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János Fodor, Monika Gönczi, Monika Sztretye, Beatrix Dienes, Tamás Oláh, et al.. Altered expression of triadin 95 causes parallel changes in localized Ca^{2+} release events and global Ca^{2+} signals in skeletal muscle cells in culture.. The Journal of Physiology, 2008, 586 (Pt 23), pp.5803-18. 10.1113/jphysiol.2008.160457 . inserm-00516686

HAL Id: inserm-00516686

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Submitted on 10 Sep 2010

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Altered expression of triadin 95 causes parallel changes in localized Ca^{2+} release events and global Ca^{2+} signals in skeletal muscle cells in culture

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The 95 kDa triadin (Trisk 95), an integral protein of the sarcoplasmic reticular membrane in skeletal muscle, interacts with both the ryanodine receptor (RyR) and calsequestrin. While its role in the regulation of calcium homeostasis has been extensively studied, data are not available on whether the overexpression or the interference with the expression of Trisk 95 would affect calcium sparks the localized events of calcium release (LCRE). In the present study LCRE and calcium transients were studied using laser scanning confocal microscopy on C2C12 cells and on primary cultures of skeletal muscle. Liposome- or adenovirus-mediated Trisk 95 overexpression and shRNA interference with triadin translation were used to modify the level of the protein. Stable overexpression in C2C12 cells significantly decreased the amplitude and frequency of calcium sparks, and the frequency of embers. In line with these observations, depolarization-evoked calcium transients were also suppressed. Similarly, adenoviral transfection of Trisk 95 into cultured mouse skeletal muscle cells significantly decreased both the frequency and amplitude of spontaneous global calcium transients. Inhibition of endogenous triadin expression by RNA interference caused opposite effects. Primary cultures of rat skeletal muscle cells expressing endogenous Trisk 95 readily generated spontaneous calcium transients but rarely produced calcium sparks. Their transfection with specific shRNA sequence significantly reduced the triadin-specific immunoreactivity. Functional experiments on these cells revealed that while caffeine-evoked calcium transients were reduced, LCRE appeared with higher frequency. These results suggest that Trisk 95 negatively regulates RyR function by suppressing localized calcium release events and global calcium signals in cultured muscle cells.

(Received 24 July 2008; accepted after revision 2 October 2008; first published online 9 October 2008)

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In skeletal muscle shortening is initiated by the increase in intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) following the depolarization of the surface and transverse (T-) tubular membranes. Calcium ions are released from the sarcoplasmic reticulum (SR), a specialized intracellular calcium store, through ryanodine receptor (RyR) calcium channels. The depolarization is conveyed to the opening of RyR via a direct mechanical link to the dihydropyridine receptor (DHPR) of the T-tubular membrane.

The opening of a single or a group of calcium release channels gives rise to localized calcium release events (LCRE) termed calcium sparks (Tsugorka *et al.* 1995; Klein *et al.* 1996) and embers (Gonzalez *et al.* 2000).

Although these events have been studied in detail and their characteristics described in both adult (Kirsch *et al.* 2001) and embryonic skeletal muscle (Chun *et al.* 2003) certain properties of LCRE remain unclear. Specifically, the termination of the inherent positive feed-back from calcium-induced calcium release involved in these events, has not been resolved completely (see e.g. Szentesi *et al.* 2004; Miller *et al.* 2005).

In addition, while calcium sparks are readily observable in skeletal muscle fibres from amphibians and in mammalian skeletal muscle cells in culture, skeletal muscle fibres from adult mammals lack these events (e.g. Shirokova *et al.* 1998). The observation that mammalian

fibres with disrupted surface and T-tubular membranes, achieved with a mild treatment with saponin, readily produce calcium sparks (Kirsch *et al.* 2001; Zhou *et al.* 2003) led to the suggestion that a structural element is involved in the suppression of these events under physiological conditions. This was strengthened by the finding that calcium sparks are restrained to areas without T-tubules in cultured muscle cells (Zhou *et al.* 2006).

Triadin, an integral membrane protein of the SR (Brandt *et al.* 1990; Caswell *et al.* 1991), has a short N-terminal part in the cytosol and a long C-terminal inside the lumen of the SR (Marty *et al.* 1995). Its 95 kDa skeletal isoform (Trisk 95) has been localized to the junctional membrane of the terminal cisternae in the SR (Marty *et al.* 1995) and suggested to bind to both calsequestrin (CSQ) and to RyR (Knudson *et al.* 1993; Caswell *et al.* 1999; Kobayashi *et al.* 2000). As a link between CSQ and RyR it could be the molecule that conveys the control associated with calsequestrin to the calcium release channel (Beard *et al.* 2002).

Recent evidence has, however, both questioned and strengthened the role of triadin in excitation–contraction (E–C) coupling. The lack of an obvious phenotype and the absence of contractile dysfunction in triadin knock-out mice led to the suggestion that triadins are not essential for E–C coupling (Shen *et al.* 2007). Nevertheless, RyRs with altered C-terminal luminal sequences, which interfered with Trisk 95 binding, gave rise to reduced SR calcium release in response to both depolarization and RyR agonists (Goonasekera *et al.* 2007) indicating that triadin binding is crucial for normal RyR function.

Here we demonstrate that altered expression of Trisk 95 results in a parallel modification in both the calcium transients and the localized calcium release events. Overexpression of the protein resulted in a decreased calcium release from the SR and in the reduction of both the frequency and amplitude of calcium sparks. Interference with the translation of the protein, on the other hand, increased the frequency and amplitude of these localized events of calcium release. Our observations clearly support the idea that triadin plays an important role as a negative regulator of RyR opening.

Some aspects of this work were presented to the Biophysical Society (Csernoch *et al.* 2007).

Methods

Cell cultures and transfection

C2C12 skeletal muscle cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U ml⁻¹ penicillin, and 50 µg ml⁻¹ streptomycin and were incubated at 37°C in a humidified incubator with 5% CO₂–95% O₂ as described in our earlier studies (Deli *et al.* 2007). Full length coding sequence of Trisk 95 was ligated into the *Eco*R1

restriction site of pcDNA 3.1 expression vector (Marty *et al.* 2000). Transfection was performed in Opti-MEM reduced serum content medium using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) for 2.5 h at 37°C. Cells were allowed to express the introduced genes for 48 h in growth medium then were selected in DMEM containing 1000 µg ml⁻¹ geneticin. After 14–18 days, single colonies were isolated and experiments were carried out on pools of transfected cells. Differentiation of transfected cells was induced at 80% confluency by changing the culture medium for DMEM supplemented with 5% horse serum (HS), 2% FBS and penicillin–streptomycin. The efficiency of Trisk 95 overexpression was monitored by immunostaining, using specific Trisk 95-antibody labelling the C-terminus of the protein (Marty *et al.* 2000). Functional experiments were carried out on 5- to 7-day-old differentiated myotubes.

Primary cultures of skeletal muscle cells were obtained from 1-day-old mice or 5- to 10-day-old rats as described earlier (Szappanos *et al.* 2004). Animals were killed following approved protocols (14/2004.DE MAB and 15/2004.DE MAB) of the Animal Care Committee of the University of Debrecen. They were anaesthetized by ether and killed with cervical dislocation. Skeletal muscle was collected from the hindlimbs, put in Hank's solution (Sigma Chemical Co., St Louis, MO, USA) and then cut into small pieces. The muscle tissue was dissociated at 37°C using 0.75 mg ml⁻¹ collagenase type I, 1.5 mg ml⁻¹ trypsin in a calcium/magnesium free phosphate buffer. The enzymatic reaction was inhibited with the addition of 10% HS. After filtration through a multilayer nylon membrane, the cells were collected by centrifugation at 150 g for 10 min and seeded onto coverslips in a proliferation medium (Ham's F12 medium supplemented with 15% FBS and penicillin–streptomycin). On the third day of culturing the medium was changed to DMEM supplemented with 5% HS to allow differentiation of the myotubes. In this system adenoviral transfection was used to modify triadin expression. Two types of adenovirus were used (both from Gene Vector Production Network, at Genethon III; Évry, France): a control virus (rAdV5-DsRed) with the cDNA of the red fluorescent protein and the test containing the full-length sequence of skeletal muscle Trisk 95 (rAdV5-Trisk 95-ires-DsRed). All the transgenes were controlled by the CMV promoter. After 48 h proliferation, cells were incubated with the viruses for 10 h in Ham's F12 medium without serum (the cell nucleus to virus ratio was 1:40), and then differentiation was induced by changing the medium. Control cells were infected with the control virus (rAdV5-DsRed), and Trisk 95 overexpressing myotubes consisted of cells infected with rAdV-Trisk 95-ires-DsRed. Confocal microscopy studies were performed after 55–60 h of infection/differentiation.

The presence of Trisk 95 was determined by immunostaining at different differentiation stages (2, 3, 4 days in differentiation medium). The RNA interference technique was applied in order to reduce endogenous Trisk 95 expression. The shRNA was engineered by RNAi Construct Builder (Genscript, Piscataway, NJ, USA) and produced by SigmaGenosys. The chosen shRNA cassette sequence besides the sense (5'GGCAAAGATGTAAAGCCTAAA) and antisense (5'TTTAGGCTTTACATCTTTGCC) region contains a loop and termination sequence resulting in a hairpin siRNA. Blast filtering ensured that this sequence has homology only with Trisk 95 and not with any other known gene. Scrambled shRNA was used to demonstrate that the target specific shRNS did not induce a non-specific effect on gene expression. The shRNA transfection was carried out after 30–36 h of differentiation with application of Lipofectamine 2000 reagent during 2.5 h in Opti-MEM transfection medium at 37°C, 5% CO₂. Immunostaining was performed after 3–4 days of differentiation in order to monitor the efficiency of RNA interference. Calcium imaging studies were also performed at this stage using a confocal microscope.

Immunostaining

Immunolabelling was performed the same way on C2C12 cells and on primary cultures. Cultured cells were washed with ice-cold phosphate-buffered saline (PBS; 0.02 M NaH₂PO₄, 0.1 M NaCl), fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked with 1% BSA diluted in PBS (blocking solution) for 30 min at room temperature. The cells were then incubated with the anti-Trisk 95 primary antibody (dilution was 1:500 in blocking solution; the primary rabbit antibody was directed against the C-terminus of Trisk 95, the specific epitope corresponds to amino acids 673–687 of Trisk 95) for 2 h at room temperature and with fluorescein labelled anti-rabbit secondary antibody for 1 h at RT. Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) was used to make the nuclei visible. Images were obtained using fluorescence and confocal microscopy. Fluorescence intensities were calculated from regions of interest (10 on each cell) of fluorescent images with Image pro Plus software (Media Cybernetics Inc., Bethesda, MD, USA).

Whole cell calcium measurement

Changes in $[Ca^{2+}]_i$ were measured using the calcium sensitive fluorescent dye Fura-2 as reported earlier (Szapannos *et al.* 2004). Myotubes were incubated with Fura-2-AM (10 μ M) for 1 h (37°C, 5% CO₂) in DMEM

supplemented with 10% FBS and neostigmin to inhibit choline-esterase. C2C12 myotubes were then placed on the stage of an inverted fluorescence microscope. Measurements were performed in normal Tyrode solution (in mM: 137 NaCl, 5.4 KCl, 0.5 MgCl₂, 1.8 CaCl₂, 11.8 Hepes, 1 g l⁻¹ glucose, pH 7.4) or calcium-free Tyrode solution. To obtain a calcium-free Tyrode solution, no CaCl₂ was added in the presence of 5 mM EGTA. In the depolarizing solution 120 mM NaCl was exchanged for KCl. Caffeine was prepared as a stock solution in Tyrode buffer and used at a final concentration up to 30 mM. Excitation wavelength was alternated between 340 and 380 nm by a dual wavelength monochromator (Deltascan, Photon Technology International, New Brunswick, NJ, USA), while the emission was monitored at 510 nm using a photomultiplier. $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence intensities ($R = F_{340}/F_{380}$) using an *in vivo* calibration ($R_{min} = 0.2045$, $R_{max} = 8.315$, $K_d\beta = 1183$).

Confocal measurements

Localized calcium release events were monitored with the LSM 510 META confocal laser scanning microscope (Zeiss, Oberkochen, Germany) as described earlier (Szentesi *et al.* 2004). In brief, C2C12 myotubes were treated with a modified relaxing solution (in mM: 125 potassium glutamate, 10 Hepes, 1 EGTA, 5 MgCl₂, 5 Na-ATP, 10 sodium phosphocreatine, 10 glucose, 0.13 CaCl₂ and 8% dextran) containing 0.002% saponin for 2–3 min. Permeabilization of the surface membrane was monitored by the addition of 50 μ M Fluo-3 into the solution and imaging the fibre. This solution was then exchanged with an internal medium (in mM: 125 potassium glutamate, 10 Hepes, 1 EGTA, 5 MgCl₂, 5 Na-ATP, 10 sodium phosphocreatin, 10 glucose, 0.13 CaCl₂, 0.1 Fluo-3 and 8% dextran).

Myotubes from primary cultures were incubated with 10 μ M Fluo-4-AM for 1 h at 37°C. Calcium imaging was performed in normal Tyrode solution. Two dimensional (x - y) and line-scan images were used to monitor the fluorescence intensity. Line-scan images were recorded at 1.54 ms per line and 512 pixels per line using a 63 $\times$ water immersion objective. Fluo-3 or -4 was excited with an argon ion laser. Images were analysed using an automatic event detection program (Szentesi *et al.* 2004), which calculated the amplitude ($\Delta F/F_0$), full width at half-maximum (FWHM), rise time and duration of the identified events. Events longer than 40 pixels (60 ms) and displaying steady fluorescence were considered as 'embers'; otherwise they were taken as 'sparks' or 'sparks with embers'. Note that rise time was calculated only for sparks, while only average amplitude was obtained for embers (Szentesi *et al.* 2004).

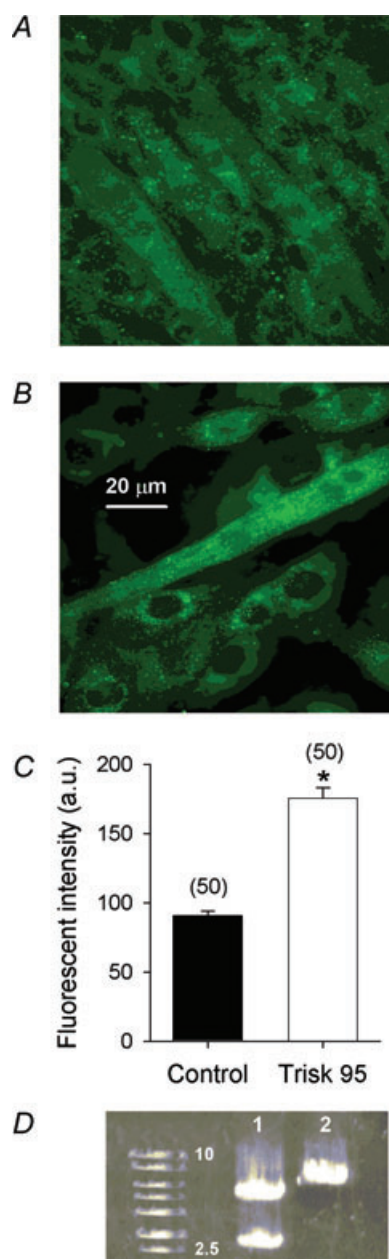


Figure 1. Overexpression of Trisk 95 in C2C12 cells

Either the pcDNA3.1 vector alone or that encoding for Trisk 95 was transfected into C2C12 cells and clones stably overexpressing the protein were selected. The presence and the amount of the protein were visualized using a triadin specific antibody. *A*, endogenous triadin 95 expression in C2C12 myotubes stably transfected with the pcDNA3.1 vector. *B*, C2C12 clones stably overexpressing Trisk 95. Vertical scale is 20 μm for the two images. *C*, pooled data of triadin expression in control and transfected cells determined by comparing the fluorescence intensities of identical areas. Asterisk marks significant difference as compared to control. Numbers in parentheses give the number of cells examined. *D*, ethidium bromide stained agarose gel showing the cDNA for Trisk 95 and the pcDNA3.1 vector before (lane 1) and after (lane 2) cloning. Left calibration shows 1 kb DNA ladder.

Statistical evaluation

Data are displayed as means $\pm$ S.E.M. To assess the significance of the differences, Student's *t* test or for very asymmetric distributions the non-parametric Mann–Whitney test (SPSS statistical software for Windows, SPSS Inc., Chicago, IL, USA) was used. A difference was regarded as significant when $P < 0.05$.

Results

Calcium transients in triadin overexpressing C2C12 cells

The effect of triadin 95 overexpression was examined in a mouse skeletal muscle cell line. Endogenous expression of this regulatory protein could be demonstrated in C2C12 myotubes (Fig. 1*A*). Trisk 95 protein synthesis has been amplified using a pCDNA3.1 plasmid-vector system. The successful ligation of the plasmid vector and triadin 95 cDNA was checked prior to transfection using agarose gel electrophoresis (Fig. 1*D*). The stable overexpression of the protein in the selected clones was confirmed with immunocytochemistry at the myotube stage (Fig. 1*B*). Quantitative analysis of the fluorescence intensities revealed a significant ($P < 0.01$) 94% overexpression of the protein as compared to C2C12 cells transfected with the empty pCDNA3.1 plasmid vector (Fig. 1*C*).

Control and Trisk 95-transfected, differentiated (5–7 days old) C2C12 myotubes were used for functional experiments. First we examined whether the protein overexpression alters the calcium homeostasis of the myotubes. The resting intracellular calcium concentration was similar ($P > 0.2$) in both groups (77.4 ± 5.2 nM, $n = 48$ in control and 71.3 ± 4.8 nM, $n = 54$ in Trisk 95 overexpressing cells). The presence of functional ryanodine receptors and the presence of releasable calcium in the SR were investigated using 15 mM caffeine (Fig. 2*A*). No significant difference was observed in the amplitude of caffeine-induced transient elevation of $[\text{Ca}^{2+}]_i$ between control and Trisk 95 overexpressing myotubes (142 ± 10 nM, $n = 41$ and 123 ± 11 nM, $n = 44$, respectively). Calcium transients could also be evoked by depolarization using 120 mM KCl both in normal (extracellular calcium concentration 1.8 mM; Fig. 2*B*) and in a Ca^{2+} -free bathing solution (Fig. 2*C*). In the presence of extracellular calcium the amplitude of KCl-induced calcium transients were similar in control and in triadin overexpressing myotubes (168 ± 10 nM, $n = 48$ and 200 ± 31 nM, $n = 54$, respectively). In contrast, the amplitude of the KCl-induced calcium transients in Ca^{2+} -free solution was significantly reduced in Trisk 95 overexpressing myotubes (98 ± 10 nM, $n = 42$) as compared to control cells (170 ± 14 nM, $n = 29$).

Figure 2*D* plots pooled data to confirm that triadin overexpression had little, if any, effects on the caffeine-evoked calcium transients suggesting that the calcium content of the SR was unaffected under these conditions. On the other hand, depolarization-evoked calcium transients in the absence of external calcium, that is when calcium release from the SR was the sole source of the calcium ions appearing in the myoplasm, were significantly reduced in Trisk 95 overexpressing cells as observed previously on rat primary skeletal muscle cell culture (Rezgui *et al.* 2005).

Localized calcium release events in triadin overexpressing C2C12 cells

To understand the underlying reasons for the suppressed SR calcium release, localized calcium release events were examined in control and in triadin overexpressing cells using laser scanning confocal microscopy. Control myotubes readily displayed both calcium sparks (calcium release events with large amplitude and short duration; Fig. 3*A*, left panel) and embers (events with small amplitude and long duration; Fig. 3*A*, right panel).

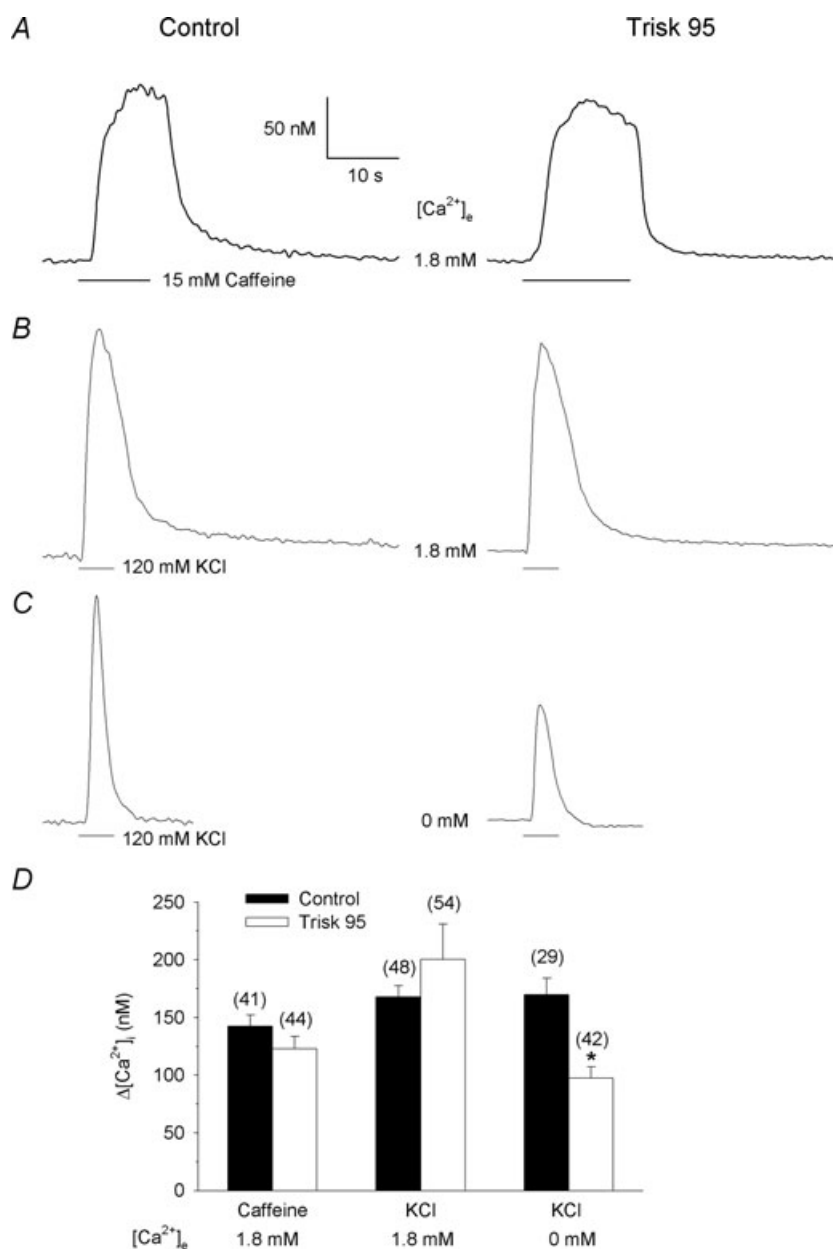
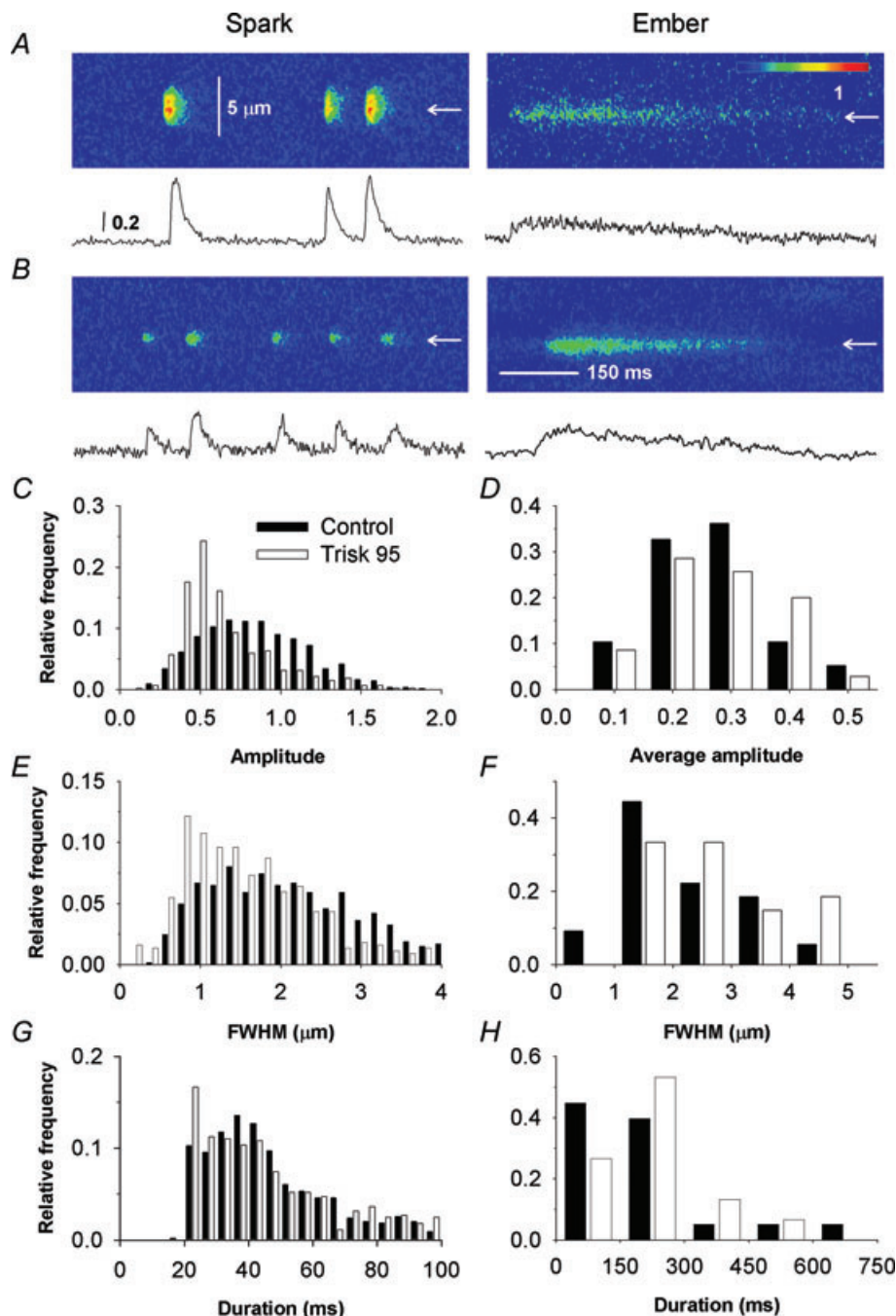


Figure 2. Whole cell Ca^{2+} transients in control and in Trisk 95 overexpressing myotubes

A–C, representative Ca^{2+} transients evoked by the addition of either caffeine (15 mM; *A*) or KCl (120 mM; *B* and *C*) detected using Fura-2 in control (left column) and in triadin-overexpressing (right column) cells. KCl-evoked calcium transients were measured both in the presence (*B*) and in the absence of external calcium (*C*). *D*, pooled data for the amplitude of the calcium transients ($\Delta[Ca^{2+}]_i$). Numbers in parentheses give the number of cells, while the asterisk denotes statistically significant difference as compared to control.

It should be noted, however, that the number of calcium sparks was far greater than that of embers. Although, myotubes following the transfection with the triadin-plasmid generated calcium sparks and embers as

control cells did (Fig. 3B), the characteristic parameters of these events were altered. Plotting the amplitude histograms of LCRE (Fig. 3C and D) revealed that triadin overexpression increased the relative frequency of



calcium sparks with small amplitudes while the relative frequency of calcium sparks with large amplitudes were reduced as compared to control myotubes (Fig. 3C). The distribution of the average amplitude of embers, on the other hand, was not altered in triadin overexpressing C2C12 cells (Fig. 3D). Similarly, the histograms of FWHM demonstrate that the overexpression of triadin shifted the distribution to the left for sparks (Fig. 3E), whereas that of the embers remained unaffected (Fig. 3F). The distribution of event duration, on the other hand, was altered neither for sparks (Fig. 3G) nor for embers (Fig. 3H) by the overexpression of Trisk 95.

In accordance with the above, pooled data show that on average the amplitude of the calcium sparks was significantly smaller in triadin overexpressing myotubes than in control cells (0.59 ± 0.01 versus 0.81 ± 0.01 ; $P < 0.001$), while the average amplitude for the embers was only slightly affected in Trisk 95 overexpressing myotubes (0.22 ± 0.01 versus 0.26 ± 0.02 ; $P > 0.07$). While we could not observe any significant difference between the duration (47.1 ± 0.9 versus 51.1 ± 1.8 ms for sparks and 215 ± 21 versus 224 ± 21 ms for embers in control and triadin overexpressing cells, respectively; $P > 0.3$) or rise time (14.1 ± 0.4 versus 15.7 ± 0.6 ms; $P > 0.1$) of events in the two groups, FWHM for calcium sparks, but not for embers, was significantly smaller in triadin transfected myotubes ($1.26 \pm 0.07 \mu\text{m}$; $P < 0.01$) than in control cells ($1.86 \pm 0.06 \mu\text{m}$). In addition, the frequency of the spontaneous calcium release events was also significantly ($P < 0.05$) reduced in Trisk 95 overexpressing myotubes (1.04 ± 0.14 Hz for sparks and 0.09 ± 0.04 Hz for embers) as compared to the control group (1.85 ± 0.21 Hz for sparks and 0.19 ± 0.02 for embers).

Triadin overexpression in mouse skeletal muscle cells in primary culture

The overexpression of triadin in primary cultured skeletal muscle cells of mice was carried out using an adenoviral transfection. Differentiated myotubes were infected either with the virus encoding only the DsRed label (control, Fig. 4A) or with the virus containing both the Trisk 95 and DsRed cDNA (Fig. 4B). Experiments were carried out on six control and four Trisk 95 overexpressing differentiated myotubes. Both control and triadin overexpressing myotubes generated calcium transients (Fig. 4C and D, respectively), probably triggered by spontaneous action potentials as tests on some cells showed that they could be blocked by $0.3 \mu\text{M}$ tetrodotoxin (see online Supplemental Material). These calcium transients were visualized using laser scanning confocal microscopy in the line-scan mode. The analysis of these images revealed that both the amplitude and the frequency of the calcium

transients in triadin overexpressing myotubes were significantly reduced (0.56 ± 0.03 and 0.26 ± 0.01 Hz, respectively, $n = 71$; $P < 0.02$ and 0.001 , respectively; Fig. 4D) as compared to controls (0.68 ± 0.02 and 0.75 ± 0.04 Hz, respectively, $n = 169$; Fig. 4C).

Calcium transients were also elicited using external electrical stimulation as shown in Fig. 4E and F. These transients, as expected, did not differ substantially in their amplitude and kinetics from their spontaneous counterparts. The observations also confirmed the significant ($P < 0.05$) reduction in the amplitude following the overexpression of Trisk 95, from a control value of 0.64 ± 0.09 ($n = 17$, four cells) to 0.46 ± 0.05 ($n = 45$, nine cells).

Inhibition of endogenous triadin expression using specific shRNAs in rat primary cultured skeletal muscle cells

In contrast to the measurements on triadin overexpression, to study the effects of reduced Trisk 95 expression a system where the frequency of LCRE is relatively low under control conditions – rat myotubes in primary culture (see Fig. 7) – was selected. Immunocytochemical analysis at different stages of culturing revealed that endogenous expression of triadin 95 in these cells started from the third day after switching to differentiating medium. When large, multinucleated myotubes were formed (Fig. 5B), usually on the third day of culture, the distribution of triadin displayed a characteristic dotted pattern (Fig. 5C). Differentiating myotubes were transfected with Rhodamine-red labelled Trisk 95 specific shRNA using a liposome-induced transfection protocol. Two days after transfection the shRNA positive cells were easily identified by their red fluorescence (Fig. 5A). However, red fluorescence was not homogeneous within the myotubes, rather, brighter and darker areas were clearly distinguishable. Importantly, areas where intense red fluorescence was observed showed suppressed triadin expression as assessed by their less intense labelling for Trisk 95 (Fig. 5C). This correlation, although to various degrees, was present in all cultures ($n = 6$) examined (Fig. 5E).

In some experiments we utilized this observation by comparing data measured in cells (subcellular areas) with intense red fluorescence, assumed to have suppressed Trisk 95 expression, to those obtained from cells with low red fluorescence. This gave, in addition to the measurements with scrambled shRNA, a possibility to obtain 'endogenous' controls.

Calcium transients in cultured rat skeletal muscle cells with suppressed triadin expression

As a first step global calcium transients were measured on shRNA transfected cells. Addition of 30 mM caffeine

to the bathing medium induced a transient elevation in $[Ca^{2+}]_i$ both on control myotubes and on myotubes with suppressed Trisk 95 expression (Fig. 6A). The latter transients were always smaller than those in control suggestive of a reduced SR calcium content. This was confirmed by the pooled data for the amplitude of the caffeine-induced calcium transients demonstrating a clear and significant reduction following the trans-

fection with the shRNA (Fig. 6C). In line with this observation, the resting $[Ca^{2+}]_i$ was found to be increased in myotubes with reduced Trisk 95 expression if held in a calcium free extracellular solution (64.9 ± 3.3 versus 85.7 ± 6.9 nM; $n = 15$ and 12; control myotubes and myotubes with reduced triadin expression, respectively). In addition, depolarization, induced by the addition of 120 mM KCl in the absence of external calcium, could

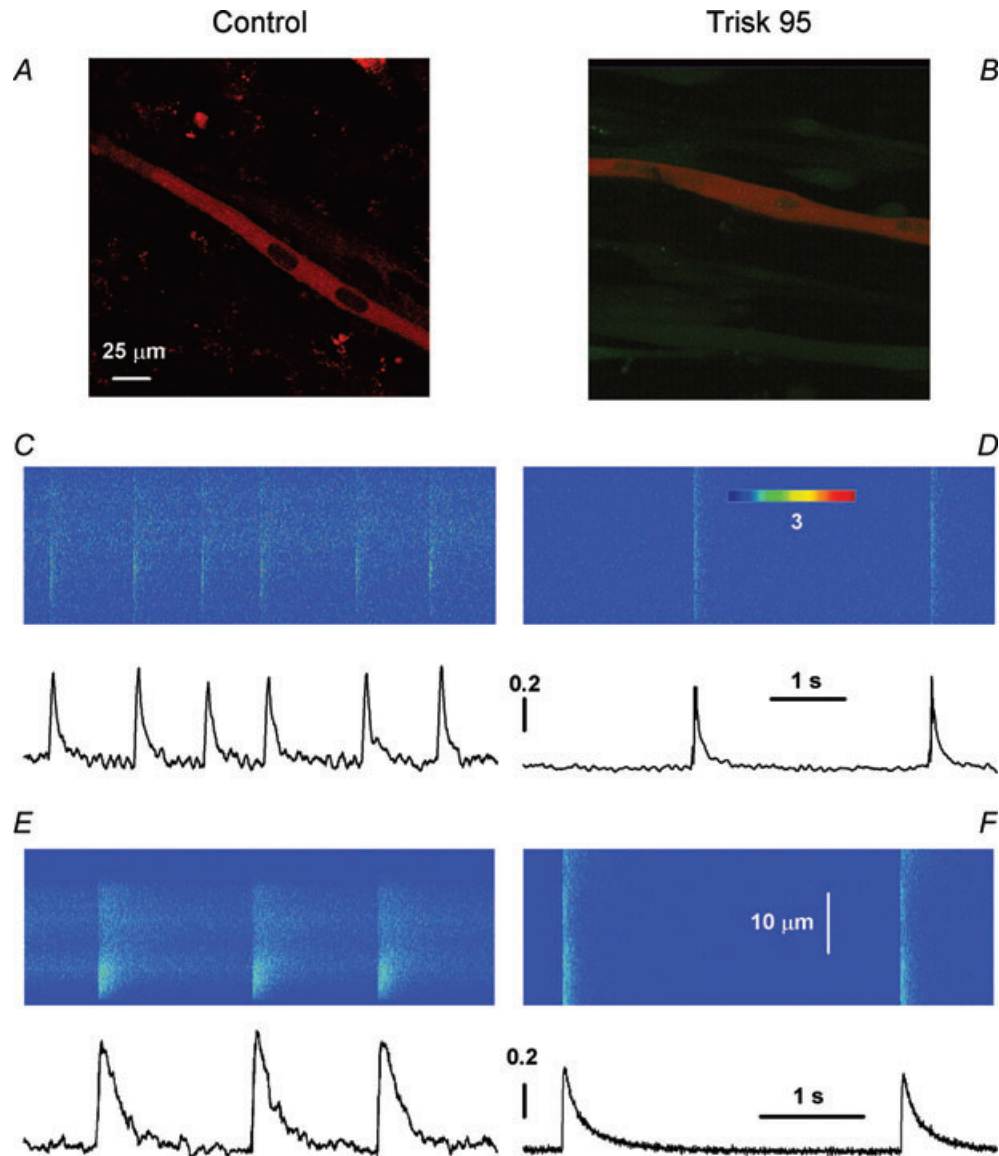


Figure 4. Spontaneous Ca^{2+} transients in mouse myotubes infected with an adenovirus vector encoding either DsRed only or Trisk 95 and DsRed

A and B, images of infected myotubes were visualized by their red fluorescence. Scaling bar represents $25 \mu\text{m}$ for both images in panels A and B. Green fluorescence is from Fluo-3. C and D, line-scan images of spontaneous Ca^{2+} transients detected in control (expressing DsRed only; C) and in Trisk 95 overexpressing myotubes (D). The traces below the images are spatially averaged time courses of Fluo-3 fluorescence normalized to baseline fluorescence (F/F_0). Note the difference in time scales for the control and the triadin-overexpressing myotube. E and F, line-scan images of electrically evoked Ca^{2+} transients measured on control (E) and on Trisk 95 overexpressing myotubes (F). The traces below the images were generated as in C and D. Vertical scaling bar represents $15 \mu\text{m}$ for all images in panels C–F.

readily induce calcium release from the SR (Fig. 6B) clearly demonstrating that the reduced triadin expression did not interfere with the normal E-C coupling. However, in contrast to what was seen for triadin overexpressing cells, myotubes with reduced Trisk 95 expression had, in spite of the reduced SR calcium content, identical KCl-evoked calcium transients to those measured in control cells (Fig. 6C).

Calcium release events in cells with suppressed Trisk 95 expression

The results obtained from the global changes in $[Ca^{2+}]_i$ suggested that suppressed triadin expression might

have the opposite effect to that seen with Trisk 95 overexpression. To test this hypothesis spontaneous calcium transients and localized calcium release events were recorded using laser scanning confocal microscopy. Control myotubes readily produced spontaneous calcium transients visible both in $x-y$ (Fig. 7A) and in line-scan images (Fig. 7B). These transients had an average amplitude of 0.62 ± 0.05 ($n = 553$ on 23 myotubes) and appeared with an average frequency of 1.01 ± 0.22 Hz. On the other hand, LCRE were scarce on these cells. A large fraction of images (225 out of 279), as the one presented in Fig. 7B (upper image), were devoid of such events. Although both sparks and embers were occasionally observed (see e.g. Fig. 7B, lower image) their numbers were low; altogether 143 sparks and 23

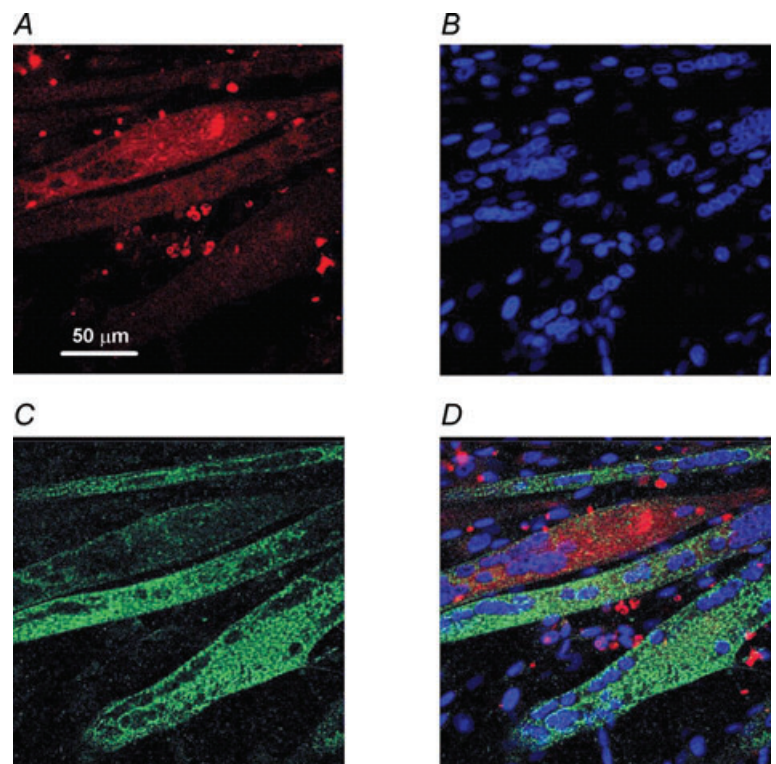
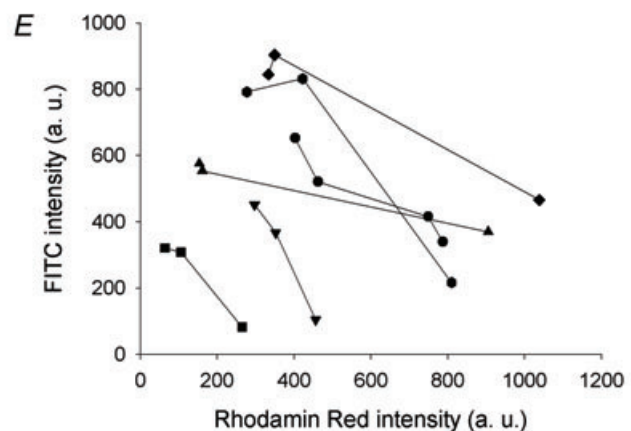


Figure 5. Suppressed Trisk 95 expression in rat primary myotubes transfected with a rhodamine red labelled shRNA

A, transfected myotubes are visualized by their red fluorescence. B, cell nuclei stained with DAPI. Note the large number of nuclei in the differentiated myotubes. C, triadin expression visualized by immunocytochemistry using a Trisk 95 primary and an anti-rabbit secondary antibody labelled with FITC. D, overlay image of those presented in panels A–C. Note the characteristic dotted pattern of Trisk 95 labelling in non-transfected cells as compared to the suppressed expression in the transfected myotube. Horizontal scaling bar is $50 \mu\text{m}$ for all images. E, effectiveness of the suppression of triadin expression. The intensity of Trisk 95 associated green fluorescence (FITC) was correlated with the shRNA associated red fluorescence (rhodamine) of the same area. Altogether 6 cultures and 19 areas were analysed; different symbols denote different cultures.



embers (in 54 images) were detected. This accounts for an average frequency of $0.0032 \pm 0.0019 \text{ s}^{-1} \mu\text{m}^{-1}$ and $0.0024 \pm 0.0014 \text{ s}^{-1} \mu\text{m}^{-1}$, respectively.

The suppression of triadin expression in these myotubes had dramatic effects on the appearance of LCRE. Ca^{2+} sparks and embers became readily observable in both x - y (Fig. 7C) and in line-scan images ($n = 649$ and 117 for sparks and embers, respectively, from 5 myotubes, 218 out of 385 images; Fig. 7D). Not only LCRE but spontaneous calcium transients were also present on these cells as visible in the image in Fig. 7D. They appeared with approximately the same frequency ($0.99 \pm 0.16 \text{ Hz}$; $n = 888$ on 20 myotubes) but with a larger amplitude (1.39 ± 0.08 ; $P < 0.01$) than those in control (see above).

Not only was the frequency of LCRE altered in myotubes with suppressed Trisk 95 expression but their characteristic parameters were also changed. Figure 8A and B present

two typical Ca^{2+} sparks measured in a control and in a triadin-suppressed myotube, respectively. These events were selected to represent the top 10% of events with respect to their amplitude. As evidenced from these two sparks and from the amplitude histograms presented in Fig. 8C, the proportion of Ca^{2+} sparks with large amplitude was increased in myotubes with suppressed Trisk 95 expression. As a result, the average amplitude of all sparks was significantly increased, from 0.49 ± 0.03 in control to 0.89 ± 0.04 ($P < 0.01$) in these cells. Not only the amplitude of sparks, but the average amplitude of embers was also increased, from 0.27 ± 0.05 to 0.49 ± 0.03 ($P < 0.01$; Fig. 8D). This was mainly due to the appearance of events with amplitudes greater than 0.6, hardly ever observed on control myotubes and never on adult fibres. On the other hand, neither the average duration of embers (229 ± 33 versus $198 \pm 13 \text{ ms}$ in control and in

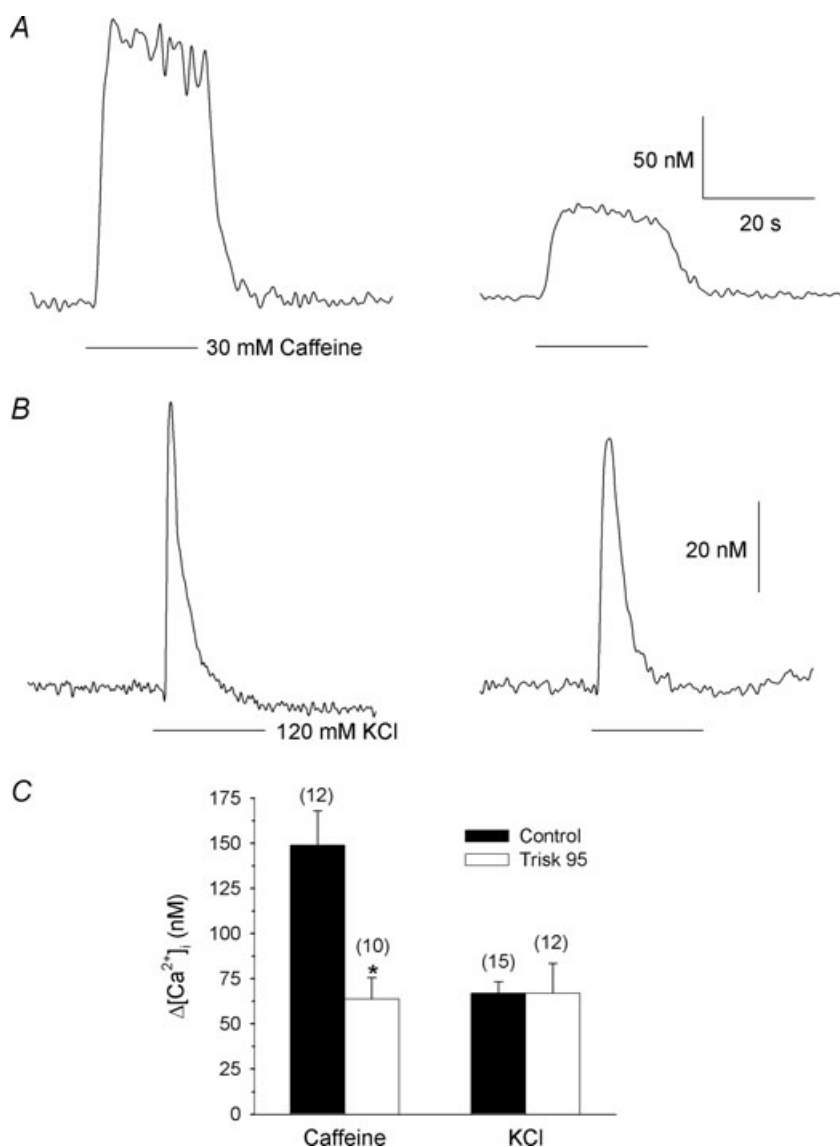


Figure 6. Calcium transients from control myotubes and from cells transfected with Trisk 95 specific shRNA

A and B, representative calcium transients evoked by either caffeine (30 mM; A) or by KCl-evoked depolarization (120 mM; B). Cells were kept in normal (A) or Ca^{2+} -free Tyrode solution (B) during the measurement. C, pooled data for $\Delta[\text{Ca}^{2+}]_i$. Note that control represents cells (subcellular areas) where Trisk 95 specific shRNA-associated red fluorescence was weak as compared to other cells of the same culture or cells from cultures treated with scrambled shRNA sequence. Asterisk marks significant difference as compared to control while the numbers in parentheses give the number of cells investigated.

shRNA treated myotubes, respectively; see also Fig. 8F) nor that of sparks (39.7 ± 4.3 versus 44.0 ± 2.5 ms) was altered significantly ($P > 0.3$ and 0.07 , respectively). In contrast, the spatial spread (from 1.67 ± 0.16 to 1.38 ± 0.07 ; $P < 0.02$) was reduced, while the rise time (from 15.4 ± 2.2 to 22.0 ± 2.9 ms; $P < 0.01$) of Ca^{2+} sparks was increased in myotubes with suppressed triadin expression.

Discussion

Triadin, an integral SR membrane protein, has previously been shown to interact with both the calcium release channel and calsequestrin, the low affinity calcium binding protein of the SR lumen. They seem to form a tri-, or maybe a tetra-molecular complex which also includes junctin (Beard *et al.* 2002; Györke *et al.* 2004; Wei *et al.*

2006). This close association suggests that triadin could influence RyR function. Indeed, triadin overexpression was shown to suppress calcium transients in cultured rat myotubes (Rezgui *et al.* 2005). However, no information is available on whether triadin overexpression might affect the localized calcium release events alterations in which are likely to reflect modifications in RyR function. Furthermore, data on the effects of transiently suppressed triadin expression on the calcium transients or on LCRE are also missing.

Here we demonstrate that both the increase and the decrease in Trisk 95 expression will affect the calcium transients and the localized calcium release events in cultured skeletal muscle cells. These effects, as expected, are opposite, demonstrating a clear and specific role of triadin in regulating RyR function. When overexpressed triadin reduced the amplitude of spontaneous and

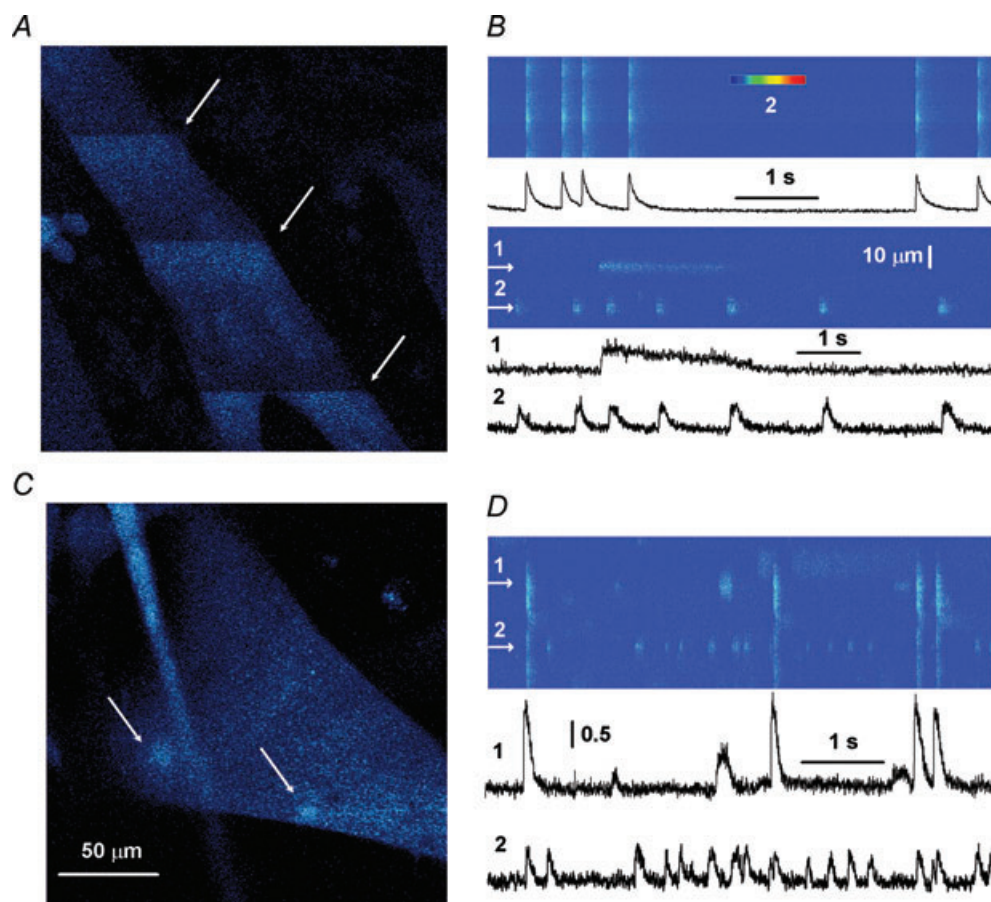


Figure 7. Spontaneous Ca^{2+} transients and Ca^{2+} release events in rat primary myotubes

A, an x-y image of a control myotube loaded with Fluo-3 displaying spontaneous Ca^{2+} transients (arrows). B, line-scan images of rat primary myotubes generating spontaneous Ca^{2+} transients. The traces below the images are spatially averaged time courses of Fluo-3 fluorescence from either the entire image (upper image) or from the lines marked by arrows. Note the absence of localized events on the upper image. C, x-y image of shRNA transfected myotubes. Note the localized Ca^{2+} release events marked by arrows. D, line-scan image of rat primary myotubes with suppressed Trisk 95 expression generating spontaneous Ca^{2+} release events. Traces below the images were generated as those in panel B. Note the large number of events. Vertical calibrations are the same for the images and for all traces in B and D.

depolarization-evoked calcium transients and suppressed the appearance of LCRE by reducing their number and size. The interference with its expression, on the other hand, increased the amplitude of the calcium transient and of LCRE together with increasing the number of the latter.

Since increased expression of triadin was accompanied by suppression, while decreased expression was followed by an augmentation of calcium release, we propose that triadin *in situ* is exerting a tonic negative effect on the opening of RyR.

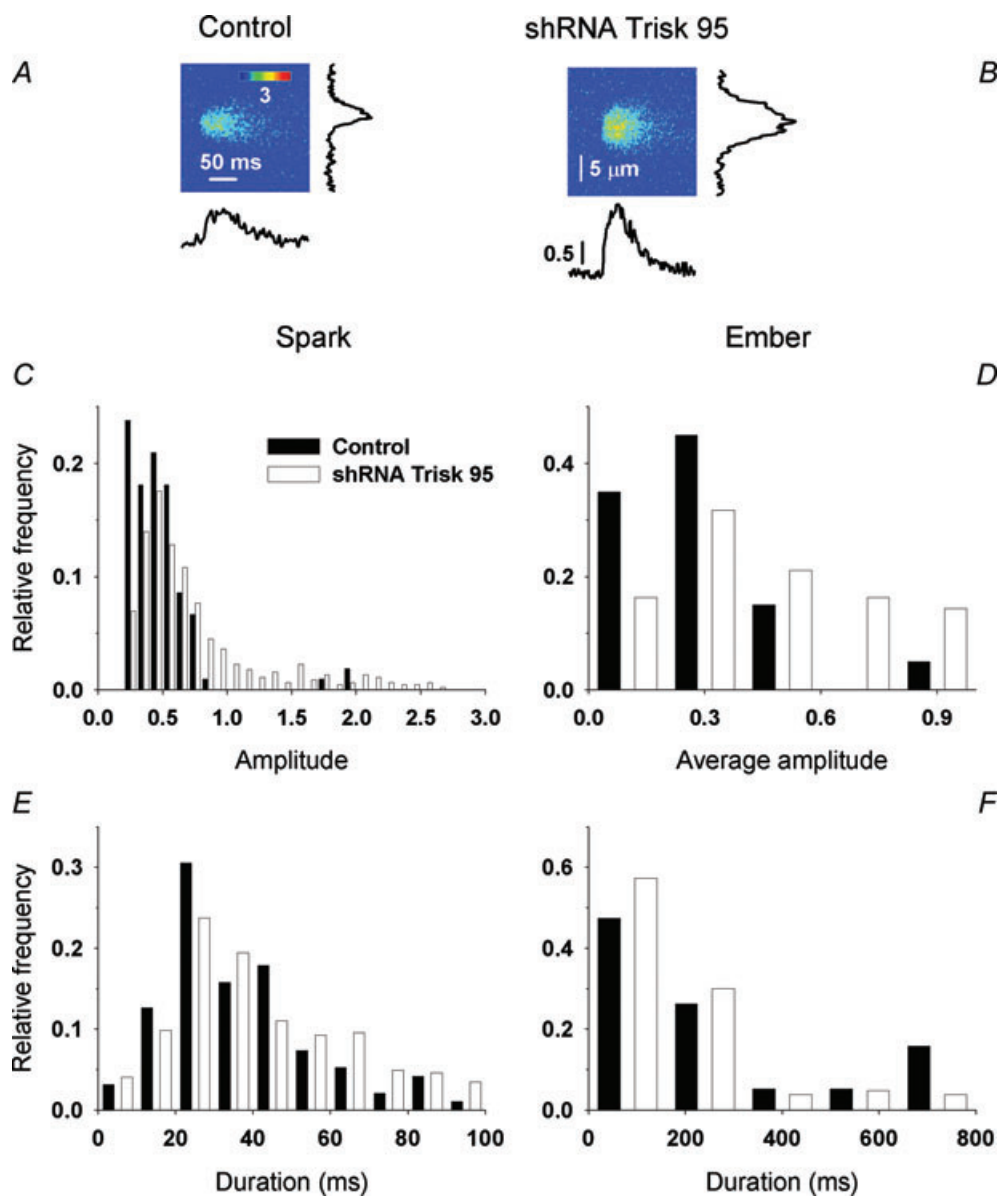


Figure 8. Parameters of Ca^{2+} sparks from control rat primary myotubes and from myotubes with suppressed Trisk 95 expression

A and B, typical Ca^{2+} sparks from a control myotube (A) and from a cell with suppressed triadin expression (B). Traces below and next to the images give the time course and the spatial distribution of fluorescence, respectively, measured at the position of the peak. Horizontal and vertical calibrations correspond to both images and to all four traces in A and B. C, amplitude histogram of Ca^{2+} sparks from control myotubes and from myotubes with suppressed triadin expression. Note the larger scatter for the control data due to the relatively low number of events (143 versus 649; control and Trisk 95 suppressed, respectively). D, histogram of the average amplitude of embers generated as in panel C. Note the significant contribution from events with large average amplitude in Trisk 95 suppressed myotubes. E and F, histograms of event duration presented as that in panels C and D for Ca^{2+} sparks (E) and embers (F).

Calcium transients in myotubes with altered triadin expression

The changes in whole-cell calcium transients following altered Trisk 95 expression, whether evoked by KCl-induced depolarization or by spontaneously occurring or electrically evoked action potentials, are clearly in line with the hypothesis that triadin serves as a tonic negative regulator of RyR function. Data reported here as well as those published earlier (Rezgui *et al.* 2005; Csernoch *et al.* 2007) established that Trisk 95 overexpression suppresses the depolarization-evoked calcium transients. It should be noted, however, that calcium transients in the presence of external calcium were only suppressed if evoked by action potentials (Fig. 4) and not if the addition of 120 mM KCl was used (Fig. 2D) to initiate calcium release. This apparent contradiction can be resolved with either of the two following possibilities. One might assume that external calcium influx gives a large contribution to the global change in $[Ca^{2+}]_i$ only if the depolarization is long enough. Although this seems as an intriguing possibility as L-type calcium current is activating slowly in skeletal myotubes (e.g. O'Connell & Dirksen, 2000), simple calculations show that the amount of calcium that enters into the myoplasm through these channels is not enough to explain the above phenomenon (Szappanos *et al.* 2004). The other and definitely more likely possibility is that the calcium entering through the L-type channels serves as a trigger for the activation of RyR (calcium-induced calcium release) and thus overrides the inhibition exerted by the overexpressed triadin. This would also explain why action potential-evoked transients are suppressed under these conditions, since the action potential is too short to allow substantial influx of external calcium.

It should also be noted that in case of action potential-evoked calcium transients the alteration in calcium release could result from the modification of the action potential itself. While there is evidence that links Trisk 95 to surface membrane channels (see below), none exists that would suggest an interaction between triadin and either the sodium or potassium channels responsible for the generation of the action potential in skeletal muscle.

Recently calcium transients from muscles with reduced triadin expression were derived from triadin knock-out animals (Shen *et al.* 2007). In these mice both the higher – 95 and 60 kDa – and lower molecular weight triadins – 35–40 kDa – were absent. While exhibiting no clear phenotype, the animals were characterized by an elevated resting intracellular Ca^{2+} concentration while reduced SR calcium content and calcium transients in response to caffeine or K^+ -induced depolarization. In addition, reduced expression of certain junctional proteins, junctin, junctophilin-1 and CSQ, with no clear alteration in the expression of type 1 RyR (RyR1) was observed in adult

fast twitch muscles. Interestingly, no alteration in the expression of junctin or CSQ was detected in myotubes.

Our results – from cells transfected with the appropriate shRNA and thus with reduced Trisk 95 expression – showing an increased resting $[Ca^{2+}]_i$ and reduced response to caffeine are essentially identical to those of Shen *et al.* (2007). Similar reduction in agonist-induced SR calcium release was reported for cells, where the interaction of RyR and triadin was interfered with (Goonasekera *et al.* 2007). On the other hand, we found identical calcium transients for KCl-evoked depolarization in control cells and in cells with reduced triadin expression (Fig. 6). This was observed in spite of the reduced caffeine response indicative of a reduced SR calcium content for the latter cells. This finding is, nevertheless, in line with the results obtained here both with the parameters of LCRE on these cells (see below) and with the data with Trisk 95 overexpression. To explain the apparent discrepancy one might consider the fact that in the reports of Shen *et al.* (2007) and Goonasekera *et al.* (2007) the RyR–triadin interaction is completely disrupted while in our experiments it is only reduced. One could then assume that the depletion of Ca^{2+} in the SR in our experiments where the Trisk 95 expression is not completely eliminated is less than that in the report of Shen *et al.* (2007). This would explain how the increased activation of RyR – due to the partial absence of triadin – could compensate for the reduced SR content.

Interestingly not only the amplitude of the spontaneous calcium transients but also their frequency was altered by altered triadin expression; reduced expression increased while increased expression reduced the frequency. We have no clear explanation at the moment whether triadin expression would alter membrane excitability, although several reports link triadin to surface membrane channels. Early reports suggested that triadin might bind to the DHPR itself and serve as a bridge between the SR and the T-tubular membranes (Caswell *et al.* 1991). This, however, has later been questioned based on the topology – short, only 46 amino acids long, intracellular segment – of triadin (Groh *et al.* 1999). In addition, recent reports linked triadin expression to store-operated calcium influx (Vassilopoulos *et al.* 2007), and the functional interaction between TRPC channels and RyR was connected to triadin expression (Lee *et al.* 2006).

Localized calcium release events with altered triadin expression

To our knowledge no previous reports have investigated whether Trisk 95 overexpression or the inhibition of its translation would affect the localized events of calcium release to assess the *in situ* effects of triadin on RyR. However, the addition of triadin to RyRs reconstituted into planar lipid bilayers resulted in a decrease of channel open

probability (Groh *et al.* 1999). Similarly, triadin inhibited [^3H]ryanodine binding to heavy SR vesicles (Guo & Campbell, 1995). These observations clearly suggested that triadin would suppress the calcium release channel function in line with the data on whole-cell calcium transients.

These assumptions were clearly confirmed by our findings that suppressed Trisk 95 expression was accompanied by an increase while the overexpression of triadin was followed by a reduction in the frequency of LCRE. Not only were the number of events altered, their amplitude and spatial parameters were also affected together with the modified expression of Trisk 95. In triadin overexpressing cells sparks were smaller, both the amplitude and FWHM, indicating that the number of channels involved in the generation of an event was reduced in these cells. In line with this assumption, and with the observation that SR calcium load was not affected, the characteristic parameters of embers were essentially identical with those in control cells. On the other hand, suppression of Trisk 95 increased not only the frequency but also the amplitude of the sparks in line with more channels opening during an event. Interestingly, embers with average amplitudes greater than 0.6 were readily observed in myotubes with reduced triadin expression. These events suggest that either the conductance of the channel or the SR calcium load is increased or more than a single channel was opened during the event. While all above possibilities, or their combination could explain the observations, the most likely scenario is the change in the number of channels since mutations interfering with triadin binding had no effect on single channel conductance in planar lipid bilayers (Goonasekera *et al.* 2007) and we saw a reduction in SR load in whole-cell calcium measurements (Fig. 6).

Recently the importance of type 3 RyR (RyR3) as well as that of the inositol trisphosphate receptor (IP₃R) in the generation of LCRE has been documented (Pouvreau *et al.* 2007 and Balghi *et al.* 2006, respectively). This raises the possibility that the observations presented in this study could correspond to interactions of Trisk 95 with either RyR3 or IP₃R and not the type 1 or skeletal isoform of RyR, thus explaining some of the differences between the results seen here and with knock-out animals (Shen *et al.* 2007; see above). While the involvement of IP₃R in the generation of LCRE seems established in mouse myotubes lacking dystrophin, they were reported to be less important if a mini-dystrophin is expressed in these cells (Balghi *et al.* 2006). Although not conclusive, it is likely that the involvement of IP₃R would be even smaller in cells expressing normal amounts of dystrophin. In addition, the localization of Trisk 95 and IP₃R seem to be distinct. The distribution of the 95 kDa isoform of triadin shows a clear dotted pattern (see e.g. Fig. 5) and the protein is confined to the junctional region. In

contrast, IP₃R shows a more diffuse distribution and seems to colocalize with the 32 kDa isoform member of the triadin family (Vassilopoulos *et al.* 2007). Although RyR3 is found in the triadic junction and could therefore interact with Trisk 95 no such protein–protein interaction has been described so far. One cannot, however, exclude the possibility that the overexpression of triadin could result in the appearance of the protein at locations other than those where it is physiologically present. This could, at least partially, explain why calcium release events and calcium transients are suppressed if Trisk 95 is overexpressed. It could not, however, account for the opposite effects seen with shRNA since it is unlikely the triadin would appear at altered locations if its expression is reduced.

Finally, it should be considered whether interfering with the expression of Trisk 95 altered the expression levels of other key proteins, e.g. RyR and CSQ, of excitation–contraction coupling thus resulting in the modification of calcium release. We have therefore checked the expression level of these two proteins in our cultures and found no significant alteration in their expression (in line with the report of Shen *et al.* 2007; see also Supplemental Material). This is in line with the report of Perez *et al.* (2005) who found that the overexpression of neither RyR1 nor RyR3 influenced the expression of triadin.

Taken together our data clearly establish that Trisk 95 regulates LCRE in cultured skeletal myotubes by exerting a tonic negative effect. Although these localized events have only been indirectly linked to normal E–C coupling in adult mammalian skeletal muscle (see e.g. Csernoch, 2007) the channels involved in their generation are most likely to be the same as those activated by membrane depolarization, suggestive of a role for Trisk 95 in regulating calcium release channel function *in situ*.

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Acknowledgements

The authors wish to thank R. Öri for the excellent technical assistance. This work was supported by grants from the Hungarian Science Fund (OTKA T049151, NK61412), and from Association Française contre les Myopathies (AFM) to I.M. MG was a recipient of an OTKA postdoctoral fellowship.