Published in final edited form as:
Circulation. 2008 November 25; 118(22): 2225–2234. doi:10.1161/CIRCULATIONAHA.108.788067.

Proarrhythmic defects in Timothy Syndrome require calmodulin kinase II

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Abstract

Background—Timothy Syndrome (TS) is a disease of excessive cellular Ca^{2+} entry and life-threatening arrhythmias due to a mutation in the primary cardiac L-type Ca^{2+} channel ($Ca_V1.2$). The TS mutation causes loss of normal voltage-dependent inactivation (VDI) of $Ca_V1.2$ current (I_{Ca}). During cellular Ca^{2+} overload the calmodulin-dependent protein kinase II (CaMKII) causes arrhythmias. We hypothesized that CaMKII is a part of the proarrhythmic mechanism in TS.

Methods and Results—We developed an adult rat ventricular myocyte model of TS (G406R) by lenti virus-mediated transfer of wild type (WT) and TS $Ca_V1.2$. The exogenous $Ca_V1.2$ contained a mutation (T1066Y) conferring dihydropyridine resistance, so we could silence endogenous $Ca_V1.2$ with nifedipine and maintain peak I_{Ca} at control levels in infected cells. TS $Ca_V1.2$ infected ventricular myocytes exhibited the signature VDI loss under Ca^{2+} buffering conditions, not permissive for CaMKII activation. In physiological Ca^{2+} solutions, TS $Ca_V1.2$ expressing ventricular myocytes exhibited increased CaMKII activity and a proarrhythmic phenotype that included action potential prolongation, increased I_{Ca} facilitation and afterdepolarizations. Intracellular dialysis of a CaMKII inhibitory peptide, but not a control peptide, reversed increases in I_{Ca} facilitation, normalized the action potential and prevented afterdepolarizations. We developed a revised mathematical model that accounts for CaMKII-dependent and CaMKII-independent effects of the TS mutation.

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Conflict of Interest Disclosures none

Conclusions—In TS the loss of VDI is an upstream initiating event for arrhythmia phenotypes that are ultimately dependent on CaMKII activation.

Keywords

action potentials; calcium; ion channels; myocytes

Introduction

Timothy Syndrome (TS) is an autosomal genetic disease of the primary voltage-gated cardiac Ca^{2+} channel ($Ca_V1.2$) consisting of a missense mutation in the pore forming α_{1c} subunit protein[1]. TS is associated with three individual mutations that include G406R on exon 8a[2], G406R on exon 8[1] and G402S[1]. The G406R mutation on exon 8 used for our model of TS is believed to be the most severe form of TS[1]. TS patients have an average life expectancy of only 2.5 years due to severe cardiac disease. TS is also known as long QT syndrome 8 (LQT8) and the prolonged QT intervals in TS patients are thought to cause cardiac arrhythmias and sudden death. TS disease phenotypes are apparently initiated by excessive Ca^{2+} entry, at least in part, due to impaired voltage dependence of inactivation (VDI) of $Ca_V1.2$ current (I_{Ca})[1, 2] Mathematical modeling predicts that intracellular Ca^{2+} overload and action potential prolongation stimulate afterdepolarizations that are the cellular mechanism for triggering ventricular arrhythmias in TS[1, 2]. However, these predictions have not been directly tested in ventricular myocytes.

In ventricular myocytes multiple signaling pathways are activated by increased intracellular Ca^{2+} entry, including the multifunctional Ca^{2+} and calmodulin dependent kinase II (CaMKII)[3], a procardiomyopathic and proarrhythmic signaling molecule[4]. Increased CaMKII activity causes action potential prolongation and arrhythmias, similar to the observed phenotypes in TS patients, in part by increasing sarcoplasmic reticulum (SR) Ca^{2+} leak and I_{Ca} facilitation [5, 6]. On the other hand, CaMKII inhibition restores normal intracellular Ca^{2+} homeostasis and suppresses arrhythmias[4, 5]. Based upon these concepts, we hypothesized that increased Ca^{2+} entry in TS ventricular myocytes enhances CaMKII actions and that activated CaMKII recruitment is important for the proarrhythmic cellular phenotype in TS. To test this hypothesis, we created an adult rat ventricular myocyte model of TS by lenti viral infection of a dihydropyridine-resistant $Ca_V1.2$ α_{1c} subunit[7] harboring the TS mutation. Our studies show that TS mutation requires CaMKII activity to cause important proarrhythmic phenotypes in adult ventricular myocytes.

Methods

Cloning

The plasmids pLentiNB CaV1.2 DHP^R HA WT and pLentiNB CaV1.2 DHP^R HA TS are described in Supplementary Methods.

Lenti virus

Lenti virus was prepared using the manufacturer's protocol (Invitrogen) and as described in Supplementary Methods.

Ventricular myocyte isolation, culturing and viral transduction

Adult male Sprague-Dawley rat (250-300g) ventricular myocytes were isolated as previously published[8] and cultured as described in Supplementary Methods. Procedures were in accordance with the Institutional Animal Care and Use Committee of the University

of Iowa. Lenti virus was added to cells at a multiplicity of infection (MOI) of 1-3, and cultures were maintained for 24-36 hours.

Electrophysiology

Electrophysiology for HEK293 cells and myocytes is detailed in the Supplementary Methods including pipette solutions, bath solutions, voltage clamp protocols, current clamp protocols and data analysis.

Immunoflourescence

HEK293 and myocytes were fixed, permeabilized, incubated with primary antibody Ig and a fluorescent secondary antibody Ig. Confocal images were collect on a Zeiss 510 Meta confocal microscope (Carl Zeiss). Detailed information on cell preparation, antibodies and acquisition of confocal images is available in Supplementary Methods.

Calcium imaging

Ca^{2+} transients, SR content and sparks were acquired by confocal laser scanning from myocytes loaded with Flou-3. Supplementary methods contain details on cell preparation and confocal Ca^{2+} imaging.

Mathematical modeling

Mathematical models of the WT and TS myocytes are based on the Luo-Rudy dynamic model of the mammalian ventricular action potential[9, 10]. See Supplementary Methods for equations that differ from the published model.

Statistics

Data presented as means with SEM. Sigma Stat was used to compare two groups with a Student T-test and multiple groups with an ANOVA. Significance was set at a P value < 0.05. Categorical data between two groups was compared using a 2-tailed Fisher Exact Test with significance set at $P<0.05$.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

An adult ventricular myocyte TS model

We marked exogenous $Ca_V1.2$ by the addition of an extracellular hemaglutanin (HA) epitope[11] (Figure 1A, green circle) and introduced a validated dihydropyridine-insensitivity mutation [7] (Figure 1A, black circle). The dihydropyridine-insensitivity mutation allows the virally introduced $Ca_V1.2$ to remain functional while using nifedipine to inhibit endogenous $Ca_V1.2$ [7]. Exogenous $Ca_V1.2$ expression was confirmed by immunoblot (Figure 1B) and immunofluorescence (Figure 1C) in transduced HEK293T cells. The function of $Ca_V1.2$ wild type (WT) and TS (G406R exon 8) were confirmed by recording I_{Ca} using whole cell voltage clamp in HEK293T cells. I_{Ca} recorded from TS expressing HEK293T cells exhibited a significant loss of VDI (Figure 1D), as previously published[1, 2, 12].

Over-expression of $Ca_V1.2$ in ventricular myocytes yielded 33.7% increase in peak I_{Ca} (Figure 1F) and an average 31.9% increase in total $Ca_V1.2$ protein (Supplemental Figure 1). Due to the dihydropyridine-resistance mutation[7], peak I_{Ca} in $Ca_V1.2$ infected ventricular myocytes was significantly resistant to nifedipine, as compared to uninfected cells (Figure 1E,F). In TS and WT infected ventricular myocytes 10nM nifedipine resulted in a peak I_{Ca}

(WT 6.6±0.7 pA/pF N=5, TS 6.9±0.7 pA/pF N=6) that was similar to the peak I_{Ca} (6.7±1.0 pA/pF N=8) measured in non-infected myocytes recorded without nifedipine (Figure 1E,F). This nifedipine engineered balance of endogenous and exogenous $Ca_V1.2$ allowed us to determine the effects of the TS mutation on cardiac electrophysiology independent of over-expression induced changes in peak I_{Ca}.

TS ventricular myocytes exhibit increased CaMKII autophosphorylation

We confirmed expression of exogenous $Ca_V1.2$ in cultured adult ventricular myocytes by immuno-staining for the HA epitope (Figure 2D,G). Virally introduced $Ca_V1.2$ was properly targeted to the transverse-tubule (T-tubule) network, based upon the punctate appearance and 1.8 μm spacing of the HA immunofluorescence that is consistent with known distances between T-tubules in a resting sarcomere[13]. No HA immuno-staining was detected in uninfected ventricular myocytes (Figure 2A).

We immuno-stained for the CaMKII autophosphorylation site, Thr 286, which is a marker of CaMKII activation[14]. TS ventricular myocytes (Figure 2H,I) exhibited greater levels of CaMKII autophosphorylation compared to both WT (Figure 2E,F) and uninfected ventricular myocytes (Figure 2B,C). Total CaMKII immuno-staining revealed no changes in CaMKII protein levels between WT, TS and uninfected ventricular myocytes (Supplemental Figure 2). These data show that activated CaMKII is recruited in TS $Ca_V1.2$ expressing ventricular myocytes and suggest that CaMKII activity may contribute to the cellular arrhythmia phenotypes in TS.

Action potential prolongation in TS ventricular myocytes is reversed by CaMKII inhibition

Stimulated action potentials (arrow head, Figure 3A) were recorded in nifedipine treated (10nM) WT and TS ventricular myocytes. Compared to WT, the TS mutation significantly prolonged the action potential duration (Figure 3A,B) as determined by the time to 90% repolarization (APD90). Excessive action potential prolongation favors the generation of afterdepolarizations[15]. We observed afterdepolarizations from TS ventricular myocytes (5 out of 10 cells, Figure 3A,C), whereas none were observed in any of the WT cells (0 out of 10 cells, Figure 3A,C). Most afterdepolarizations were delayed afterdepolarizations (DADs), but early afterdepolarizations (EADs) were also recorded from TS ventricular myocytes. DADs are favored by increased diastolic Ca^{2+} leak from the sarcoplasmic reticulum (SR)[16, 17] and EADs are caused by increased I_{Ca} facilitation [18]. The action potential prolongation and the tendency for afterdepolarizations in TS ventricular myocytes are consistent with predictions from computational modeling[1, 2].

Action potential durations from WT and TS ventricular myocytes in 1μM nifedipine were reduced to equivalent times and neither WT nor TS ventricular myocytes exhibited afterdepolarizations under these conditions (Figure 3B,C). The 1μM nifedipine bath solution overcomes the dihydropyridine resistance mutation and inhibited the total peak I_{Ca} by >50% (Figure 1F, double arrows). These findings indicate that the observed TS phenotypes were initiated by increased I_{Ca}.

We tested the role of CaMKII activity in the observed proarrhythmic cellular phenotypes observed from TS ventricular myocytes by dialysis of AC3-I, a selective CaMKII inhibitory peptide [4, 19]. AC3-I normalized the action potential duration in TS to WT levels (P=0.40, Figure 3D,E). The inactive control peptide, AC3-C[4, 19], had no effect, suggesting that CaMKII-dependent increases in I_{Ca} contributed to action potential prolongation in TS. The CaMKII inhibitory peptide also eliminated afterdepolarizations in TS ventricular myocytes (P=1.0, Figure 3D,F), whereas AC3-C did not (P=0.04, Figure 3D,F). These data support the

concept that CaMKII activity is required for the proarrhythmic electrophysiological phenotypes in TS ventricular myocytes.

In WT ventricular myocytes the CaMKII inhibitory peptide, AC3-I, resulted in a non-significant (P=0.28) shortening of the action potential duration (Figure 3E) compared to WT ventricular myocytes dialyzed with the control peptide, AC3-C. WT ventricular myocytes did not exhibit afterdepolarizations after dialysis with AC3-I or AC3-C (Figure 3F). We assessed additional action potential parameters, including resting cell membrane potential and peak cell membrane depolarization amplitude. Both TS and WT ventricular myocytes exhibited equivalent resting membrane potentials and peak action potential amplitudes (Supplemental Table 1). Action potential parameters from WT ventricular myocytes, in the presence of 10nM nifedipine, were similar to uninfected ventricular myocytes, cultured for the same time period (24-36 hours) and recorded without nifedipine (Supplemental Table 1). These controls suggest that viral expression of $Ca_V1.2$ does not alter the action potential when peak I_{Ca} is adjusted to normal levels (by 10nM nifedipine) and that the proarrhythmic phenotype observed in TS ventricular myocytes was due to the TS mutation.

Taken together, these findings are the first to demonstrate experimentally that the action potential phenotypes observed in TS ventricular myocytes were dependent upon increased Ca^{2+} entry through $Ca_V1.2$. Our findings suggest that the TS VDI defect is insufficient, in the absence of increased CaMKII activity, to cause significant action potential prolongation in ventricular myocytes.

TS reduces VDI in ventricular myocytes independent of CaMKII activity

Expression of TS $Ca_V1.2$ in *Xenopus* oocytes[1, 2] and heterologous cells[1, 2, 12] (Figure 1D) showed a loss of $Ca_V1.2$ VDI. The *Xenopus ooctye* experiments[1, 2] included Ca^{2+} independent conditions that would not favor CaMKII activation because Ba^{2+} substituted Ca^{2+} as the charge carrier. To test the effect of the TS mutation on VDI in ventricular myocytes under conditions not permissive to CaMKII activation, we recorded I_{Ca} from TS and WT ventricular myocytes (10nM nifedipine) using Ba^{2+} (1.8mM) as the charge carrier and under high intracellular Ca^{2+} buffering (20mM BAPTA). TS ventricular myocytes exhibited a loss of VDI as a significant ($p = 0.008$, Figure 4A) rightward shift compared to WT. The TS $V_{1/2}$ (-30.75 mV) shifted to more positive potentials compared to WT $V_{1/2}$ (-35.89 mV). In contrast, the peak I_{Ca} elicited by the conditioning pulses showed no difference between WT and TS (Figure 4B), confirming equivalent expression of exogenous WT and TS $Ca_V1.2$. No differences were observed in peak I_{Ca} or VDI recorded from adult ventricular myocytes expressing WT dihydropyridine-resistant $Ca_V1.2$, with 10nM nifedipine, compared to uninfected adult ventricular myocytes, without nifedipine (Supplemental Table 2). These findings show that TS causes a loss of $Ca_V1.2$ VDI in ventricular myocytes, establishing the initial requirement for increased cellular Ca^{2+} entry necessary to recruit CaMKII.

CaMKII is required for TS effects on I_{Ca}

To test the importance of CaMKII for additional I_{Ca} changes other than VDI in our TS model, we measured CaMKII dependent I_{Ca} facilitation [18, 20]. I_{Ca} facilitation consists of dynamic increases in peak I_{Ca} and slowing of inactivation with repetitive depolarizations[21, 22]. TS ventricular myocytes exhibited maximal peak I_{Ca} during the first depolarization, whereas WT attained peak I_{Ca} after the initial depolarization (Figure 5A, Supplemental Figure 3). Subsequent depolarizations showed no difference in peak I_{Ca} between TS and WT (Figure 5A, Supplemental Figure 3). To measure the effects of I_{Ca} facilitation on cellular Ca^{2+} entry, we integrated total I_{Ca} during the voltage clamp command step. Integrated I_{Ca} was significantly greater in TS compared to WT during all

depolarization steps (First step P=0.029, Remaining steps P<0.001, Figure 5B). We found the fast component of I_{Ca} inactivation (τ_{fast}) was slower in TS compared to WT (First step P=0.006, Remaining steps P<0.001, Figure 5C), consistent with increased I_{Ca} facilitation and augmented cellular Ca^{2+} entry in TS ventricular myocytes.

AC3-I restored the dynamic response characteristics of integrated I_{Ca} and τ_{fast} in TS to levels recorded from WT cells (integrated I_{Ca} P=0.522, τ_{fast} P=0.294, Figure 5E,F). In contrast, dialysis of AC3-C had no effect of τ_{fast} or integrated I_{Ca}. Dialysis of the CaMKII inhibitory peptide prevented I_{Ca} facilitation in WT ventricular myocytes (Supplemental Figure 3), whereas the control peptide had no effect on WT ventricular myocyte I_{Ca} facilitation. These measurements show that CaMKII is a significant determinant of I_{Ca} from TS mutant channels, and that CaMKII actions are distinct from the previously reported shift in VDI.

TS augments intracellular Ca^{2+}

Mathematical modeling studies predicted alterations in intracellular Ca^{2+} handling in TS, including increased Ca^{2+} transient amplitude and increased SR Ca^{2+} content[1]. We recorded Ca^{2+} transients (Figure 6A) from WT and TS ventricular myocytes loaded with fluo-3 AM and field stimulated at 1Hz[23]. TS caused a significant increase in the peak Ca^{2+} transient compared to WT (P=0.04, Figure 6B), which is consistent with computer models[1, 23, 24]. Interestingly, the 50% decay time for Ca^{2+} transients in TS was significantly shortened over WT (P=0.02, Figure 6C). A faster decay time implicates increased SERCA activity[25], which was not predicted by modeling studies, but is associated with CaMKII signaling[26-29]. These experimental data reveal that TS alters intracellular Ca^{2+} handling by increasing the peak Ca^{2+} transient amplitude and enhancing the decay of the intracellular Ca^{2+} transient.

Mathematical modeling also predicted increased SR Ca^{2+} content with TS, due to enhanced I_{Ca} from TS $Ca_V1.2$. Surprisingly, we found that TS SR Ca^{2+} content was not different than WT (P=0.55, Figure 6D). We considered that increased SR Ca^{2+} leak in TS balances faster SR Ca^{2+} uptake[25], thereby preventing a net increase in SR Ca^{2+} content compared to WT. Increased SR Ca^{2+} leak is implicated in CaMKII signaling[6, 30, 31] and in triggering DADs[16, 17], a prominent feature of the TS ventricular myocytes (Figure 3A,C). We assessed diastolic SR Ca^{2+} leak by measuring spontaneous Ca^{2+} sparks from TS and WT ventricular myocytes (Figure 6E)[32]. The SR Ca^{2+} sparks were significantly increased in TS compared to WT (P=0.001, Figure 6E,F), indicating increased SR Ca^{2+} leak in TS. The spark amplitude for TS was significantly greater than WT (P=0.002, Supplemental Table 3), consistent with the increase in peak Ca^{2+} transient observed with TS. The effects of TS on intracellular Ca^{2+} handling had two unexpected results, first the faster decay time and second the increase in spark frequency. Taken together, these data suggest that SR Ca^{2+} cycling is enhanced in TS ventricular myocytes, resulting in significantly increased SR Ca^{2+} uptake and diastolic Ca^{2+} leak, but without a change in SR Ca^{2+} content.

In contrast to TS, WT exhibited no difference in the Ca^{2+} transient peak amplitude or decay time as compared to uninfected ventricular myocytes (Supplemental Table 3). No significant changes were observed in the width or duration of the Ca^{2+} sparks (Supplemental Table 3) between WT, TS and uninfected cells. The spark frequency and profile of individual sparks showed no difference between WT and uninfected ventricular myocytes (Supplemental Table 3)[33].

Revised TS mathematical modeling

Several studies have modeled the impact of TS on myocardial electrophysiology by using a shift in $Ca_V1.2$ VDI estimated from measurements in non-myocytes[1, 2, 24]. Using data from

our TS ventricular myocyte model, we developed a new mathematical model of TS incorporating CaMKII signaling (Figure 7A). As the basis for our new model of TS, we used the Luo-Rudy dynamic model (LRd), [9, 10] because of its established utility in studying cardiac arrhythmia mechanisms.

Our model of TS incorporated three modifications to match our experimental observations. First, we shifted the $Ca_V1.2$ steady-state VDI in the LRd model to simulate the measured TS defect on channel gating. Second, we simulated the downstream CaMKII effect on $Ca_V1.2$ I_{Ca} facilitation associated with TS by slowing I_{Ca} inactivation to increase integrated I_{Ca} as measured experimentally (Supplemental Figure 4). Third, we simulated the CaMKII actions on intracellular Ca^{2+} handling associated with TS by increasing the mean open time of the ryanodine receptor SR Ca^{2+} release channels, decreasing the threshold for spontaneous SR Ca^{2+} release and increasing SR Ca^{2+} release. Consistent with our experimental measurements, the new model of TS predicted an increase in the intracellular Ca^{2+} transient amplitude without any change in SR Ca^{2+} load compared to WT (Supplemental Figure 4). The model also predicted an increase in action potential duration (Figure 7B) and afterdepolarizations (Figure 7C) during a pause after pacing. We also simulated CaMKII inhibition using the TS LRd model by reversing the simulated downstream CaMKII effects, but leaving in place the shift in $Ca_V1.2$ VDI we measured under conditions not permissive for CaMKII activity (Figure 4A). The resulting TS LRd model with 'CaMKII inhibition' prevented action potential prolongation and afterdepolarizations (Figure 7C). Our mathematical models of TS, with and without CaMKII inhibition, are consistent with our experimental data from our TS ventricular myocyte model.

Discussion

TS is the first arrhythmia syndrome (LQT8) due to a genetic mutation in the $Ca_V1.2$ pore-forming α subunit.[1, 2] In comparison to cardiac Na^+ and K^+ channels, $Ca_V1.2$ has proven to be remarkably resistant to genetic disease. One key difference between Ca^{2+}, Na^+ and K^+ is the prominent role Ca^{2+} plays as a second messenger. TS patients not only have extremely profound QT interval prolongation, but also structural cardiac abnormalities, which are not typical of Na^+ or K^+ channel gene-related long QT syndrome patients. QT interval prolongation reflects increased duration of the ventricular action potential. The action potential duration prolongation in TS was attributed entirely to the defect in VDI[1], but this defect in TS VDI was ascertained in heterologous (non-myocardial) cells, where action potentials could not be directly measured. Furthermore, heterologous cells lack the highly ordered ultrastructure that is present in ventricular myocytes, for Ca^{2+} homeostasis and excitation-contraction coupling. The ventricular myocyte TS model allowed us to measure electrophysiological, intracellular Ca^{2+} handling and Ca^{2+} mediated signaling changes that occur downstream to the loss of VDI.

Despite the relatively modest reduction in $Ca_V1.2$ VDI measured in our TS model, we found action potential prolongation and spontaneous afterdepolarizations that were due to secondary activation of CaMKII. We conclude that the shift in VDI provides the initial stimulus to trigger intracellular Ca^{2+} signaling that includes CaMKII activation. Increased CaMKII activity appears to be necessary for the cellular phenotype of prolonged action potentials and afterdepolarizations, in so far as CaMKII inhibition prevents these phenotypes. CaMKII inhibition may be a viable alternative therapeutic approach for TS patients treated with the I_{Ca} antagonist verapamil[34]. Our results showed that CaMKII amplifies Ca^{2+} entry through $Ca_V1.2$ in TS, by slowing τ_{fast}, and shifting the $V_{1/2}$ of I_{Ca} inactivation. Our studies do not exclude the possibility that CaMKII inhibition could also affect other depolarizing or repolarizing currents, such as Na^+ current[35] or K^+ current[36]. Our findings that SR Ca^{2+} leak is increased in TS is consistent with other reports that show

proarrhythmic actions of CaMKII are due to increasing SR Ca^{2+} leak[37], thereby enabling a transient inward current[16] (I_{NCX}) that triggers DADs. Thus, our data support the concept that the ryanodine receptor is a secondary proarrhythmic target for excessive CaMKII activity in TS. Our data highlight how small changes in cellular Ca^{2+} entry through $Ca_V1.2$ can lead to unanticipated, maladaptive and far-reaching changes in Ca^{2+} activated signaling.

Interestingly a connection between CaMKII and a TS mutation was suggested based upon single channel recordings from heterologous expression of TS $Ca_V1.2$ in baby hamster kidney 6 cells[38]. These experiments found that TS $Ca_V1.2$ were more likely than WT to exhibit frequent, long openings, so called mode 2 gating that are the single channel mechanism underlying CaMKII-mediated I_{Ca} facilitation[20]. Our new studies add to evidence supporting a connection between TS and CaMKII by showing that CaMKII is critical for increased I_{Ca} facilitation action potential prolongation and afterdepolarizations in our TS ventricular myocyte model. Enhanced CaMKII activity increases I_{Ca} facilitation,[17] which may cause generation of EADs[5].

Although major Ca^{2+} homeostatic proteins are conserved in ventricular myocytes across mammalian species, differences exist between species regarding the quantitative contribution of these components to the action potential [39]. Thus, one goal of future studies should be to determine if CaMKII, or other Ca^{2+}-activated signaling molecules, contribute to TS phenotypes in ventricular myocytes from other species. However, the use of our TS adult ventricular myocyte model has contributed new insights about arrhythmia mechanisms in TS, by illustrating how a concise defect in $Ca_V1.2$ gating can initiate downstream recruitment of CaMKII that ultimately enables the electrophysiological cellular disease phenotype in TS. The CNS defects of TS patients may also be due to secondary recruitment of Ca^{2+} activated signaling molecules, including CaMKII. Over-expression of CaMKII is known to interfere with neuronal growth and differentiation[40] and a constitutively active CaMKII within the mouse brain causes significantly impaired spatial memory[41]. CaMKII recruitment in TS ventricular myocytes also suggests the possibility that other disease phenotypes in TS patients (e.g. structural heart disease or mental retardation), may be initiated by defects in VDI but carried forward, indirectly, by recruitment of Ca^{2+}-dependent signaling molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the University of Iowa Gene Transfer Vector Core (an NIH-funded resource) for their help in preparing the lenti virus, in particular Maria Scheel and Dalyz Ochoa for their assistance and expertise.

Funding Sources Work funded by NIH R01 HL 079031, R01 HL 62494, and R01 HL 70250 (MEA); NIH R01 HL084583, R01 HL083422, and Pew Scholars Trust (PJM); the University of Iowa Cardiovascular Center Interdisciplinary Research Fellowship (TH) and the University of Iowa Research Foundation. William Thiel was supported in part by an American Heart Association pre-doctoral fellowship award.

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Clinical Perspective

Timothy Syndrome (TS) is a genetic disorder causing excessive cellular Ca^{2+} entry due to defective voltage dependent inactivation (VDI) of the predominant myocardial L-type Ca^{2+} channel ($Ca_V1.2$) current (I_{Ca}). Timothy Syndrome patients die on average at 2.5 years, due to malignant cardiac arrhythmias. TS is a 'model' disease whereby a concise biophysical defect in I_{Ca} activates a cellular signaling cascade that is required for the cardiac disease phenotypes. Our studies showed that loss of VDI leads to cellular arrhythmias by recruiting activity of the calmodulin dependent protein kinase II (CaMKII). The role of CaMKII was not anticipated by previous computer models that relied on data obtained from non-excitable, heterologous cells. Our studies show that the TS VDI defect activated CaMKII and CaMKII was the feed forward signal required for the proarrhythmic cellular phenotypes in TS. These findings have potentially broad implications for other pathological phenotypes in Timothy Syndrome, such as autism, and for other genetic diseases affecting Ca^{2+} channels, such as migraine headache, myasthenia, ataxia and malignant hyperthermia. Diseases associated with excitable cells, where ion channels frequently constitute a final common pathway, require careful consideration of the connections between ion channel gating and signaling cascades in order to more comprehensively understand underlying mechanisms.

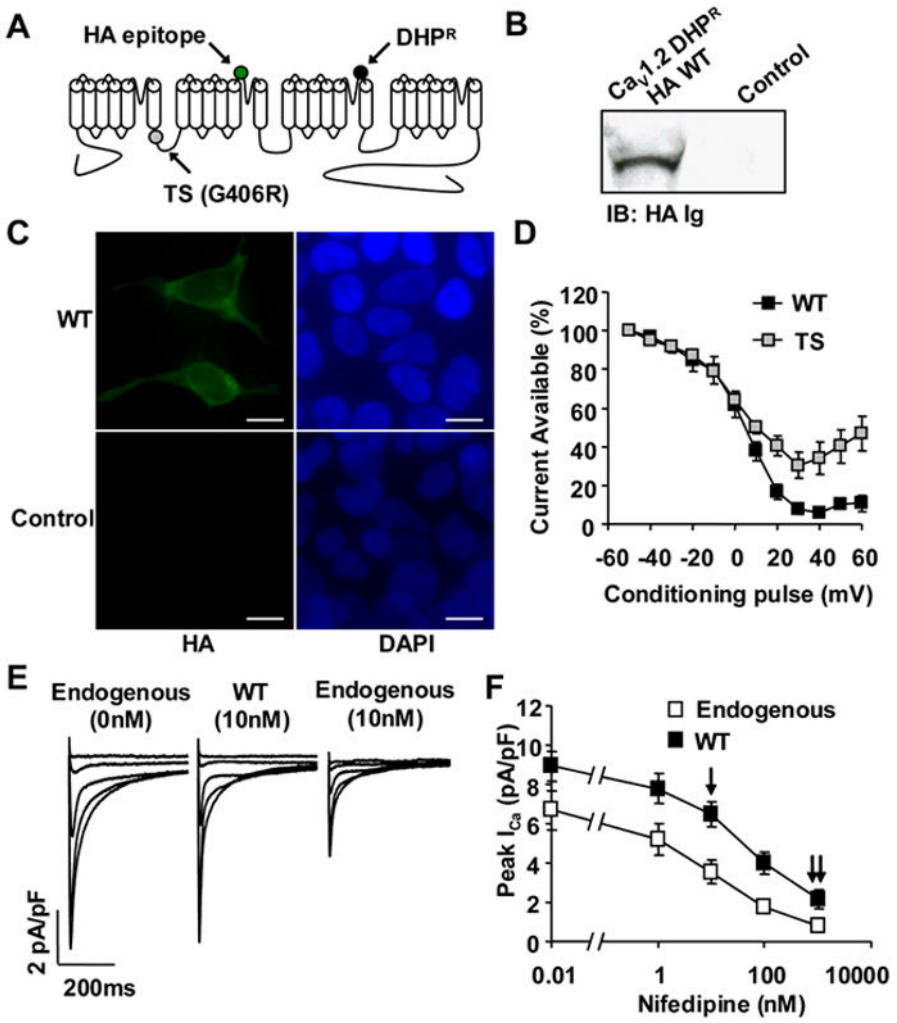

Figure 1.

Dihydropyridine-resistant $Ca_V1.2$ α subunit Timothy Syndrome (TS) model. **(A)** A topology diagram of $Ca_V1.2$ depicting dihydropyridine resistance mutation (DHP^R, black circle), extracellular hemaglutanin epitope (HA, green circle) and the TS mutation (G406R, gray circle) on the I-II intracellular loop. **(B)** Immunoblot (HA Ig) of HEK293T cells expressing the modified $Ca_V1.2$ or empty vector control. **(C)** FITC immunofluorescence (HA Ig) of HEk293T cells expressing the modified $Ca_V1.2$ with corresponding nuclear stain by DAPI (Scale bar, 10μm). **(D)** $Ca_V1.2$ TS expressing HEK293T cells show a reduction in VDI as compared to HEK293T cells transfected with $Ca_V1.2$ WT (N=5 cells/point). **(E)** Exogenous $Ca_V1.2$ with dihydropyridine mutation is resistant to nifedipine. **(F)** Preserved I_{Ca} during exposure to nifedipine. The single arrow indicates the nifedipine concentration (10nM) used to study the cellular consequences of the TS mutation, and the double arrow indicates the nifedipine concentration (1μM) to overcome dihydropyridine resistance and block the majority of I_{Ca} (N=5-8 cells/point, $P<0.05$ at each nifedipine concentration).

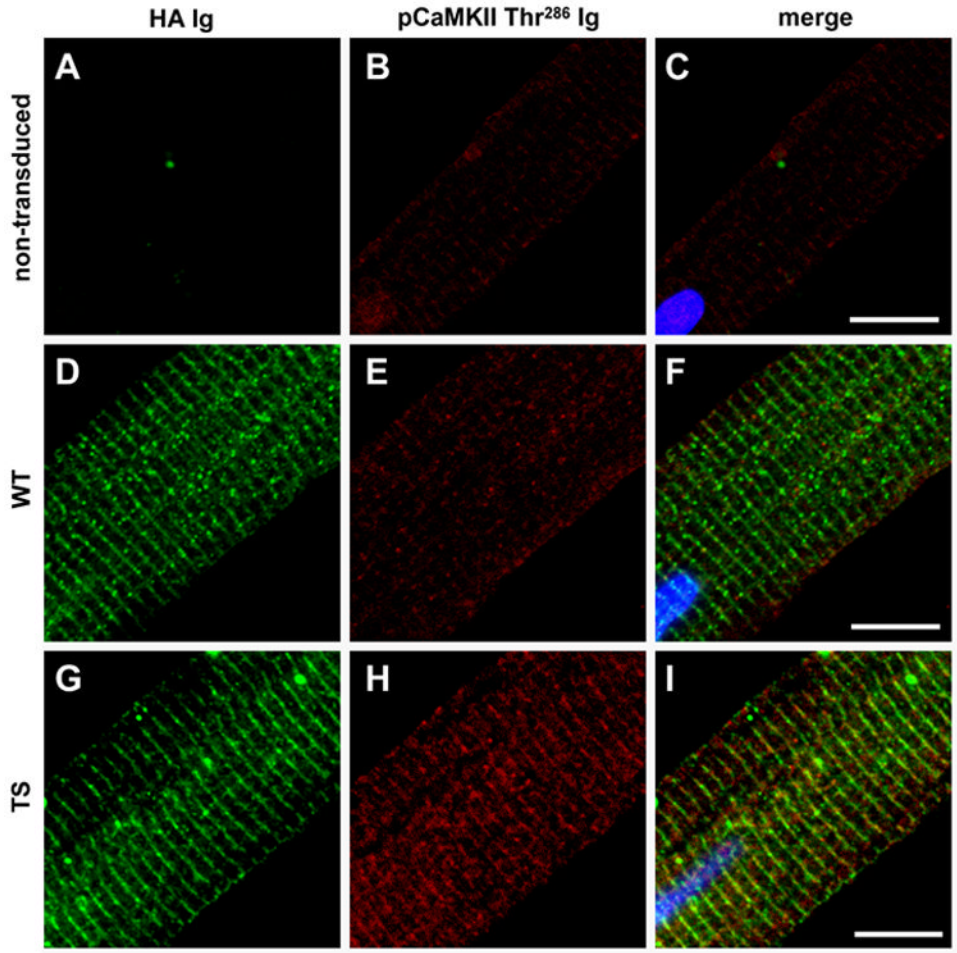

Figure 2.

CaMKII recruitment in the TS adult ventricular myocyte model. **(A-C)** Non-transduced, **(D-F)** WT and **(G-I)** TS adult ventricular myocytes (field stimulated 1Hz for 5 minutes in Tyrodes with 1.8mM $CaCl_2$). **(A,D,G)** Exogenous $Ca_V1.2$ channels are expressed in regularly distributed punctae across ventricular myocytes as shown by HA immunostaining. Both WT and TS $Ca_V1.2$ show spacing consistent with T-tubule network localization. HA immunofluorescence section of $Ca_V1.2$ WT, TS mutation, and uninfected negative control. **(H)** More activated CaMKII (pCaMKII Thr^{286}) immuno-stained with **(I)** TS ventricular myocytes as compared to WT **(E and F)** and non-transduced **(B and C)** ventricular myocytes. (Scale bar, 10μm)

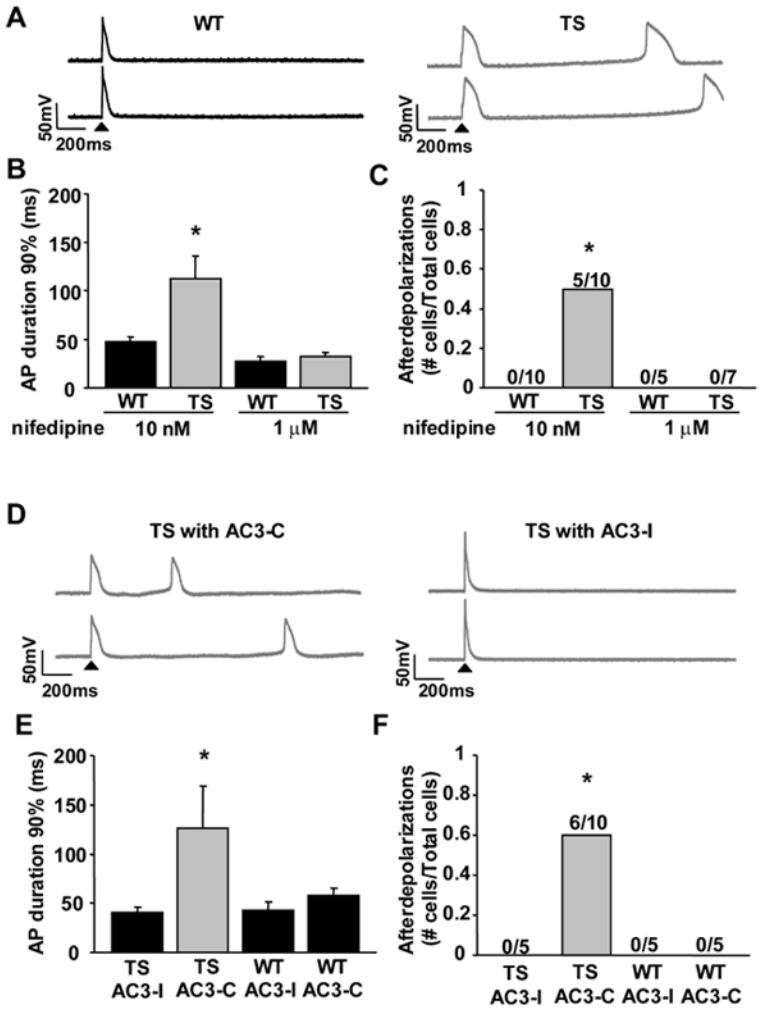

Figure 3.

CaMKII inhibition reverses TS ventricular myocyte action potential (AP) prolongation and afterdepolarizations. **(A)** Action potential recordings from WT and TS ventricular myocytes. The first action potential for each sweep was initiated by injected current (arrow head), but the subsequent action potentials in TS arose from spontaneous afterdepolarizations. **(B)** $Ca_V1.2$ TS results in an increased action potential duration (N=5-10 cells/group, *P=0.018) and **(C)** afterdepolarizations (N=5-10 cells/group, *P=0.033). Numerals indicate the fraction of cells studied with afterdepolarizations. 1μm Nifedipine inhibited a majority of I_{Ca} and so prevented the $Ca_V1.2$ TS increase in **(B)** action potential duration (N=5-7 cells/group, P-0.39) and **(C)** frequency of afterdepolarizations (N=5-7 cells/group, P=1.0). **(D)** Action potential recordings from TS ventricular myocytes with either the CaMKII inhibitory peptide, AC3-I, or a control peptide, AC3-C. **(E and F)** Dialyzing AC3-I restored action potential duration in TS to WT levels and prevented afterdepolarizations (N=5-10 cells/group, TS with AC3-I compared to WT: APD90% P=0.403, afterdepolarizations P=1.0). AC3-I resulted in a non-significant shortening of the WT AP duration (N=5 cells/group, P=0.25) and no significant change in afterdepolarizations (N=5 cells/group, P=1.0) compared to WT with no peptide. **(D-F)** Dialyzing the control peptide, AC3-C, did not alter the TS mutation affects on action potential duration or afterdepolarizations (N=5-10 cells/group, TS with AC3-C compared to TS with AC3-I: APD90% *P=0.017,

afterdepolarizations *P=0.044). AC3-C did not alter the AP duration (N=5 cells/group, P=0.28) or afterdepolarizations (N=5 cells/group, P=1.0) of WT with no peptide.

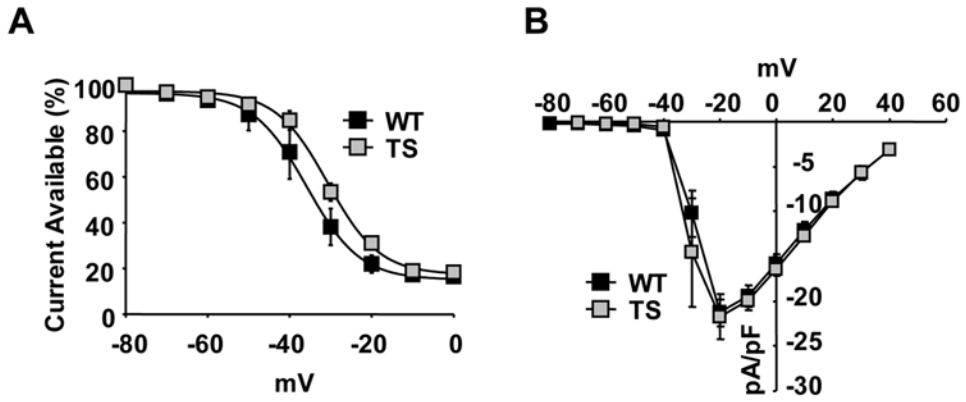

Figure 4.

TS mutation shifts the VDI independent of Ca^{2+} signaling. **(A)** The TS mutation shifts the $Ca_V1.2$ I_{Ba} VDI (N=5 cells/point, *P=0.008), **(B)** without changing the current-voltage (IV) relationship (N=5 cells/group, P=0.88).

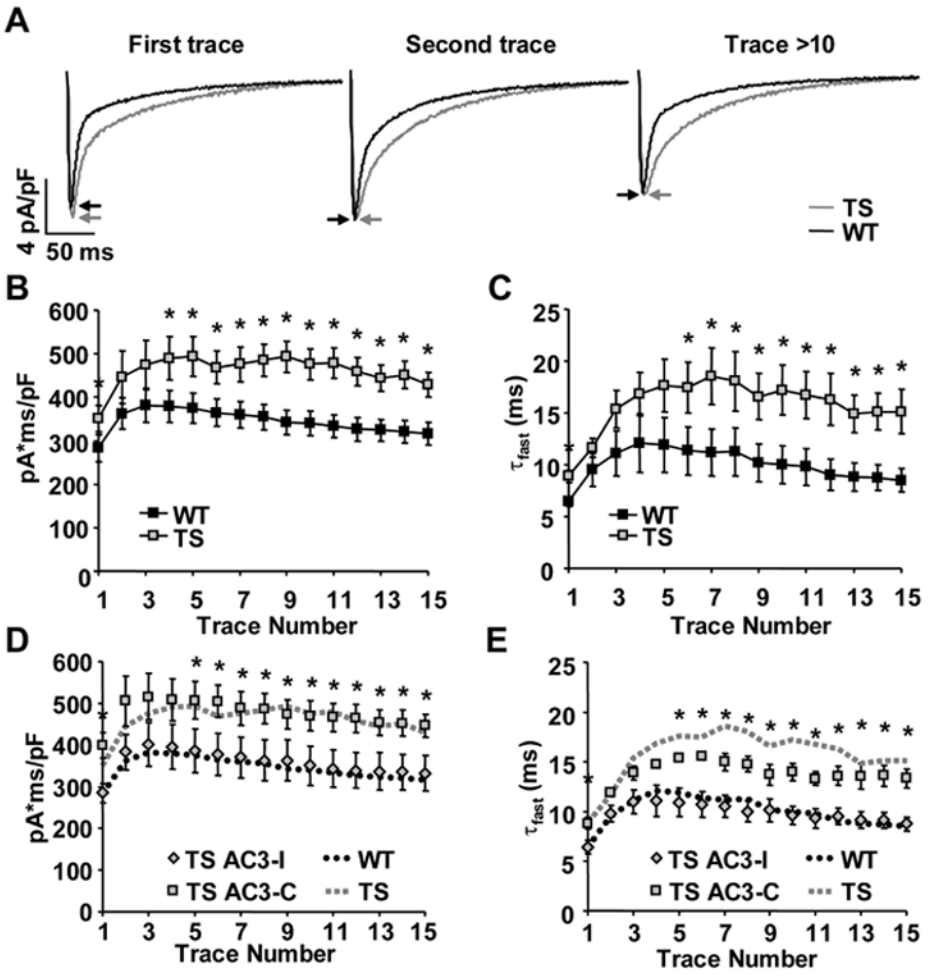

Figure 5.

TS mutation enhances I_{Ca} facilitation. **(A)** TS ventricular myocytes exhibit increased peak I_{Ca} (arrows) during the first depolarizing voltage clamp command step (-80mV to 0mV, 300ms, 0.5Hz) and slowing of inactivation during all depolarizing steps. **(B)** Integrated I_{Ca} evoked by repetitive depolarizing voltage command steps (as in **A** above) is greater in TS mutation than WT (N=6-7 cells/point, ANOVA P<0.001, * P<0.05). **(C)** The time constant of the fast component of I_{Ca} inactivation (τ_{fast}) is significantly slower in TS ventricular myocytes than WT (N=6-7 cells/point, ANOVA P<0.001, * P<0.05). **(D and E)** Integrated I_{Ca} and τ_{fast} were restored to WT levels in TS ventricular myocytes dialyzed with the CaMKII inhibitory peptide, AC3-I (N=5-6 cells/point, TS with AC3-I compared to WT: integrated I_{Ca} ANOVA P=0.522, τ_{fast} ANOVA P=0.294). Dialyzing the control peptide, AC3-C, did not alter the TS mutation affects on I_{Ca} facilitation (N=5 cells/group, TS with AC3-C compared to TS with AC3-I: integrated I_{Ca} ANOVA P<0.001, * P<0.05; τ_{fast} ANOVA P<0.001, * P<0.05).

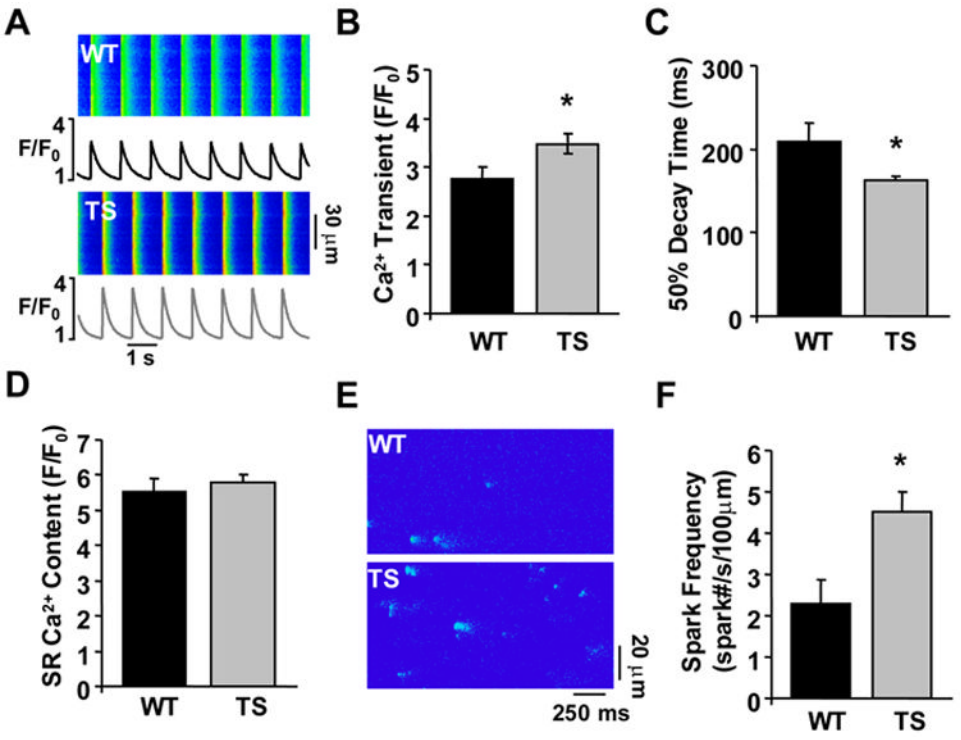

Figure 6.

The TS mutation augments intracellular Ca^{2+} handling. **(A)** Confocal Ca^{2+} transient recordings from WT and TS ventricular myocytes. **(B)** Summary data showing TS mutation causes an increase in the peak Ca^{2+} transient during 1Hz stimulations (N=14-28 cells/group, *P=0.042). **(C)** Summary data showing the 50% decay time of the whole cell Ca^{2+} transients were faster in TS ventricular myocytes (N=14-28 cells/group, *P=0.047). **(D)** No difference was observed in SR Ca^{2+} content between TS and WT ventricular myocytes (N=14-28 cells/group, P=0.524). **(E)** Ca^{2+} sparks recorded from WT and TS ventricular myocytes. **(F)** Summary data showing TS infected ventricular myocytes exhibited an increased frequency of Ca^{2+} sparks during diastole (N=22-37 cells/group, *P=0.001).

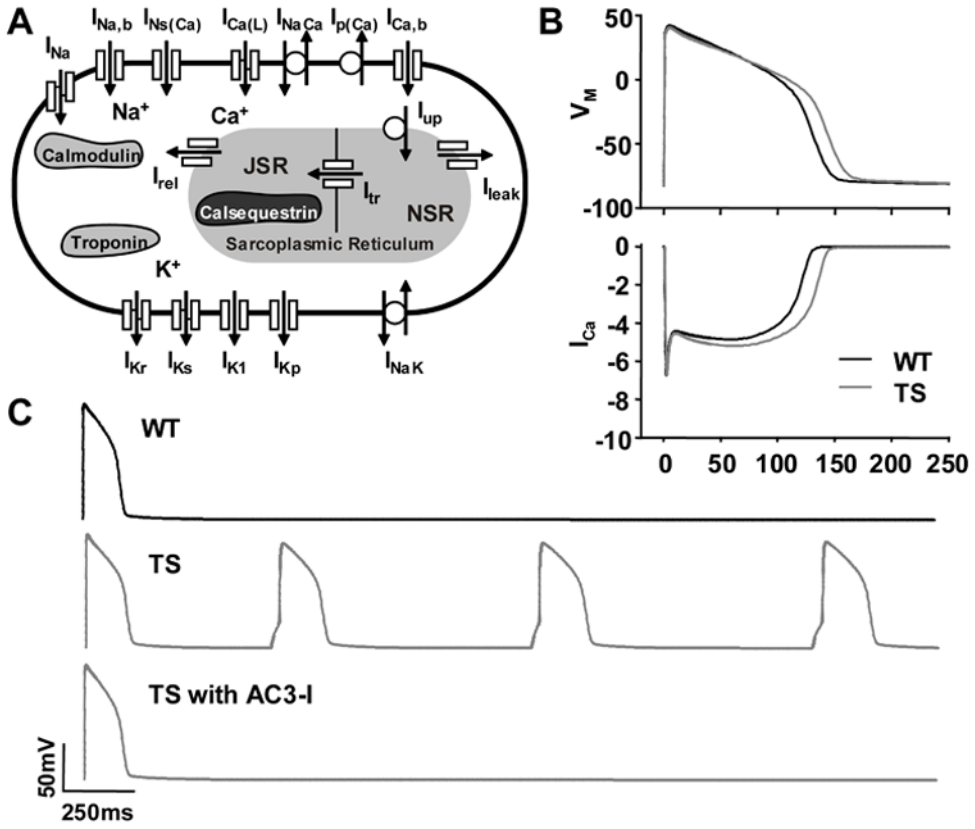

Figure 7.

LRd modeling of WT, TS and TS with AC3-I based upon experimental data from ventricular myocytes. **(A)** Schematic of LRd model. **(B)** LRd model indicates CaMKII activation in TS causes increased I_{Ca} and action potential prolongation (CL = 700ms) and **(C)** afterdepolarizations.