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Reduced gain of excitation contraction coupling in triadin-null myotubes is mediated by the disruption of FKBP12/RyR1 interaction

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Summary

Several studies have suggested that triadin (Tdn) may be a critical component of skeletal EC-coupling. However, using Tdn-null mice we have shown that triadin ablation results in no significant disruption of skeletal EC-coupling. To analyze the role of triadin in EC-coupling signaling here we used whole-cell voltage clamp and simultaneous recording of intracellular Ca^{2+} release to characterize the retrograde and orthograde signaling between RyR1 and DHPR in cultured myotubes. DHPR Ca^{2+} currents elicited by depolarization of Wt and Tdn-null myotubes displayed similar current densities and voltage dependence. However, kinetic analysis of the Ca^{2+} current shows that activation time constant of the slow component was slightly decreased in Tdn-null cells. Voltage-evoked Ca^{2+} transient of Tdn-null myotubes showed small but significant reduction in peak fluorescence amplitude but no differences in voltage dependence. This difference in Ca^{2+} amplitude was averted by over-expression of FKBP12.6. Our results show that bi-directional signaling between DHPR and RyR1 is preserved nearly intact in Tdn-null myotubes and that the effect of triadin ablation on Ca^{2+} transients appears to be secondary to the reduced FKBP12 binding capacity of RyR1 in Tdn-null myotubes. These data suggest that skeletal triadins do not play a direct role in skeletal EC-coupling.

1-INTRODUCTION

In mammalian skeletal muscle a structural and functional interaction between the voltage-dependent, dihydropyridine-sensitive, L-type calcium channel (DHPR) located in the plasma membrane and the ryanodine-sensitive/calcium release channel (RyR1) located in the junctional face membrane of the sarcoplasmic reticulum (SR) has been shown to be absolutely required for excitation-contraction (EC) coupling. It is currently accepted that the skeletal type (or external Ca^{2+} independent) EC-coupling process is the result of bi-directional signaling between the DHPR and RyR1 in which DHPR delivers the Ca^{2+}

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release signal (also known as orthograde signal) to RyR1 and RyR1 in turn enhances DHPR Ca^{2+} channel activity (also known as retrograde signal).

Although there seems to be a consensus that a direct physical interaction between the DHPR and RyR1 plays a pivotal role in the transmission of the calcium release signal to the ryanodine receptor the molecular basis of how the conformational change of DHPR is sensed by RyR1 is still unknown. Based in their ability to interact or influence the activity of either or both of the two channels several regulatory proteins have been proposed as potential candidates to mediate or modulate skeletal EC-coupling, among which triadin has generated recent interest.

Triadins are integral components of the SR membrane and are the product of alternative splicing of a single gene [1,2]. Several isoforms of triadin had been identified in skeletal muscle with all of them sharing a common N-terminal domain but a unique C-terminal region [2–5] that likely prompts them for specific functions.

Studies in rat skeletal muscle have shown that two of the five isoforms of triadin currently identified, Trisk 95 and Trisk 51 (95 kDa and 51 kDa, respectively), are primarily located at the junctional SR [5,6] and, therefore, may meet the criteria for an EC-coupling modulator. Accordingly, over-expression of Trisk 95, but not Trisk 51, has been shown to significantly reduce the amplitude of K^{+} -induced Ca^{2+} transients of cultured myotubes [7], suggesting a role for Trisk 95 on modulating RyR/DHPR interaction and, therefore, EC-coupling. A more recent study in cultured mouse myotubes seems to support this idea as the expression of mutant RyRs that lack triadin-binding ability in dyspedic myotubes dramatically impaired electrically-evoked Ca^{2+} transients [8].

In a different series of studies aimed at preventing triadin expression in cultured cells, the role for triadin in modulating skeletal EC-coupling seems less apparent. Using siRNAs to knockdown expression of triadin in cultured myotubes Wang et al [9] reported that, unlike over-expression, disruption of triadin expression resulted in a rather modest reduction of the amplitude of K^{+} -induced Ca^{2+} transients. Likewise, using a similar approach Fodor et al [10] was unable to detect significant differences in the amplitude of K^{+} -induced Ca^{2+} transients between Wt- and triadin knockdown rat myotubes. Consistent with these data we have shown that, despite a slight reduction in the amplitude of Ca^{2+} transients, cultured myotubes from Tdn-null mice show nearly normal skeletal EC-coupling in response to either K^{+} depolarization or electrical field stimulation [11]. Based on these results we have proposed that the lack of triadin expression (either acute or chronic) only marginally affects the functional communication between DHPR and RyR1 and, therefore, that triadins may not be critical components of skeletal EC-coupling. Recently, a study of a new triadin knockout mouse model has reported similar findings on the effect of triadin in EC-coupling. However, triadin ablation resulted in significant decay in strength of skeletal muscles, suggesting that triadins do play a modulatory role in skeletal muscle function [12].

These seemingly conflicting results reveal the complexity and balance of the protein interactions at the junctional SR and underline the importance of a comprehensive analysis of the role of triadins in the cross-talk between the DHPRs and the RyRs during skeletal EC-coupling.

In an attempt to determine whether triadin plays a direct role in skeletal EC-coupling here we examined the impact of triadin ablation on bi-directional signaling between RyR1 and DHPR. Using whole-cell patch clamp and Tdn-null cultured myotubes we demonstrate that despite the lack of triadin expression null myotubes display normal DHPR Ca^{2+} current densities (retrograde signal) and strong voltage-dependent Ca^{2+} release activity (orthograde signal). However, ablation of triadin did result in moderate alterations of DHPR Ca^{2+}

current kinetics and the amplitude of the voltage-dependent Ca^{2+} release. Our data suggest that these changes are secondary to the disruption of the FKBP12/RyR1 complex induced by ablation of triadin [13] and can be overcome by over-expression of FKBP12.6. These results support the idea that triadins are not mandatory components of skeletal EC-coupling, but suggest that they may play an indirect role supporting the functional interaction between RyR1 and DHPR and other accessory proteins (i.e. FKBP12) at the junctional SR.

2-MATERIAL AND METHODS

2.1 Immunofluorescence labeling and Western blot

Differentiated myotubes were cultured and quick fixed in cold methanol as described previously [11,14]. Cells were then immunostained with anti-DHPR α_{1S} ($\text{Ca}_{V1.1}$) antibody (MA3-921, Thermo Scientific, Rockford IL) and anti-RyRs antibody (34C, ISHB, University of Iowa). Controls omitting primary antibody were routinely performed. Images were recorded on an Olympus IX70 microscope using a SPOT/RT3 digital camera (Diagnostic Instruments Inc.) and processed with Adobe® Photoshop CS3 (Version 10.0.1). For immunoblots whole cell crude homogenates were prepared by sonication in hypotonic buffer (5mM HEPES, pH 7.4, 2mM EDTA) supplemented with protease inhibitors cocktail (Complete, EDTA free, Roche). Proteins were separated in SDS-polyacrylamide gel electrophoresis [15] and transferred to PVDF membrane. Expression of RyR1 and $\text{Ca}_{V1.1}$ was confirmed with the same antibodies used for immuno-staining. Expression of FKBP12 and Triadin was detected with monoclonal antibodies PA1-026A and MA3-927, respectively (Thermo Scientific, Rockford IL). Membranes were then incubated with either anti-mouse or anti-rabbit horseradish-peroxidase-conjugated goat, secondary antibody and developed with SuperSignal ultra chemiluminescence substrate (Pierce, Rockford, IL) and the intensity of the signal collected using a Kodak Imaging Station 4000MM PRO. Band identification and densitometry of the identified proteins was performed using Kodak MI Software (version 4.5.1 ES). Band intensities were normalized to GAPDH expression (FL-335, SCBT, Santa Cruz CA) to correct for protein loading.

2.2 Measurement of ionic currents

Macroscopic Ca^{2+} currents were measured using the whole-cell patch clamp technique according to previously described protocols [16]. Myotubes were voltage-clamped with the use of an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Effective series resistance was compensated up to the point of amplifier oscillation with the Axopatch circuit. All experiments were performed at room temperature. The composition of the external bath solution was 145mM TEA-Cl, 10mM CaCl_2 , 1mM MgCl_2 , 10mM HEPES titrated with TEA(OH) to pH 7.4. The patch pipette internal solution consisted of 140mM Cs-Aspartate, 5mM MgCl_2 , 0.1mM Cs-EGTA and 10mM HEPES titrated with CsOH to pH 7.2. Patch pipettes had a resistance of 1.5–3.0 M Ω when filled with the pipette solution. For intracellular Ca^{2+} transient measurements the internal solution was adjusted to: 135mM Cs-Aspartate, 5mM CsCl, 5mM ATP-Mg, 30 μM Fluo3-K5, 0.1mM Cs-EGTA, 10mM HEPES titrated with CsOH to pH 7.2. Fluorescence signals during voltage-patch recordings were photometrically acquired with a PIN photodiode connected to a headstage of a second patch-clamp amplifier (Axopatch-1C, Axon Instruments) and the photo-current filtered at 2kHz, as previously described [17].

The voltage dependence of the Ca^{2+} currents was fitted to the following equation.

$$I = G_{\max} (V - V_r) / [1 + \exp((V_{1/2} - V)/k)] \quad (\text{Eq. 1})$$

The voltage dependence of Ca^{2+} transients was fitted to:

$$\Delta F/F_o = (\Delta F/F_o)_{\max} / [1 + \exp((V_{1/2} - V)/k)] \quad (\text{Eq. 2})$$

Where $G_{\max}$ and $(\Delta F/F_o)_{\max}$ are the maximal conductance and maximal fluorescence amplitude, respectively. V corresponds to the test potentials, $V_{1/2}$ is the potential at which $G=1/2G_{\max}$ and $(\Delta F/F_o)=1/2 (\Delta F/F_o)_{\max}$. k represents a slope parameter and V_r the reversion potential.

2.3 Calcium Imaging

Primary myoblasts were grown and differentiated in 96well/plate format as described previously [11]. Ca^{2+} imaging was performed 5 days after differentiation in myotubes loaded with $5\mu\text{M}$ Fura2-AM (Molecular Probes, OR) in imaging buffer (125mM NaCl, 5mM KCl, 2mM CaCl_2 , 1.2mM MgSO_4 , 6mM Glucose, and 25mM Hepes/Tris, pH 7.4). Cells were imaged with a Stanford Photonics 12 bit digital intensified CCD using a DG4 multi-wavelength light source. Myotubes were excited alternatively at 340nm and 380nm and fluorescence emission at 510nm was then captured from regions of interest within each myotube at 6 frames per second. Data was displayed and analyzed using QED imaging software (QED Software, Pittsburgh PA)

2.4 Data Analysis

Statistical significant differences among the data were evaluated using One-way Tukey's ANOVA (nonparametric) analysis and Student's t test (GraphPad Software, San Diego, Ca). Data are presented as mean $\pm$ standard error (SEM), unless indicated otherwise.

3-RESULTS AND DISCUSSION

3.1 Expression and junctional SR targeting of $\text{Ca}_{v1.1}$ and RyR1

To assess whether lack of triadin expression alters expression pattern or targeting of RyR1 and $\text{Ca}_{v1.1}$ in cultured myotubes we compared Wt and Tdn-null cells using Western blot and immuno-labeling analysis. Fig. 1A, shows average band intensity, expressed as fraction of the Wt signal, of RyR1, $\text{Ca}_{v1.1}$, FKBP12 and Triadin detected in crude membrane homogenates of Wt and Tdn-null myotubes. Western blot analysis revealed no significant difference in $\text{Ca}_{v1.1}$ expression between phenotypes, a result that is consistent with the expression levels observed in crude membrane homogenates of adult Tdn-null muscle [11]. Although, these results seem to support the idea that triadin ablation does not affect $\text{Ca}_{v1.1}$ expression studies in a newly developed triadin knockout model [12] suggest that $\text{Ca}_{v1.1}$ expression of triadin-KO muscles is differently affected in fast and slow twitch fibers, where $\text{Ca}_{v1.1}$ was found to be up- and down-regulated, respectively. Whereas the effect of triadin on DHPR expression of adult Tdn-null fibers may need further study our results suggest that ablation of triadin does not appear to affect relative expression levels of $\text{Ca}_{v1.1}$ in cultured cells. Lack of effect of triadin in $\text{Ca}_{v1.1}$ expression is also supported by previous reports in rat cultured myotubes showing that severe dysregulation of triadin expression did not result in changes in DHPR Ca^{2+} current density [7]. Unlike $\text{Ca}_{v1.1}$, there was a significant up-regulation in the expression of RyR1 to $150 \pm 20\%$ of the Wt levels in null myotubes. This increased expression of RyR1 is consistent with our previous finding showing that Tdn-null muscles displayed both significant up-regulation of RyR1 in adult Soleus muscle and increased ^3H -ryanodine binding densities in total adult muscle homogenates [11]. In an apparent disagreement with our results Oddoux et al [12] reported no statistical differences in RyR1 expression between Wt and triadin KO muscles, a difference that could be related to a higher dispersion in densitometric data in Oddoux's report. As reported previously [13],

expression of FKBP12 was also up-regulated in Tdn-null myotubes to $216 \pm 21\%$ of the Wt expression levels.

Fig 1B compares representative immuno-pattern of expression of RyR1 and $\text{Ca}_v1.1$ between Wt and Tdn-null myotubes. Both Wt and Tdn-null cells displayed identical punctate pattern of labeling characteristic of channels properly localized at peripheral junctions, strongly suggesting that lack of triadin expression had not detectable effect on the sub-cellular localization of either receptor. This is consistent with immuno-fluorescence studies in triadin KO muscles that found no difference in co-localization pattern of $\text{Ca}_v1.1$ and RyR1 expression in EDL muscle fibers of Wt and KO mice [12]. Altogether, these results confirm that the lack of triadin expression seems to have a negligible effect on targeting and interaction of the $\text{Ca}_v1.1$ -RyR1 pair.

3.2 Triadin expression does not alter DHPR Ca^{2+} current densities but it modulates its kinetic of activation

It is accepted that DHPR Ca^{2+} current densities are highly dependent on a physical coupling with RyR1 as a result of retrograde signals sent to the DHPR from RyR1 [18,19]. Although the above results suggest that overall physical integrity of the DHPR/RyR1 interaction appear to be unaffected in Tdn-null myotubes it was important to determine whether the lack of triadin could affect the functional cross-talk between these two channels. To determine this, whole-cell patch clamp records of Wt and Tdn-null myotubes were obtained in response to a series of voltage potential pulses. Each cell was subjected to a total of 13 voltage steps of increasing amplitude and a constant duration of 200 ms starting from a holding potential of -50mV . Fig. 2A shows representative records of Ca^{2+} current for both of Wt and Tdn-null cell displaying Ca^{2+} currents typical of cultured myotubes [20–22] with a slow kinetic of activation and peak current at approximately $+30\text{mV}$. The voltage-dependence of the Ca^{2+} conductance displayed by each phenotype is shown in Fig. 2B. The curves are a Boltzmann fit of the population mean with parameters indicated in the figure legend. In addition to these data, the Boltzmann parameters of the Ca^{2+} conductance averaged for 11 cells in each case are shown in Table I. The overall data indicates that both the maximum Ca^{2+} current density and the voltage-dependence of Tdn-null cell were similar to those of Wt cells. However, $V_{1/2}$ of Tdn-null myotubes had a slight but significant shift toward more negative potentials ($p < 0.05$). In addition, a close examination of the Ca^{2+} current traces revealed an apparent difference in kinetic of activation between Wt and Tdn-null cells was (Fig. 3A). Kinetic analysis of the Ca^{2+} currents shows that activation time constant of the fast (τ_{fast}) component of the current was not significantly affected in Tdn-null cells, however, the activation time constant of the slow component (τ_{slow}) was significantly reduced from 37.2 ± 2.5 ms in Wt to 29.5 ± 2.5 ms in Tdn-null myotubes ($p < 0.05$, Fig. 3B). This change in activation kinetics in Tdn-null cells resulted in a change in the relative proportions of the two components with the fast component accounting for a larger fraction of the total current in Tdn-null myotubes than in Wt cells (Fig. 3C). Therefore, the kinetic analysis reveals that triadin expression confers unique activation kinetics to the α_{1S} subunit, suggesting that the DHPR activity is not insensitive to the loss of triadin. Interestingly, the effects of triadin ablation on DHPR Ca^{2+} current kinetics seem to resemble those induced by the loss of RyR1 expression in dyspedic myotubes [23,24], raising the possibility that the effects of triadin in current kinetics may, to some extent, be mediated by changes in the interaction between DHPR and RyR1. Overall, these results suggest for the most part that the function of DHPR as Ca^{2+} channel in Tdn-null myotubes appears to be unaffected. However, both the shift in voltage-dependence and the change in Ca^{2+} current kinetics displayed by Tdn-null skeletal myotubes suggest that to some extent the DHPR Ca^{2+} channel activity is susceptible to modulation by skeletal triadin.

3.3 Triadin ablation does not prevent orthograde signaling between RyR1 and DHPR

To examine the effect of triadin expression on orthograde signaling between DHPR and RyR1 whole-cell patch clamp analysis of L-type Ca^{2+} currents was combined with simultaneous fluorometric recordings of intracellular Ca^{2+} release. Myotubes were loaded with 5 μM Fluo-3 in the patch pipette and then depolarized with a series of potential steps of 10 mV starting from a holding potential of -50 mV. Fig. 4A shows that whereas myotubes from both genotypes responded to depolarization with robust Ca^{2+} transients small differences in voltage dependence and peak transient amplitude ($\Delta F/F_0$) were apparent between Wt and Tdn-null cells. Like the voltage dependence of DHPR Ca^{2+} current the voltage dependence of Ca^{2+} transients of Tdn-null myotubes was slightly shifted toward more negative potentials with half maximal activation ($V_{1/2}$) of -8.9 ± 1.0 mV and -11.2 ± 1.3 mV for Wt and Tdn-null myotubes, respectively. However, this difference was not statistically significant ($p > 0.05$, Fig. 4B and Table II), suggesting that the changes in voltage dependence of Ca^{2+} currents did not affect intracellular Ca^{2+} release. This is consistent with the well-documented insensitivity of skeletal EC-coupling to extracellular Ca^{2+} entry [22,25,26]. Maximal intracellular Ca^{2+} release amplitude ($\Delta F/F_0$) was also reduced from 0.83 ± 0.05 in Wt myotubes to 0.62 ± 0.07 in Tdn-null cells, a reduction of approximately 25% ($p < 0.05$, Fig. 3C.) similar to the difference observed previously using K^+ depolarization [11].

Since no detectable differences in $\text{Ca}_v1.1$ expression levels, its targeting to the plasma membrane or the overall DHPR Ca^{2+} current density were observed between Wt- and Tdn-null cells it seems unlikely that the reduced orthograde signal displayed by Tdn-null cells could be attributed to alteration of the DHPR function. Instead, an impairment of either the allosteric transmission of the orthograde signal to RyR1 or RyR1-mediated Ca^{2+} release itself seem more feasible. In this regard, our previous studies have shown that both a significant disruption FKBP12/RyR1 interaction and a sizable reduction in SR Ca^{2+} content are associated with triadin ablation [13] and either of these effects by itself could account for the reduction in amplitude of the Ca^{2+} release signal seen in triadin-null myotubes.

3.4 FKBP12.6 expression prevents the effect of triadin ablation on orthograde signaling

Previously we have shown that over-expression of FKBP12.6 in Tdn-null myotubes almost completely ablates the effects that the loss of triadin has on Ca^{2+} homeostasis. Because of its higher affinity for RyR1, over-expression of FKBP12.6 seems to overcome the defective interaction of endogenous FKBP12, reducing SR Ca^{2+} leak and restoring normal SR Ca^{2+} load [13]. To assess whether the alteration of the FKBP12/RyR1 interaction plays any role in diminishing orthograde signaling of Tdn-null myotubes we tested the effect FKBP12.6 over-expression on the amplitude of depolarization-induced Ca^{2+} transients in cultured cells permanently over-expressing FKBP12.6 (herein Wt_{12.6} and Tdn_{12.6}). Fig 5A shows average peak Ca^{2+} transient of Fura2-AM loaded control Wt and Tdn-null myotubes stimulated by K^+ -induced depolarization. Fluorescence signals are expressed as 340/380 ratios to allow for comparable estimation of the amount of Ca^{2+} released. As previously observed using electrically-evoked depolarization (Fig. 4) Tdn-null myotubes exposed to K^+ depolarization displayed significantly smaller peak Ca^{2+} amplitude than that of Wt cells. As seen in Fig. 5A, at 60mM K^+ Wt and Tdn-null myotubes displayed a peak 340/380 ratio of 1.32 ± 0.03 ($N=76$ cells) and 1.10 ± 0.03 ($N=31$ cells), respectively ($p < 0.0001$, unpaired t test with Welch's correction). Over-expression of FKBP12.6, however, eliminated this difference with Wt_{12.6} and Tdn_{12.6} displaying nearly identical peak responses to K^+ (Fig. 5B). Whereas over-expression of FKBP12.6 resulted in significant increase of the peak Ca^{2+} amplitude of Tdn_{12.6} myotubes the expression of FKBP12.6 had an apparent opposite effect in Wt_{12.6} cells where it caused a slight but statistically significant reduction in peak Ca^{2+} amplitude (Fig 5B). This seemingly opposite effect of FKBP12.6, however, is consistent with both the

inhibitory action that FKBP12.6 exerts on RyR1 channel activity and the subsequent effect on the replenishment of the SR Ca^{2+} stores in Tdn_{12.6} but not in Wt_{12.6} [13].

In addition to having a similar amplitude in peak Ca^{2+} release, both Wt_{12.6} and Tdn_{12.6} myotubes showed an increased sensitivity to K^{+} depolarization, as is apparent from the leftward shift in K^{+} -sensitivity from $24.1 \pm 0.7 \text{ mM K}^{+}$ (N=70 cells) and $23.8 \pm 0.7 \text{ mM K}^{+}$ (N=30 cells) in Wt and Tdn-null cells, to $18.0 \pm 0.6 \text{ mM K}^{+}$ (N=68 cells) and $17.4 \pm 0.6 \text{ mM K}^{+}$ (N=43 cells) in Wt_{12.6} and Tdn_{12.6}, respectively (Fig 5B, $p < 0.05$). In this regard, since FKBP12.6 also seems to interact with sites in RyR1 different from that of FKBP12 [27,28] it is not unlikely that some of the functional effects observed on myotubes over-expressing FKBP12.6 may not be dependent on triadin. Interaction of FKBP12.6 with a site distinct from that of FKBP12 may possibly explain the shift in K^{+} -sensitivity and the slight reduction of the peak Ca^{2+} transients observed in Wt_{12.6} cells.

Overall these results strongly suggest that over-expression of FKBP12.6 in Tdn-null cells restored FKBP/RyR interactions that are important for EC-coupling, sensitizing Ca^{2+} release to K^{+} depolarization to levels similar of Wt_{12.6} myotubes. These results are consistent with Avila et al. [29], who has shown that disruption of FKBP binding site of RyR1 severely compromise voltage-gated SR Ca^{2+} release (orthograde coupling) without altering RyR1-mediated enhancement of L-channel activity (retrograde coupling). More importantly, over-expression of FKBP12.6 completely restored normal voltage-gated Ca^{2+} release of the myotubes expressing FKBP-binding deficient RyR1. The striking similarities of the EC-coupling signaling between Tdn-null and the FKBP-deficient RyR1 phenotype strongly suggest that the effect of triadin ablation on SR Ca^{2+} release may be primarily, if not exclusively, mediated by disruption of the FKBP/RyR1 interaction observed in Tdn-null cells. However, unlike Avila et al. [29] where FKBP12.6 over-expression did not seem to affect voltage-dependence sensitivity, the shift in K^{+} -sensitivity observed in Wt_{12.6} and Tdn_{12.6} cells suggests a significant sensitization of the orthograde signaling in our FKBP12.6-expressing cells, a difference that might result from the dissimilarity in the depolarization protocol used (electrical vs K^{+}). In addition, it is worth noting that disruption of the FKBP/RyR1 interaction in Tdn-null myotubes was also associated with significant increase of myoplasmic resting free Ca^{2+} concentration and reduced SR Ca^{2+} load [13], two phenomena that apparently were not affected in Avila's report, which suggests that triadin ablation may result in physiological effects that go beyond the effects expected from the sole disruption of the FKBP/RyR1 interaction.

The use of triadin binding-deficient RyR1 mutant channels expressed in dyspedic myotubes has suggested that triadin is a critical component of EC-coupling [8]. In agreement with our data triadin binding-deficient RyR1 did not significantly affect DHPR Ca^{2+} current density (retrograde signaling) or voltage-dependence. However, the mutated RyR1 channels showed a reduction in depolarization-induced Ca^{2+} release signal (orthograde signal) of up to 90%, depending on severity of disruption of the triadin/RyR1 interaction. Whereas our voltage clamp and K^{+} -induced depolarization data in Tdn-null myotubes did confirm a 30% reduction of Ca^{2+} release associated with triadin ablation (Fig. 5A) this effect was significantly milder than that reported by Goonesekera et al. [8]. Our data are also consistent with the result of others where triadin expression in skeletal myotubes was transiently knocked down using siRNA [9,10] or knocked out by homologous recombination [12]. An intriguing difference between these studies is that while the use of siRNA and knock out systems abrogate the expression of triadin Goonesekera's report on triadin binding-deficient RyR1 myotubes was conducted in the presence of endogenous triadin. This approach, in essence, creates an excess of RyR1-free triadin that may favor interactions with other triadic components (i.e. Calsequestrin, Histidine-rich Ca^{2+} binding-proteins and triadin itself) that normally may be prevented by its association with RyR1 and that indirectly may modulate

the efficiency of Ca^{2+} release. Triadin has been reported to inhibit RyR1 channel activity through an interaction involving the cytoplasmic domains of both proteins [30,31], this interaction should have remained intact in Goonasekera's study that targeted the triadin-binding site at the intra-luminal domain of RyR1. Accordingly, over-expression of skeletal triadin in cultured myotubes has shown to cause severe impairment of the EC-coupling signaling similar to that observed in cells expressing triadin binding-deficient RyR1 [7]. This, added to the extended effects of triadin ablation on myoplasmic resting Ca^{2+} and SR Ca^{2+} content [13] seems to support the idea that triadin may engage in a broad range of protein-protein interactions with proteins other than RyR1 [32,33].

Overall, our data reveal minimal, or not effect, of triadin ablation in DHPR Ca^{2+} current, suggesting that the effects of triadin in EC-coupling signaling are located downstream of depolarization. Since Tdn-null RyRs channels show no differences in sensitivity to activation by agonists with wt-RyRs [11] our results seem to point out to a defect in the allosteric transmission of the depolarization signal to the RyRs. Full restoration of EC-coupling signal of Tdn-null myotubes by over-expression of FKBP12.6 strongly suggests that the effects of triadin on the allosteric transmission are not direct but indirectly mediated by the disruption of the FKBP/RyR1 interaction that we have recently described in Tdn-null cells. In conclusion, it appears that triadin is not directly involved in bi-directional coupling between DHPR and RyR1. Therefore, as its non-lethal phenotype seems to suggest, triadins would not be mandatory component of skeletal EC-coupling. However, since moderate changes in DHPR Ca^{2+} current kinetics were evident in Tdn-null myotubes our results cannot exclude the possibility that triadins may play some role in modulating the cross-talk between the DHPR and RyR1.

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Fig. 1. Effect of triadin ablation on expression and targeting of DHPR and RyR1

A) Equivalent amounts of crude membrane homogenates (30 μ g total protein/lane) from Wt and triadin-null myotubes were loaded and immunoblotted for expression of RyR1, DHPR α_{1S} , FKBP12 and triadin. Band intensity is expressed as fraction of the Wt signal (dotted line). Numbers in the bars indicate the number of blots analyzed per protein. Data presented as mean $\pm$ SEM. *: $p < 0.05$, **: $p < 0.01$. B) Immuno-staining pattern of Wt and Tdn-null myotubes incubated with anti-RyR1 and anti- $\text{Ca}_v1.1$ antibodies. Pictures depict a focal plane near to the outside surface of the myotubes, bar: 10 μ M.

Fig. 2. L-type Ca^{2+} current densities are not affected in Tdn-null myotubes

A) Representative recordings of L-type Ca^{2+} currents elicited by 200-ms depolarizations to the indicated test potentials displayed by Wt and Tdn-null myotubes. B) Comparison of average current-voltage dependence of peak current densities of Wt (black) and Tdn-null (grey) myotubes fitted according to Equation 1. The currents were evoked by a trend of 200 ms pulses starting from a holding potential of -50mV in steps 10mV after a pre-pulse protocol. Parameters for each plot are presented in Table 1. Data expressed as mean $\pm$ SEM.

Fig. 3. Kinetics of activation of Ca^{2+} currents displayed by Wt and Tdn-null myotubes
 A) Representative normalized Ca^{2+} currents of Wt and Tdn-null cells in response to a 200 ms depolarization pulse to +30 mV from a holding potential of -50 mV. All currents were fitted to a bi-exponential curve and their corresponding average τ slow and τ fast at +30 mV are shown in (B). τ slow for Wt and Tdn-null myotubes are: 37.2 ± 2.5 ms (N=11) and 29.5 ± 2.5 ms (N=11), respectively. τ fast for Wt and Tdn-null myotubes are: 2.4 ± 1.8 ms (N=9) and 4.1 ± 0.6 (N=10), respectively. C) Average contribution of the fast and slow components of the bi-exponential fit. Values are expressed as fraction of the total amplitude. Data presented as mean $\pm$ SEM, *:p<0.05.

Fig. 4. Orthograde signaling is weakened in Tdn-null myotubes

A) Representative traces of depolarization-induced intracellular Ca^{2+} transients from Wt and Tdn-null myotubes. Step depolarizations from -40 to $+40$ mV were applied in 10 mV increments (200 ms test pulses) from a holding potential of -50 mV. $\Delta F/F_0$ indicates increments of Ca^{2+} -induced Fluo-3 fluorescence (F) with respect to basal fluorescence (F_0). Depolarization protocol is shown at the bottom. B) Comparison of the average voltage dependence of depolarization-induced Ca^{2+} transients of Wt and Tdn-null myotubes. Maximal amplitude of transient elevations of cytoplasmic Ca^{2+} from Tdn-null cells were significantly reduced in comparison to Wt cells ($p < 0.05$). Parameters for each plot are presented in Table 2. Values represented as mean $\pm$ SEM.

Fig. 5. Expression of FKBP12.6 restores orthograde signaling to Tdn-null myotubes

A) Average peak fluorescent amplitude of K^+ -induced Ca^{2+} release transients observed in Fura-2 loaded Wt (black circles) and Tdn-null (grey open circles) myotubes. B) Effect of co-expression of FKBP12.6 on the average peak amplitude of K^+ -induced Ca^{2+} transients of Wt_{12.6} (black open circles) and Tdn_{12.6} (grey circles) myotubes. Dotted lines correspond to the K^+ dose responses plotted in Fig 5A. Data presented as mean $\pm$ SEM. Values of EC_{50} are based on fits of averaged data (n=2 experiments, 31–76 cells) to a sigmoidal dose-response.

Table ICa²⁺ conductance displayed by Wt and Tdn-null myotubes

	$G_{\max}$ (pS/pF)	$V_{1/2}$ (mV)	k (mV)	n
Wt	223 ± 13	22.2 ± 0.9*	5.8 ± 0.2	11
Tdn-null	226 ± 24	16.6 ± 1.1*	6.6 ± 0.2	11

Mean ± SEM of Boltzmann parameters fitted to each individual cell using Eq. 1.

*
p<0.001, Student's t test (Welch's correction).

Table IIPeak Ca^{2+} transient amplitude of Wt and Tdn-null cells

	$F_{\max} (\Delta F/F_0)$	$V_{1/2} (\text{mV})$	$K (\text{mV})$	n
Wt	$0.83 \pm 0.05^*$	-8.9 ± 1.0	5.5 ± 0.4	8
Tdn-null	$0.62 \pm 0.07^*$	-11.2 ± 1.3	5.5 ± 0.4	8

Mean $\pm$ SEM of Boltzmann parameters fitted to each individual cell using Eq. 2. Parameters are shown for 200 ms depolarization.

* $P < 0.05$, Student's t test (Welch's correction)