Triadin Overexpression Stimulates Excitation-Contraction Coupling and Increases Predisposition to Cellular Arrhythmia in Cardiac Myocytes

Dmitry Terentyev, Steven E. Cala, Timothy D. Houle, Serge Viatchenko-Karpinski, Inna Gyorke, Radmila Terentyeva, Simon C. Williams, Sandor Gyorke

Abstract—Triadin 1 (TRD) is an integral membrane protein that associates with the ryanodine receptor (RyR2), calsequestrin (CASQ2) and junctin to form a macromolecular Ca signaling complex in the cardiac junctional sarcoplasmic reticulum (SR). To define the functional role of TRD, we examined the effects of adenoviral-mediated overexpression of the wild-type protein (TRDWT) or a TRD mutant lacking the putative CASQ2 interaction domain residues 200 to 224 (TRDDel.200–224) on intracellular Ca signaling in adult rat ventricular myocytes. Overexpression of TRDWT reduced the amplitude of ICaL induced Ca transients (at 0 mV) but voltage dependency of the Ca transients was markedly widened and flattened such that even small ICaL at low- and high depolarizations triggered maximal Ca transients. The frequency of spontaneous Ca sparks was significantly increased in TRDWT myocytes, whereas the amplitude of individual sparks was reduced. Consistent with these changes in Ca release signals, SR Ca content was decreased in TRDWT myocytes. Periodic electrical stimulation of TRDWT myocytes resulted in irregular, spontaneous Ca transients and arrhythmic oscillations of the membrane potential. Expression of TRDDel.200–224 failed to produce any of the effects of the wild-type protein. The lipid bilayer technique was used to record the activity of single RyR2 channels using micropore samples obtained from control, TRDWT, and TRDDel.200–224 myocytes. Elevation of TRDWT levels increased the open probability of RyR2 channels, whereas expression of the mutant protein did not affect RyR2 activity. We conclude that TRD enhances cardiac excitation-contraction coupling by directly stimulating the RyR2. Interaction of TRD with RyR2 may involve amino acids 200 to 224 in C-terminal domain of TRD. (Circ Res. 2005;96:000-000.)

Key Words: triadin ▪ calsequestrin ▪ ryanodine receptor ▪ calcium-induced calcium release ▪ catecholaminergic polymorphic ventricular tachycardia.

The process of excitation-contraction (EC) coupling in cardiac muscle relies on inward Ca currents through the dihydropyridine receptor Ca channels that trigger Ca-induced Ca release (CICR) from the sarcoplasmic reticulum.1,2 After rapid activation, the release of SR Ca is efficiently terminated. Termination of release appears to involve luminal Ca-dependent deactivation, a mechanism in which a decline in free [Ca] inside the SR causes the RyR2 channels to close.3,4 Despite significant recent progress in the elucidation of the functional aspects of EC coupling, the precise molecular mechanisms that regulate this process remain poorly understood.5 Apart from RyR2, the EC coupling machinery is composed of a number of other proteins including the low-affinity Ca binding protein, calsequestrin (CASQ2), and the integral membrane proteins triadin 1 and junctin. Recently, mutations in protein components of this multimolecular Ca signaling complex, including RyR26–7 and CASQ2,8,9 have been linked to a form of inherited effort-induced arrhythmia termed catecholaminergic ventricular tachycardia (CPVT). However, the specific cellular and molecular mechanisms whereby genetic defects in these proteins lead to arrhythmia are only beginning to emerge.

Overexpression of CASQ2, Triadin 1 (TRD), or junctin can result in cardiac hypertrophy10–12 and failure13 in transgenic animal models. However, when induced by other factors, these disease states are characterized with unchanged CASQ2 and TRD abundance,1,14 although junctin levels have been reported to decline in a model of heart failure induced by overexpression of the β1-adrenergic receptor in mouse.15

Triadin 1 and junctin are transmembrane proteins in the junctional SR that appear to bind directly to both the RyR2 and CASQ2, thereby anchoring CASQ2 to the RyR2 channel.16–18 Three isoforms of triadin (35, 40, and 92 kDa) have been described of which triadin 1 (40 kDa) is predominant.
form in cardiac myocytes, representing more than 95% of the total amount of triadin in these cells.17,19 Junctin was identified as a 26-kDa CASQ2 binding protein in cardiac and skeletal muscle.16 The domain structures of TRD and junctin are similar, being composed of a single membrane-spanning domain, a short cytoplasmic N-terminal segment and a long, highly positively charged C-terminal domain extending into the lumen of the SR. The luminal domains of both proteins are characterized by frequent occurrence of long stretches of alternating positively and negatively charged residues known as KEKE motifs.16,18 These regions, in general denoted as protein-protein binding motifs,20 are thought to be involved in interactions between TRD and junctin themselves and with RyR2 and CASQ2.21 The CASQ2-binding domain of TRD has been localized to a single KEKE motif composed of 25 residues (200 to 224)18 and the same region has been predicted from in vitro studies to mediate interaction of triadin with ryanodine receptors.22 The functional relevance of these interactions inside living cardiomyocytes, however, is yet to be defined.

The functions of TRD and junctin have been studied using transgenic animal models,11,12,23 Although yielding important results, the interpretation of these data in terms of the physiological roles of these proteins is complicated by various adaptive and pathological changes, such as the hypertrophy and heart failure that accompanied chronic TRD overexpression.11 Elucidation of the precise role of TRD in myocardial intracellular Ca handling in more acute settings is yet to be defined.

Materials and Methods

Electrophysiological Recordings

Ventricular myocytes were obtained from adult male Sprague-Dawley rat hearts by enzymatic dissociation.4 Whole-cell patch-clamp recordings of transmembrane ionic currents were performed with an Axopatch 200B amplifier (Axon Instruments). The myocytes were stimulated by application of 400-ms–long voltage pulses to specified membrane potentials from a holding potential of −50 mV to 50 mV at 1-s intervals. The external solution contained (in mM/L): 140 NaCl, 5.4 KCl, 1.0 CaCl2, 0.5 MgCl2, 10 HEPES, and 5.6 glucose; pH 7.3. Patch pipettes (tip resistance of 1 to 3 MΩ) were filled with a solution that contained (in mM/L): 90 Cs-aspartate, 50 CsCl, 3 NaATP, 3.5 MgCl2, 10 HEPES, and 0.05 Fluo-3 K-salt; pH 7.3.3 In some experiments, myocytes were electrically stimulated and their membrane potential measured in the current-clamp configuration. In these experiments, Cs+ in the pipette solution was replaced with K+.4

Confocal Ca Measurements

Intracellular Ca imaging was performed using a Bio-Rad Laser Scanning Confocal System (Bio-Rad MRC-1024ES) interfaced to an Olympus IX-70 inverted microscope and equipped with an Olympus 60×1.4 N.A. oil objective) as described previously.3 Fluo-3 was excited by the 488-nm line of an argon-ion laser and the fluorescence was acquired at wavelengths >515 nm in the line-scan mode of the confocal system at the rate of 2 ms per scan. Ca spark parameters were quantified with a detection/analysis computer algorithm.3

Single RyR Channel Recordings

Heavy SR microsomes were isolated from rat myocytes infected with the adenoviral constructs. Myocytes were homogenized in Kraft-Brune (KB) solution containing (in mM/L) 110 potassium glutamate, 10 KH2PO4, 25 KCl, 2 MgSO4, 20 tauroine, 5 creatine, 0.5 EGTA, 20 glucose, and 5 HEPES (pH 7.4) at 20 000 rpm for 3 minutes, followed by differential centrifugation. Single RyRs were reconstituted by fusing SR microsomes into planar lipid bilayers and single channel currents were recorded as described previously.24 Channel incorporation was performed in solutions containing (in mM/L) 350 CsCH3SO3, 0.02 CaCl2, 20 HEPES (pH 7.4), on the cytosolic (cis) side of the bilayer, and 20 CsCH3SO3, 0.02 CaCl2, 20 HEPES (pH 7.4) on the luminal (trans) side of the bilayer. Single channel currents were recorded at room temperature (21 to 23°C) with an Axopatch 200A (Axon Instruments) patch-clamp amplifier. Data were digitized at 5 to 10 kHz and filtered at 2 kHz. Acquisition and analysis of data were performed using pClamp 6.01 software (Axon Instruments).

Adenoviral Gene Transfer

Isolated myocytes were suspended in serum-free medium 199 containing 25 mM/L NaHCO3, 5 mM/L creatine, 5 mM/L taurine, 10 U/mL penicillin, 10 µg/mL streptomycin, and 10 µg/mL gentamycin (pH 7.3), and plated at a density of 105 cells/cm2 on laminin-coated glass coverslips. Cells were kept at 37°C in a humidified atmosphere with 95% air/5% CO2. After 2 hours, unattached cells were removed and myocytes were infected with adenoviruses at multiplicity of infection (MOI) 10. After 3 hours, media was changed and myocytes were cultured for 48 hours before analysis. Efficiency of infection, which constituted virtually 100%, was determined using an adenoviral construct for expression of GFP (Ad-GFP) at the same MOI.4

Construction of Recombinant Adenoviruses

Canine cardiac triadin 1 (Clone 4c5z5) was kindly provided by Drs Larry R. Jones and Yvonne M. Kobayashi, Indiana University, Bloomington, Ind, and subcloned into pBluescript (Stratagene). The TRD deletion mutant was amplified from wild-type construct using PCR primers encoding sequence on either side of residues 200 to 224, the sequence to be deleted. The resulting 3760-bp product was self-ligated to produce TRDΔ200–224, TRDΔ and TRDΔ200–224 were sequenced in pBluescript and both fragments were released from pBluescript with Kpn1 and Xba1 and ligated into pShuttle-CMV. The pShuttle-CMV/TRDV224 and pShuttle-CMV/ TRDΔ200–224 plasmids were transformed to BJ183-Ad competent cells (Stratagene) per manufacturer specifications, and recombinant clones identified by PacI digestion.

Western Blotting

The levels of WT and mutant dog TRD as well as rat TRD, CASQ2, junctin, phospholamban (PLB), SR (ER) Ca-ATPase (SERCA2a), Na+–Ca exchanger (NCX), and RyR2 proteins were determined by immunoblot analysis. Cell lysates proteins (10 µg) were subjected to SDS 4% to 20% PAGE, blotted onto nitrocellulose membranes (Bio-Rad Labs), and probed with antibodies specific for these proteins. Anti-CASQ2, -SERCA2a, and -RyR2 antibodies were from Affinity Bioreagents; anti-PLB antibody was from Upstate Biotechnology and anti-NX3 from Chemicon Int. Rabbit antisera was raised to canine cardiac triadin-1 peptide (257KGGHSE-EVAGGSKR) and canine junctin (195SKHTHSAKGNNQKRKN) coupled to keyhole limpet hemocyanin via an N-terminal Cys and affinity purified as previously described.25 Anti-rat TRD antibody was a generous gift from Dr M. Periasamy. Blots were developed with Super Signal West Pico (PIERCE) and quantified using a Visage 2000 Blot Scanning and Analysis system (BioImage Systems Corporation).

Statistical Analysis

Data are presented as mean±SEM from individual experiments. Comparisons were performed by using unpaired Student t test or one-way ANOVA test, when appropriate, and significance was defined at P<0.05.
Results

Adenoviral-Mediated Expression of WT and Mutant TRD

To overexpress triadin 1 in rat ventricular myocytes, we constructed an adenoviral vector carrying the full coding sequence of canine cardiac triadin 1 (Ad-TRDWT). In vitro binding studies identified a specific region of TRD (amino acids 200 to 224) that incorporates a single KEKE motif that appears to mediate interactions between TRD and other components of the SR junction. To further evaluate the role of this specific region in TRD function, we also constructed an adenoviral vector encoding a form of TRD (Ad-TRDDel.200–224) that lacks residues 200 to 224. Control myocytes were infected with a viral construct carrying truncated segment of the CASQ2 coding region (Ad-Control).4 Ectopic expression of both TRDWT and Ad-TRDDel.200–224 was confirmed by Western blot analysis using an antibody specific for dog TRD. Assuming both types of antibodies interact with comparable strength with their respective targets (online Figure in the online data supplement available at http://circres.ahajournals.org), dog TRD was present in amounts ~3-fold higher than the native protein in myocytes infected with Ad-TRDWT and Ad-TRDDel.200–224. Importantly, ectopic expression of either form of TRD did not result in changes in the steady state levels of other major Ca handling proteins including SERCA2, PLB, NCX, junctin, and RyR2.

I_Ca and Intracellular Ca Transients

We initially determined the effects of overexpression of TRDWT and Ad-TRDDel.200–224 proteins on intracellular Ca handling in rat ventricular myocytes. L-type Ca currents and intracellular Ca transients were simultaneously measured in cardiac myocytes depolarized to various membrane potentials in cardiomyocytes infected with Ad-TRDWT, Ad-TRDDel.200–224, and Ad-Control vectors. Voltage protocols are illustrated at the top. B and C, Voltage dependencies of Ca transients (B) and I_Ca (C) in myocytes infected with Ad-Control (black), Ad-TRDWT (red), and Ad-TRDDel.200–224 (blue) vectors. Data are mean±SE from 5 to 8 experiments performed in myocytes from nine heart preparations. Comparisons were performed by using one way ANOVA. *Significance was defined at P<0.05.
characteristic of normal cardiac EC coupling (Figure 2B). Small and very large depolarization steps that elicited small \( I_{Ca} \) triggered small Ca transients. The amplitude of the transients was highest near 0 mV were \( I_{Ca} \) amplitude also is at its maximum. In TRD\textsuperscript{WT} myocytes, the average amplitude of the maximal \( I_{Ca} \)-triggered Ca transients near 0 mV was reduced with respect to control. However, Ca transient amplitudes in these myocytes were significantly increased compared with control at small and high depolarizing pulses (ie, at \(-30 \) and \(+60 \) mV, respectively), resulting in a flattened voltage-dependence curve in Ad-TRD\textsuperscript{WT} myocytes. It is also worth mentioning that for voltage-clamp steps to highly positive membrane potentials Ca transients in TRD-overexpressing myocytes showed a pronounced sustained component, which was likely due to enhanced Na\textsuperscript{+}-Ca exchange-mediated Ca influx at these membrane potentials. All these changes in Ca transients occurred without any alterations in the peak amplitude of \( I_{Ca} \), the trigger signal for SR Ca release, at different membrane potentials (Figure 2C). Notably, \( I_{Ca} \) in TRD-overexpressing myocytes exhibited an accelerated Ca-dependent inactivation at low membrane potentials (eg, \(-30 \) mV) apparently owing to the larger Ca release at these membrane potentials (online Table). In myocytes expressing TRD\textsuperscript{Del.200–224}, the properties of both Ca transients and \( I_{Ca} \) were similar to control. We observed similar effects of TRD overexpression on voltage dependency of SR Ca release when myocytes were dialyzed with solutions containing K\textsuperscript{+} instead of Cs\textsuperscript{+} (data not shown). These results seem to indicate that (1) TRD enhances the functional activity of RyR2s, so that even a small Ca trigger signal during depolarizations to low and high membrane potentials leads to maximal regenerative CICR; and that (2) the functional effects of TRD may involve residues 200 to 224 in its C-terminal tail.

**SR Ca Content**

We hypothesized that the reduced amplitude of Ca transients at 0 mV in TRD\textsuperscript{WT} myocytes could be due to TRD-dependent increases in RyR2 activity that lead to a reduction in SR Ca content. To test this hypothesis, we used caffeine applications to assess the SR Ca content in myocytes expressing TRD\textsuperscript{WT} and TRD\textsuperscript{Del.200–224}. The relative amount of Ca released from the SR to the cytosol in response to caffeine was determined from both Fluo-3 fluorescence and Na\textsuperscript{+}-Ca exchange current (I\textsubscript{NCX}) (Figure 3). The amplitude of Ca transients was reduced to \( \approx 70\% \), and I\textsubscript{NCX} integral decreased to \( \approx 60\% \) of control in TRD\textsuperscript{WT} myocytes, indicating a substantial reduction in the SR Ca content in these cells. In contrast, expression of TRD\textsuperscript{Del.200–224} did not affect the SR Ca load, consistent with the results of the \( I_{Ca} \) and Ca transient measurements.

**Ca Sparks**

To further evaluate the effects of TRD\textsuperscript{WT} and TRD\textsuperscript{Del.200–224} on the properties of SR Ca release, we measured spontaneous Ca sparks in myocytes permeabilized with saponin. In these experiments, [Ca] was buffered at 100 nmol/L. As shown in Figure 4, increased TRD\textsuperscript{WT} abundance resulted in a small but significant reduction of Ca spark amplitude. At the same time, spark frequency increased by \( \approx 60\% \) in myocytes overexpressing TRD\textsuperscript{WT}. Expression of TRD\textsuperscript{Del.200–224} did not affect Ca spark amplitude and frequency. No significant differences were detected in the duration of the rising phase and overall duration of Ca sparks among the three different cell types. Again caffeine applications (10 mmol/L) were used to assess the SR Ca content in permeabilized myocytes (not shown). Consistent with results obtained in patch-clamped myocytes, the amplitude (F/F\textsubscript{0}) of caffeine-induced Ca transients was reduced significantly in TRD overexpressing myocytes and remained unchanged in TRD\textsuperscript{Del.200–224} expressing myocytes with respect to control after their permeabilization (3.37\( \pm \)0.15, 2.62\( \pm \)0.25, and 3.25\( \pm \)0.18 in myocytes infected with Ad-Control, Ad-TRD\textsuperscript{WT}, and Ad-TRD\textsuperscript{Del.200–224} vectors, respectively, n from 5 to 6, n of heart preparation 4). Thus, the observed effects of TRD\textsuperscript{WT} on cell-averaged Ca \( I_{Ca} \), and caffeine-induced Ca transients can be ascribed to increased functional activity of individual Ca release sites, ie, RyR2 clusters.

**Single RyR2 Activity**

We next used the planar lipid bilayer technique to study directly the effects of TRD\textsuperscript{WT} and TRD\textsuperscript{Del.200–224} on the activity of single RyR2 channels.\textsuperscript{24} Heavy SR microsomes were prepared from myocytes infected with Ad-TRD\textsuperscript{WT}, Ad-TRD\textsuperscript{Del.200–224}, and Ad-Control viral constructs. Microsomes were fused into lipid bilayers and single RyR2 channel currents were measured using Cs\textsuperscript{+} as the charge carrier at a cytosolic (cis) [Ca] of 5 \( \mu \)mol/L in the presence of physiological concentrations of ATP and Mg\textsuperscript{2+}.
The open probability \((P_o)\) of RyR2s was approximately 3-fold higher for channels isolated from TRDWT myocytes compared with control cells (Figure 5). On the other hand, the \(P_o\) of RyR2s obtained from myocytes expressing TRDDel.200–224 was similar to control. We conclude that TRD enhances the functional activity of the RyR2 channel through a direct interaction that might depend on residues 200 to 224 in the luminal tail of TRD.

**TRD Overexpression Induces Cellular Arrhythmia**

It is known that generation of delayed afterdepolarizations (DADs) involves Ca-dependent inward Ca currents and that these deflections of the membrane potential can trigger arrhythmias.\(^{26–29}\) We recently showed that exposure to isoproterenol (ISO) caused disturbances in periodic Ca transients and arrhythmic DADs in cardiac myocytes underexpressing CASQ2 or expressing a CASQ2 mutant specifically linked to cholinergically-induced tachycardia.\(^{4,30}\) We hypothesized that overexpression of TRDWT might lead to the same pathological phenotype by disrupting normal molecular interactions within the junctional Ca signaling complex. To determine whether increased TRDWT abundance enhanced the predisposition of myocytes to cellular arrhythmia, we subjected TRD-overexpressing or control myocytes to periodic electrical stimulation in the presence of ISO. As shown in Figure 6, TRDWT myocytes exhibited altered Ca transients and DADs characteristic of myocytes overexpressing a CPVT-associated CASQ2 mutant protein.\(^{30}\) Similar results were obtained in five other experiments. Neither control nor TRDDel.200–224 myocytes exhibited such a behavior under identical conditions. These results indicate that TRD is an important determinant of the functional activity of the Ca release channels in cardiac myocytes and that altered TRD levels result in abnormal intracellular Ca cycling.

**Discussion**

In the present study, we investigated the effects of acute overexpression of the SR junctional protein TRD on intracellular Ca handling in cardiac myocytes. Using a comprehensive approach that involved measurements of both global and local Ca transients and recordings from single RyR2 channels, we provide evidence that TRD enhances cardiac EC coupling by stimulating the activity of the RyR2 Ca release channel. Importantly, TRDWT-overexpressing myocytes ex-
hibrated characteristic disturbances in intracellular [Ca] oscillations and membrane potential similar to those described previously in myocytes expressing a CPVT-linked CASQ2 mutant. These effects of TRD required a complete primary structure that included the region of the protein encompassing amino acids 200 to 224 in its luminal tail, as expression of a mutant protein missing this domain failed to produce any of the effects observed with its WT counterpart. This study is the first to demonstrate a direct role for TRD in EC coupling using acute overexpression of this protein in cardiomyocytes.

TRD Function
In general, two possibilities have been discussed in the literature concerning TRD function in cardiac muscle. One possibility is that TRD plays merely an anchoring role by concentrating CASQ2 near the junctional face of the SR. In this scaffolding capacity, TRD is presumed to facilitate SR Ca release indirectly by permitting CASQ2 to buffer Ca in the vicinity of the release sites. The other possibility is that TRD regulates directly the activity of the RyR2 Ca release channel. In particular, we recently showed that reassociation of purified RyR2 with TRD and junctin increases channel open probability in lipid bilayers. Interestingly, these effects of TRD and junctin could be reversed by CASQ2 in a Ca-dependent fashion. Specifically, CASQ2 inhibited the RyR2/TRD/Junctin complex at low and intermediate luminal Ca (≤5 mmol/L) but not at high Ca (>5 mmol/L). These results were interpreted to suggest that a dual regulation of RyR2s by TRD (or junctin) and CASQ2 accounts for the ability of RyR2s to sense and respond to changes in the SR luminal Ca. The present study in a physiologically relevant experimental cell system represents a significant expansion of our previous in vitro data. Our data clearly demonstrate that TRD stimulates RyR2 channel activity both in vitro and inside life myocytes. Moreover, coexpression of CASQ2 with TRD reduced the stimulatory effects of TRD on SR Ca release consistent with the hypothesis of dual regulation of RyR2 function by TRD and CASQ2 (not shown). Thus, we conclude that TRD modulates RyR2 function directly and this molecular control mechanism may involve other proteins including CASQ2.

TRD-RyR2 Stoichiometry
Although little or no data exists on this subject to our knowledge, for the cardiac release complex, standard models based on analysis of in vitro binding interactions typically assume that both TRD and junctin interact with RyR2 in a 1:1:1 stoichiometry. It is assumed that these complexes are dynamic structures, and so are subject to standard mass action considerations. That is, if higher concentrations of TRD are present, the amount of TRD bound to RyR2 at any time reflect the effective concentration of TRD available for binding to RyR. Such a model might also permit TRD binding to sites normally occupied by junctin, because similar KEKE motifs are predicted to account for junctin binding. The data reported from our study in general are consistent with these views and present a new and important paradigm for constructing a dynamic model of the multiprotein junctional complex in living myocytes.

Structural Basis of TRD Interaction With Functional SR Components
By expressing a TRD deletion mutant lacking residues 200 to 224, we found that the functional effects of TRD in cardiac myocytes depended on the presence of these residues in the luminal tail of the protein. Previously, this stretch of 25 amino acids, containing a single KEKE motive, was identified as the CASQ2 binding domain of TRD. A segment encompassing the same sequence has been implicated in binding of TRD to a highly conserved region of the skeletal isoform of RyR. The lack of functional effect of TRDDel.200–224 is consistent with the importance of this domain for protein-protein interactions of TRD with other elements of the junctional SR. Potential alterations in folding or processing of the mutant protein, however, may also contribute to this result. Studies with additional deletion and substitution TRD mutants are under way to systematically address the specific amino acids involved in situ interaction of TRD with RyR2 and CASQ2.

Relevance to CPVT
An important finding of the present study is that increased expression of TRD led to extrasystolic Ca transients and arrhythmic DADs specifically in the presence of ISO, thus replicating the effects of overexpressing a CPVT-linked CASQ2 mutant protein in cardiac myocytes. Two genetic forms of CPVT have been described, one that is linked to mutations in RyR2 and a second that is caused by mutations in CASQ2. Given their similar clinical manifestations as well the fact that RyR2 and CASQ2 are components of the same macromolecular signaling complex, it is possible that the pathogenic causes of these two forms of CPVT converge to the same underlying mechanisms. Although it is clear that arrhythmias in both cases are triggered by spontaneous Ca release from the SR inducing DADs, the specific mechanisms whereby the genetic defects in these proteins cause abnormal SR Ca release remains a matter of debate. It has been reported that CPVT-linked RyR2 mutations disrupt the
interaction of RyR2 with the auxiliary protein FKBP12.6 thereby sensitizing the RyR2 channel to cytosolic Ca$^{2+}$. Consequently, it has been proposed that in CPVT an increased sensitivity of RyR2s to cytosolic Ca enhances the predisposition of cardiac muscle to spontaneous Ca release. However, other investigators were unable to detect differences in FKBP12.6 binding affinity between normal and mutant RyR2s. Alternatively, it has been shown that arrhythmic spontaneous Ca release occurs as a result of abnormal modulation of RyR2s by luminal Ca, due to either altered dynamics of free luminal Ca or abnormal responsiveness of the channel to luminal Ca in myocytes expressing a CPVT mutant. Recently, a similar mechanism was proposed to rationalize the increased predisposition to spontaneous Ca release in HEK cells expressing CPVT-associated RyR2 mutant proteins. Because TRD appears to be an integral part of the luminal Ca sensor our results provide strong support for the hypothesis that altered regulation of the RyR2 channel complex by luminal Ca is a common mechanism for the different genetic forms of CPVT linked to RyR2 and CASQ2. Further support of this hypothesis comes from recent findings that a CPVT-associated CASQ2 mutant protein (D307H) does not undergo Ca$^{2+}$-dependent conformational changes seen with the wild-type CASQ2 protein and that the inability to undergo these conformational changes is associated with the loss of TRD and junctin binding.

Conclusions

Our results reveal a functional role for TRD in cardiac EC coupling. We find that TRD acts to increase RyR2 channel activity both in vitro and in situ, demonstrating a consistent set of effects in enhancing EC coupling efficacy in cardiac myocytes. Overexpression of TRD also exerted a clear destabilizing influence on CICR, leading to arrhythmic oscillations in Ca transients and DADs similar to those described previously in myocytes expressing CPVT-related CASQ2 mutants. These results provide new insights into the molecular basis of cardiac Ca signaling and important clues regarding the pathogenesis of cardiac arrhythmia associated with genetic defects within components of the EC coupling machinery. An important clinical implication of our findings is that they predict that certain forms of CPVT may be associated with mutations in the TRD and junctin genes that result in altered abundance or functional activity of these proteins. Careful examination of these genes in CPVT patients should address this possibility.

Acknowledgments

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Table 1  Effects of overexpression of TRDWT or TRDDel.200-224 on parameters of I_Ca in rat ventricular myocytes. Data are means ± SE from 4-8 experiments. * P<0.01 vs. Control, one way ANOVA.

Figure Legends

Figure 1. Equal amounts of microsomal protein from either canine or rat hearts were analyzed on immunoblots with the two respective TRD antibodies using 5, 10, and 20 µg of microsomal protein loaded onto gels (A). The degree of immunoreactivity of the two antibodies was determined by densitometric analysis of the X-ray film (B). This analysis showed similar TRD-specific staining intensities for the rat and dog microsomal samples. Assuming TRD is present in comparable amounts in these samples this result suggests that TRD rat and dog antibodies bind with comparable strengths to their respective targets. n=4, not significant at P < 0.05, one way ANOVA. 4 different rat heart preparations of microsomes have been used, same for dog.
A

37 kDa

µg of protein

Rat

Dog

TRD Rat

TRD Dog

B

Optical Density, a.u.

µg of protein

0 5 10 20 5 10 20

0 5 10 20
Parameters of $I_{Ca}$

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**Table 1** Effects of overexpression of TRD$^{WT}$ or TRD$^{Del.200-224}$ on parameters of $I_{Ca}$ in rat ventricular myocytes. Data are means ± SE from 4-8 experiments. * P<0.01 vs. Control, one way ANOVA.

**Figure Legends**

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A

37 kDa

µg of protein

5 10 20

Rat

Dog

B

[Graph showing optical density vs. µg of protein for TRD Rat and TRD Dog]

Optical Density, a.u.

0 5 10 20

µg of protein