6\)) (Chagot et al., 2009). Calcium sensitivity is imparted in Na\textsubscript{v}1.5 via calmodulin, which binds to the IQ motif (\(\alpha_6\)) via its C-lobe or N-lobe depending on cytosolic calcium levels (Shah et al., 2006; Sarhan et al., 2009, 2012). During diastole or systole, calmodulin is calcified to different extents at its N-lobe (Ben-Johny et al., 2014). Calcified calmodulin has a lower affinity for the IQ motif and binds, via its C-lobe, to the DIII-DIV linker, forming a tripartite complex. This interaction is thought to prevent the DIII-DIV linker fast inactivation particle from occluding the pore, increasing channel availability near resting potential (Shah et al., 2006; Sarhan et al., 2012). With depolarized potentials, the calmodulin C-lobe stabilizes the fast inactivation particle, suppressing late
\[ I_{Na} \], as in \( \Delta \)KPQ and other mixed syndrome mutants (Van Petegem et al., 2012; Potet et al., 2015; Abdelsayed et al., 2017). Some studies refute the tripartite complex formation and favor a CaV1.2-like regulation of inactivation in Na\( v \)1.5 (Ben-Johny et al., 2014; Pitt & Lee, 2016). In those studies, the calcium-calmodulin complex is localized to the CTD (Petegem et al., 2005; Ben-Johny & Yue, 2014). The Na\( v \)Pas structure showed intermolecular interactions between the DIII-DIV linker, CTD, and DISV4 (Shen et al., 2017). Motoike et al. (2004) confirm calmodulin-independent interactions between the inter-linkers in Na\( v \)1.5, suggesting that calmodulin acts as an auxiliary channel modifier during a calcium signal (Pitt & Lee, 2016). Calcium regulation in Na\( v \)1.5 is mediated by calmodulin since the dual EF-like hand domains in the CTD do not bind calcium (Ben-Johny et al., 2014; Pitt & Lee, 2016). In light of these structural models, we speculate that E1784K decouples both the calcium-dependent and calcium-independent interactions between the DIII-DIV linker and the CTD. Thus, E1784K inhibits calcium-dependent facilitation (CDF) and calcium-dependent inactivation (CDI) in Na\( v \)1.5. We propose that the decoupling in the CTD caused by E1784K may create a high entropy, unstable structure. Upon a calcium signal, the calcified calmodulin has reduced affinity for the IQ motif, thus augmenting the CTD entropy (Sarhan et al., 2012).

Both mechanisms (1) and (2) may occur simultaneously in E1784K. The calcium effects in Na\( v \)1.5 are localized to the CTD. No reports have shown direct interaction between calcium-calmodulin and DISV4, so if mechanism (2) occurs, it may be via an indirect effect on DISV4.

In light of the discussed structural insights, we speculate that ranolazine can easily access the inner vestibule with non-calcified calmodulin, since the molecule binds tightly to the IQ motif. Ranolazine efficacy, however, is hampered by cytosolic calcium, suggesting an interaction between the drug and the channel at the CTD. The high entropy CTD in calcified calmodulin seems to physically hinder ranolazine from entering into the inner vestibule.

### 4.5.3. Physiological and Medical Implications

Elevated temperature and cytosolic calcium are two of many other physiological triggers that occur during exercise and are common to other pathophysiological states, such as myocardial ischemia or infarction, and heart failure (Lukas & Antzelevitch, 1996;...
Rivera-Fernández et al., 2014). The majority of patients with SCN5a mutations show ameliorated LQT3 phenotype during exercise (Schwartz et al., 1995). Functional studies have correlated this to a stimulation frequency or calcium-induced reduction in late $I_{Na}$ (Potet et al., 2015). However, it is clear from our study, focusing on E1784K, that the SCN5a mutation response to triggers can be unique (Abdelsayed et al., 2015, 2017). Thus, it is necessary to study antiarrhythmics in SCN5a cohorts during different physiological states as the mutant-trigger effect may determine drug efficacy.

Our AP simulations clearly show pro-arrhythmic effects of ranolazine, which are exacerbated by febrile temperatures. Electrical restitution curves clearly show a critical diastolic interval at which alternans are triggered. Our AP simulations provide evidence for ranolazine arrhythmogenicity, as it does not shorten APD$_{90}$ in E1784K at high heart rates. At low heart rates and at body core temperature, the drug shortens APD$_{90}$ in cardiac cells. However, with normal and elevated heart rates, the drug induces alternans, an effect exacerbated at febrile temperature. The critical diastolic intervals at which alternans are caused by the drug appear earlier (at higher BCLs) at febrile temperature.

E1784K induces alternans with higher prevalence in epicardial cells at low heart rates. This result coincides with the phase 2 re-entry phenomenon constituting the repolarization hypothesis in BrS1 (Lukas & Antzelevitch, 1996). The high $I_{Kto}$ density, especially in the right epicardium, results in complete action potential dome failure (Di Diego et al., 1996). E1784K channels are less available for activation due to the hyperpolarized SSFI-V$_{1/2}$. This seems to be the main mechanism behind the decrease in AP upstroke velocity in cardiac cells, especially the epicardial cells, despite the mutant and triggers-exacerbated increased late $I_{Na}$. Thus, E1784K expresses both gain- and loss-of-function at the electrical level in cardiac cells. However, this expressivity is finely tuned by channel switches, like temperature and cytosolic calcium.

Our previous and current data suggest exercise, and its accompanying physiological triggers, differentially affect mixed syndrome mutations, especially E1784K (Makita et al., 2008a; Abdelsayed et al., 2015; Peters et al., 2016; Abdelsayed et al., 2017). The action of different antiarrhythmics appear to differ depending on physiological state.
4.6. Conclusions

Appropriate management of cardiac arrhythmias in patients with SCN5a mutations requires careful investigation of antiarrhythmic drug efficacy under various physiological states. Our results suggest that ranolazine may increase the susceptibility for arrhythmia development in E1784K carriers at sinus rhythm and tachycardia. The risk is augmented under febrile conditions. Although exercise is commonly associated with high heart rates, other pathophysiological states share common triggers, as in heart failure or myocardial ischemia and infarction. Other antiarrhythmics should also be screened against E1784K and other channel mutants under various physiological conditions.

4.7. Acknowledgments

We thank Dr. Elizabeth M. Cherry for helping in AP simulation analysis. We are also grateful to both Mr. Colin Peters and Mr. Mohammed-Reza Ghovanloo for their valuable input and support.
Chapter 5. General Discussion and Future Directions

5.1. What was the point?

My goal was to study how physiological triggers like elevated temperature and cytosolic calcium affect E1784K channel properties separately or together with ranolazine. Multiple cascades and signalling molecules are activated during exercise and pathophysiological states accompanied with elevated heart rates, such as myocardial ischemia/infarction or heart failure. Some factors may exacerbate or ameliorate the E1784K channotype. I studied two factors which both exacerbate the biophysical defects caused by E1784K. The physiological and clinical implications made from the biophysical data should be treated with caution as not all channel-modifying factors were characterized. Thus, it is fair to assume that not all E1784K carriers will display similar phenotypes since genotype-channotype-phenotype correlations in SCN5A mutations are highly complex. Furthermore, additional caveats exist in the experimental approach used in these studies which potentially obfuscates establishing a clear channotype-phenotype relationship in E1784K. Despite these limitations, many interesting biophysical interpretations can be derived from the data presented in this thesis, which ultimately have physiological and pharmacological implications.

The functional data presented in the past three chapters all support past findings on the biophysical defects in Na\textsubscript{v}1.5 caused by E1784K. The mutant depolarizes activation (GV) and hyperpolarizes steady-state fast inactivation (SSFI) midpoints (V\textsubscript{1/2}). The mutant significantly accelerates fast inactivation kinetics at all voltages and elevates late I\textsubscript{Na}. Elevated temperature affected E1784K fast inactivation voltage-dependence differently depending on the heterologous expression system: fast inactivation was destabilized in HEK293 cells (Chapter 3) compared to CHOK1 cells when temperature was raised from 22 °C to 34 °C. Intriguingly, elevated temperature had no impact on E1784K fast inactivation kinetics, especially near resting potential (as in Chapter 1 with CHOK1 cells). E1784K seems to lower fast inactivation activation energy, thereby accelerating onset and recovery kinetics. Unfortunately, I could not measure activation energy directly using the Arrhenius relationship since Na\textsubscript{v} follows a multi-activation energy profile (Schwarz, 1979; Collins & Rojas, 1982). As speculated, E1784K
dramatically lowers DIV S4 (inactivation segment) activation energy barrier, thus speeding its mobility, as supported by the Peters-Ruben 2017 model (Peters et al., 2017). Additional thermal energy has minimal effects on fast inactivation kinetics, since the activation energy is substantially lowered by E1784K. The stabilized fast-inactivated state in E1784K suggests that the inactivation segments is pre-activate, thereby depleting the available E1784K pool at resting potential. Reduced channel availability leads to a decrease in the action potential (AP) upstroke velocity, as observed in the AP simulations. This effect is usually correlated with conduction abnormalities related to heart block and BrS1 (Veltmann et al., 2016).

The exacerbated late $I_{\text{Na}}$ measured in E1784K occurs at very depolarized potentials. The destabilized open fast-inactivated state in E1784K suggests that the inactivation particle (IFMT motif) has reduced affinity for its binding sites in the pore. E1784K seems to perturb the latent ‘latching’ role served by the C-terminal domain (CTD) in stabilizing the fast inactivation particle during open-state. Recently, the American cockroach Na, Pas structure reveals native electrostatic and van der Waals interactions between the DIII-DIV linker and the CTD. The homology model solved for Na, 1.5 based on Na, Pas is shown in Figure 5-1. The structure does not contain any intracellular interactions between Na, 1.5 and associated molecules, like calmodulin or Fibroblast Homologous Factor (FHF) (Wang et al., 2012; Gabelli et al., 2014; Ben-Johny et al., 2015). However, the middle $\alpha$-helix in the DIII-DIV linker, following the IFMT motif, is a key spot for CTD interaction in Na, Pas. Multiple salt bridges are formed between the highly acidic EF-like hand domain (EFLD) in the CTD and the DIII-DIV linker positively charged residues (Figure 5-1). E1784 is located prior to the EFLD and is in close proximity with K1493 in the DIII-DIV linker, potentially engaging in an ionic bond (Figure 5-1). I speculate that E1784K disrupts the salt bridge with K1493, thus perturbing fast inactivation stability (C.H. Peters, personal communication, October 21, 2017). Many Na, 1.5 mutants occur in the DIII-DIV linker and the CTD. Generalizing E1784K biophysical results to other Na, 1.5 mutants is futile since most Na, 1.5 mutants characterized have a unique response to external triggers (Chapter 2). Nevertheless, predictions on how Na, 1.5 mutants affect channel behavior can be made from the Na, 1.5 homology modelling included in this section (Shen et al., 2017). However, the homology model is a very limited molecular depiction of the channel’s highly dynamic structure.
Figure 5-1 Nav1.5-NaVPas DIII-DIV Linker and CTD Homology Model
Panel I shows the DIII-DIV linker (orange) and the fast inactivation particle, IFMT motif (yellow), interacting with residues in the CTD (cyan). The basis of this interaction lies in the salt bridges formed between many of the positively (red color) and negatively (blue color) charged residues found in both structures (side view in Panel AI and top view in Panel AII). Van der Waals interactions exist between Y1494, Y1495, and F1879 residues (green) as shown in the top view in Panel AII. E1784 forms a salt bridge with K1493 found in the middle helix in the DIII-DIV linker (Panel II).

E1784K is distinguished from other mutants in its ability to resist calcium-induced block of late INa, which is thought to be therapeutic in ameliorating LQT3 in exercising ΔKPQ patients (Potet et al., 2015). Disrupted CTD integrity may decrease calcium-calmodulin effects on NaV1.5. Mutants like 1795insD or Q1909R seem to strengthen the CTD integrity compared to E1784K. Elevated cytosolic calcium in 1795insD ameliorates defective fast inactivation by supressing late INa. 1795insD is located in the EFLD-α1 helix in NaV1.5 CTD. Although this domain is unable to chelate calcium in NaV1.5, mutants like 1795insD introduce an additional negative residue in the EFLD. An aspartate residue in the analogous region in NaV1.4 heightens the channels sensitivity to calcium compared to NaV1.5 (Ben-Johny et al., 2014). This mechanism may potentially reconfigure the NaV1.5 EFLD to operate as a functional calcium chelator, as thought to occur in NaV1.4 (Deschenes, 2002).

My data on calcium effects in NaV1.5 mutants are consistent with the tripartite complex for calcium-calmodulin regulation. Although several reports refute the interaction between calmodulin and the DIII-DIV linker, others suggest that disrupting
this inherent interaction is the underpinning for biophysical defects associated with BrS1 and LQT3 mutants (Van Petegem et al., 2012; Potet et al., 2015). Although calmodulin does not interact with the DIII-DIV linker in CaV1.2, it does in CaV1.3 (Ben-Johny et al., 2014). The new NaVPas structure confirms the inherent non-calmodulin based interaction between the CTD and the DIII-DIV linker (Shen et al., 2017). It seems reasonable to assume that the close proximity between these two channel structures would be affected by calmodulin regulation, which binds to the CTD IQ-domain (IQD). The calcified C-lobe in calmodulin specifically binds to the DIII-DIV linker IQD nested within the central α-helix. The putative binding site for the calcified C-lobe in calmodulin is a double tyrosine motif, Y1494 and Y1495, immediately downstream from K1493 (Figure 5-2) (Sarhan et al., 2012). As speculated above, the perturbed electrostatic interaction between the CTD and the DIII-DIV linker in E1784K, may prevent calmodulin from binding to its putative binding sites in the DIII-DIV linker. These binding sites also interact closely with the CTD even in calmodulin’s absence (Figure 5-1). Thus, E1784K seems to decouple the inactivation complex, which naturally conjugates via both calcium-dependent and calcium-independent interactions to modulate fast inactivation. In addition to distorting inter-CTD interactions, E1784K is thought to disturb intra-CTD interactions, which establish a firm anchorage for calmodulin binding and regulation. Thus, calmodulin may not be even near the inactivation complex vicinity in E1784K.

Besides calmodulin binding to the CTD or the DIII-DIV linker IQD, intracellular FHFs bind to the EFLD in NaV1.5, forming a ternary complex (Wang et al., 2012). FHF binds to the CTD via α4, α5, and the loop between α5 and β4 (Pitt & Lee, 2016). FHF determines the points of interaction between non-calcified and calcified calmodulin with the CTD (Pitt & Lee, 2016). It would seem plausible that E1784K may affect the normal FHF-mediated regulation in the CTD since it is near the EFLD. However, this observation remains to be confirmed.

The predicted instability in E1784K CTD is thought to increase the overall entropy in the intracellular milieu near the channel. The disentangled CTD in E1784K may expose the calmodulin in the binary complex along with FHF in the ternary complex to the inner vestibule, thereby affecting class I antiarrhythmic binding in NaV1.5. Alternative routes like the fenestrations allow for drugs to enter into the channel’s pore (Kaczmarski & Corry, 2014); however, these drug routes may be modulated by channotype.
Figure 5-2  Calmodulin C-lobe interaction with the DIII-DIV linker in Nav1.5
The two EF-hand domains (N-lobe is green and C-lobe is blue) in calmodulin chelate four calcium ions (light green). The calcified C-lobe in calmodulin interacts with two Tyrosines in the DIII-DIV linker (light grey). BrS1 and LQT3 mutants are included. Reproduced from Van Petegam et al., (2012).

Elevated temperature exacerbates late $I_{\text{Na}}$ in E1784K. These thermal effects are not synergistically heightened with elevated cytosolic calcium. Rather, the interaction between temperature and calcium clearly reduces the normalized late $I_{\text{Na}}$, indicating a lower E1784K channel availability at open-state potentials. This reduction was owed to a decrease in peak $I_{\text{Na}}$. Late $I_{\text{Na}}$ density, however, was still large in E1784K with elevated temperature and cytosolic calcium. Elevated temperatures may increase the rate by which the inactivation gate unfastens in E1784K. Inactivation gate mobility is expected to increase when there are less constraints faced from inherent CTD regulation. However, various limitations arise from the methods used in this thesis, which may undermine the above speculations.
5.2. Possible Limitations

5.2.1. Patching using Immortal cell lines

The heterologous expression systems used in this study are an important caveat. To study sodium channels in vivo as opposed to in vitro may have been more relevant to the goal of this thesis, which was to elucidate how triggers which accompany exercise affect E1784K. Native Na\textsubscript{v}1.5 in cardiac tissue are not the same as in CHOK1 and HEK293. Differences are, in part, due to lipid-membrane composition, post-translational modifications, and the intracellular protein composition of channel modifiers. Many researchers favor using native or iPSC-derived cardiomyocytes, especially when making physiological inferences as not all in vitro measurements are reproducible in vivo. For instance, the D1275N mutant displays slowed conduction velocity, atrial fibrillation, and other arrhythmias in vivo but displays no loss-of-function when biophysically characterized in vitro (Watanabe et al., 2011). Other studies, however, show that Na\textsubscript{v} in heterologous expression systems are comparable to those in native cardiomyocytes, e.g. the augmented late I\textsubscript{Na} in ΔKPQ (Nuyens et al., 2001; Knollmann, 2013). I chose to isolate Na\textsubscript{v}1.5 in heterologous cells since this removes the need for potentially contaminating pharmacological agents required to block other channels. However, my predictions on arrhythmogenicity may be further strengthened with current-clamp recordings from cardiomyocytes, to help understand E1784K dynamic response to increased stimulation frequency.

Another limitation in my whole-cell recordings was using fluoride ions in the intracellular solution. Although used to stabilize and maintain the giga-ohm seal, fluoride ions shift SSFI-V\textsubscript{1/2}. To minimize this effect, activation and fast inactivation voltage-dependences were measured immediately after intracellular solution equilibration. Fluoride is also a phosphatase inhibitor; thus, channels in the whole-cell configuration were mainly phosphorylated. Although this effect may bias the results, it may further validate the biophysical and physiological inferences.

5.2.2. Calcium Buffering

The cytosolic calcium buffer used was EGTA. This calcium chelator is suitable for the calcium concentrations (0 µM – 2.5 µM) buffered in the patch-clamp experiments.
According to Bers et al. (2010), selecting the appropriate calcium buffer to chelate cytosolic calcium depends on the buffer’s dissociation constant ($K_d$), which should lie away from the desired calcium concentration by a factor of $10^1$ (Bers et al., 2010). Following this rule, we selected EGTA, which at pH 7.4 (physiological pH) has a $K_d \approx 67$ nM (Ben-Johny & Yue, 2014). Thus, both 500 nM and 2500 nM cytosolic calcium were strongly buffered by EGTA since these concentrations are above the $K_d$ value. Although buffering 2500 nM cytosolic calcium lies above the range allowed by Bers et al. (2010), I did not consider this a significant issue for several reasons: (1) At 2500 nM cytosolic calcium, no shifts were observed in the wild-type (WT) SSFI-V1/2, similar to previous reports showing no SSFI-V1/2 shifts at 0.3 µM cytosolic calcium, also buffered with EGTA (Sarhan et al., 2012). (2) Former studies using EGTA did not necessarily show calcium-induced biophysical shifts, despite surpassing the permissible concentration limit by 10$^x$-folds ($x=2,3,4$). The reported free cytosolic calcium in most studies is inaccurate given the presence of endogenous calcium in heterologous expression systems and cardiomyocytes. Past studies used EGTA and BAPTA to buffer cytosolic calcium between 1 and 10 µM, which is well above the concentration range used in this study (Wingo et al., 2004; Shah et al., 2006; Potet et al., 2009; Biswas et al., 2009; Sarhan et al., 2012). When the calcium chelator, HEDTA, is used in buffering 0 µM to 10 µM cytosolic calcium, no shifts are observed in WT SSFI-V1/2 (Ben-Johny & Yue, 2014). The SSFI-V1/2 is depolarized when cytosolic calcium is elevated from 0 µM to 10 µM and buffered with BAPTA, which has a relatively high affinity for calcium (low $K_d$), compared to EDTA, and is supposedly super-saturated with very high calcium concentrations.

HEDTA, like EDTA (except that the former is highly soluble in water) has a $K_d \approx 4$ µM. HEDTA affinity for calcium far exceeds the desired concentrations I used in my experiments (500 nM and 2500 nM), making HEDTA an inappropriate chelator. No shifts in WT SSFI-V1/2 are caused by free cytosolic calcium (10 µM) when buffered with HEDTA (Ben-Johny et al., 2014). In addition, HEDTA’s affinity for calcium is very weak compared to EGTA. EGTA is highly selective for calcium. EGTA-$K_d$ for magnesium is in the millimolar range (9 mM) compared to EDTA which has a higher affinity for magnesium (2.5 µM) (Parsons et al., 1996).

BAPTA was not used in this study since it is usually used for chelating transient calcium currents, prevalent in iPSC-derived cardiomyocytes, due to its rapid forward and reverse chelation kinetics (BAPTA has a $K_d \approx 192$ nM, similar to EGTA’s). My objective
was to study the long-term cytosolic calcium effects on channel properties that take a longer time to develop, such as the non-inactivating state, underlying late $I_{Na}$, or the slow-inactivated state. The biophysical protocols simulated were long enough providing sufficient time for EGTA to execute its buffering effects. In addition, upon whole-cell formation, I waited 10 min to allow for the seal to stabilize and for the biophysical shifts to equilibrate with fluoride ions. Thus, allowing sufficient time for EGTA to buffer calcium. An additional culprit in this study arises from using fluoride ions, since they avidly bind to calcium ($K_{sp} \approx 3.45 \times 10^{-11}$), potentially decreasing cytosolic calcium levels.

The debatable shift in SSFI-$V_{1/2}$ caused by elevated cytosolic calcium can be attributed to the inconsistencies in experimentation between patch clampers. The contemporary thought in the field leans towards the notion that calcium does not regulate fast inactivation in NaV1.5, since recent NaV1.5-CTD crystal structures showed a more CaV1.2--like mode of csalcium-calmodulin regulation in NaV1.5 (Pitt & Lee, 2016) compared to the earlier models (Shah et al., 2006; Sarhan et al., 2012).

A major limitation in this study, which affected dynamic action potential simulations, is the inability to study NaV1.5 response to subspace calcium levels, which can rise to 10 µMs or more. Although proven to not affect WT NaV1.5, as confirmed using photo-uncaging and calcium spillover techniques, subspace calcium can modulate mutant channotype.

Many of the mechanistic speculations on calcium effects in NaV1.5, mediated by its association with calmodulin, require experimental validation. To test the legitimacy of the calcium-calmodulin speculations in Chapter 2 and Chapter 3, the free calcium in the intracellular pipette can be replaced with barium, which weakly binds to calmodulin. The calcium effects on mixed NaV1.5 mutants can also be tested with calmodulin mutants caused by CALM1 and CAM2 genes, which underlie LQT15 and LQT16, respectively.

5.2.3. The β1-Subunit

The purpose of co-transfecting NaV1.5 α subunit with the β1-subunit was to enhance channel expression in CHOK1 and HEK293 cells. However, certain doubts may arise about whether maximal transfection efficiency is obtained. All the cells patch-clamped were glowing and expressed $I_{Na}$, which suggests close to 100% co-transfection
of eGFP and the Na\(v\)1.5 \(\alpha\)-subunit. I assumed that, since both the \(\alpha\)-subunit and eGFP were successfully co-transfected into the cells, the \(\beta\)1-subunit, which was present in the mixture, is also expressed. To further confirm \(\beta\)1-subunit co-expression, I compared my biophysical results (from **Chapter 2**) with past reports.

Vilin et al. (1999) compared fast and slow inactivation between skeletal (Na\(v\)1.4) and cardiac (Na\(v\)1.5) voltage-gated sodium channels, in which the co-expression proportion was 1:1 for \(\alpha\) and \(\beta\)1-subunits (Vilin et al., 1999). \(\beta\)1-subunit co-expression has a minor effect on fast inactivation voltage-dependence in Na\(v\)1.5 compared to Na\(v\)1.4, although another study shows a depolarized shift in SSFI-V\(1/2\) (Maltsev et al., 2009). The steady-state slow inactivation midpoint (SSSI-V\(1/2\)) is negatively shifted in Na\(v\)1.4 by the \(\beta\)1-subunit compared to a negligible shift in Na\(v\)1.5. Slow inactivation kinetics are accelerated by the \(\beta\)1-subunit in Na\(v\)1.5 near SSSI-V\(1/2\) potentials. In one study, TsA201 cells were co-transfected with either \(\alpha + \beta\)1 or \(\alpha + \beta\)2 subunits (Maltsev et al., 2009). The co-transfected \(\beta\)1-subunit results in slower sodium current decay and a larger amplitude in late \(I_{\text{Na}}\) compared to peak \(I_{\text{Na}}\). These results were compared to the minimal shifts observed in the \(\alpha + \beta\)2-subunits or the \(\alpha\)-subunit conditions. When co-expressed with the \(\beta\)1-subunit in Xenopus Oocytes, Na\(v\)1.5 expression is increased with no modifications in gating (Qu et al., 1995). It is clear then that the heterologous expression system used impacts the interaction between Na\(v\)1.5 \(\alpha\)-subunit and \(\beta\)1-subunit.

Since I did not perform experiments in the absence of \(\beta\)1, I compare in **Table 5-1** the HEK 293 WT \(\alpha\)-subunit + \(\beta\)1-subunit + eGFP results (from **Chapter 2**) to HEK 293 WT \(\alpha\)-subunit (performed with 0 nM cytosolic calcium, pH 7.4, and at room temperature) retrieved from Vilin et al. (2012). All differences can be attributed to the \(\beta\)1-subunit.
Table 5-1 Differences between α + β1 and α – β1 subunits

<table>
<thead>
<tr>
<th>Parameters</th>
<th>α + β1 (Chapter 2)</th>
<th>α – β1 (Vilin et al., 2012)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GV-V1/2</td>
<td>-43.8 ± 1.10</td>
<td>-32.6 ± 2.8</td>
</tr>
<tr>
<td>GV-z</td>
<td>4.92 ± 0.26</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>SSFI-V1/2</td>
<td>-89.2 ± 1.97</td>
<td>-80.6 ± 1.3</td>
</tr>
<tr>
<td>SSFI-z</td>
<td>-3.72 ± 0.28</td>
<td>-4.4 ± 0.1</td>
</tr>
<tr>
<td>SSSI-V1/2</td>
<td>-70.8 ± 2.28</td>
<td>-71.4 ± 3.2</td>
</tr>
<tr>
<td>SSSI-z</td>
<td>-1.34 ± 0.07</td>
<td>1.9 ± 0.12</td>
</tr>
<tr>
<td>SSSI-y0</td>
<td>0.30 ± 0.03</td>
<td>0.36 ± 0.05</td>
</tr>
<tr>
<td>Fast Inactivation τmax*</td>
<td>65</td>
<td>109</td>
</tr>
<tr>
<td>SI onset T1 (-50 mV)</td>
<td>0.54 ± 0.41</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>SI onset T2 (-50 mV)</td>
<td>14.6 ± 1.44</td>
<td>6.0 ± 1.1</td>
</tr>
<tr>
<td>SI recovery T1 (-110 mV)</td>
<td>0.04 ± 0.02</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>SI recovery T2 (-110 mV)</td>
<td>2.06 ± 0.31</td>
<td>7.9 ± 2.9</td>
</tr>
</tbody>
</table>

*This parameter is the maximum amplitude of the Gaussian distribution fit to the fast inactivation time constant versus voltage curve. GV, conductance; SSFI, steady-state fast inactivation; SSSI, steady-state slow inactivation; SI, slow inactivation.

The accelerated slow inactivation kinetics observed in Vilin et al. (1999) coincides with the observed shifts in slow inactivation recovery (both τ1 and τ2) and onset (τ1) time constants (Table 5-1). The negative shift in activation and fast inactivation voltage-dependences along with the enhanced fast inactivation kinetics caused by the β1-subunit is comparable with the well-known shifts also caused by the β1-subunit in adult rat brain type IIA, embryonic rat brain type III, and rat skeletal muscle NaV channels (Patton et al., 1994). Thus, the patch-clamp assay proved the β1-subunit expression. Immunocytochemistry would not be ideal to test for the presence of the β1-subunit since HEK293 cells tend to cluster and are difficult to isolate for visual assays.

Whether the temperature or cytosolic calcium effects reported in this thesis were partly due to the β1-subunit is unknown; however, the β1-subunit is not known to directly interact with the DIII-DIV linker and the CTD (Qu et al., 1995; Chen et al., 2012; Gilchrist et al., 2013). The β1-subunit aggravates the biophysical defects induced by the DIV S3-S4 linker NaV1.5 mutant, T1620M: fast inactivation is destabilized and its recovery kinetics are accelerated (Makita et al., 2000). The non-covalent interactions between the β1-subunit and NaV1.5 may be directly affected by this extracellular linker mutant. However, it is unknown whether the intracellular mutants studied in this thesis affect upper stream channel structures engaged with the β1-subunit.
5.2.4. Action Potential Modelling

The Tüsscher-Noble-Noble-Panfilov (TNNP) model used in Chapter 2 does not show the expected E1784K-induced prolongation in APD compared to the O'Hara-Rudy (O'Rd) Model (Chapter 3 and Chapter 4). The transient outward ($I_{KtO}$) and slow delayed rectifier ($I_{KS}$) potassium currents in the TNNP model are approximately 11-folds and 4-folds greater than in the O'Rd model, respectively. Thus, the depolarizing current is greatly offset by the immense repolarizing current in the TNNP model. Additionally, the rapid delayed rectifier ($I_{Kr}$) to slow delayed rectifier ($I_{KS}$) proportions are approximately equal in the TNNP model compared to ~9:1 ratio in the O'Rd model. Reduced $I_{KS}$ underlies prolonged midmyocardial action potentials in LQT3, the reason why prolonged APDs caused by $\Delta KpQ$, E1784K, 1795insD, and Q1909R mutants were observed in the O'Rd model (Shimizu & Antzelevitch, 1998).

The disruption of the AP waveforms is more prominent in E1784K in the O'Rd model. The elevated late $I_{Na}$ in E1784K is high at resting potentials compared to the other mutants studied in Chapter 2. Although there is minimal sodium conductance at resting potentials, the normalized late $I_{Na}$ in E1784K is high, suggesting a readily available sodium channel pool. The diastolic calcium at rest is sufficient to attenuate the late $I_{Na}$ in other mutants with higher sensitivity to cytosolic calcium, compared to E1784K. DADs and EADs due to exacerbated late $I_{Na}$ were observed in APs simulated with E1784K.

The reduced dV/dt in E1784K is due to decreased channel availability at resting potential, caused by the depolarized and hyperpolarized voltage-dependence of activation and fast inactivation, respectively. Previous studies show that the decrease in 1795inD upstroke velocity is caused by a channel trafficking defect (Veldkamp et al., 2000a; Clancy & Rudy, 2002). Unfortunately, the absolute channel conductance was not quantified in the AP models since I did not measure channel trafficking/expression.

5.2.5. Physiological and Medical Implications

To date, only one study shows that an E1784K carrier’s response to exercise is like other patients with SCN5a mutations displaying QT shortening during exercise (Schwartz et al., 1995). This result may contradict the main results presented in this
thesis showing the unique response of E1784K to physiological triggers compared to other mixed syndrome mutants. However, other factors, which were not studied, determine E1784K arrhythmogenicity at the clinical and molecular levels. At the clinical level, factors such as ethnicity, sex, and age may contribute to the differential responses exhibited by patients with SCN5a mutations to exercise stress testing. I previously collaborated with clinicians with access to data on the response of pediatric patients with SCN5a mutations to exercise stress tests: E1784K carriers had a highly variable response to exercise with a few E1784K carriers showing QT shortening during peak exercise and prolongation during recovery from exercise. In other carriers, a prolonged QTc is induced by exercise, which recovers to baseline during the recovery phase (A-E. Baruteau, personal communication, February 3, 2016).

In this thesis, my aim was to elucidate potential sources of arrhythmogenesis at the molecular level. Other factors, aside from temperature and cytosolic calcium, may determine arrhythmogenicity: various intracellular kinases are activated during heightened sympathetic stimulation, having the potential to sway E1784K channotype to loss-of-function or gain-of-function. This variability may correlate with the phenotypic heterogeneity observed in carriers. CaMKIIδc and PKA both decrease Na\textsubscript{v}1.5 availability by stabilizing the slow-inactivated state (Qu et al., 1996; Herren et al., 2013). Intriguingly, CaMKIIδc also augments the late $I_{Na}$ in Na\textsubscript{v}1.5, thereby inducing mixed channel behavior (Herren et al., 2013). CaMKIIδc activation may increase risk for arrhythmias, especially with mixed syndrome Na\textsubscript{v}1.5 mutants. At high heart rates, the inherent rise in sodium channel use-dependence may be further exacerbated by these kinases in E1784K, thereby ameliorating the gain-of-function induced by temperature and cytosolic calcium factors. Thus, the E1784K channotype pendulum can be acted upon by various external forces. The effects of channel modulators on the mutants must be further examined to appropriately target the channel biophysical defects with suitable pharmacological agent(s).

Although I show that ranolazine does not suppress late $I_{Na}$ in E1784K, under elevated temperature and cytosolic calcium, the drug may be used to clinically screen for genotype-positive patients while exercising. It is clear that traditional provocative drugs used to unmask arrhythmias may not necessarily be provocative under different physiological states. The channotype combined with the external trigger affects how the
drug interacts with the channel. Thus, exercise stress tests and provocative drug tests can be potentially combined to fully elucidate disease entity.

Ranolazine, as suggested in Chapter 3, may be resisted by the high entropy CTD in E1784K due to its large size compared to other classic antiarrhythmics. Follow-up experiments using Lidocaine could test the validity of this speculation, since the drug is smaller and shares 50% identity of ranolazine. One risk associated with screening classic antiarrhythmics against E1784K and other Na\(_v\)1.5 mutants is that these drugs usually have high potency to block peak I\(_{Nax}\). Reducing sodium conductance may exacerbate biophysical defects underpinning channel loss-of-function. Thus, newer approaches in treating gain-of-function should be pursued as with the novel aryl sulfonamides, stabilizing fast inactivation extracellularly, or GS-6615 (Eleclazine), the highly selective late I\(_{Nax}\) blocker (Rajamani et al., 2016).

5.3. Conclusions and Future Directions

Mixed syndrome mutants, like E1784K, are uniquely modulated by physiological and pharmacological switches. Genotype-phenotype correlations generally constitute the basis for treatment for arrhythmias, such as in LQT3. However, it is clear from this study that the channotype-trigger interaction further complicates the intricate genotype-phenotype relationship in SCN5a mutation carriers. Thus, prescribing the appropriate medication for arrhythmia treatment in patients is difficult. In fact, a rigorous analysis of channotype may indicate that no suitable therapeutics currently exist for ameliorating cardiac disease. This is supported by the current focus on use of device therapy with implantable cardioverter defibrillators (ICDs). Future drug discoveries should strategically be aimed at treating both gain-of-function and loss-of-function in Na\(_v\)1.5. Although challenging, most BrS1 and LQT3 mutants display mixed channel defects, which are modulated by external factors. Unmasking these biophysical abnormalities can vary between individuals and depend on the physiological state. Thus, alternating phenotypes are observed in 1795insD carriers at different heart rates. Dividing Brugada and Long-QT syndromes as separate clinical entities may be beneficial for diagnosis but may not be the correct approach for personalized treatment. The solution then may be to administer more than one drug type for mixed syndrome patients: one targeting loss-of-function and the other for gain-of-function. However, there are currently no Na\(_v\)-selective drugs targeting loss-of-function. To minimize the risk for blocking peak I\(_{Nax}\),
pharmaceutical companies should design drugs that target the channel extracellularly, thereby minimizing drug interaction near the channel inner vestibule and pore where most mixed syndrome mutants exist. Aryl sulfonamides have been shown to stabilize fast inactivation in NaV1.7. Developing NaV1.5-specific aryl sulfonamides will help ameliorate LQT3-related biophysical defects. Furthermore, understanding the structural gating correlates in slow inactivation is pivotal in designing drugs which target and prevent slow inactivation. Limiting slow inactivation will prevent channel loss-of-function caused by elevated frequency and other channel modifiers. Fully treating SCN5a mutations is an important pharmaceutical goal, and keenly inspecting SCN5a channotype-trigger interactions is a first step toward precision arrhythmia management and treatment.
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