Proarrhythmic Defects in Timothy Syndrome Require Calmodulin Kinase II

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Background—Timothy syndrome (TS) is a disease of excessive cellular Ca\(^{2+}\) entry and life-threatening arrhythmias caused by a mutation in the primary cardiac L-type Ca\(^{2+}\) channel (Ca\(_{\alpha1.2}\)). The TS mutation causes loss of normal voltage-dependent inactivation of Ca\(_{\alpha1.2}\) current (I\(_{\text{Ca}}\)). 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 lentivirus-mediated transfer of wild-type and TS Ca\(_{\alpha1.2}\). The exogenous Ca\(_{\alpha1.2}\) contained a mutation (T1066Y) conferring dihydropyridine resistance, so we could silence endogenous Ca\(_{\alpha1.2}\) with nifedipine and maintain peak I\(_{\text{Ca}}\) at control levels in infected cells. TS Ca\(_{\alpha1.2}\)–infected ventricular myocytes exhibited the signature voltage-dependent inactivation loss under Ca\(^{2+}\) buffering conditions, not permissive for CaMKII activation. In physiological Ca\(^{2+}\) solutions, TS Ca\(_{\alpha1.2}\)–expressing ventricular myocytes exhibited increased CaMKII activity and a proarrhythmic phenotype that included action potential prolongation, increased I\(_{\text{Ca}}\) facilitation, and afterdepolarizations. Intracellular dialysis of a CaMKII inhibitory peptide, but not a control peptide, reversed increases in I\(_{\text{Ca}}\) 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.

Conclusion—In TS, the loss of voltage-dependent inactivation is an upstream initiating event for arrhythmia phenotypes that are ultimately dependent on CaMKII activation. (Circulation. 2008;118:2225-2234.)

Key Words: action potentials ▪ calcium ▪ ion channels ▪ long-QT syndrome ▪ myocytes

Timothy syndrome (TS) is an autosomal-dominant genetic disease of the primary voltage-gated cardiac Ca\(^{2+}\) channel (Ca\(_{\alpha1.2}\)) consisting of a missense mutation in the pore-forming \(\alpha_{1.2}\) subunit protein. TS is associated with 3 individual mutations: G406R on exon 8a, G406R on exon 8, and G402S. The G406R mutation on exon 8 with 3 individual mutations: G406R on exon 8a, G406R on exon 8, and G402S. The G406R mutation on exon 8 used for our model of TS is believed to be the most severe form of TS. TS patients have an average life expectancy of only 2.5 years because of severe cardiac disease. TS also is known as long-QT syndrome 8, 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 due, at least in part, to impaired voltage-dependent inactivation (VDI) of Ca\(_{\alpha1.2}\) current (I\(_{\text{Ca}}\)). Mathematical modeling predicts that intracellular Ca\(^{2+}\) overload and action potential prolongation stimulate afterdepolarizations, which are the cellular mechanism for triggering ventricular arrhythmias in TS. However, these predictions have not been directly tested in ventricular myocytes.

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Ca_{1.2} \alpha_{1C} subunit harboring the TS mutation. Our studies show that TS mutation requires CaMKII activity to cause important proarhythmic phenotypes in adult ventricular myocytes.

**Methods**

**Cloning**

The plasmids pLentiNB CaV1.2 DHP\textsuperscript{a} hemaglutanin wild-type (WT) TS and pLentiNB CaV1.2 DHP\textsuperscript{a} hemaglutanin TS are described in the Methods section of the online-only Data Supplement.

**Lentivirus**

Lentivirus was prepared from the manufacturer’s (Invitrogen, Carlsbad, Calif) protocol and as described in the online-only Data Supplement Methods.

**Ventricular Myocyte Isolation, Culturing, and Viral Transduction**

Adult male Sprague-Dawley rat (250 to 300 g) ventricular myocytes were isolated as previously published\textsuperscript{8} and cultured as described in the online-only Data Supplement Methods. Procedures were in accordance with the Institutional Animal Care and Use Committee of the University of Iowa. Lentivirus was added to cells at a multiplicity of infection of 1 to 3, and cultures were maintained for 24 to 36 hours.

**Electrophysiology**

Electrophysiology for HEK293 cells and myocytes, including pipette solutions, bath solutions, voltage clamp protocols, current clamp protocols, and data analysis, is detailed in the online-only Data Supplement Methods.

**Immunofluorescence**

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

**Calcium Imaging**

Ca\textsuperscript{2+} transients, SR content, and sparks were acquired by confocal laser scanning from myocytes loaded with Fluo-3. The online-only Data Supplement Methods contain details on cell preparation and confocal Ca\textsuperscript{2+} imaging.

**Mathematical Modeling**

Mathematical models of the WT and TS myocytes are based on the Luo-Rudy dynamic (LRd) model of the mammalian ventricular action potential.\textsuperscript{9,10} See the online-only Data Supplement Methods for equations that differ from the published model.

**Statistics**

Data are presented as means with SEM. Sigma Stat was used to compare 2 groups with Student t test and multiple groups with ANOVA. Significance was set at a value of \( P<0.05 \). Categorical data between 2 groups were compared by use of 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\textsubscript{1.2} by adding an extracellular hemaglutanin epitope\textsuperscript{11} (Figure 1A, green circle) and introduced a validated dihydropyridine-insensitivity mutation\textsuperscript{7} (Figure 1A, black circle). The dihydropyridine-insensitivity mutation allows the virally introduced Ca\textsubscript{1.2} to remain functional while using nifedipine to inhibit endogenous Ca\textsubscript{1.2}.\textsuperscript{7} Exogenous Ca\textsubscript{1.2} expression was confirmed by immunoblot (Figure 1B) and immunofluorescence (Figure 1C) in transduced HEK293T cells. The functions of Ca\textsubscript{1.2} 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.\textsuperscript{1,2,12}

Overexpression of Ca\textsubscript{1.2} in ventricular myocytes yielded a 33.7% increase in peak \( I_{Ca} \) (Figure 1F) and an average 31.9% increase in total Ca\textsubscript{1.2} protein (online-only Data Supplement Figure I). Because of the dihydropyridine-resistance mutation,\textsuperscript{7} peak \( I_{Ca} \) in Ca\textsubscript{1.2}-infected ventricular myocytes was significantly resistant to nifedipine compared with uninfected cells (Figure 1E and 1F). In TS and WT infected ventricular myocytes, 10 nmol/L 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 noninfected myocytes recorded without nifedipine (Figure 1E and 1F). This nifedipine-engineered balance of endogenous and exogenous Ca\textsubscript{1.2} allowed us to determine the effects of the TS mutation on cardiac electrophysiology independently of overexpression-induced changes in peak \( I_{Ca} \).

**TS Ventricular Myocytes Exhibit Increased CaMKII Autophosphorylation**

We confirmed expression of exogenous Ca\textsubscript{1.2} in cultured adult ventricular myocytes by immunostaining for the hemaglutanin epitope (Figure 2D and 2G). Virally introduced Ca\textsubscript{1.2} was properly targeted to the transverse-tubule (T-tubule) network on the basis of the punctate appearance and 1.8-µm spacing of the hemaglutanin immunofluorescence that is consistent with known distances between T tubules in a resting sarcomere.\textsuperscript{13} No hemaglutanin immunostaining was detected in uninfected ventricular myocytes (Figure 2A).

We immunostained for the CaMKII autophosphorylation site, Thr 286, which is a marker of CaMKII activation.\textsuperscript{14} TS ventricular myocytes (Figure 2H and 2I) exhibited greater levels of CaMKII autophosphorylation compared with both WT (Figure 2E and 2F) and uninfected (Figure 2B and 2C) ventricular myocytes. Total CaMKII immunostaining revealed no changes in CaMKII protein levels between WT, TS, and uninfected ventricular myocytes (online-only Data Supplement Figure II). These data show that activated CaMKII is recruited in TS Ca\textsubscript{1.2}–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 (arrowhead in Figure 3A) were recorded in nifedipine-treated (10 nmol/L) WT and TS...
ventricular myocytes. Compared with WT, the TS mutation significantly prolonged the action potential duration (Figure 3A and 3B) as determined by the time to 90% repolarization. Excessive action potential prolongation favors the generation of afterdepolarizations.15 We observed afterdepolarizations from TS ventricular myocytes (5 of 10 cells; Figure 3A and 3C), whereas none were observed in any of the WT cells (0 of 10 cells; Figure 3A and 3C). Most afterdepolarizations were delayed afterdepolarizations, but early afterdepolarizations also were recorded from TS ventricular myocytes. Delayed afterdepolarizations are favored by increased diastolic Ca\(^{2+}\) leak from the SR;16,17 early afterdepolarizations are caused by increased I\(_{\text{Ca}}\) 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 1 \(\mu\)mol/L nifedipine were reduced to equivalent times, and neither WT nor TS ventricular myocytes exhibited afterdepolarizations under these conditions (Figure 3B and 3C). The 1\(\mu\)mol/L nifedipine bath solution overcomes the dihydropyridine resistance mutation and inhibited the total peak I\(_{\text{Ca}}\) by >50% (Figure 1F, double arrows). These findings indicate that the observed TS phenotypes were initiated by increased I\(_{\text{Ca}}\).

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 and 3E). The inactive control peptide, AC3-C,4,19 had no effect, suggesting that CaMKII-dependent increases in I\(_{\text{Ca}}\) contributed to action potential prolongation in TS. The CaMKII inhibitory peptide also eliminated afterdepolarizations in TS ventricular myocytes (P=1.0; Figure 3D and 3F), whereas AC3-C did not (P=0.04; Figure 3D and 3F). 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 nonsignificant (P=0.28) shortening of the action potential duration (Figure 3E) compared with 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 (online-only Data Supplement Table I). Action potential parameters from WT ventric-
ular myocytes in the presence of 10 nmol/L nifedipine were similar to uninfected ventricular myocytes cultured for the same time period (24 to 36 hours) and recorded without nifedipine (online-only Data Supplement Table I). These controls suggest that viral expression of CaV1.2 does not alter the action potential when peak ICa is adjusted to normal levels (by 10 nmol/L 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 on increased Ca2+ entry through CaV1.2. 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 Independently of CaMKII Activity

Expression of TS CaV1.2 in Xenopus oocytes1,2 and heterologous cells1,2,12 (Figure 1D) showed a loss of CaV1.2 VDI. The Xenopus oocyte experiments1,2 included Ca2+ independent conditions that would not favor CaMKII activation because Ba2+ replaced Ca2+ 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 ICa from TS and WT ventricular myocytes (10 nmol/L nifedipine) using Ba2+ (1.8 mmol/L) as the charge carrier and under high intracellular Ca2+ buffering (20 mmol/L BAPTA). TS ventricular myocytes exhibited a loss of VDI as a significant (P=0.008; Figure 4A) rightward shift compared with WT. The TS V1/2 (−30.75 mV) shifted to more positive potentials compared with WT V1/2 (−35.89 mV). In contrast, the peak ICa elicited by the conditioning pulses showed no difference between WT and TS (Figure 4B), confirming equivalent expression of exogenous WT and TS CaV1.2. No differences were observed in peak ICa or VDI recorded from adult ventricular myocytes expressing WT dihydropyridine-resistant CaV1.2 with 10 nmol/L nifedipine compared with uninfected adult ventricular myocytes without nifedipine (online-only Data Supplement Table II). These findings show that TS causes a loss of CaV1.2 VDI in ventricular myocytes, establishing the initial requirement for increased cellular Ca2+ entry necessary to recruit CaMKII.

CaMKII Is Required for TS Effects on ICa

to test the importance of CaMKII for ICa changes other than VDI in our TS model, we measured CaMKII-dependent ICa facilitation.18,20 ICa facilitation consists of dynamic increases in peak ICa and slowing of inactivation with repetitive depolarizations.21,22 TS ventricular myocytes exhibited maximal peak ICa during the first depolarization, whereas WT attained peak ICa after the initial depolarization (Figure 5A and online-only Data Supplement Figure III). Subsequent depolarizations showed no difference in peak ICa between TS and WT (Figure 5A and online-only Data Supplement Figure III). To measure the effects of ICa facilitation on cellular Ca2+ entry, we integrated total ICa during the voltage clamp command step. Integrated ICa was significantly greater in TS compared with WT during all depolarization steps (first step, P=0.029; remaining steps, P<0.001; Figure 5B). We found

Figure 2. CaMKII recruitment in the TS adult ventricular myocyte model. Nontransduced (A through C), WT (D through F), and TS (G through I) adult ventricular myocytes (field stimulated at 1 Hz for 5 minutes in Tyrode solution with 1.8 mmol/L CaCl2). A, D, and G, Exogenous CaV1.2 channels are expressed in regularly distributed punctate across ventricular myocytes as shown by hemaglutinin (HA) immunostaining. Both WT CaV1.2 and TS CaV1.2 show spacing consistent with T-tubule network localization. HA immunofluorescence section of CaV1.2 WT, TS mutation, and uninfected negative control. H, More activated CaMKII (pCaMKII Thr286) immunostained with TS ventricular myocytes (I) compared with WT (E and F) and nontransduced (B and C) ventricular myocytes. Scale bar=10 μm.
that the fast component of $I_{Ca}$ inactivation ($\tau_{\text{fast}}$) was slower in TS compared with 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 $\tau_{\text{fast}}$ in TS to levels recorded from WT cells (integrated $I_{Ca}$, $P=0.522$; $\tau_{\text{fast}}$, $P=0.294$; Figure 5E and 5F). In contrast, dialysis of AC3-C had no effect on $\tau_{\text{fast}}$ or integrated $I_{Ca}$. Dialysis of the CaMKII inhibitory peptide prevented $I_{Ca}$ facilitation in WT ventricular myocytes (online-only Data Supplement Figure III), 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. We recorded
Ca\(^{2+}\) transients (Figure 6A) from WT and TS ventricular myocytes loaded with Fluo-3 AM and field stimulated at 1 Hz.\(^2\) TS caused a significant increase in the peak Ca\(^{2+}\) transient compared with WT (P=0.04; Figure 6B), which is consistent with computer models.\(^1,2,23\) Interestingly, the 50% decay time for Ca\(^{2+}\) transients in TS was significantly shortened compared with WT (P=0.02; Figure 6C). A faster decay time implicates increased SR/endoplasmic reticulum calcium ATPase activity,\(^25\) which was not predicted by modeling studies but is associated with CaMKII signal-

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**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 (−80 to 0 mV, 300 ms, 0.5 Hz) and slowing of inactivation during all depolarizing steps. *, P \(< 0.05\), ANOVA). B, Integrated \(I_{Ca}\) evoked by repetitive depolarizing voltage command steps (as in A) is greater in TS mutation than WT (n=6 to 7 cells per point; *P \(< 0.001\), P \(< 0.05\), ANOVA). C, \(\tau_{fast}\) is significantly slower in TS ventricular myocytes than WT (n=6 to 7 cells per point; *P \(< 0.001\), **P \(< 0.05\), ANOVA). D and E, Integrated \(I_{Ca}\) and \(\tau_{fast}\) were restored to WT levels in TS ventricular myocytes dialyzed with the CaMKII inhibitory peptide, AC3-I (n=5 to 6 cells per point; TS with AC3-I vs WT: integrated \(I_{Ca}\), *P \(< 0.001\), \(\tau_{fast}\), P = 0.522 \([\text{ANOVA}]\); **P \(< 0.001\), ANOVA). Dialyzing the control peptide, AC3-C, did not alter the TS mutation effects on \(I_{Ca}\) facilitation (n=5 cells per group; TS with AC3-C vs TS with AC3-I: integrated \(I_{Ca}\), *P \(< 0.001\), \(\tau_{fast}\); P \(< 0.001\), *P \(< 0.05\) \([\text{ANOVA}]\), **P \(< 0.05\) \([\text{ANOVA}]\).
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 as a result of enhanced IC\(a\) from TS Ca\(V_{1.2}\).

Surprisingly, we found that TS SR Ca\(^{2+}\) content was not different from WT (\(P = 0.55\); Figure 6D). We considered that increased SR Ca\(^{2+}\) leak in TS balances faster SR Ca\(^{2+}\) uptake, thereby preventing a net increase in SR Ca\(^{2+}\) content compared with WT. Increased SR Ca\(^{2+}\) leak is implicated in CaMKII signaling and in triggering delayed afterdepolarizations, a prominent feature of the TS ventricular myocytes (Figure 3A and 3C). We assessed diastolic SR Ca\(^{2+}\) leak by measuring spontaneous Ca\(^{2+}\) sparks from TS and WT ventricular myocytes (Figure 6E). The SR Ca\(^{2+}\) sparks were significantly increased in TS compared with WT. Increased SR Ca\(^{2+}\) leak is implicated in CaMKII signaling and in triggering delayed afterdepolarizations, and in triggering delayed afterdepolarizations, a prominent feature of the TS ventricular myocytes (Figure 3A and 3C).

Revised TS Mathematical Modeling

Several studies have modeled the impact of TS on myocardial electrophysiology by using a shift in Ca\(V_{1.2}\) VDI estimated from measurements in nonmyocytes. 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 LRd model because of its established utility in studying cardiac arrhythmia mechanisms.

Our model of TS incorporated 3 modifications to match our experimental observations. First, we shifted the Ca\(V_{1.2}\) 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\(V_{1.2}\) IC\(a\) facilitation associ-
ated with TS by slowing $I_{Ca}$ inactivation to increase integrated $I_{Ca}$ as measured experimentally (online-only Data Supplement Figure IV). 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 with WT (online-only Data Supplement Figure IV). 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 CaV1.2 VDI that 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 (long-QT syndrome 8) resulting from a genetic mutation in the CaV1.2 pore-forming α subunit. Compared with cardiac Na$^{+}$ and K$^{+}$ channels, CaV1.2 has proved to be remarkably resistant to genetic disease. One key difference between Cr$^{2+}$, Na$^{+}$, and K$^{+}$ is the prominent role that Ca$^{2+}$ plays as a second messenger. TS patients have not only extremely profound QT interval prolongation but also structural cardiac abnormalities that 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, but this defect in TS VDI was ascertained in heterologous (nonmyocardial) cells in which 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 of the loss of VDI.

Despite the relatively modest reduction in CaV1.2 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 insofar 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. Our results showed that CaMKII amplifies Ca$^{2+}$ entry through CaV1.2 in TS by slowing $r_{fast}$ and shifting the $V_{1/2}$ of $I_{Ca}$ inactivation. Our studies do not exclude the possibility that CaMKII inhibition also could affect other depolarizing or repolarizing currents such as Na$^{+}$ current or K$^{+}$ current. Our finding that SR Ca$^{2+}$ leak is increased in TS is consistent with other reports that show that proarrhythmic actions of CaMKII are due to increasing SR Ca$^{2+}$ leak, thereby enabling a transient inward current ($I_{Ca}$) that triggers delayed afterdepolarizations. 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 CaV1.2 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 on the basis of single-channel recordings from heterologous expression of TS CaV1.2 in baby hamster kidney 6 cells. These experiments found that TS CaV1.2 was more likely than WT to exhibit frequent long openings, so-called mode 2 gating, that are the single-channel mechanisms underlying CaMKII-mediated $I_{Ca}$ facilitation. 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, which may cause generation of early afterdepolarizations.

Although major Ca$^{2+}$ homeostatic proteins are conserved in ventricular myocytes across mammalian species, differences exist between species as to the quantitative contribution of these components to the action potential. Thus, one goal of future studies should be to determine whether 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 into arrhythmia mechanisms in TS by illustrating how a concise defect in CaV1.2 gating can initiate downstream recruitment of CaMKII that ultimately enables the electrophysiological cellular disease phenotype in TS. The central nervous system defects of TS patients also may be due to secondary recruitment of Ca$^{2+}$-activated signaling molecules, including CaMKII. Overexpression of CaMKII is known to interfere with neuronal growth and differentiation, and a constitutively active CaMKII within the mouse brain causes significantly impaired spatial memory. CaMKII recruitment in TS ventricular myocytes also suggests the possibility that other disease phenotypes in TS patients (eg, 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.

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Disclosures
None.

References


Timothy syndrome (TS) is a genetic disorder causing excessive cellular Ca\(^{2+}\) entry resulting from defective voltage-dependent inactivation of the predominant myocardial L-type Ca\(^{2+}\) channel (Ca\(_{\text{v}1.2}\)) current (I\(_{\text{Ca}}\)). TS patients die on average at 2.5 years of age as a result of malignant cardiac arrhythmias. TS is a “model” disease whereby a concise biophysical defect in I\(_{\text{Ca}}\) activates a cellular signaling cascade that is required for the cardiac disease phenotypes. Our studies showed that loss of voltage-dependent inactivation 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 nonexcitable, heterologous cells. Our studies show that the TS voltage-dependent inactivation defect activated CaMKII and that 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 TS 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 in which ion channels frequently constitute a final common pathway require careful consideration of the connections between ion channel gating and signaling cascades to more comprehensively understand the underlying mechanisms.
Supplemental Methods

Cloning

The open reading frame of CaV1.2 α1c subunit (NCBI X15539) was amplified by PCR and ligated into a modified pLenti6 plasmid (Invitrogen), pLentiNB, which had the blasticidin resistance gene and promoters of the pLenti6 plasmid removed to facilitate viral packaging. An extracellular hemagglutinin epitope was added to CaV1.2 by methods previously published1. The dihydropyridine resistance mutation (DHPR, T1066Y) and TS mutation (G406R) were introduced by using the PCR method Quikchange (Stratagene) as per manufacturer’s protocol.

HEK293 transfection

HEK293T cells were transfected with the pLentiNB CaV1.2 WT or TS with a pIRES eGFP β2a subunit using Fugene6 (Roche) as described by the manufacturer. For electrophysiology experiments, transfected HEK293T cells were detected by expression of eGFP and confirmed by inward I_{Ca}. For immunofluorescence, transfected HEK293T cells were fixed with 2% PFA and stained as described under immunofluorescence methods.

Lenti virus

The transgene plasmid pLentiNB carrying the modified CaV1.2 was transfected (Qiagen, Effectene) with the Lenti viral packaging plasmids (Invitrogen’s pLP1, pLP2 and pVSVG) into HEK293FT cells (Invitrogen). Media was collected and replaced at 24, 48 and 72 hours post-transfection. The viral containing media was concentrated by either ultrafiltration (Millipore Centricon Plus-70 30kDa) or
ultacentrifugation. Viral titer (transducing units per mL, TU/mL) was determined by serial dilution ($10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$, no virus) on HEK293 cells followed by immuno-staining (see Immunoflourescence methods) for the CaV1.2 HA epitope (anti-HA conjugated Alexa 488 Ig) and counting positively stained cells within each dilution. Viral titers achieved were between $10^5$ and $10^6$ TU/mL. Extracts from HEK293 cells used to produce virus were analyzed by SDS-PAGE and immuno-blotting with an affinity-purified HA Ig.

**Ventricular myocyte isolation, culturing and viral transduction**

Adult male Sprague-Dawley rats (250-300g) were anesthetized by Avertin (2.5%) with Heparin (55 units/mL) through IP injection (0.2mL/10g). Hearts were excised, perfused retro-aortically (Langendorff) and enzymatically digested with a mixture of Collagenase (Worthington, 250 units/mL), Hyaluronidase (Sigma, 0.01%) and Protease Type XIV (Sigma, 0.0025%) in a modified tyrodes solution (0.1mM CaCl$_2$, 10mM BDM). Dissociated cardiomyocytes were washed three times in Joklik MEM (Sigma M0518) with 1% Pen/Strep and 1X ITS (Sigma) with increasing Ca$_2^+$ (0.25mM, 0.5mM, 0.75mM). Ventricular myocytes were plated on glass cover slips (glass #1) coated with Geltrex (Invitrogen, thin layer) and allowed to attach for 1 hour. Cells were washed with a culture media consisting of a 50:50 mix of DMEM and F10 media with 1% Pen/Strep and 1X ITS. Attached cardiomyocytes were counted and the cell density was calculated. Lenti virus was added to the cells at a multiplicity of infection (MOI) of 1-3, and cells cultures were maintained for 24-36 hours. Cultured ventricular myocytes (WT, TS,
uninfected) extracts were analyzed by SDS-PAGE and immuno-blotting with a CaV1.2 Ig (ABR).

**Electrophysiology**

HEK293 I$_{\text{Ca}}$ recordings for voltage dependence of inactivation (VDI) used a two step voltage clamp protocol (repeated 0.1 Hz, resting -80mV, 25°C) with an initial conditioning step (0.8s, -50mV to +60mV, Δ10mV) followed by a test pulse (300ms, +30mV). Bath solution was in mM; 130 NMDG, 10 HEPES, 5 KCl, 15 CaCl$_2$. Pipette solution was in mM; 120 Cs methanesulfonate, 5 CaCl$_2$, 1 MgCl$_2$, 2 MgATP, 10 HEPES, 10 EGTA. Available current observed each test pulse after a given conditioning pulse was accessed a percent of the maximum current observed.

Cardiomyocyte action potentials (AP) were stimulated (2ms, 1.5-2.5nA) in current clamp mode (0.5Hz, 25°C). Bath solution was in mM; 140 NaCl, 4 HEPES, 10 Glucose, 5.4 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$. Pipette solution was in mM; 120 K aspartate, 5 HEPES, 25 KCl, 4 Na$_2$ATP, 1 MgCl$_2$, 10 EGTA, 2 Na$_2$ phosphocreatine, 1 CaCl$_2$, 2 NaGTP. Recorded APs were analyzed using ClampFit’s (Axon Instruments) event detection algorithm and statistics decay time (ms) algorithm.

Cardiomyocyte I$_{\text{Ba}}$ recordings for VDI used a two step voltage clamp protocol (repeated 0.1 Hz, resting -80mV, 25°C) with an initial conditioning pulse (2.0s, -80mV to +30mV, Δ10mV) followed by a test pulse (300ms, 0mV). To record only VDI and prevent Ca$^{2+}$ dependent inactivation, Ca$^{2+}$ was tightly buffered through the use of Ba$^{2+}$ as the charge carrier in the bath solution and
BAPTA with no Ca\textsuperscript{2+} in the pipette solution. Bath solution was in mM; 137 NMDG, 10 HEPES, 10 Glucose, 1.8 BaCl\textsubscript{2}, 0.5 MgCl\textsubscript{2}, 25 CsCl. Pipette solution was in mM; 120 CsCl, 10 TEA, 1 MgATP, 1 NaGTP, 5 phosphocreatine, 10 HEPES, 20 BAPTA. Available current observed each test pulse after a given conditioning pulse was accessed a percent of the maximum current observed.

Cardiomyocyte I\textsubscript{Ca} facilitation was recorded using a single step (300ms, 0mV) voltage clamp protocol (repeated 0.5Hz, resting -80mV, 25°C). Bath solution was in mM; 137 NMDG, 10 HEPES, 10 Glucose, 1.8 CaCl\textsubscript{2}, 0.5 MgCl\textsubscript{2}, 25 CsCl. Pipette solution was in mM; 120 CsCl, 3 CaCl\textsubscript{2}, 10 TEA, 1 MgATP, 1 NaGTP, 5 phosphocreatine, 10 HEPES, 10 EGTA. I\textsubscript{Ca} facilitation was integrated using ClampFit’s area statistics (pA*ms) algorithm and normalized to cell size (pF). Inactivation time constants were calculated using ClampFit.

Immunofluorescence

HEK293 cells, cultured on coverslips (glass #1), were gently washed with PBS and fixed for 20 minutes in 2% paraformaldehyde (25°C). Cultured adult ventricular myocytes (WT, TS and uninfected) were paced by field stimulation (Ion Optix C-pace and C-dish, 1Hz, 35V, 2ms) for 5 minutes in Tyrodes (1.8mM CaCl\textsubscript{2}, 37°C). Immediately following the pacing protocol, ventricular myocytes were fixed for 20 minutes in 2% paraformaldehyde (25°C). Fixed cells were permeabilized for 10 minutes with PBS with 0.1% Triton X-100, 2 mg/mL BSA and 2% fish gelatin. Permeabilized cells were blocked with PBS with 2 mg/mL BSA and 2% fish gelatin. Cells were incubated overnight (4°C) in one of the following; anti-HA conjugated Alexa 488 Ig (Molecular Probes), HA Ig (Santa
Cruz), pCaMKII Thr286 Ig (ABR), CaMKII Ig (Bers Lab) and washed. The cells incubated with HA Ig were then incubated in donkey anti-rabbit Alexa 488 Ig (Molecular Probes) at 4°C. Cells incubated with pCamKII Thr286 Ig were then incubated in donkey anti-mouse 568 (Molecular Probes). Cells incubated with CaMKII Ig were then incubated in donkey anti-rabbit 568 (Molecular Probes). Ventricular myocytes were mounted with glass coverslips and Vectashield (with or without DAPI; Vector Laboratories).

Ventricular myocyte images were collected on a Zeiss 510 Meta confocal microscope (Carl Zeiss), under 40x magnification (oil, 1.30 NA lens), with a pinhole of 1.0 airy disc (Carl Zeiss), using the Zeiss image acquisition software. HEK293 images were taken at 40x magnification using both the FITC filter and DAPI filter. All images were exported to Photoshop (Adobe) for cropping and linear adjustment of contrast.

**Calcium imaging**

Myocytes were loaded with Fluo-3 AM (5μM, 20 min.) at room temperature. After de-esterification, the cells were perfused with normal Tyrode solution (1.8 mM Ca^{2+}). Confocal Ca^{2+} imaging was performed with a laser scanning confocal microscope (LSM 510 Meta, Carl Zeiss) equipped with a NA=1.35, 63x lens. Line scan measurement of Ca^{2+} transients, SR content and sparks were acquired at a sampling rate of 1.93 ms/line along the longitudinal axis of the myocytes. Sparks were measured under resting conditions. Steady state Ca^{2+} transients were achieved by a 30 sec pacing at 1 Hz. SR Ca^{2+} content
was measured as a global Ca\textsuperscript{2+} release induced by 10mM caffeine. All digital images were processed with IDL 6.0 (Research System Inc).

**Mathematical modeling**

Mathematical models of the WT and TS myocytes are based on the Luo-Rudy dynamic model of the mammalian ventricular action potential\textsuperscript{2,3}. For this study, we incorporated a revised formulation for Ca\textsuperscript{2+} release from the sarcoplasmic reticulum and regulation by CaMKII based on the model of Livshitz and Rudy\textsuperscript{4}. Our model of SR Ca\textsuperscript{2+} release includes a formulation for spontaneous Ca\textsuperscript{2+} release from the sarcoplasmic reticulum, which occurs when the amount of Ca\textsuperscript{2+} bound to calsequestrin reaches threshold, as described in the original LRd model. Cells were paced to steady-state (over 15 min. pacing) at a cycle length of 700 ms using a conservative current stimulus\textsuperscript{5}. Afterdepolarization events were monitored during a pause following steady-state pacing. Ordinary differential equations in the model were integrated numerically using the Forward Euler Method and an adaptive time step. Details on the mathematics involved in the model can be found in the Supplemental Equations.
Reference List


Supplemental Tables

**Supplemental Table 1: Action potential data**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>TS</th>
<th>Uninfected</th>
<th>WT:TS</th>
<th>WT:Un</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nifedipine (nM)</strong></td>
<td>10</td>
<td>10</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Number of Cells (n)</strong></td>
<td>10</td>
<td>10</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>APD90% (ms)</strong></td>
<td>±8.02</td>
<td>±23.47</td>
<td>±12.54</td>
<td>0.018</td>
<td>0.17</td>
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<tr>
<td><strong>Afterdepolarizations (#/Total)</strong></td>
<td>0/10</td>
<td>5/10</td>
<td>0/11</td>
<td>0.033</td>
<td>1</td>
</tr>
<tr>
<td><strong>Resting Potential (mV)</strong></td>
<td>-62.60</td>
<td>-60.85</td>
<td>-67.10 ±1.02</td>
<td>0.497</td>
<td>0.966</td>
</tr>
<tr>
<td><strong>Peak Amplitude (mV)</strong></td>
<td>101.77</td>
<td>99.88</td>
<td>113.08</td>
<td>0.799</td>
<td>0.103</td>
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</table>
Supplemental Table 2: Voltage dependence of inactivation data

<table>
<thead>
<tr>
<th></th>
<th>VT</th>
<th>TS</th>
<th>Uninfected</th>
<th>WT:TS</th>
<th>WT:Un</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nifedipine (nM)</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of Cells (n)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VDI $V_{1/2}$ (mV)</td>
<td>-35.89 ±0.64</td>
<td>-30.75 ±0.76</td>
<td>-37.06 ±0.31</td>
<td><strong>0.008</strong></td>
<td>0.507</td>
<td></td>
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<tr>
<td>Peak (pA/pF)</td>
<td>21.30 ±1.45</td>
<td>21.71 ±2.48</td>
<td>19.40 ±3.56</td>
<td>0.880</td>
<td>0.583</td>
<td></td>
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</table>
Supplemental Table 3: Intracellular Ca\textsuperscript{2+} handling data

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>TS</th>
<th>Uninfected</th>
<th>WT:TS</th>
<th>WT:Un</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of Cells (n)</strong></td>
<td>14</td>
<td>20</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Calcium Transient (F/F\textsubscript{0})</strong></td>
<td>2.76 ± 0.26</td>
<td>3.48 ± 0.21</td>
<td>2.84 ± 0.18</td>
<td>0.042</td>
<td>0.792</td>
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<tr>
<td><strong>50% Decay Time (ms)</strong></td>
<td>208.47 ± 23.21</td>
<td>161.97 ± 6.09</td>
<td>193.61 ± 7.50</td>
<td>0.047</td>
<td>0.475</td>
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<tr>
<td><strong>SR calcium content (F/F\textsubscript{0})</strong></td>
<td>5.52 ± 0.38</td>
<td>5.78 ± 0.24</td>
<td>5.64 ± 0.22</td>
<td>0.524</td>
<td>0.705</td>
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<tr>
<td><strong>Spark Frequency</strong> (Sparks/ms/100\textmu m)</td>
<td>2.28 ± 0.58</td>
<td>4.52 ± 0.50</td>
<td>2.50 ± 0.51</td>
<td>0.001</td>
<td>0.354</td>
</tr>
<tr>
<td><strong>Spark Intensity (F/F\textsubscript{0})</strong></td>
<td>1.47 ± 0.03</td>
<td>1.75 ± 0.06</td>
<td>1.62 ± 0.05</td>
<td>0.002</td>
<td>0.138</td>
</tr>
<tr>
<td><strong>Spark Duration (FDHM)</strong></td>
<td>2.27 ± 0.06</td>
<td>2.22 ± 0.07</td>
<td>2.28 ± 0.08</td>
<td>0.121</td>
<td>0.056</td>
</tr>
<tr>
<td><strong>Spark Width (FWHM)</strong></td>
<td>45.02 ± 3.13</td>
<td>53.56 ± 3.80</td>
<td>44.81 ± 8.79</td>
<td>0.457</td>
<td>0.474</td>
</tr>
</tbody>
</table>
Supplemental Equations

**Revised formulation for \( I_{rel} \)**

\[
I_{rel} = O \cdot \left[ [Ca^{2+}]_{JSR} - [Ca^{2+}]_{SS} \right];
\]

Where

\[
\frac{dO}{dt} = -\frac{O}{\tau_{rel}} + O; \\
\]

and

\[
O_\infty = \frac{\alpha_{rel} \cdot I_{Ca(L)}}{1 + (K_{rel} \cdot [Ca^{2+}]_{JSR})^\nu \cdot \theta};
\]

All other parameters and equations for \( I_{rel} \) are same as in original model published by Livshitz and Rudy.

**Ca\(^{2+}\) release of JSR under Ca\(^{2+}\)-overload conditions.**

If buffered [csqn] > [csqn]\(_{th}\)

\[
O = O_\infty = 6.0; \\
\tau_{rel} = 10.0;
\]

Where [csqn]\(_{th}\) = 7.0 as in original Luo-Rudy dynamic cell model.

**Nonspecific Ca\(^{2+}\)-activated current**

The nonspecific Ca\(^{2+}\)-activated current was used according to the formulation in the original Luo-Rudy cell model with a reduced permeability.

\[
P_{N_s(Ca)} = 1.0 \times 10^{-7} \text{ cm/s};
\]

**Mathematical model of TS action potential.**

**L-type Ca\(^{2+}\) current**
To simulate the effects of the Timothy Syndrome mutation on $I_{\text{Ca}(L)}$ channel gating, the following equation was used for steady-state voltage dependent inactivation:

$$f_{\infty} = \frac{1}{1 + \exp\left(\frac{V_m + 28.86}{8.0}\right)} + \frac{0.6}{1 + \exp\left(\frac{50.0 - V_m}{20.0}\right)};$$

To simulate the CaMKII-dependent effects on $I_{\text{Ca}(L)}$ facilitation, the following equation was used for the time constant of voltage-dependent inactivation:

$$\tau_f = \frac{1.35}{0.0197 \cdot \exp\left(-0.0337 \cdot (V_m + 10.0)^2\right) + 0.02};$$

**Ca$^{2+}$ leak from the SR**

Increased leak from the SR due to CaMKII was simulated by increasing the conductance of $I_{\text{leak}}$.

$$I_{\text{leak}} = \frac{1.23 \cdot I_{\text{up}}}{\text{NSR}} \cdot [\text{Ca}^{2+}]_{\text{NSR}};$$

**SR Ca$^{2+}$ release**

$$\beta_\tau = 7.125 \text{ ms};$$

$$K_{\text{rel},c} = 0.65 \text{ mM};$$

$$[\text{csqn}]_{\text{th}} = 6.2 \text{ mM};$$
Supplemental Figure Legends

**Supplemental Figure 1:** (A) Immuno-blot for total CaV1.2 protein and from ventricular myocytes infected with CaV1.2 WT (lane 1), CaV1.2 TS (lane 3) or uninfected (lane 2) as a control. The average increase in WT and TS CaV1.2 protein relative to uninfected was 31.9% (WT=36.4%, TS=27.4%) after correcting for total protein loading observed in the (B) Coomassie stained lanes.

**Supplemental Figure 2:** Adult ventricular myocytes (A,B,C) non-transduced, infected with (D,E,F) WT CaV1.2 virus or infected with (G,H,I) TS CaV1.2 virus (scale bar 10μm). TS and WT infected ventricular myocytes show overexpressed CaV1.2 (D,G) by HA immuno-staining, but no changes in total CaMKII protein (E,H) as compared to non-transduced ventricular myocytes (B).

**Supplemental Figure 3:** TS ventricular myocyte I_{Ca} facilitation (A) TS ventricular myocytes show increased peak I_{Ca} during the first depolarizing voltage clamp command step (-80mV to 0mV, 300ms, 0.5Hz) over WT ventricular myocytes (N=6-7 cells/point, P=0.02). With the second depolarizing step WT ventricular myocytes match the peak I_{Ca} observed with TS ventricular myocytes (N=6-7 cells/point, P=0.46). The CaMKII inhibitory peptide, AC3-I, restores normal I_{Ca} facilitation to TS ventricular myocytes (N=5-6, P vs. WT=0.469), but not the control peptide AC3-C (N=5-6 cells/point, P vs. WT=0.038). (B) WT cardiomyocytes dialyzed with the CaMKII inhibitory peptide, AC3-I, loose the dynamic increase of integrated I_{Ca} and (C) the dynamic change of the fast time
constant ($\tau_{\text{fast}}$) that are associated with facilitation (N=5 cells/point; integrated $I_{\text{Ca}}$
WT AC3-C vs. WT AC3-I ANOVA $P<0.001$; $\tau_{\text{fast}}$ WT AC3-C vs. WT AC3-I
ANOVA $P<0.001$). (C and D) The control peptide, AC3-C, has no effect on $I_{\text{Ca}}$
facilitation (N=5 cells/point; integrated $I_{\text{Ca}}$ WT AC3-C vs. WT ANOVA $P=0.675$;
$\tau_{\text{fast}}$ WT AC3-C vs. WT ANOVA $P=0.85$).

**Supplemental Figure 4:** Mathematical model of WT (black) and TS (red)
myocytes. (A) Measured (left) and simulated (right) $I_{\text{Ca(L)}}$ steady-state voltage-
dependent inactivation curves. (B) Simulated $I_{\text{Ca(L)}}$ current traces during a voltage
pulse to 0 mV from a holding potential of -80 mV (left). Measured and simulated
current integrals (right) are determined during the pulse duration (300 ms). In
simulations and experiments, Ca$^{2+}$ was buffered with 10 mM EGTA. (C)
Measured and simulated Ca$^{2+}$ transient amplitude (left) and SR Ca$^{2+}$ content
(right) after steady-state pacing.