

Protein–protein interactions between triadin and calsequestrin are involved in modulation of sarcoplasmic reticulum calcium release in cardiac myocytes

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In cardiac muscle, intracellular Ca^{2+} release is controlled by a number of proteins including the ryanodine receptor (RyR2), calsequestrin (CASQ2), triadin-1 (Trd) and junctin (Jn) which form a complex in the junctional sarcoplasmic reticulum (SR) membrane. Within this complex, Trd appears to link CASQ2 to RyR2 although the functional significance of this interaction is unclear. In this study, we explored the functional importance of Trd–CASQ2 interactions for intracellular Ca^{2+} handling in rat ventricular myocytes. A peptide encompassing the homologous CASQ2 binding domain of Trd (residues 206–230 in the rat; TrdPt) was expressed in the lumen of the SR to disrupt Trd–CASQ2 interactions. Myocytes expressing TrdPt exhibited increased responsiveness of SR Ca^{2+} release to activation by I_{Ca} as manifested by flattened and broadened voltage dependency of the amplitude of cytosolic Ca^{2+} transients. Rhythmically paced, TrdPt-expressing myocytes exhibited spontaneous arrhythmogenic oscillations of intracellular Ca^{2+} and membrane potential that was not seen in control cells. In addition, the frequency of spontaneous Ca^{2+} sparks and Ca^{2+} waves was significantly increased in TrdPt-expressing, permeabilized myocytes. These alterations in SR Ca^{2+} release were accompanied by a significant decrease in basal free intra-SR $[\text{Ca}^{2+}]$ and total SR Ca^{2+} content in TrdPt-expressing cells. At the same time a synthetic peptide corresponding to the CASQ2 binding domain of Trd produced no direct effects on the activity of single RyR2 channels incorporated into lipid bilayers while interfering with the ability of CASQ2 to inhibit the RyR2 channel. These results suggest that CASQ2 stabilizes SR Ca^{2+} release by inhibiting the RyR2 channel through interaction with Trd. They also show that intracellular Ca^{2+} cycling in the heart relies on coordinated interactions between proteins of the RyR2 channel complex and that disruption of these interactions may represent a molecular mechanism for cardiac disease.

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In cardiac muscle, the release of Ca^{2+} from the sarcoplasmic reticulum (SR) mediates the process of excitation–contraction coupling causing the initiation of the heart beat. SR Ca^{2+} release is controlled by a multimolecular Ca^{2+} signalling complex, localized to the junctional SR (Zhang *et al.* 1997; Franzini-Armstrong & Protasi, 1997; Bers, 2004). The protein components of this complex include the cardiac ryanodine receptor (RyR2), the Ca^{2+} binding protein calsequestrin (CASQ2), and the junctional SR transmembrane proteins triadin 1 (Trd;

Jones *et al.* 1995) and junctin (Jn; Kobayashi & Jones, 1999). RyR2 operates as a Ca^{2+} conducting pore (Meissner, 1994; Fill & Copello, 2002) while Trd and Jn have been proposed to anchor CASQ2 to RyR2 (Jones *et al.* 1995; Kobayashi & Jones, 1999). CASQ2 is a 45 kDa, highly acidic protein that binds Ca^{2+} with high capacity (40 ions per molecule) and low affinity ($K_{\text{D}} \sim 0.6$ mM) and is located in the lumen of the SR (Mitchell *et al.* 1988; Beard *et al.* 2004). In addition to serving as a major Ca^{2+} reservoir in the SR (Jones *et al.* 1998; Terentyev *et al.* 2003; Knollmann *et al.* 2006), CASQ2 appears to modulate SR Ca^{2+} release by influencing the open probability of the RyR2 channel (Ikemoto *et al.* 1989; Györke *et al.* 2004; Knollmann *et al.*

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2006), possibly via interactions with Trd and Jn (Zhang *et al.* 1997; Gyorke *et al.* 2004). Trd (40 kDa) and Jn (30 kDa) are structurally homologous proteins that are each 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 (Jones *et al.* 1995; Kobayashi & Jones, 1999; Kobayashi *et al.* 2000). The luminal domains of both proteins are enriched in alternating positively and negatively charged amino acids, denoted as KEKE motifs, which have been proposed to be involved in protein–protein interactions. These putative protein binding domains are thought to provide the structural basis for coordinated interactions between the Trd and Jn and also with CASQ2 and RyR2 during EC coupling (Zhang *et al.* 1997). Consistent with this view, the CASQ2 binding domain of Trd was localized to a specific stretch of 25 amino acids (200–224) that encompass a single KEKE motif (Kobayashi *et al.* 2000), suggesting that the protein–protein interactions that occur between Trd and other components of the junctional complex are highly selective and organized.

Despite significant progress in defining the biochemical properties of the components of the RyR2 complex, our understanding of the functional role of the individual protein components of the junctional complex and the modes of their interactions is limited. *In vitro* reconstitution studies have revealed that CASQ2 inhibits the functional activity of RyR2 in planar lipid bilayers and these effects of CASQ2 are apparently mediated by Trd and Jn (Beard *et al.* 2004; Gyorke *et al.* 2004). As demonstrated by *in vitro* binding and reconstitution studies, the interaction of CASQ2 with Trd is Ca^{2+} dependent such that increasing $[\text{Ca}^{2+}]$ promotes dissociation of CASQ2 molecules from Trd (and possibly from Jn as well) (Zhang *et al.* 1997) and relieves the inhibition exerted by CASQ2 on RyR2 complexed with Trd and Jn (Gyorke *et al.* 2004). Ca^{2+} -dependent communications between RyR2 and the luminal auxiliary proteins have been suggested to play a role in modulation of SR Ca^{2+} release during EC coupling (Zhang *et al.* 1997; Gyorke *et al.* 2004; Rios *et al.* 2006). In particular, inhibition of the RyR2 channel by CASQ2 at reduced luminal $[\text{Ca}^{2+}]$ has been proposed to contribute to SR Ca^{2+} release termination and release channel refractoriness following SR Ca^{2+} release in cardiac muscle (Gyorke *et al.* 2004). Supporting this idea, genetic alterations in CASQ2 that affect interactions of this protein with the RyR2 channel complex can lead to deregulated SR Ca^{2+} release and cardiac arrhythmia (Houle *et al.* 2004; Terentyev *et al.* 2006). However, the specific molecular mechanisms whereby RyR2 activity is regulated by interactions with its luminal binding partners remain to be defined. In the present study we explored the consequences of SR targeted expression of a peptide that mimics the putative CASQ2 binding domain of Trd on SR Ca^{2+} release in cardiac myocytes. We hypothesized

that this peptide would act as a competitive inhibitor of Trd–CASQ2 interactions such that expression of this peptide in the SR should impair any functions that rely on these interactions. Our study shows that inhibiting CASQ2–Trd interactions resulted in hyperactive RyR2 channels and arrhythmogenic Ca^{2+} oscillations, thereby providing direct experimental evidence that Trd–CASQ2 interactions are required for regulated SR Ca^{2+} release in cardiac myocytes.

Methods

Construction of recombinant adenoviral vector

The region of the rat cardiac triadin 1 cDNA encoding the homologous CASQ2 binding domain (aas 206–230 for the rat) extending to naturally occurring proline residues (aas 180 and 255 to minimize disruption of protein structure) was amplified from rat heart cDNA using the Pfu thermostable polymerase (Stratagene, La Jolla, CA, USA) using the following primers: sense: GGCGTTCGACGAGAAGAAAGCAACTCAC; antisense: GCCGCGCCGCTGGCGTTTCTTTGTCATC). These primers attach a *SalI* site at the 5' end and *NotI* site at the 3' end of the PCR product. The PCR product was A-tailed and subcloned into pGEM-Teasy vector (Promega, Madison, WI, USA) for sequence verification. The insert was then released with *SalI* and *NotI* subcloned in the pCMV/Myc/ER (Invitrogen, Carlsbad, CA, USA) plasmid, digested with the same enzymes. The triadin sequences were amplified along with flanking elements encoding the ER retention signal (KDEL) and the Myc epitope tag with the following primers: sense: GGCTCTAGAGCCATGGGATGGAGCTGTATC; antisense: GGCGGTACCCTACAGCTCGTCCTTCTC, which introduced a 5' *XbaI* site and 3' *KpnI* site. This PCR product was again sequence verified and subcloned into the pShuttle vector (Clontech, Mountain View, CA, USA) using the *XbaI* and *KpnI* sites. The pShuttle_Trld180–255 expression plasmid was transfected into HEK cells and the expression of the Trd180–255 polypeptide was confirmed by Western blotting using an antibody that recognizes the Myc epitope (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The TrdPt-MycER coding region was transferred into the Adeno-X Viral DNA, and recombinant adenoviruses were generated according to the manufacturers instructions (Clontech). The virus was amplified and purified using the BD Adeno-X virus purification kit (BD Biosciences, San Jose, CA, USA). Rat ventricular myocytes were infected with adenoviruses at a multiplicity of infection of 100 and cultured for 48–56 h (Terentyev *et al.* 2003).

Electrophysiological recordings

The procedure for cell isolation was approved by the Institutional Animal Care and Use Committee of Ohio

State University and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). Adult 200–250 g Sprague–Dawley male rats were anaesthetized with pentobarbital sodium (25 mg kg⁻¹ i.p.). Following thoracotomy hearts were quickly excised, mounted and retrogradely perfused on a Langendorff apparatus with collagenase-containing solution at 37°C according to the method previously described (Terentyev *et al.* 2003). Sixteen hearts were used to isolate ventricular myocytes. Whole-cell patch clamp recordings of transmembrane ionic currents were performed with an Axopatch 200B amplifier (Axon Instruments). The external solution contained (mM): 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 1–3 MΩ) were filled with a solution that contained (mM): 90 caesium aspartate, 50 CsCl, 3 Na₂ATP, 3.5 MgCl₂ and 10 Hepes (pH 7.3). The myocytes were stimulated by application of 400 ms-long voltage pulses to specified membrane potentials from a holding potential of –50 mV at 1 min intervals (Viatchenko-Karpinski & Györke, 2001; Terentyev *et al.* 2003, 2005, 2006). Under these conditions, the SR Ca²⁺ content can be considered at steady state with respect to resting cytosolic [Ca²⁺] and the SR is fully loaded to a level set by the balance between SR Ca²⁺ leak and uptake. For current-clamp experiments Cs⁺ in the pipette solution was replaced with K⁺.

Confocal Ca²⁺ measurements

Intracellular Ca²⁺ imaging was performed using an Olympus Fluoview 1000 laser scanning confocal microscope equipped with an Olympus 60×, 1.4 NA oil objective. To simultaneously monitor the intra-SR and cytosolic Ca²⁺ levels, myocytes were loaded with Fluo-5N AM (10 μM for 7–9 h at 37°C, 95% air–5% CO₂) as previously described (Shannon *et al.* 2003; Kubalova *et al.* 2004). Antibiotic-free Fluo-5N loading media contained: Medium 199M; 5 mM creatine; 5 mM taurine; 25 mM NaHCO₃, 5 mM Hepes, pH 7.3. Subsequently, myocytes were permeabilized with saponin to remove the Fluo-5N from the cytoplasm and to introduce Rhod-2 (30 μM) to the internal solution for cytosolic Ca²⁺ measurements. The control experimental solution contained (mM): 120 potassium aspartate, 20 KCl, 3 MgATP, 10 phosphocreatine, 5 U ml⁻¹ creatine phosphokinase, 0.5 EGTA and 20 Hepes (pH 7.2). Calcium waves were initiated by lowering EGTA in the internal solution from 0.5 to 0.1 mM, free [Ca²⁺] ~75 nM. Fluo-5N and Rhod-2 were excited sequentially by 488 and 543 nm laser lines, and fluorescence was acquired at a rate of 5 ms per line at wavelengths of 500–530 and > 560 nm, respectively. Ca²⁺ sparks in permeabilized myocytes and

Ca²⁺ transients in patch-clamped cells were recorded using Ca²⁺ indicator Fluo-3 in intracellular solution (30 and 50 μM, respectively). Fluo-3 was excited by the 488 nm line of an argon ion laser, and the fluorescence was acquired at wavelengths > 510 nm in the line-scan mode of the confocal system at the rate of 2–5 ms per scan. For quantitative studies, the temporal dynamics in fluorescence were expressed as:

$$\Delta F/F_{\text{Caf}} = (F - F_{\text{Caf}})/F_{\text{Caf}},$$

where F represents fluorescence at time t and F_{Caf} represents the fluorescence level of the myocytes upon application of 10 mM caffeine, or:

$$\Delta F/F_0 = (F - F_0)/F_0,$$

where F_0 stands for baseline fluorescence (Kubalova *et al.* 2005). All chemicals were from Sigma and all Ca²⁺ indicators were from Molecular Probes.

Bilayer experiments

Heavy SR microsomes were isolated from canine LV tissue. Canine cardiac RyR2s were purified from SR vesicles and native or purified RyR2s reconstituted into proteoliposomes and fused into planar lipid bilayers for single-channel recordings as previously described (Gyorke *et al.* 2004). The experimental solutions contained (mM): 350 CsCH₃SO₃, 3 MgATP, 0.6 MgCl₂ (free [Mg²⁺] 0.9 mM), 0.02 CaCl₂, and 20 Hepes, pH 7.4 (*cis*), and 350 CsCH₃SO₃, 0.02 CaCl₂, and 20 Hepes, pH 7.4 (*trans*). The holding potential in all experiments was +40 mV. Single-channel currents were recorded with an Axopatch 200A patch-clamp amplifier (Axon Instruments, Union City, CA, USA). Acquisition and analysis of data were performed using pCLAMP 6.01 software (Axon Instruments). A custom peptide corresponding to amino acids 200–224 of canine Trd (sequence: KEERKAKTEEKIKKEVKGGKQEKVK) which contains the putative CASQ2-binding domain (Kobayashi *et al.* 2000) was synthesized by GenScript Corp., NJ, USA. Recombinant purified canine cardiac calsequestrin (Kobayashi *et al.* 2000; Gyorke *et al.* 2004), was obtained as described. CASQ2 was stored and added from a buffer solution containing 20 mM Mops, 150 mM NaCl (pH 7.0), and 3 mg ml⁻¹ of the protein.

Immunocytochemistry

Myocytes were fixed on glass coverslips with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100/citrate buffer (pH 7.2) containing 1% BSA. Specimens were incubated with anti-CASQ2 antibody (rabbit, PA1-913, ABR, 1:2000) and anti-Myc Tag antibody (mouse, 9B11, Cell Sign. Technol. Inc., 1:1000);

followed by secondary antibodies antirabbit Alexa Fluor 488 and antimouse Alexa Fluor 546 (A11034 and A11003, respectively, 1 : 200, Molecular Probes).

Statistical analysis of data

Data are presented as means \pm s.e.m. Paired or unpaired data sets were statistically evaluated using Student's *t* test or by one-way ANOVA test, when appropriate, and significance was defined at $P < 0.05$.

Results

A domain through which Trd interacts with CASQ2 has been localized to a homologous region in the luminal C-terminal of Trd (amino acids 200–224) which contains a single KEKE motif (Kobayashi *et al.* 2000). To investigate the functional significance of Trd–CASQ2 interactions for calcium handling, we developed a method to inhibit Trd–CASQ2 interactions by expressing a decoy peptide containing a stretch of residues corresponding to the CASQ2 binding domain of Trd (TrdPt) in cardiac myocytes. The appropriate region of Trd cDNA was inserted into an adenoviral vector together with the KDEL ER/SR targeting sequence to generate Ad-TrdPt for subsequent gene transfer into adult rat ventricular myocytes. In addition, a short Myc-tag encoding sequence was added to the transgene to permit verification of expression of the peptide in the ER/SR compartment using a Myc tag-specific antibody (Fig. 1).

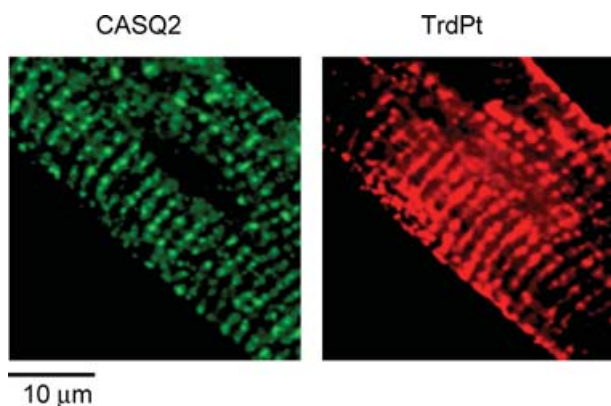


Figure 1. SR localization in ventricular myocytes of adenovirally expressed TrdPt

CASQ2 and TrdPt were coimmunostained with CASQ2 and Myc Tag-specific antibodies using green and red fluorescent tagged secondary antibodies, respectively. Images were recorded simultaneously with an Olympus Fluoview-1000 spectral confocal system. Alexa-Fluo 488 was excited with the 488 nm argon laser line and emission was collected at 500–530 nm, while Alexa Fluo-546 was excited at 543 nm and emission recorded at > 570 nm. Measurements were performed 48–56 h after infection.

I_{Ca} and intracellular Ca^{2+} transients

We examined the effects of expression of TrdPt on excitation–contraction coupling in adult rat ventricular myocytes. L-type Ca^{2+} currents and intracellular Ca^{2+} transients were simultaneously measured in myocytes depolarized to various membrane potentials (in the range from -40 to $+100$ mV) (Fig. 2). Expression of TrdPt resulted in a dramatic augmentation of the amplitude of Ca^{2+} transients at low and high depolarizing steps (where I_{Ca} amplitude is small) without a significant change in the amplitude of the maximal Ca^{2+} transients (at 0 mV) with respect to control (Fig. 2A). Accordingly, the voltage dependence of Ca^{2+} transients was broadened and flattened in TrdPt myocytes (Fig. 2B). These alterations in Ca^{2+} transients occurred without changes in the amplitude of I_{Ca} , the trigger signal for SR Ca^{2+} release, at different membrane potentials (Fig. 2C). Therefore TrdPt stimulated SR Ca^{2+} release by sensitizing it to activation by I_{Ca} .

TrdPt expression induces cellular arrhythmia

We have previously shown that ectopic expression of specific CASQ2 mutants associated with CPVT or over-expressing WT Trd lead to arrhythmic oscillations in Ca^{2+} transients and membrane potential in paced cardiac myocytes exposed to ISO (Terentyev *et al.* 2005, 2006). As both of these interventions may function by disrupting interactions between the components of the RyR2 complex, we next tested whether ectopic expression of TrdPt would cause similar alterations in cytosolic Ca^{2+} and membrane potential (MP) signals. Therefore, TrdPt-overexpressing and control myocytes were subjected to rhythmic pacing in the presence of ISO. As shown in Fig. 3, rhythmically paced, TrdPt-overexpressing myocytes exhibited profound arrhythmogenic disturbances in Ca^{2+} cycling and MP manifested by after- Ca^{2+} transients and delayed after-polarizations (DADs). Similar results were obtained in four independent TrdPt-overexpressing myocytes ($n = 5$ from 6) while control myocytes exhibited no such behaviour under identical conditions ($n = 0$ from 5). Accordingly, average peak diastolic Fluo-3 fluorescence (F/F_0) was $1.51 \pm 0.20^*$ and 1.08 ± 0.07 in TrdPt ($n = 6$) and control ($n = 5$) myocytes, respectively (*significantly different at $P < 0.05$).

Spontaneous Ca^{2+} sparks and Ca^{2+} waves

To further investigate the effects of TrdPt-expression on the properties of SR Ca^{2+} release, we recorded spontaneous Ca^{2+} sparks in myocytes permeabilized with saponin. In these experiments, $[Ca^{2+}]_i$ was buffered at ~ 75 nM with 0.5 mM EGTA. As shown in Fig. 4, ectopic expression of TrdPt resulted in a significant increase in Ca^{2+} spark

frequency. At the same time, although there was no significant difference for the average amplitude of all detected events, the magnitude of the 5% of the brightest sparks (most likely to be situated at the centre of the line scan) significantly decreased in TrdPt-expressing myocytes compared to control ($\Delta F/F_0$ was 2.52 ± 0.05 ($n = 29$) and 2.80 ± 0.07 ($n = 18$), respectively; $P < 0.05$). Thus, the observed effects of TrdPt expression on cell-averaged Ca^{2+} signals could be ascribed to increased functional activity of individual Ca^{2+} release sites, i.e. RyR2 clusters.

In addition to recording Ca^{2+} sparks we also measured spontaneous propagating Ca^{2+} waves by monitoring $[\text{Ca}^{2+}]$ in both extra- and intra-SR compartments in saponin-permeabilized myocytes. In these experiments, cytosolic $[\text{Ca}^{2+}]$ was monitored with Rhod-2 whereas $[\text{Ca}^{2+}]_{\text{SR}}$ was tracked with Fluo-5N loaded into the SR

(see below). Similar to the effects upon Ca^{2+} sparks, TrdPt expression increased the frequency of occurrence of Ca^{2+} waves (Fig. 5A and B). Additionally, the amplitude of the waves was significantly decreased in TrdPt-expressing myocytes with respect to control (Fig 5A and C). These results provide further evidence that TrdPt stimulates SR Ca^{2+} release making it susceptible to spontaneous activation.

Intra-SR $[\text{Ca}^{2+}]$

Activation of RyR2 by luminal Ca^{2+} is thought to play an important role in the generation of spontaneous Ca^{2+} release in cardiac muscle. In various pathological states, increased spontaneous Ca^{2+} release has been associated with either elevated SR Ca^{2+} content (Orchard *et al.*

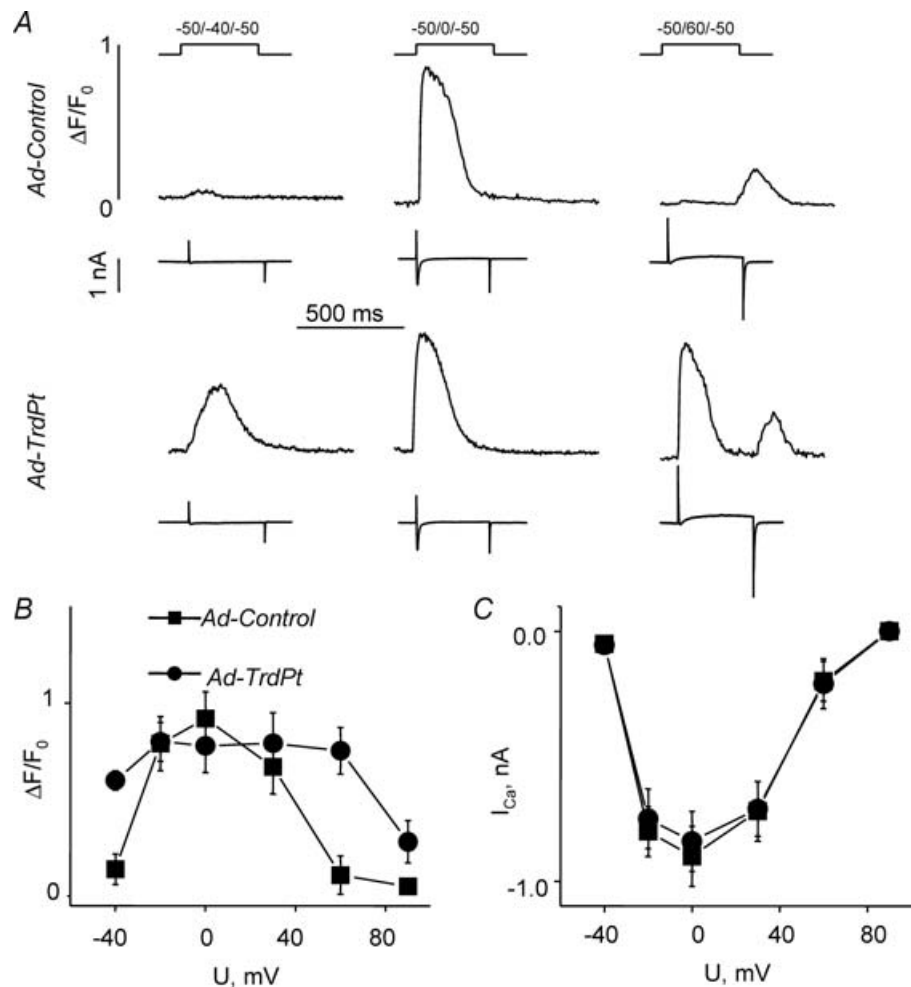


Figure 2. Loss of voltage dependency of Ca^{2+} release in cells expressing TrdPt

A, traces of I_{Ca} (lower traces) and intracellular Ca^{2+} transients (upper traces) evoked by depolarization steps from -50 mV HP to different voltages in myocytes infected with Ad-TrdPt and Ad-Control vectors. B and C, voltage dependencies of Ca^{2+} transients (B) and I_{Ca} (C) in myocytes infected with Ad-Control (■), and Ad-TrdPt (●) vectors (n from 6 to 12). Average rise times and maximal rates of rise of Ca^{2+} transients evoked by depolarization to 0 mV were not significantly different for control and TrdPt expressing myocytes (32 ± 4 ms and 0.49 ± 0.12 ms^{-1} ($n = 6$) versus 24 ± 4 ms and 0.49 ± 0.16 ms^{-1} ($n = 5$), respectively).

1983; Stern *et al.* 1988; Lipp & Niggli, 1993; Diaz *et al.* 1997) or sensitization of RyR2s to luminal Ca^{2+} (Kubalova *et al.* 2005; Jiang *et al.* 2005). Thus, we investigated the effects of ectopic expression of TrdPt on intra-SR Ca^{2+} levels in permeabilized myocytes to determine which of these mechanisms contributed to the altered Ca^{2+} release properties (Fig. 5). Free $[\text{Ca}^{2+}]_{\text{SR}}$ was monitored with SR-entrapped Fluo-5N (simultaneously with cytosolic $[\text{Ca}^{2+}]$; Fig. 5A), whereas the total SR Ca^{2+} content was estimated from the amplitude of the cytosolic Ca^{2+} transients induced by application of caffeine (Fig. 5E). In comparison with control cells, basal free intra-SR $[\text{Ca}^{2+}]$ and the extent of $[\text{Ca}^{2+}]_{\text{SR}}$ depletion (nadir) during waves were significantly reduced in TrdPt myocytes (Fig. 5D). Similarly, the total SR Ca^{2+} content was significantly lowered in TrdPt myocytes (Fig. 5F).

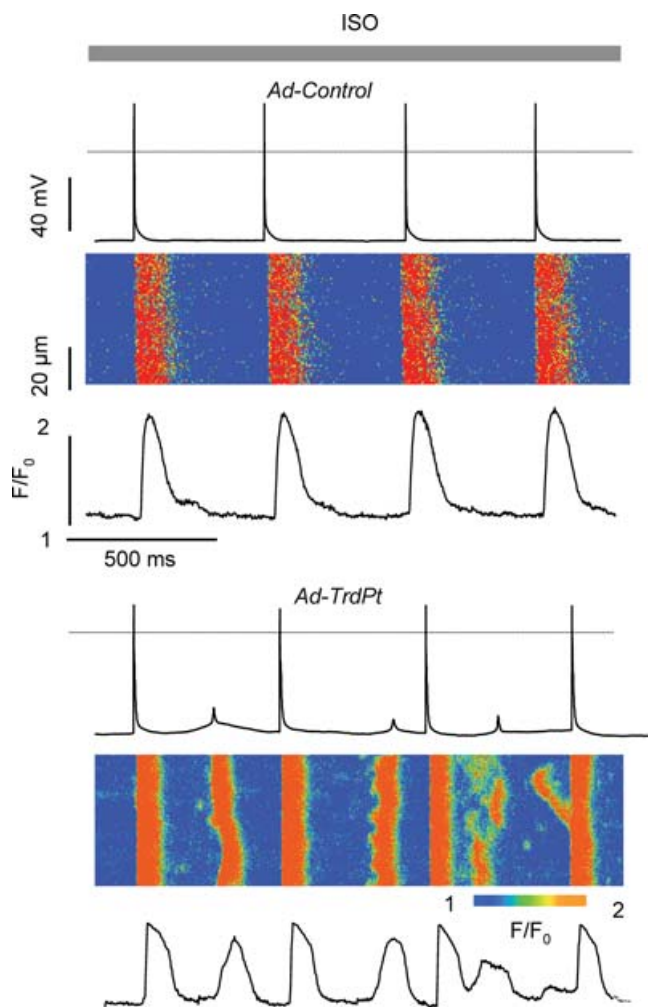


Figure 3. Arrhythmogenic disturbances in Ca^{2+} cycling in myocytes expressing TrdPt

Whole cell current clamp recordings of membrane potential (upper traces), line-scan images of Ca^{2+} transients (middle) and time dependent profiles (lower traces) in the presence of $1 \mu\text{M}$ isoproterenol. Stimulation frequency was 2 Hz.

These results suggest that the increased propensity of TrdPt-expressing myocytes for spontaneous Ca^{2+} release is attributable to sensitization of RyR2 channels to luminal Ca^{2+} rather than to increased levels of Ca^{2+} in the SR.

SERCA2a-mediated SR $[\text{Ca}^{2+}]$ uptake

Although designed to specifically inhibit CASQ2 interaction with the RyR2 complex, the TrdPt peptide could produce its effects on myocyte Ca^{2+} signalling via non-specific interactions with other targets in the SR such as SERCA2a. To test whether TrdPt had an effect on the SERCA2a pump, we performed measurements of SERCA2a-mediated SR $[\text{Ca}^{2+}]$ uptake in permeabilized myocytes using SR-entrapped Fluo-5N. In this series of experiments, the SR was first depleted with 10 mM of caffeine, then caffeine was washed out with a $[\text{Ca}^{2+}]$ -free

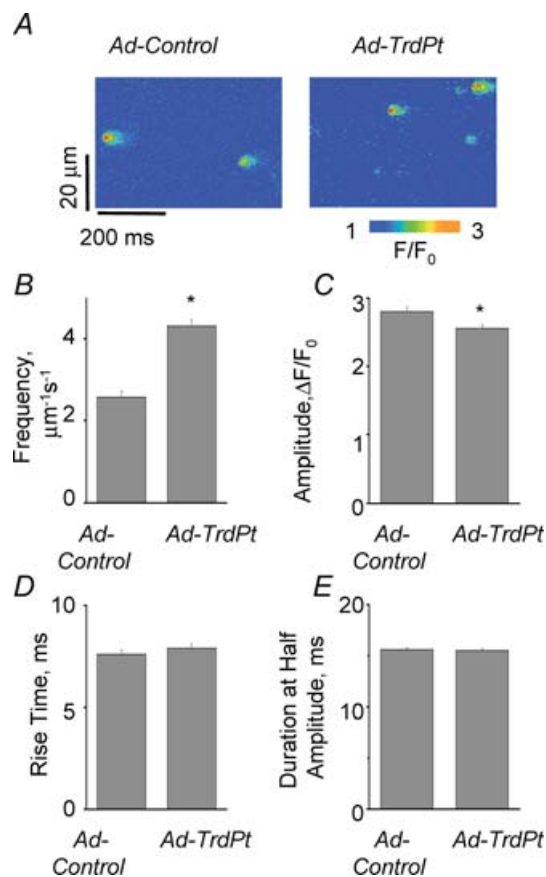


Figure 4. Expression of TrdPt leads to increased frequency of spontaneous Ca^{2+} sparks in permeabilized myocytes

A, representative line scan images of Ca^{2+} sparks in cells infected with adenoviral constructs. B, C, D and E, bar graphs of pooled values of spark frequency (B), amplitude of 5% of brightest events (C), rise time (D) and spark duration at half-amplitude (E) for the same groups of cells. *Significantly different at $P < 0.05$; one-way ANOVA (n of Ca^{2+} sparks was 388 and 565; n of cells was 73 and 76 for Ad-Control and Ad-TrdPt-infected myocytes, respectively).

solution and ruthenium red ($10 \mu\text{M}$) was applied to block the RyR2 channels. In the presence of ruthenium red, SR $[\text{Ca}^{2+}]$ uptake was initiated by application of 100 nM $[\text{Ca}^{2+}]$ and monitored by measuring the rise of SR $[\text{Ca}^{2+}]$ with Fluo-5N. Representative traces and pooled data for SR $[\text{Ca}^{2+}]$ uptake kinetics in control and TrdPt-expressing myocytes are shown in Fig. 6. In TrdPt-expressing cells the time constant of SR $[\text{Ca}^{2+}]$ was not significantly different from that measured in the control group. These results indicate that intrinsic SERCA2a Ca^{2+} transport activity was not affected by TrdPt.

Single RyR2 channel activity

In principle, the TrdPt peptide could exert its effects on SR Ca^{2+} release by direct activation of the RyR2 rather than by impairing protein–protein interactions between

Trd and CASQ2. To examine the ability of TrdPt to directly activate RyR2 through the 200–224 KEKE protein binding motif, we investigated the effects of a synthetic peptide corresponding to this region of Trd on the activity of purified single RyR2 channels in planar lipid bilayers. Single-channel currents were recorded with Cs^+ as the charge carrier (Gyorke *et al.* 2004). Addition of this peptide ($5\text{--}50 \mu\text{g per ml}$) to the luminal (*trans*) side of the channel did not significantly affect RyR2 open probability (Fig. 7A and B). To directly examine whether TrdPt acted by interfering with protein–protein interactions within the RyR2 complex, we performed experiments with combined additions of TrdPt and purified CASQ2 to the luminal side of the channel (Fig. 7C). Native RyR2s were first stripped of CASQ2 by increasing *trans* $[\text{Ca}^{2+}]$ from $20 \mu\text{M}$ to 5 mM (Gyorke *et al.* 2004). After reverting back to low *trans* $[\text{Ca}^{2+}]$, addition of CASQ2 ($5 \mu\text{g ml}^{-1}$) resulted

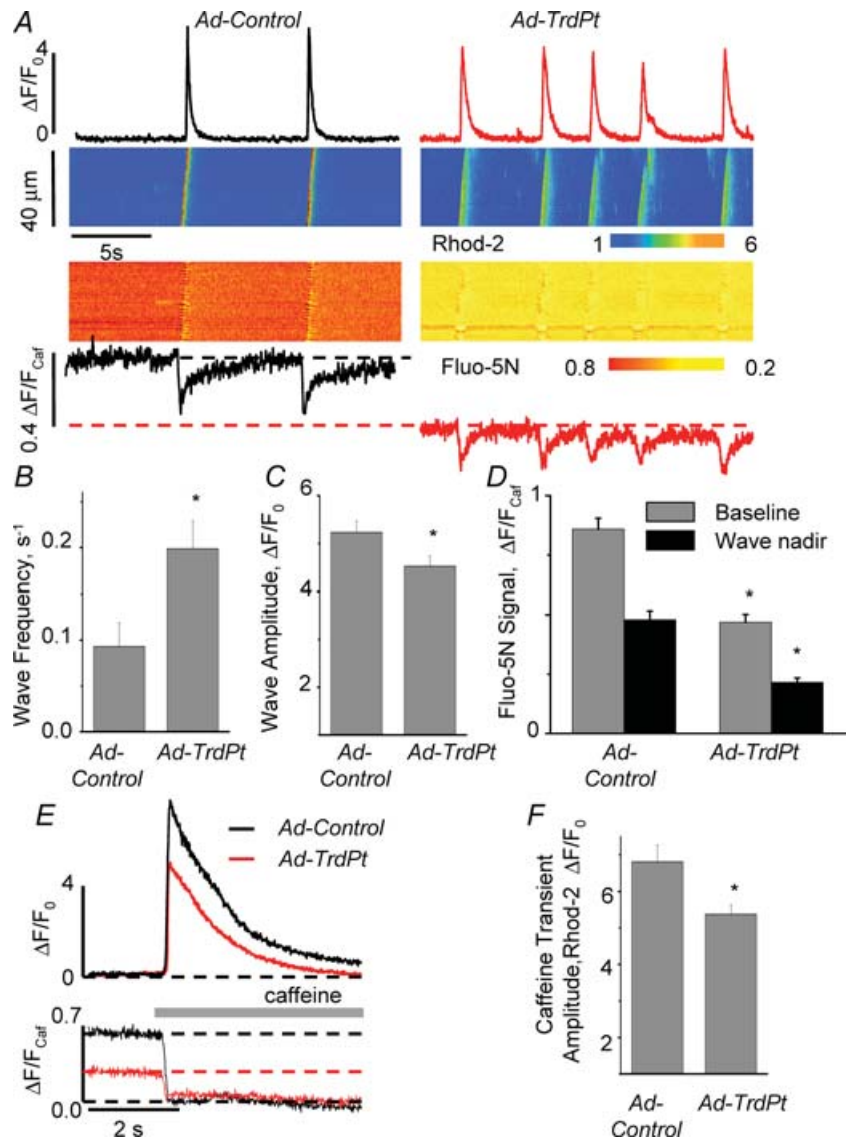


Figure 5. Effects of expression of TrdPt on properties of cytoplasmic and intra-SR Ca^{2+} waves in permeabilized myocytes

A, line-scan images along with time-dependent profiles of Rhod-2 and Fluo-5N fluorescence. B, C, D and F, pooled data on wave frequency (B), cytosolic wave amplitude measured with Rhod-2 (C), changes in the baseline $[\text{Ca}^{2+}]_{\text{SR}}$ and intra-SR $[\text{Ca}^{2+}]$ wave nadir measured with SR-entrapped Fluo-5N (D) and amplitude of cytosolic caffeine-induced Ca^{2+} transients (F). E, caffeine-evoked Ca^{2+} transients (upper panel) and depletion signals (lower panel) recorded in myocytes infected with Ad-Control and Ad-TrdPt vectors. *Significantly different at $P < 0.05$; one-way ANOVA (n from 8 to 17).

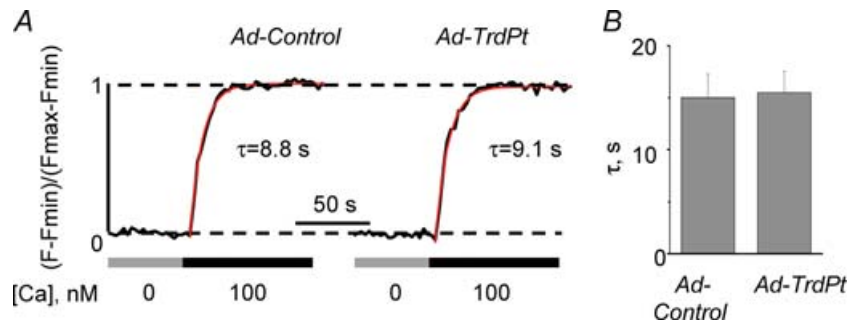


Figure 6. SERCA2a-mediated SR Ca^{2+} uptake is not significantly altered in TrdPt expressing myocytes

A, time course of SR Ca^{2+} uptake, measured with SR-entrapped Fluo-5N in permeabilized control and TrdPt-expressing myocytes in the presence of $10 \mu\text{M}$ ruthenium red. Intra-SR $[\text{Ca}^{2+}]$ was depleted with 10 mM caffeine; caffeine was washed out with a Ca^{2+} -free solution; then ruthenium red was applied and SR Ca^{2+} uptake initiated by raising cytosolic $[\text{Ca}^{2+}]$ to 100 nM $[\text{Ca}^{2+}]$. **B**, pooled data for the time constants from single exponential fits of SR Ca^{2+} uptake. n was 11 and 12 for control and TrdPt expressing cells, respectively.

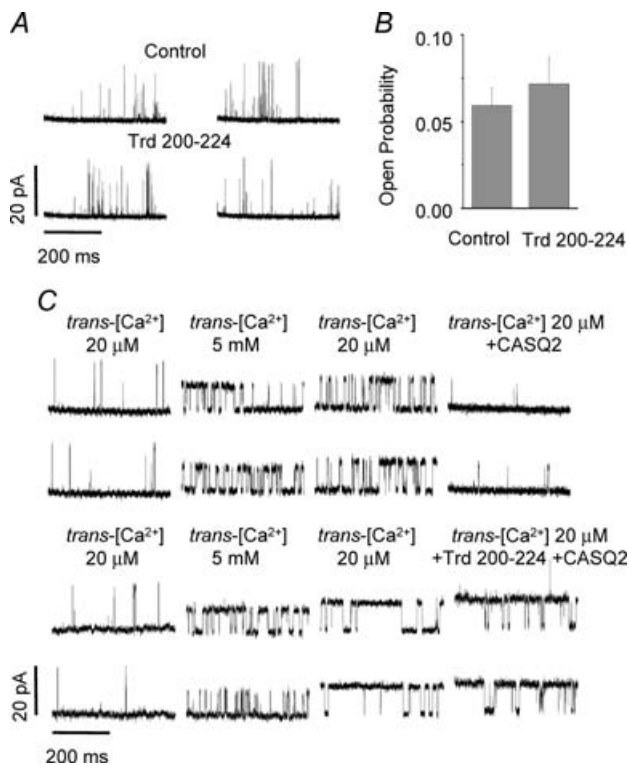


Figure 7. A synthetic decoy peptide corresponding to CASQ2 binding domain of Trd (residues 200–224) interferes with CASQ2 inhibitory action on RyR2 channel complex

Trd 200–224 does not modulate activity of purified RyR2 channels. **A**, representative single RyR2 channel traces. **B**, pooled data on open probability of purified RyR2s before and after addition to the *trans* chamber $5\text{--}50 \mu\text{M}$ of the Trd decoy peptide ($n = 8$). **C**, representative single-channel traces illustrating the irreversibility of the effects of $5\text{--}50 \mu\text{M}$ luminal Ca^{2+} and restoration of initial low activity by CASQ2 added to the *trans* chamber in native RyR2s (upper panel). Trd 200–224 interferes with the ability of CASQ2 to inhibit native RyR2s (lower panel). The P_o values were 0.06 ± 0.01 for low *trans* $[\text{Ca}^{2+}]$ ($20 \mu\text{M}$); 0.29 ± 0.07 for high *trans* $[\text{Ca}^{2+}]$ (5 mM); 0.41 ± 0.12 reverting to low *trans* $[\text{Ca}^{2+}]$ ($20 \mu\text{M}$); and 0.04 ± 0.01 ($n = 6$) versus 0.48 ± 0.08 ($n = 4$)^{*} after application of $5 \mu\text{g ml}^{-1}$ of CASQ2 alone or with $50 \mu\text{g ml}^{-1}$ of Trd 200–224. The data are presented as means \pm s.e.m. ^{*}Significantly different versus CASQ2 alone at $P < 0.05$ (one-way ANOVA).

in reduction of RyR2 open probability, as previously described (Gyorke *et al.* 2004; Terentyev *et al.* 2006). However, when the introduction of CASQ2 was preceded by addition of TrdPt ($50 \mu\text{g ml}^{-1}$), CASQ2 failed to inhibit the RyR2 channel. Therefore, TrdPt appears to lack the ability to activate the RyR2 channel directly and rather acts by interfering with the ability of CASQ2 to inhibit the RyR2.

Discussion

Growing evidence suggest that interactions of the RyR2 channel with the luminal auxiliary proteins CASQ2 and Trd might be involved in regulation of SR Ca^{2+} release in cardiac muscle. However, the exact roles of these proteins in cardiac EC coupling remain to be defined. We have studied the functional significance of interactions between CASQ2 and Trd by expression of a peptide that mimics the CASQ2 binding domain of Trd and thus should act as a competitive inhibitor of CASQ2–Trd interactions in cardiac myocytes. Our data demonstrate that this decoy peptide stimulates the functional activity of the Ca^{2+} release channels, leading to deregulated SR Ca^{2+} release and arrhythmogenic delayed afterdepolarizations (DADs). These results provide new insights into the molecular events involved in regulation of SR Ca^{2+} release and show how interference with these events can lead to pathological states such as cardiac arrhythmia.

SR-targeted expression of the TrdPt peptide compromised the ability of the Ca^{2+} release mechanism to respond to I_{Ca} in a graded manner and increased the frequency of spontaneous Ca^{2+} sparks and Ca^{2+} waves in intact and permeabilized myocytes. TrdPt expression did not affect SERCA2a Ca^{2+} transport activity but led to a significant decrease in both free and total SR Ca^{2+} content consistent with increased Ca^{2+} leakage from the SR. These effects are indicative of a stimulatory influence of the peptide on the RyR2 channel. Since by design, the sequence of the peptide corresponds to the

CASQ2 binding domain of Trd (Kobayashi *et al.* 2000), we attribute these effects to competitive inhibition by the peptide of CASQ2–Trd interaction in myocytes. This interpretation is strongly supported by the results of our *in vitro* reconstitution experiments on RyR2s incorporated into lipid bilayers. According to previous studies (Wang *et al.* 2000; Gyorke *et al.* 2004), these experiments showed that CASQ2 added to the luminal side of RyR2s stripped of CASQ2 inhibits their open probability. Notably, a synthetic peptide corresponding to the CASQ2 binding domain of Trd (residues 200–224) prevented CASQ2 from inhibiting RyR2s without affecting RyR2 activity directly. Therefore, collectively, our results suggest that the TrdPt affects SR Ca²⁺ release by altering interactions between CASQ2 and Trd.

The functional effects of TrdPt expression on SR Ca²⁺ release were qualitatively similar to those observed previously in myocytes overexpressing the full-length wild-type Trd protein (Terentyev *et al.* 2005). A likely explanation for these results is that expressions of the TrdPt and the full-length protein have similar effects on the function of the junctional complex, presumably involving the disruption of normal protein–protein interactions mediated by the KEKE motif within the Trd decoy peptide. In support of this interpretation, we previously demonstrated that a Trd deletion mutant protein that lacks the fragment encompassing 200–224 failed to exert the functional effects on SR Ca²⁺ release and RyR2 activity observed with the WT protein or the TrdPt peptide (Terentyev *et al.* 2005). Although more difficult to interpret due to various adaptive changes, the results obtained with chronic overexpression or ablation of Trd, and Jn in transgenic mouse models also indicated that normal intracellular Ca²⁺ handling relies on an intricate balance between the components of the RyR2 complex including Jn Trd and CASQ2 (Kirchhefer *et al.* 2001; Muller *et al.* 2002; Yuan *et al.* 2007).

Based on our *in vitro* reconstitution studies we have previously proposed that CASQ2 modulates the activity of the RyR2 channel in a Ca²⁺-dependent manner through interaction with Trd and Jn (Gyorke *et al.* 2004). In particular, CASQ2 inhibited RyR2 complexed with Trd at low luminal [Ca²⁺] whereas this inhibitory function was not observed at elevated luminal [Ca²⁺]. The results of the present study, which show that disruption of CASQ2–Trd interactions leads to enhanced and destabilized SR Ca²⁺ release, are consistent with this mechanism and present, to our knowledge, the first experimental evidence for functionally relevant interactions between CASQ2 and Trd in intact myocytes. These results also predict that genetic defects in CASQ2 and Trd that alter normal communication between these proteins could lead to cardiac arrhythmia. In support of this prediction, we recently showed that a specific CPVT-linked mutation in CASQ2 (CASQ2^{R33Q}) compromised the ability of CASQ2

to inhibit RyR2 activity and produced effects similar to those exerted by the TrdPt peptide when expressed in myocytes (Terentyev *et al.* 2006).

In summary, our results provide evidence for functionally relevant structure-specific interactions between the SR luminal proteins CASQ2 and Trd in cardiac excitation–contraction coupling. Since disruption of these interactions resulted in potentiation of the Ca²⁺ release channels, we conclude that CASQ2 exert an inhibitory influence on the RyR2 channel through interaction with the luminal domain of Trd. Thus, our results show that intracellular Ca²⁺ cycling in heart relies on an intricate interplay between proteins of the RyR2 channel complex and disruption of these interactions may present a molecular mechanism for cardiac disease.

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Supplemental material

Online supplemental material for this paper can be accessed at: <http://jp.physoc.org/cgi/content/full/jphysiol.2007.136879/DC1> and <http://www.blackwell-synergy.com/doi/suppl/10.1113/jphysiol.2007.136879>