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Calcium Channel Mutations in Cardiac Arrhythmia Syndromes

Matthew J. Betzenhauser, PhD[1], **Geoffrey S. Pitt, MD, PhD**[2], and **Charles Antzelevitch, PhD**[1]

[1]Experimental Cardiology Program, Masonic Medical Research Laboratory, Utica, NY

[2]Departments of Medicine (Cardiology), Pharmacology and Cancer Biology, and Neurobiology, Duke University School of Medicine, Durham, NC

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Introduction

Calcium influx through cardiac voltage gated calcium channels (VGCC) is essential for proper cardiac function. Calcium entering the cardiomyocyte via L-type VGCC (LTCC) provides the initiating event in cardiac excitation-contraction (EC) coupling. The calcium that enters the cell during the action potential serves to trigger release of Ca^{2+} from internal sarcoplasmic reticulum (SR) stores via activation of type 2 ryanodine receptors (RyR2). By virtue of its activation and inactivation kinetics, inward current driven by L-type VGCC is also a major contributor to the action potential plateau phase. As such, the channel is a key mediator of cardiac AP shape. Given the central importance of L-type VGCC in cardiac physiology, it is not surprising that subtle perturbations of its function lead to profound cardiac diseases. In this review, our principal focus will be on genetic mutations affecting the subunits of the cardiac L-type calcium channel (LTCC) and their impact on inherited cardiac arrhythmia syndromes. Tables 1 and 2 show all of the mutations associated with the long QT(LQTS), Brugada (BrS), short QT (SQTS) and early repolarization (ERS) syndromes in chronological order of their discovery, highlighting linkage to genes comprising the cardiac L-type calcium channel. Figure 1 shows missense mutations in $Ca_V1.2$ and auxiliary subunits associated with these various phenotypes.

I. Structure of Voltage-gated Calcium Channels

VGCC are comprised of macromolecular complexes of protein subunits. The main pore forming subunits are called α1-subunits and the accessory proteins are referred to as β, α2δ and γ-subunits. VGCC are also characterized by their activation profiles. Three classes of VGCC are present in cardiac tissue. T-type channels, made up of $Ca_V3.1$ α1G-subunits and lacking β subunits, are present in nodal tissue where they contribute to pacemaker function. P-type channels, made up of $Ca_V2.1$ α1B-subunits, are a minor constituent of cardiac VGCC. By far the most prevalent class of VGCC in the myocardium is the L-type current

Address for correspondence: Charles Antzelevitch, PhD, FACC, FAHA, FHRS, Masonic Medical Research Laboratory, 2150 Bleecker Street, Utica, New York 13501-1787, Phone: (315) 735-2217, ext 129, cantzelevitch@gmail.com.

carried by $Ca_V1.2$ α1C- subunits. $Ca_V1.2$ associates with auxiliary subunits including α2δ, β and possibly γ subunits.

A. α1 Subunit of the Cardiac LTCC

The human *CACNA1C* gene located on chromosome 12 codes[1] for the pore-forming $Ca_V1.2$ subunit protein of the cardiac LTCC.[1–2] *CACNA1C* spans over 500 kb and as many as 19 of the 55 known exons are subject to alternative splicing, thus generating considerable possible diversity of expression.[3] While most subunit splice variants have not been functionally characterized, some alternative splicing events are known to alter the biophysical properties or modulation of the cardiac L-type current. As such, mutations in the *CACNA1C* gene could have different functional consequences depending on which splice variant is expressed. $Ca_V1.2$ is a 2100 amino acid, ~220kDa protein and it shares structural homology with voltage gated sodium channel family members with four structural repeats. Activation of $Ca_V1.2$ is rapid and voltage dependent, with inactivation occurring more slowly by both voltage (VDI) and calcium (CDI)-dependent mechanisms. $Ca_V1.2$ subunits also contain the necessary structural information required for voltage-dependent inhibition by dihyropyridines (DHPs) and for Ca^{2+}-dependent facilitation found in cardiac myocytes. $Ca_V1.2$ proteins are subject to multiple modes of regulation that determine the number of available channels via trafficking or by modulating channel activation or inactivation via protein kinases and accessory proteins. As such, there are numerous variables that determine L-type channel activity and therefore multiple possible modes of dysfunction via gene mutations.

B. Cardiac auxiliary subunits

While the pore forming α1-subunits are sufficient for voltage-gated calcium channel activity, at least three other subunits have been demonstrated to modulate channel activity or membrane localization (α2δ, β and γ).[4] There are multiple isoforms for each of these proteins leading to still more diversity in L-type channel structure. To date, mutations associated with arrhythmias have been identified in Ca_Vα2δ-1 and Ca_Vβ2b genes.

There are four known α2δ genes, but the α2δ-1 isoform encoded by *CACNA2D1* is the only one to be identified in heart tissue to date [5]. The α2δ-1 subunit is required for full expression of L-type current along with β2b and $Ca_V1.2$ in heterologous expression systems. Expression of α2δ-1 was found to increase the amount of $Ca_V1.2$ associated with the plasma membrane in *Xenopus* oocytes, suggesting a positive influence on trafficking of $Ca_V1.2$. [6] Most reports suggest a minor role for α2δ-1 in regulating $Ca_V1.2$ gating and channel kinetics.

There are four distinct genes for calcium channel β subunits, each with various transcript variants.[7] Expression of Ca_Vβ subunits 1–3 has been identified in human heart at both the mRNA and protein levels. Of these, Cavβ2b encoded by *CACNB2* is the most prevalent in ventricular myocardium and this isoform faithfully recapitulates the properties of cardiac L-type channels when expressed with CaV1.2 and CaVα2δ-1. Ca_Vβ subunits can exert effects on VGCC activity via multiple routes. The predominant effect of Ca_Vβ2 is to promote proper trafficking of $Ca_V1.2$ to the sarcolemmal membrane in myocytes. Binding of Ca_Vβ2

to the alpha interacting domain (AID) in $Ca_V1.2$ promotes trafficking of the channel complex to the cell surface. Besides these effects, $Ca_V\beta2$ is known to impact both L-type channel activation and inactivation. Because of these pleotropic effects, it is important to perform exhaustive functional analyses before determining the mechanistic effects of gain or loss of function mutations.

Ca^{2+} channel γ subunits, consisting of 4 transmembrane domains were long thought to be absent in the heart. There are 8 isoforms of the γ subunit, The γ1 subunit is present in the skeletal muscle $Ca_V1.1$ channel complex, but is not detected in cardiac muscle. Recent work by Yang et al.[8] identified γ subunits in the cardiac muscle, thus further increasing the functional diversity of cardiac LTCC. They showed that γ4, γ6, γ7, and γ8 subunits, encoded by *CACNG4, 6, 7 and 8*, physically interact with the cardiac $Ca_V1.2$ complex. These γ subunits were shown to modulate Ca^{2+} channel function when co-expressed with the β1b and α2/δ-1 subunits in HEK cells, altering both activation and inactivation characteristics. The effects of the γ subunit on $Ca_V1.2$ function were found to dependent on the subtype of the β subunit.

II. Gain of Function Mutations in LTCC Genes

A. Syndromic *CACNA1C* mutations: Long QT associated with extracardiac phenotypes (Timothy syndrome)

Given LTCC's fundamental importance, it was somewhat surprising that, during the first decade after ion channel genes were discovered as the major loci underlying inherited cardiac arrhythmias, no mutations were identified in LTCC. A 2004 report identifying the first calcium channel mutation in an arrhythmia syndrome provided a rationale for this rarity. That report described a sporadic heterozygous identical single point mutation in the $Ca_V1.2$-encoding *CACNA1C* as the cause for a multisystem disorder (called Timothy Syndrome) characterized by an invariant long QT syndrome and syndactyly, as well as variable penetration of phenotypes such as autism spectrum disorders, craniofacial abnormalities, and hypoglycemia, in 17 subjects [9]. A spontaneous mutation in 15 of the subjects and a mutation inherited from an asymptomatic parent with mosaicism in 2 other subjects affected a single amino acid, G406R, in the alternatively spliced exon 8. Ten of the 17 patients died (average age of death was 2.5 years) of arrhythmogenic sudden death even though expression of this alternatively spliced mutated variant contributes only 11.5% of the $Ca_V1.2$ channels in the heart in the heterozygous state. A closely following report described an analogous *de novo* G406R mutation in the alternatively spliced exon 8A in one patient and a second *de novo* mutation G402S also in exon 8A in a second patient, both with long QT syndrome [10]. In comparison to the original Timothy Syndrome cohort (now referred to as TS1), these new patients (now referred to as TS2) experienced severe arrhythmias along with an overlapping but distinct set of extracardiac phenotypes. Thus, mutations in *CACNA1C* may cause severe cardiac arrhythmias that limit survival and therefore the overall prevalence of these mutations.

The severity of the arrhythmia in TS patients is explained by the single case in the original report in which the G406R mutation was inherited from an asymptomatic carrier (mother) was mosaic for the *CACNA1C* mutation. This suggests that mutation arose during

development after specification of cardiomyocytes, and she was able to transmit the mutation only because she did not suffer life-threatening arrhythmias. Four additional mosaicism subjects have subsequently been documented [1112]. Only one had a prolonged QT interval.

The molecular mechanism by which the TS1 or TS2 mutations cause Long QT syndrome was revealed by biophysical analyses of the mutant channels in heterologous expression systems, which showed a prominent loss of voltage-dependent inactivation (VDI). *In silico* modeling predicts that, as a result of the diminished VDI, calcium mutant channels fail to close during the plateau phase of the ventricular action potential, thereby increasing the depolarizing current and resulting in a marked delay in action potential repolarization [9–10]. G406 sits just distal to the end of the IS6 transmembrane segment. This is just upstream of the obligate calcium channel beta subunit binding site, which sits within the channel's intracellular linker between domains I and II. Biochemical and functional analyses suggest that this region forms a rigid alpha-helix that helps properly orient the auxiliary beta subunit, a major regulator of the channel by VDI. Mutation of G406 disrupts this rigid alpha-helix. Reorientation of the beta subunit relative to the rest of the channel is hypothesized to perturb VDI mediated by the beta subunit. [13].

The diminished VDI caused by the G406R mutation also provides an explanation for some of the extracardiac phenotypes observed in TS patients. For example, the $Ca_V1.2$ calcium channel is a major contributor to Ca^{2+}-dependent insulin secretion from pancreatic beta cells [14]. The gain-of-function mutation therefore contributes to excess insulin secretion and the hypoglycemia reported in one-third of the original cohort. The gain-of-function defect and resulting excess Ca^{2+} influx also provides a rational for the craniofacial abnormalities present in half of the original cohort. Most prominent is macrognathia (abnormally large mandible). Zebrafish and mouse models demonstrate that excess Ca^{2+} influx through the mutant $Ca_V1.2$ channels in the chondrocytes within the developing mandible drive hypertrophy and hyperplasia [15]. The target for excess Ca^{2+} influx may be the downstream Ca^{2+}-dependent phosphatase calcineurin, which then triggers a NFAT-dependent gene expression program that has been associated with hypertrophy and hyperplasia.

Some of the TS phenotypes reported in the original cohort may be independent of the Ca^{2+} permeating properties of the $Ca_V1.2$ calcium channel, however. The autism spectrum disorders present in over three quarters of the original cohort have been hypothesized to result at least in part from dendritic retraction in neurons that was observed after expression of the mutant channel in cultured neurons [16]. Dendritic retraction also occurred in neurons after expression of a compound $Ca_V1.2$ mutant channel that bore the G406R mutation as well as mutations in the pore that rendered the channel unable to pass Ca^{2+}. Instead of a Ca^{2+}-dependent signaling process, the authors hypothesize that, upon depolarization, a conformational change in the G406R TS mutant channel activates ectopic RhoA signaling that then initiates cytoskeletal rearrangements associated with dendritic retraction. An analogous Ca^{2+}-independent process has been suggested to underlie the mechanism by which the TS mutant $Ca_V1.2$ channel affects hair follicle stem cells [17], and thereby explain why TS patients are born bald.

The consistent and potent effect of the G406R mutation across a number of unrelated patients provides strong support for gain-of-function mutations in *CACNA1C* as a cause for long QT syndrome and for extracardiac effects as a part of a syndromic presentation. On the basis of this evidence, *CACNA1C* has been subjected to candidate sequencing in cases of unexplained long QT syndromes, especially when accompanied by other phenotypes such as syndactyly, which has led to the presumptive identification of new *CACNA1C* mutations associated with syndromic presentations or only with long QT syndrome. Additionally, *CACNA1C* variants discovered during whole exome sequencing have sparked attention. Because the number of affected subjects is small (often a single subject) compared to the TS1 G406R mutation, the evidence supporting any of these new *CACNA1C* mutations as causative for the studied phenotypes is less clear. Nevertheless, several of these new reports present compelling data from multiple levels of investigation.

Two *CACNA1C* mutations associated with long QT and extracardiac phenotypes have been reported. Targeted sequencing in a child with a markedly prolonged QT interval, dysmorphic facial features, syndactyly, and joint contractures identified a *de novo* A1473G mutation of $Ca_V1.2$ at the end of IVS6 of $Ca_V1.2$ [18]. Analysis of the biophysical effect of this mutation was not reported. It has been shown, however, that the $Ca_V1.2$ C-terminus can interact with the I–II intracellular linker ([18]), the location of the TS1 and TS2 mutations, thus providing a possible rationale for a gain-of-function effect similar to the TS1 and TS2 mutations. A I1166T mutation within the III–IV linker was discovered through whole exome sequencing in a subject with long QT syndrome, intellectual impairment, a patent ductus arteriosus, facial dysmorphisms, various digit abnormalities (clinodactyly and short thumbs), and tooth decay [19]. These phenotypes, reported for a single patient, only partially overlap those reported for TS1 or TS2. However, biophysical analysis of the mutant $Ca_V1.2$ channel in a heterologous expression shows that I1166T causes a gain-of-function effect upon Ca^{2+} current by increasing the “window” current. *In silico* analysis demonstrates how the specific kinetics effects lead to an increase in action potential duration and development of after hyperpolarizations that could trigger arrhythmias. Although there is a general absence of an understanding of how *CACNA1C* participates during development and therefore how the I1166T mutation may contribute to the reported extracardiac phenotypes, it is interesting to speculate the different set of phenotypes associated with this I1166T mutant compared to the G406R mutation in TS1 may correlate with the different biophysical defects between the mutant channels (increased window current *versus* decreased VDI).

B. Non-syndromic *CACNA1C* mutations: Long QT syndrome without extracardiac phenotypes

Whole exome sequencing, applied to a pedigree with isolated long QT syndrome (no extracardiac manifestations) identified another candidate *CACNA1C* gain-of-function mutation, P857R [20]. Heterologous expression of the mutant $Ca_V1.2$ channel showed increased current density. The authors speculate that the mutated Pro857, which sits within the intracellular linker between domains II and III, affects a “PEST” domain important for regulating rapid protein degradation; the mutation may slow degradation and thereby prolong the life of $Ca_V1.2$ channels at the cell surface. Another group performed targeted sequencing of *CACNA1C* in 278 subjects with inherited long QT syndrome and no obvious

extracardiac defects in whom genetic testing for the major long QT loci was negative [21]. Five novel mutations in seven unrelated probands were identified, representing a surprisingly high prevalence (2.5%) of *CACNA1C* mutations among Long QT subjects who were genotype-negative for other established causative loci. The *CACNA1C* mutant was assessed in the proband's relatives and genotype and phenotype correlated well for these novel *CACNA1C* mutations. Because these cohorts are relatively small, causation cannot be easily established. The authors analyzed each of the five mutations in a heterologous expression system, providing supportive information (regarding causality) for two of the mutants. One of them, R858H, showed increased current density in an analogous manner to the nearly adjacent P857H mutation discussed above. The similar effects of these two mutations observed in separate studies substantiates the hypothesis that perturbation of the PEST domain in the $Ca_V1.2$ II–III linker can increase the number of channels at the cell surface, increase inward Ca^{2+} current, and lead to a gain-of-function phenotype. Another mutant, A582D in domain II, displayed a different gain-of-function effect. Channel inactivation kinetics, recorded in a solution with extracellular Ca^{2+}, were slowed. This effect seems more analogous to the loss of VDI observed with the G406R and G402S TS mutations; however, the specific recording conditions do not allow dissection of VDI from CDI. Testing of the three other mutations did not reveal an obvious gain-of-function effect. Why these three mutations (P857H, R858H, and A582D) are associated with an isolated long QT syndrome and why the patients did not manifest any extracardiac effects is not known. It is reasonable to speculate that the increase in total current generated by more channels at the cell surface (for P857H or R858H) is not sufficient to affect development based on work examining how the canonical G406R mutant produces craniofacial dysmorphia [15]. In that study, expression of the G406R mutant in jaw precursors produced macrognathia that recapitulates the phenotype observed in TS1 patients. However, over-expression of wild type $Ca_V1.2$ channel had no effect. These data suggest that, during jaw development, the failure of $Ca_V1.2$ channels to inactivate is a more potent perturbation than augmenting Ca^{2+} influx by increasing the number of normally inactivating channels. Nevertheless, this argument does not explain why the A582D mutant, which slows channel inactivation, does not exert an effect similar to what was observed with TS1. Perhaps additional electrophysiological analysis of A582D will reveal differences with G406R that point to mechanism.

III. Loss of Function Mutations in LTCC Genes: Brugada, Early Repolarization and Short QT syndromes

The electrocardiographic J wave, also referred to as an Osborn wave, was first described in an ECG recorded from an accidentally frozen human in 1938[22] Prominent J wave are typically observed in clinical cases of hypothermia,[23] hypercalcemia,[24] and have more recently been suggested as a marker for substrates capable of generating life-threatening VT/VF associated with both the Brugada (BrS) and early repolarization (ERS) syndromes.[25]

An early repolarization ECG pattern, consisting of a distinct J wave or J point elevation, a notch or slur of the terminal part of the QRS with or without an ST segment elevation, is generally found in healthy young males. Although traditionally viewed as benign ECG

manifestations,[26] prominent J waves have in recent years been shown to predispose to life-threatening arrhythmias in experimental models[25, 27] as well as in clinical cases of ERS.[28]

Because accentuated J waves characterize both BrS and ERS, these syndromes have been grouped under the heading of J wave syndromes.[25] ERS and BrS share common ECG characteristics, clinical outcomes, risk factors as well as a common arrhythmic platform related to amplification of I_{to}-mediated J waves. They differ with respect to the magnitude and lead location of abnormal J waves and can be considered to represent a continuous spectrum of phenotypic expression.

BrS and ERS are caused by a preferential accentuation of the action potential notch in right and left ventricular epicardium, respectively, caused by an outward shift in the balance of current active during the early phases of the epicardial action potential secondary to either an increase in outward current, such as the transient outward (I_{to}), or a decrease inward currents, including sodium (I_{Na}) and calcium (I_{Ca}) channel currents. [29] Indeed, a majority of mutations associated with BrS to date have been found in the voltage-gated sodium channel $Na_V1.5$. However, $Na_V1.5$ mutations account for ~20% of known BrS cases. Multiple groups have undertaken candidate-gene, whole exome, and GWAS approaches to uncover additional genes responsible for BrS.

The rationale for exploring Ca^{2+} channel genes as candidates for BrS derived from experimental models of BrS generated using coronary-perfused RV wedge preparations, in which the BrS phenotype could be induced using Ca^{2+} channel inhibitors.[30] Treatment with verapamil was shown to eliminate the dome normally present in phase 2 of the RV epicardial action potential. Under these conditions, I_{to} is left unopposed causing loss of the action potential dome, thus providing a substrate for development of phase 2 reentry.

In the process of looking for BrS candidate genes, Antzelevitch and colleagues[31] screened a cohort of 82 BrS patients for mutations in *CACNA1C* and *CACNB2B*. In doing so, they were the first to propose L-type Ca^{2+} channel genes as causative for BrS with associated short QT intervals. Seven patients (8.5%) harbored mutations in either *CACNA1C* or *CACNB2B*. Three of the 7 patients exhibited clinically significant short QT intervals. Two of the probands had substitutions in *CACNA1C* leading to A39V or G490R substitutions in $Ca_V1.2$ and one patient had a mutation leading to a S481L substitution in $Ca_V\beta2b$. In order to determine the functional consequences of these substitutions, the variants were co-expressed with wild type constructs in CHO cells. Under these conditions, all three mutations produced dramatic loss of function in I_{Ca}.

As described above, loss of function in peak I_{Ca} could result from changes in gating, permeation or trafficking. Monitoring the apparent surface expression of EYFP-tagged Cav1.2 provides a useful tool for determining the impact of mutations on alpha subunit trafficking. The EYFP-A39V mutations was found concentrated in the perinuclear region, suggesting impaired trafficking. Conversely, expression of the G490R substitution in $Ca_V1.2$ or the S481L substitution in $Ca_V\beta2b$ produced EYFP-CaV1.2 fluorescence patterns similar to WT. [31]

In a separate study, Cordeiro, et al. examined the functional consequences of another *CACNB2b* mutation leading to T11I substitution in $Ca_V\beta 2b$. [32] The proband in this case exhibited a positive BrS sign and VF under procainamide challenge. Unlike the S481L $Ca_V\beta 2b$ variant, the T11I substitute channel exhibited a similar current-voltage relationship to WT, suggesting no major loss of function. However, upon further biophysical analysis, the T11I channel exhibited a marked increase in the rate of inactivation. In this case, a significant hastening of inactivation would result in few channels open during phase 2 of the action potential. In order to test this hypothesis, the WT and T11I channels were examined under AP clamp using waveforms recorded from canine epicardial and endocardial myocytes. Indeed, the mutant channels produced a 50% loss in total charge under the epicardial waveform, but no difference in total charge when stimulated by the endocardial waveform. These results provided mechanistic insight into the impact of a loss of epicardial I_{Ca} in the BrS phenotype.

Since these initial studies, more cases of BrS patients harboring mutations in calcium channel genes have been reported. In the largest study to date, Burashnikov et al. described 23 distinct mutations in a cohort of 205 probands with BrS, IVF and ERS.[33] Of these, 7 were novel mutations in *CACNA1C*, 8 were newly identified substitutions in *CACNB2* subunits. The remaining 4 were the first ever described mutations in *CACNA2D1* in patients with inherited arrhythmia syndromes. In this study, two of the novel mutations were analyzed in heterologous expressions systems. Both the V2014I variant and the construct harboring a duplication at Q1833 produced a loss of peak I_{Ca} when compared to WT in I–V comparisons. Of note, the patient harboring the V2104I variant also carried the common $Ca_V\beta 2$ polymorphism D601E. When the two were co-expressed, a marked increase in the amount of I_{Ca} present at the end of a 500 ms long pulse was observed. This increase in so-called "late" I_{Ca} may account for the normal QT interval observed in that patient.

A more recent study of a Japanese BrS and IVF cohort identified 6 *CACNA1C* mutations in 7 probands out of 312 screened.[34] The authors also screened for variation in *CACNB2b* but did not detect any. Of the patients with *CACNA1C* mutations, 6 presented with BrS without short QT intervals and one patient was diagnosed with IVF. The authors performed a phenotypic comparison of the clinical characteristics of BrS patients with *CACNA1C* mutations to those with *SCN5A* mutations. They found no differences in male predominance, age, symptoms or ECG characteristics including HR, PR, QRS and QTc values. The lack of significant difference, particularly with respect to PR interval is likely due to the small cohort.

The current yield for identification of a genotype in BrS probands is over 50%, whereas the yield for calcium channel gene mutations in approximately 13% (Table 1, Figure 1). While these studies examined the prevalence of L-type channel mutations in BrS populations, Risgaard et al. examined the distribution of BrS variants in the general population. The authors used data from the NHLBI GO Exome Sequencing Project (ESP) to determine the prevalence of proposed BrS causative mutations in apparently healthy controls. In total, they identified 355 of the variants in the database of 6500 samples for a genotype prevalence of 1:23. Four previously identified *CACNA1C* and *CACNB2* variants were found in the samples as well as two previously identified *CACNA2D1*. While it is not possible to know whether

any of these samples were from patients with BrS or other arrhythmias, the study highlights the importance of screening control subjects. Unfortunately, most of the variants found in the control population have not been functionally characterized.

The finding that some BrS patients with Ca^{2+} channel mutations also presented with SQT prompted the search for calcium channel genes mutations in cases of congenital SQT. Inherited SQT has been associated with gain of function mutations in repolarizing currents. Templin et al. identified a *CACNA2D1* mutation in a SQT patient who had previously screened negative for known SQT genes leading to a S755T substitution in $Ca_V\alpha 2\delta$. When expressed with WT $Ca_V1.2$ and $Ca_V\beta 2b$, this variant produced a profound loss of currents with barium as the charge carrier. Surprisingly, no defect in trafficking was found as the authors found equivalent amounts of Cav1.2 in surface biotinylation experiments. However, there were positive shifts in the activation and inactivation curves consistent with those found in cells lacking $Ca_V\alpha 2\delta 1$.

IV. Limitations and Future Directions for the Study of LTCC Mutations

It is important to point out that only a small fraction of identified genetic variants in the genes associated with BrS and ERS have been investigated functionally to establish causality. Very few have been studied in genetically engineered animal model or native cardiac cells so as to provide a plausible case for a contribution to pathogenesis. Computational tools have been developed to predict the functional consequences of mutations, but none have been rigorously tested. The lack of functional or biological validation of mutation effects remains an important limitation of genetic test interpretation.[35] This limitation is compounded in cases in which a susceptibility gene has been identified on the basis of a single proband, with absence of familial segregation data.

Additional studies are clearly needed to examine causality and the mechanisms associated with both gain and loss of function mutations. Most approaches to date have utilized heterologous systems expressing WT vs. mutated channels. Functional expression studies are, however, limited since they are undertaken in cultured cells that do not fully re-capitulate the cardiac myocyte phenotype. Another limitation of heterologous expression studies for Ca^{2+} channels is that they require expression of multiple subunits at the proper stoichiometry, which is usually not well established. β subunits are also known to form hetero- and homo-oligomers and mutations may alter the oligomerization process. Many cultured cell lines used for heterologous expression also contain endogenous α2δ and β subunits, making it difficult to properly determine the underlying mechanisms leading to a loss or gain of function.

Cultured cell lines also lack the highly organized structure of cardiomyocytes where $Ca_V1.2$ channels cluster together in t-tubules.[36] Mutations that disrupt this localization or clustering behavior might not be evident in heterologous systems. Similarly, mutations that alter the proportion of small number of channels activated during an action potential would need to be tested in myocytes. Given these limitations, researchers have and should continue to explore additional approaches including viral expression of mutants in adult myocytes, gene

knock-in and transgenic overexpression of Timothy Syndrome mutations in mice as well as induced pluripotent stem cell derived cardiomyocytes from TS patients. [37]

Overexpression of $Ca_V1.2$ harboring the Timothy Syndrome mutation G406R in adult myocytes is a promising approach that has provided evidence for alternative explanations for the cardiac phenotype. Thiel et al. utilized lentiviral overexpression of WT and G406R Cav1.2 containing T1066Yvariants to remove DHP sensitivity. [38] Application of nifedipine suppressed the endogenous currents leaving the virally transduced constructs as the only L-type current measured. Under these conditions, the authors found that the increased APD and after-depolarizations brought on by the G406R mutations could be suppressed by inhibition of CaMKII. Cheng et al. generated a transgenic mouse line expressing the G406R mutant selectively in cardiac tissue. [39] They found that the mutation brought about an aberrant coupling to WT channels via an A-Kinase Anchoring Protein (AKAP79/150). Furthermore, they were able to show that ablation of AKAP79/150 ameliorated the pro-arrhythmic effects of the G406R mutation.

Examining the consequences of Cav1.2 mutations on cardiac arrhythmias *in vivo* has been hampered by a lack of suitable animal models. Expression of the G406R mutation in exon 8 led to neonatal death in mice in both heterozygous and homozygous situations.[11a] However, mice that retained the neomycin cassette (TS2-Neo) survived to adulthood presumably due to a reduced expression of the G406R mutation. While the impact of the TS mutation brought about mouse behavioral defects consistent with autism, the cardiac consequences of the G406R mutation have not been examined *in viv*o.

Mouse models harboring genetic knock-in of human arrhythmia related mutations offer the potential to study the effects of mutations on cardiac arrhythmias *in vivo, ex vivo* as well as at the cellular and molecular level. Unfortunately, murine models have serious limitations that preclude widespread use for studying calcium channel-induced arrhythmias. First and foremost, the mouse cardiac action potential lacks the classical spike and dome morphology, evident in higher mammals including humans. The repolarizing K^+ currents also differ between mouse and humans. As such extrapolating information on arrhythmia mechanisms from mouse studies is fraught with danger. These limitations, notwithstanding, knock-in mice would likely help explain some of the multi-system manifestations of calcium channel mutations.

With the advent of induced pluripotent stem cell (iPS) technology, it has become possible to examine human cardiac myocytes from patients harboring *CACNA1C* mutations tied to Timothy Syndrome. Yazawa et al. reported on of two such patient-derived iPS-derived cardiomyocyte (iPS-CM) cell lines [37]. The iPS-CMs were from two patients with the G406R mutation. [40] The mutation disrupted spontaneous beating and calcium handling in the iPS-CM single cells and beating clusters. The L-type channels recorded in Timothy Syndrome iPS-CM also exhibited impaired VDI as expected. The authors also demonstrated the efficacy of roscovatine to restore VDI as well as prevent spontaneous after-depolarizations, altered beating patterns and calcium handling defects in Timothy Syndrome cells.

To date, no loss of function Ca^{2+} mutations have been reported in studies involving iPS-CM from affected patients. While studies of this type could prove useful in examining the cellular basis of cardiac dysfunction, these studies are likely to be hampered by the fact that action potential profiles of iPS-CM do not fully recapitulate the adult phenotype. In particular, they lack the spike and dome morphology needed for manifestation of the BrS and ERS phenotypes. Examination of iPS-CM from SQT patients harboring mutations in Ca^{2+} channel genes may be more fruitful given the success of modeling LQT in iPS-CM.

Since the first description of a mutation in *CACNA1C* leading to Timothy Syndrome in 2004, much has been learned about the effects of calcium channel mutations on cardiac arrhythmias. Ample targets for possible candidates are present in the ever-increasing number of proteins that comprise the $Ca_V1.2$ multiprotein complex. Moreover, the mechanistic bases for loss of function mutations need additional characterization to facilitate development of novel treatment strategies for J-wave syndromes and SQT. Development of more appropriate systems to study these mutations is also needed to fully understand to role of $Ca_V1.2$ mutations in cardiac arrhythmias syndromes.

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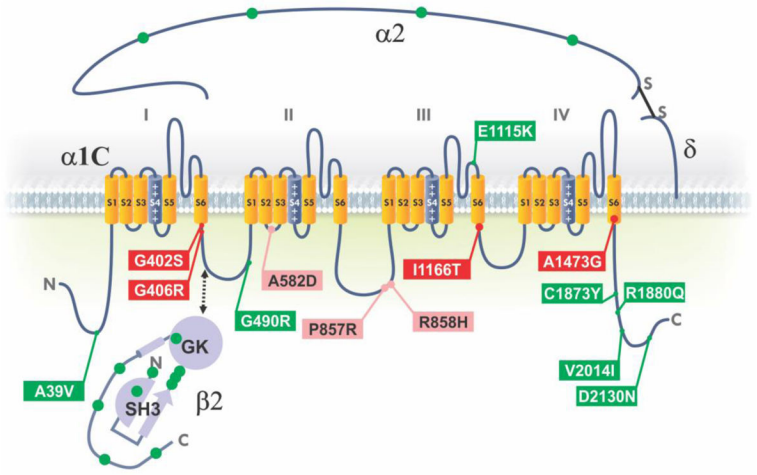

Figure 1.

Schematic of the L-type cardiac calcium channel showing missense mutation associated with loss of function, contributing to BrS and/or ERS and/or SQTS (green), those associated with a gain of function with extracardiac manifestations contributing to Timothy syndrome and Timothy-like syndromes (red), and gain of function mutations without extracardiac manifestations (rose). The auxiliary subunits $Ca_V\beta 2$ and $\alpha 2\delta 1$ are also shown, and the locations of loss of function mutations contributing to BrS and/or ERS and/or SQTS and indicated (green dots). For clarity, the mutations in the auxiliary subunits are not identified by amino acid.

Table 1

Gene defects responsible for LQTS and Brugada Syndrome.

Genetic defects of LQTS				
	Chromosome	**Gene/Protein**	**Ion Channel**	
LQT1	11	*KCNQ1, KvLQT1*	↓I_{Ks}	30–35%
LQT2	7	*KCNH2, HERG*	↓I_{Kr}	20–25%
LQT3	3	*SCN5A, $Na_v1.5$*	↑Late I_{Na}	5–10%
LQT4	4	*Ankyrin-B, ANK2*	↑Ca_i, ↑Late I_{Na}	1–2%
LQT5	21	*KCNE1, MinK*	↓I_{Ks}	1%
LQT6	21	*KCNE2, MiRP1*	↓I_{Kr}	Rare
LQT7 *	17	*KCNJ2, Kir 2.1*	↓I_{K1}	Rare
LQT8 **	12	*CACNA1C, $Ca_v1.2$*	↑I_{Ca}	Rare
LQT9	3	*CAV3, Caveolin-3*	↑Late I_{Na}	Rare
LQT10	11	*SCN4B, NavB4*	↑Late I_{Na}	Rare
LQT11	7	*AKAP9, Yatiao*	↓I_{Ks}	Rare
LQT12	20	*SNTA1, a1 Syntrophin*	↑Late I_{Na}	Rare
LQT13	11	*KCNJ5, Kir 3.4*	↓$I_{K\text{-}ACh}$	Rare
LQT14	14	*CALM1, Calmodulin*		Rare
LQT15	2	CALM2, *Calmodulin*		Rare
Genetic defects of Brugada Syndrome				
	Locus	**Gene/Protein**	**Ion Channel**	
BrS1	3p21	*SCN5A, $Na_v1.5$*	↓ I_{Na}	11–28%
BrS2	3p24	*GPD1L*	↓ I_{Na}	Rare
BrS3	12p13.3	*CACNA1C, $Ca_v1.2$*	↓ I_{Ca}	6.6%
BrS4	10p12.33	*CACNB2b, $Ca_v\beta2b$*	↓ I_{Ca}	4.8%
BrS5	19q13.1	*SCN1B, $Na_v\beta1$*	↓ I_{Na}	1.1%
BrS6	11q13-14	*KCNE3, MiRP2*	↑ I_{to}	Rare
BrS7	11q23.3	*SCN3B, $Na_v\beta3$*	↓ I_{Na}	Rare
BrS8	12p11.23	*KCNJ8, Kir6.1*	↑ $I_{K\text{-}ATP}$	2%
BrS9	7q21.11	*CACNA2D1, $Ca_v\alpha2\delta$*	↓ I_{Ca}	1.8%
BrS10	1p13.2	*KCND3, $K_v4.3$*	↑ I_{to}	Rare
BrS11	17p13.1	*RANGRF, MOG1*	↓ I_{Na}	Rare
BrS12	3p21.2-p14.3	*SLMAP*	↓ I_{Na}	Rare
BrS13	12p12.1	*ABCC9, SUR2A*	↑ $I_{K\text{-}ATP}$	Rare
BrS14	11q23	*SCN2B, $Na_v\beta2$*	↓ I_{Na}	Rare
BrS15	12p11	*PKP2, Plakophillin-2*	↓ I_{Na}	Rare
BrS16	3q28	*FGF12, FHAF1*	↓ I_{Na}	Rare

Genetic defects of LQTS				
	Chromosome	**Gene/Protein**	**Ion Channel**	
BrS17	3p22.2	*SCN10A, $Na_v1.8$*	$\downarrow I_{Na}$	16.7%
BrS18	6q	*HEY2 (transcriptional factor)*	$\uparrow I_{Na}$	Rare
BrS19	7p12.1	*SEMA3A, Semaphorin*	$\uparrow I_{to}$	Rare

* Andersen-Tawill Syndrome,

** Timothy Syndrome.

Table 2

Gene defects responsible for Early Repolarization Short QT syndromes

Genetic defects of Short QT Syndrome			
	Chromosome	**Gene/Protein**	**Ion Channel**
SQT1	7	*KCNH2, HERG*	↑I_{Kr}
SQT2	11	*KCNQ1, KvLQT1*	↑I_{Ks}
SQT3	17	*KCNJ2, Kir 2.1*	↑I_{K1}
SQT4	12	*CACNA1C, $Ca_v1.2$*	↓ I_{Ca}
SQT5	10	*CACNB2b, $Ca_v\beta2b$*	↓ I_{Ca}
SQT6	7	*CACNA2D1, $Ca_v a2\delta$*	↓ I_{Ca}
Genetic defects of Early Repolarization Syndrome			
	Chromosome	**Gene/Protein**	**Ion Channel**
ERS1	12	*KCNJ8, Kir6.1*	↑ IK-ATP
ERS2	12	*CACNA1C, $Ca_v1.2$*	↓ I_{Ca}
ERS3	10	*CACNB2b, $Ca_v\beta2b$*	↓ I_{Ca}
ERS4	7	*CACNA2D1, $Ca_v a2\delta$*	↓ I_{Ca}
ERS5	12	*ABCC9, SUR2A*	↑ IK-ATP
ERS6	3	*SCN5A, NaV1.5*	↓ I_{Na}
ERS7	3	*SCN10A,* NaV1.8	↓ I_{Na}