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Altered stored calcium release in skeletal myotubes deficient of triadin and junctin

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Summary

Triadin and junctin are integral sarcoplasmic reticulum membrane proteins that form a macromolecular complex with the skeletal muscle ryanodine receptor (RyR1) but their roles in skeletal muscle calcium homeostasis remain incompletely understood. Here we report that delivery of siRNAs specific for triadin or junctin into C_2C_{12} skeletal myoblasts reduced the expression of triadin and junctin in 8-day-old myotubes by 80 and 100%, respectively. Knocking down either triadin or junctin in these cells reduced Ca^{2+} release induced by depolarization (10 mM KCl) by 20–25%. Unlike triadin knockdown myotubes, junctin knockdown and junctin/triadin double knockdown myotubes also had reduced Ca^{2+} release induced by 400 μM 4-chloro-*m*-cresol, 10 mM caffeine, 400 μM UTP, or 1 μM thapsigargin. Thus, knocking down junctin compromised the Ca^{2+} stores in the sarcoplasmic reticulum of these cells. Our subsequent studies showed that in junctin knockdown myotubes at least two sarcoplasmic reticulum proteins (RyR1 and skeletal muscle calsequestrin) were down-regulated while these proteins' mRNA expression was not affected. The results suggest that triadin has a role in facilitating KCl depolarization-induced Ca^{2+} release in contrast to junctin which has a role in maintaining sarcoplasmic reticulum Ca^{2+} store size in C_2C_{12} myotubes.

Keywords

C2C12 myotubes; Ryanodine receptor; Sarcoplasmic reticulum; Calcium release; Triadin; Junctin

Introduction

In the specialized triad region of skeletal muscle, the voltage-dependent L-type Ca^{2+} channels localized to the invaginations of the cell membrane (t-tubules) are physically coupled to the juxtaposing intracellular Ca^{2+} release channels/ryanodine receptors (RyR1s) in the junctional

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Conflicts of interest None.

sarcoplasmic reticulum (SR). Through this physical coupling, depolarization of t-tubules sensed by the L-type Ca^{2+} channels provokes RyR1 to release Ca^{2+} stored in the SR into the cytosol, thereby initiating muscle contraction [1]. This process, known as excitation–contraction (EC) coupling, therefore requires both dihydropyridine-sensitive L-type Ca^{2+} channel ($Ca_v1.1$, DHPR) and RyR1. In the SR, RyR1 is also physically associated with the two integral membrane proteins junctin and triadin to form a macromolecular Ca^{2+} signaling complex [2-5]. However, the roles of triadin and junctin in SR Ca^{2+} release of skeletal muscle remain incompletely understood.

Triadin is a glycoprotein with several splice variants that have molecular weights ranging from 35 to 95 kDa [6-11]. Expressed in both heart and skeletal muscle, triadin was initially isolated from skeletal muscle as a 95-kDa splice variant linked to both the α1 subunit of L-type Ca^{2+} channel and RyR1 [12,13]. Triadin has a short amino-terminal cytoplasmic domain, one transmembrane spanning segment, followed by a longer C-terminal luminal domain [6]. The luminal region of triadin is enriched in clusters of alternating lysine and glutamic acid residues that interact with skeletal muscle calsequestrin (CSQ1) [14] and with negatively charged amino acids of RyR [5]. Although it has been reported that the interruption between the luminal domains of triadin and RyR1 impairs RyR1 function and SR Ca^{2+} release [4,15], triadin also has been reported to inhibit channel activity of RyR1 through its cytoplasmic domain by reducing channel open probability [16,17]. Consistent with the inhibitory role of triadin, over-expression of triadin in skeletal myotubes inhibited depolarization-induced SR Ca^{2+} release [18]. Thus, whether triadin has a positive, negative or neutral effect on skeletal muscle EC coupling remains unclear. However, in a recent study triadin-deficient mice survived to adulthood, which showed that triadin is not essential for EC coupling [19]. Skeletal myotubes from triadin-deficient mice had reduced depolarization-induced Ca^{2+} release that was mainly attributed to reduced Ca^{2+} store size.

Junctin is a smaller protein (26 kDa) with a short 21 amino acid cytoplasmic N-terminal and a single membrane-spanning domain that is followed by a longer, highly charged C-terminal domain [20]. Like triadin, junctin is also expressed in both skeletal and cardiac muscle [20, 21]. Junctin-deficient mice also survived to adulthood but were susceptible to ventricular arrhythmias and sudden death due to aberrant Ca^{2+} homeostasis in the heart [22]. Junctin's role in skeletal muscle EC coupling is not well characterized.

Using recombinant adeno-associated virus (rAAV) delivered siRNAs, we have previously reported the roles of SR skeletal muscle calsequestrin (CSQ1) and cardiac muscle calsequestrin (CSQ2) isoforms in stored Ca^{2+} release of C_2C_{12} skeletal myotubes [23]. Using the same knockdown strategy, we assessed the roles of triadin and junctin in SR Ca^{2+} release induced by depolarization (10 mM KCl) in C_2C_{12} skeletal myotubes. Here we report that knocking down either triadin or junctin in these cells reproducibly reduced SR Ca^{2+} release induced by depolarization by 20–25%. Moreover, the effect of knocking down both proteins is additive, as Ca^{2+} release induced by 10 mM KCl is decreased by ~35% in double-knockdown C_2C_{12} myotubes. Our subsequent studies showed that triadin specifically facilitates depolarization-induced Ca^{2+} release but has no noticeable role in maintaining Ca^{2+} store size of SR. In contrast, junctin appears to maintain Ca^{2+} store size of SR in C_2C_{12} myotubes, as junctin-knockdown myotubes also have reduced stored Ca^{2+} release provoked by pharmacological agents that do not cause membrane depolarization or activate RyR1. A preliminary report of this work has been presented in abstract form [24].

Materials and methods

Materials

Immature C_2C_{12} skeletal muscle cells (myoblasts) derived from normal adult C_3H mouse leg muscles were purchased from ATCC (Manassas, VA). Double-strand recombinant adeno-associated viral (rAAV) vector was generously provided by Dr. Douglas McCarty (University of North Carolina, Chapel Hill). Rabbit polyclonal anti-junctin raised against an amino acid region specific for junctin and C-terminal cysteine (MAEDKEAKHGGHKNGRR GC) was prepared by ProSci Incorporated (San Diego, CA). Rabbit polyclonal anti-RyR1 antibody was prepared as described previously [25]. Monoclonal anti-triadin antibody and all other primary antibodies were purchased from Affinity Bioreagents (Golden, CO). [^{3}H]ryanodine was obtained from PerkinElmer Life Sciences. Fluo 4-AM, Alexa 488 and Alexa 647-labeled secondary antibodies were purchased from Molecular Probes (Eugene, OR) and Fura 2-AM was from TEF LABS (Austin, TX). All other chemicals were obtained from Sigma unless specified otherwise.

Construction of vector, packaging, and purification of rAAV

The sequences of the triadin and junctin siRNAs were 5′-GGAAATGCATCGACAACCATTCAAGAGATGGTTGTCGATG CATTTCC-3′ and 5′-CTGACAAGAGTTCCAAGTCTATTCAAGAGATAGACTTGGAACTCTT GTCAG-3′, respectively. BLAST searches confirmed that the selected oligonucleotide sequences were not homologous to any other genes. A control sequence of 5′-TTCTCCGAACGTGTCA CGTTTCAAGAGAACGTGACACGTTCGGAGAA-3′ was used to construct rAAV-control as a negative control. The oligonucleotides encoding the specific siRNA for junctin were inserted into pSilencer-1.0 vector (Ambion, Austin TX) downstream of the U6 promoter using ApaI and EcoRI sites. For triadin, the sequence of the oligonucleotide is near 5′ end of triadin which is homologous to the known triadin isoforms. U6 promoter-driven expression cassettes were inserted into rAAV vector (ptrs-U1a-RFP-U6) using the KpnI and NotI sites [23]. The resulting vectors are termed rAAV-triadin, rAAV-junctin and rAAV-control. Serum type 2 double strand rAAVs were produced by the triple plasmid cotransfection method and purified by ammonium sulfate precipitation and on cesium chloride gradients [26,27].

Cell culture and recombinant AAV transduction

C_2C_{12} myoblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1 × antibiotics/antimycotics. Myoblasts were seeded at a concentration of 6 × 105 cells per 10-cm plate or 1×10^5 per well in 6-well plates and cultured for 48 h to reach 100% confluence (defined as day 0 myotubes). To induce myogenic differentiation, the growth medium was changed to differentiation medium (Dulbecco's modified Eagle's medium supplemented with 2% horse serum and 1 × antibiotics/antimycotics) on day 0. For rAAV transduction, C_2C_{12} myoblasts were infected with rAAV carrying the control, junctin and/or triadin siRNA silencing cassette at 1×10^4 particles/cell about 24 h after seeding as described previously [23].

Stored Ca^{2+} release

Stored Ca^{2+} release was determined using the fluorescent Ca^{2+} indicator dye Fluo 4-AM as described previously [23]. Briefly, 8-day-old C_2C_{12} myotubes grown on glass coverslips were washed three times with PBS buffer and loaded with 5 μM Fluo 4-AM for 1 h at 37 °C in Krebs–Ringer–Henseleit (KRH) buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 6 mM glucose, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, and 25 mM HEPES, pH 7.4). After loading, cells were rinsed with KRH buffer to remove non-hydrolyzed fluophore and kept in KRH buffer for 30 min to complete de-esterification. Individual cells were defined as region of interest, and

average fluorescence was measured using the program ImageMaster (Photon Technology International, Lawrenceville, NJ). Resting calcium levels were monitored with Fura-2 as described [23].

Depolarization-induced Ca^{2+} transients were provoked by addition of 10 mM KCl to Ca^{2+}-free KRH buffer (2 mM $CaCl_2$ was replaced by 0.5 mM EGTA). In other experiments, stored Ca^{2+} release was induced by 400 μM 4-chloro-*m*-cresol, 10 mM caffeine, 400 μM UTP, or 1 μM thapsigargin.

Immunoblot analyses

8-Day-old C_2C_{12} myotubes grown on six-well plates were harvested, washed twice with cold phosphate-buffered saline, lysed in RIPA buffer plus protease inhibitors (Complete Mini, Roche Diagnostics), and centrifuged at 12,000 × *g* for 10 min to remove insoluble material. Protein concentrations were determined using BCA assay. Twenty microgram of lysates were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with antibodies for skeletal muscle triadin 95, junctin, CSQ1, CSQ2, DHPR-α1 subunit of the L-type Ca^{2+} channel (DHPR-α1), RyR1, and SERCA1. Western blots were developed using 3,3′-diaminobenzidine or enhanced chemiluminescence and quantified using Kodak Digital Science ID Image Analysis Software.

Immunofluorescence analyses

8-Day-old C_2C_{12} myotubes fixed with 3% paraformaldehyde were permeabilized with buffer containing 20 mM HEPES, pH 7.4, 0.3 M sucrose, 50 mM NaCl, 3 mM $MgCl_2$ and 0.5% Triton-X100. The myotubes were then incubated with antibodies specific for triadin and junctin, followed by incubation with an appropriate Alexa 488-labeled goat anti-mouse or Alexa 647-labeled goat anti-rabbit antibody. The prepared slides were examined using a Carl Zeiss LSM 510 laser scanning confocal microscopy system attached to an Axiovert 100 inverted microscope (Carl Zeiss, Jena, Germany) as described [23]. Argon and helium/neon lasers provided the excitation light beams (488 and 633 nm, respectively) for the confocal microscopy system. The images (512 × 512 pixels) obtained with a 100 × objective (Plan-Neofluar) were recorded using LSM Image software.

Quantitative RT-PCR

C_2C_{12} cells were harvested, and total RNA was isolated using an RNeasy mini kit per the manufacturer's instructions (Qiagen, Valencia, CA). The gene expression levels of RyR1, RyR3, CSQ1, CSQ2 and SERCA1 were determined by quantitative RT-PCR [28]. The primer and probe sequences of RyR1 are 5′-AGAATTCTGGGGTGAACTG, 3′-AACCGCAGTGTGTAGA AATTC. Probe sequence is (FAM) AGAGAGTGAAATTCTTGAACTACTTGTCG(TAMRA). The corresponding sequences are for RyR3: 5′-CCAACTTCTTCAAAGGGCTG, 3′-GAACCTCA GGTTGTAGAAATTC and (FAM) TCAGACCAAGTTATTGCACTACCTGGC(TAMRA); for DHPR-α1: 5′-CTCATAGGGTTGCTTGCAAG, 3′-AGGGTGGAGTTGAAGTGAAC and (FAM) TCCTGCGGATGGACCTGCCGGTA(TAMRA); for CSQ1: 5′-TACGAGGCCTTCATG GAAGA, 3′-GTTTCCTCAGGGTTGATCTC and (FAM) TGACCATCCCAGACAAGCCCAACA (TAMRA); for CSQ2: 5′-AAGAGT CACCCAGATGGCTA, 3′-ATGCTCAAGTCAGGATTGTCA and (FAM) CCTAGAGATCCTGAAACAGGTTGCCC(TAMRA); and for SERCA1: 5′-TCACCA CCAACCAGATGTCA, 3′-GAGAACTCGTTCAGTGAGCA and (FAM) ATCCCCATCCA CCTTTCAATGATG (TAMRA).

[^{3}H]ryanodine binding

Ca^{2+} dependent activity of RyRs was determined by a [^{3}H]ryanodine binding assay as described [23]. Crude membrane fractions prepared from C_2C_{12} cells were incubated for 20 h at 24 °C with 2.5 nM [^{3}H]ryanodine in 20 mM imidazole, pH 7.0, 250 mM KCl, 150 mM sucrose, 1 mM glutathione (oxidized), protease inhibitors, and the indicated free Ca^{2+} concentrations. B_{max} values of [^{3}H]ryanodine binding were determined by incubating membranes for 4 h at 24 °C with a saturating concentration of [^{3}H]ryanodine (30 nM) in 20 mM imidazole, pH 7.0, 0.6 M KCl, 0.15 M sucrose, 20 μM leupeptin, 200 μM Pefabloc, and 100 μM Ca^{2+}.

$^{45}Ca^{2+}$ uptake

ATP-dependent $^{45}Ca^{2+}$ uptake by C_2C_{12} total particulate matter was determined using a filtration method. $^{45}Ca^{2+}$ uptake was initiated by placing membranes in 0.15 M KCl, 20 mM imidazole, pH 7.0, solution containing 5 mM ATP, 8 mM Mg^{2+}, 5 mM Koxalate (a Ca^{2+} precipitating agent to increase Ca^{2+} uptake capacity [29], 10 μM ruthenium red (to inhibit RyRs [30], 5 mM NaN_3 (to inhibit mitochondrial Ca^{2+} uptake), 1 mM EGTA, and $^{45}Ca^{2+}$ to yield a free Ca^{2+} concentration of 0.5 μM. To obtain $^{45}Ca^{2+}$ uptake rates, aliquots were placed at 2.5, 5, and 10 min on 0.45 μm Millipore filters under vacuum and rinsed with three 3-ml volumes of ice-cold 0.175 M KCl, 5 mM imidazole, pH 7.0, solution. Radioactivity remaining with the vesicles on the filters was determined by liquid scintillation counting.

Biochemical assays and data analyses

Free Ca^{2+} concentrations were obtained by including in the solution the appropriate amounts of Ca^{2+} and EGTA as determined using the stability constants and published computer program [31]. Free Ca^{2+} concentrations were verified with the use of a Ca^{2+} selective electrode.

Results are given as mean ± S.E. Significance of differences in data ($p < 0.05$) were determined using Student's *t*-test.

Results

Efficacies of triadin and junctin knockdown in C_2C_{12} myotubes

The efficacies of knocking down triadin and junctin in singly and doubly infected C_2C_{12} myotubes were first investigated by immunoblot analyses. As shown in Fig. 1A and B, the expression of junctin and triadin in 8-day-old C_2C_{12} skeletal myotubes, as assessed by a densitometry method, was reduced by approximately 100 and 80%, respectively, by their corresponding siRNAs. Knocking down junctin did not cause a compensatory over-expression of triadin and vice versa. We also assessed the expression of these two proteins using confocal microscopy (Fig. 2). Compared to myotubes infected with rAAV vector that contained the control oligonucleotide sequences, myotubes infected with rAAV-junctin siRNA knocked down the protein to background levels. Myotubes infected with rAAV-triadin siRNA maintained a weak level of fluorescence, in agreement with an 80% reduction in protein level (Fig. 1). There were no significant differences between the resting cytosolic Ca^{2+} levels among the control, triadin knockdown, junctin knockdown, and double knockdown myotubes using the fluorescent Ca^{2+} indicator Fura-2 [23] (data not shown).

Depolarization-induced stored Ca^{2+} release in triadin and junctin knockdown myotubes

The individual roles of triadin and junctin in EC coupling of 8-day-old C_2C_{12} myotubes were assessed by measuring stored Ca^{2+} release provoked by addition of a submaximally activating KCl concentration of 10 mM in the absence of extracellular Ca^{2+}. Stored Ca^{2+} release, measured as the ratio of peak fluo-4 fluorescence over baseline fluorescence ratio (F/F_0), was

reduced by 19 and 25% in the junctin and triadin knockdown groups, respectively (Fig. 3A and Table 1). These results suggest that a submaximally activating KCl concentration was sufficient to induce stored Ca^{2+} release in both triadin and junctin knockdown myotubes. Moreover, we noted that the effect of knocking down both proteins is additive of knocking down each protein individually, as stored Ca^{2+} release by double-knockdown myotubes exposed to 10 mM KCl was reduced by ~35% as compared to the control myotubes (Fig. 3A and Table 1).

Stored Ca^{2+} release induced by non-depolarizing agents in triadin and junctin knockdown myotubes

To determine whether triadin and/or junctin specifically facilitate KCl-depolarization induced Ca^{2+} release in C_2C_{12} myotubes or have a less specific effect on stored Ca^{2+} release, we also exposed the four groups of C_2C_{12} myotubes to caffeine (which activates RyR1 and RyR3), 4-chloro-*m*-cresol (which activates RyR1 but not RyR3 [32], or UTP (which generates inositol 1,4,5-trisphosphate and activates its receptor) in the absence of extracellular Ca^{2+}. As shown in Fig. 3B and C and Table 1, knocking down triadin had no significant effects on the stored Ca^{2+} release of 8-day-old C_2C_{12} myotubes provoked by caffeine, 4-chloro-*m*-cresol or UTP, thus indicating that Ca^{2+} store size was maintained. In contrast, junctin and junctin/triadin knockdown showed a significantly reduced Fluo-4 fluorescence signal in response to the three agents (Fig. 3B and C, Table 1). Thus, unlike triadin, junctin knockdown appeared to reduce Ca^{2+} store size. In support of this conclusion, thapsigargin – a SERCA inhibitor which empties Ca^{2+} stores independent of RyR – and inositol 1,4,5-trisphosphate receptor also provoked significantly less fluorescence responses in junctin and double knockdown myotubes as compared to control and triadin knockdown myotubes (Table 1). Taken together, data of Fig. 3 and Table 1 suggest that triadin knockdown impairs KCl-induced Ca^{2+} release without reducing SR Ca^{2+} store, whereas junctin knockdown decreases SR Ca^{2+} store thereby causing impaired KCl-induced Ca^{2+}release.

Expression and function of Ca^{2+} handling proteins in junctin and triadin knockdown myotubes

We had previously shown that knocking down CSQ2 but not CSQ1 reduced SR Ca^{2+} uptake and KCl-induced Ca^{2+} release by affecting the expression of both RyR1 and SERCA1 in C_2C_{12} myotubes [23]. In initial experiments, we measured the mRNA levels of RyR1, RyR3, DHPR-α1, CSQ1, CSQ2 and SERCA1 in 8-day triadin, junctin and triadin/junctin knockdown myotubes, as determined by quantitative RT-PCR. None differed substantially from those in myotubes infected with the control vector (Table 2). To determine whether the reduction in stored Ca^{2+} release of junctin knockdown myotubes was due to a reduction of Ca^{2+} handling proteins in SR, we performed immunoblot analyses of the four groups of C_2C_{12} myotubes. CSQ1 and RyR1 were reduced by about 40 and 30% in junctin knockdown myotubes, respectively, but maintained in triadin knockdown myotubes at levels comparable to control myotubes (Fig. 4A and B). Since it is possible that the small fraction of triadin (~20%) not eliminated by our knockdown approach may still be sufficient to exert its effect, we attempted immunoprecipitation experiments to determine whether the amount of triadin interacting with RyR1 is affected by triadin knockdown. However, despite multiple attempts the relatively small amount of membrane fractions isolated from C_2C_{12} myotubes was not sufficient for us to conclusively address this question. No significant changes of protein expression levels of SERCA1, CSQ2 or DHPR-α1 were detected among the four groups of myotubes.

In agreement with immunoblot analyses that indicate a reduction of RyR1 protein in junctin knockdown and double knockdown myotubes, the maximal [^{3}H]ryanodine binding capacity (B_{max}) of crude membrane fractions isolated from junctin knockdown (but not from triadin knockdown) myotubes was significantly reduced (Table 3). A small (not significant) decrease

in B_{max} of [^{3}H]ryanodine binding was also observed in double-knockdown myotubes. We tested whether junctin and triadin knockdown altered the Ca^{2+} dependence of RyR1 activity, as determined by a ligand binding assay [33]. As shown in Fig. 5, knockdown of triadin, junctin and both proteins did not significantly alter the bimodal Ca^{2+} dependence of [^{3}H]ryanodine binding. Although the immunoblot analyses did not suggest a reduction in SERCA1 protein in any of the four groups, we nevertheless measured $^{45}Ca^{2+}$ uptake rates by membrane fractions isolated from each of the four groups of myotubes to determine whether a reduction in Ca^{2+} uptake could explain the non-specific reduction in stored Ca^{2+} release of junctin knockdown myotubes. The measured $^{45}Ca^{2+}$ uptake rates (Table 3) confirmed that knocking down junctin, triadin or both proteins did not affect Ca^{2+} uptake in these cells that could lead to a reduction of Ca^{2+} storage.

Discussion

To better understand the functional significance of the two ryanodine receptor associated proteins junctin and triadin in a skeletal muscle derived cell line, we used a knockdown approach that effectively reduced the two proteins by at least 80% in C_2C_{12} myotubes (Figs. 1 and 2). We had previously shown that the transduction of rAVV by itself did not lead to alterations of other Ca^{2+} handling proteins in 8-day-old C_2C_{12} myotubes and these cells released normal amounts of stored Ca^{2+} in response to KCl depolarization [23].

In a previous study, acute over-expression of 95-kDa triadin in skeletal myotubes inhibited depolarization-induced SR Ca^{2+} release [18]. These data implicated that the role of triadin could be to prevent excessive stored Ca^{2+} release during cell membrane depolarization. In contrast, our data (Fig. 3 and Table 1) indicate that triadin, through its physical association with RyR1 [4,15] and possibly DHPRs [12,13], actually facilitates depolarization-induced Ca^{2+} release in skeletal muscle. However, our data is not as dramatic as a study that showed expression of the triadin-binding deficient RyR1-D4878A/D4907A/E4908A mutant in dyspedic myotubes eliminated electrically evoked Ca^{2+} release [4]. Our data is more comparable to data obtained from myotubes of triadin-deficient mice, as we found that triadin knockdown myotubes exposed to KCl-induced depolarization had a reduction in stored Ca^{2+} release by only ~20%, as compared to control infected myotubes [19]. But in contrast to the study of skeletal myotubes from triadin-deficient mice, our triadin knockdown myotubes had preserved Ca^{2+} store size, as stored Ca^{2+} release provoked by RyR-specific agents (caffeine and 4-chloro-*m*-cresol) or by other agents (UTP and thapsigargin) that release stored Ca^{2+} via different mechanisms was not affected by triadin knockdown. The difference between our study and that of triadin-deficient mice could be due to the formation of a less well organized cell membrane and sarcoplasmic reticulum system in C_2C_{12} myotubes compared to skeletal muscle fibers [34-36].

An alternative possibility is that the longer and complete elimination of triadin in triadin knockout mice can lead to reduced Ca^{2+} store size in muscle fibers.

Immunoprecipitation studies with a RyR1 mutant (D4878A/D4907A/E4908A) that lacked triadin binding showed junctin binding, which suggests that triadin and junctin bind to different sites on RyR1 [4]. Although the functional effects of over-expressing and abolishing junctin on cardiac function have been extensively studied [22,37-40], the physiological role of junctin in skeletal muscle Ca^{2+} release is less well understood. Because we found that junctin knockdown myotubes had reduced stored Ca^{2+} release provoked by KCl as well as pharmacological agents that do not affect RyR1 (UTP and thapsigargin), we conclude that knocking down junctin reduces the SR Ca^{2+} store in C_2C_{12} myotubes. The expression of at least two SR proteins (RyR1 and CSQ1) was reduced in junctin knockdown myotubes. Therefore, it is conceivable that knockdown of junctin may have resulted in the disruption of

the macromolecular Ca^{2+} signaling complex that include RyR1 and CSQ1. However, the reduction in Ca^{2+} stores in junctin knockdown C_2C_{12} myotubes is unlikely due to the reduction of CSQ1, as we previously showed that knockdown of CSQ1 did not reduce the Ca^{2+} store in C_2C_{12} myotubes [23]. We also note that in junctin knockdown myotubes no noticeable changes in the mRNA levels of several key Ca^{2+} handling proteins were observed including RyR1 and CSQ1 (Table 2). Thus, knocking down junctin may increase degradation of RyR1 and CSQ1 and possibly other SR proteins. How junctin knockdown reduces SR Ca^{2+} store size and the concentrations of certain SR proteins will require further studies.

The present study describes for the first time the effects of the combined knockdown of junctin and triadin. C_2C_{12} myotubes infected with siRNAs specific to junctin and triadin exhibited reduced Ca^{2+} transients in response to pharmacological agents similar to those in junctin knockdown myotubes. Ca^{2+} transients were lower in double knockdown myotubes in response to KCl depolarization than in response to pharmacological agents in agreement with triadin knockdown myotubes. The data support the notion that knockdown of junctin decreases SR Ca^{2+} store size, whereas reduction in triadin decreased depolarization-induced stored Ca^{2+} release without affecting Ca^{2+} store size. Taken together, our results suggest that the two structurally related proteins do not have a redundant role in forming a macromolecular Ca^{2+} signaling complex in a skeletal muscle derived cell line.

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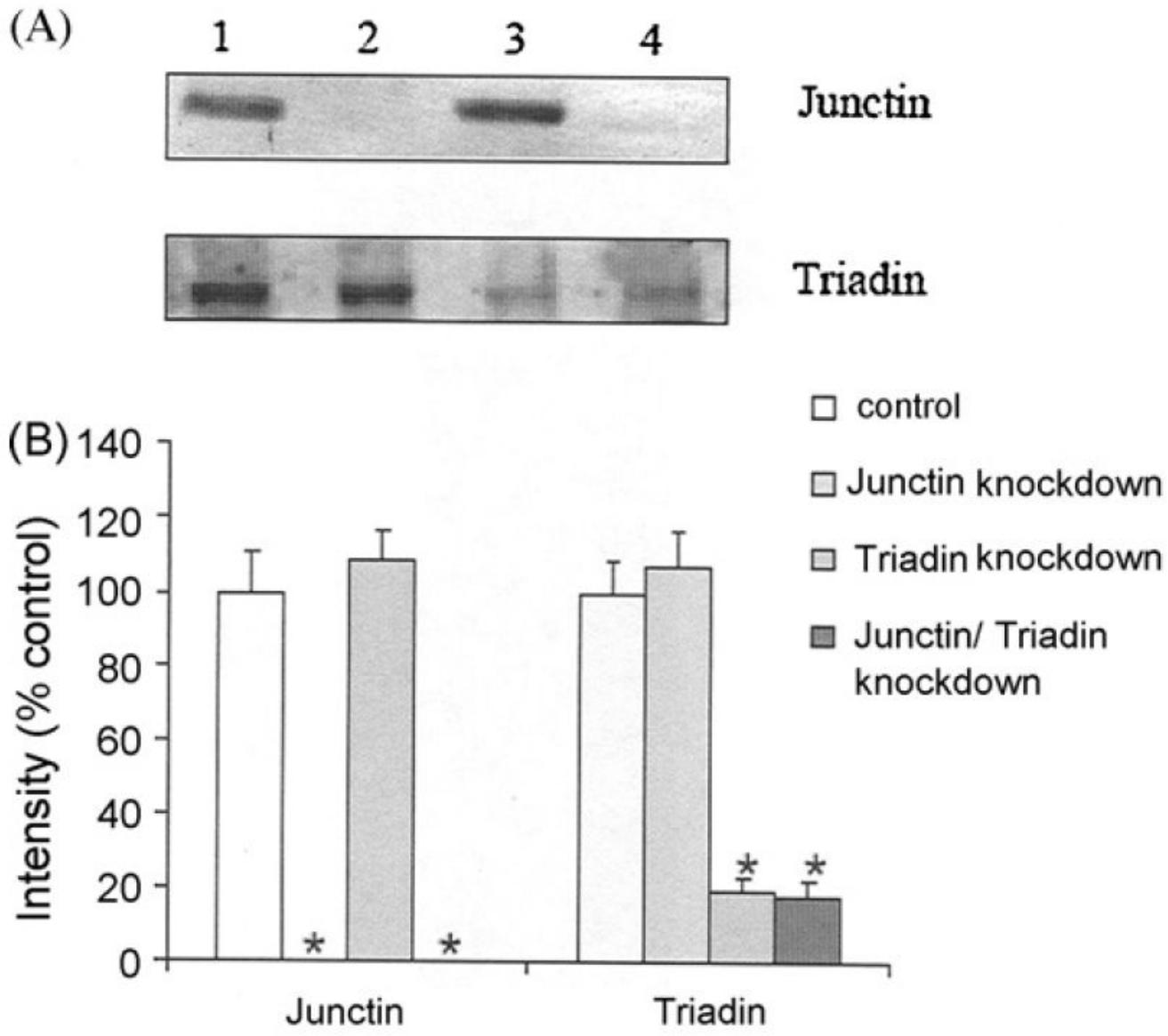

Figure 1.

Immunoblot analysis of C_2C_{12} myotubes. (A and B) Junctin and triadin protein levels in C_2C_{12} myotubes infected with rAAV-control (lane 1), rAAV-junctin (lane 2), rAAV-triadin (lane 3), and both rAAV-junctin and rAAV-triadin (lane 4). Immuoblots were performed on 8-day-old C_2C_{12} myotube lysates. (C) Intensities of protein bands were normalized by comparing them to respective control bands. Data are the mean ± S.E. of three experiments. *$p < 0.05$ compared to controls.

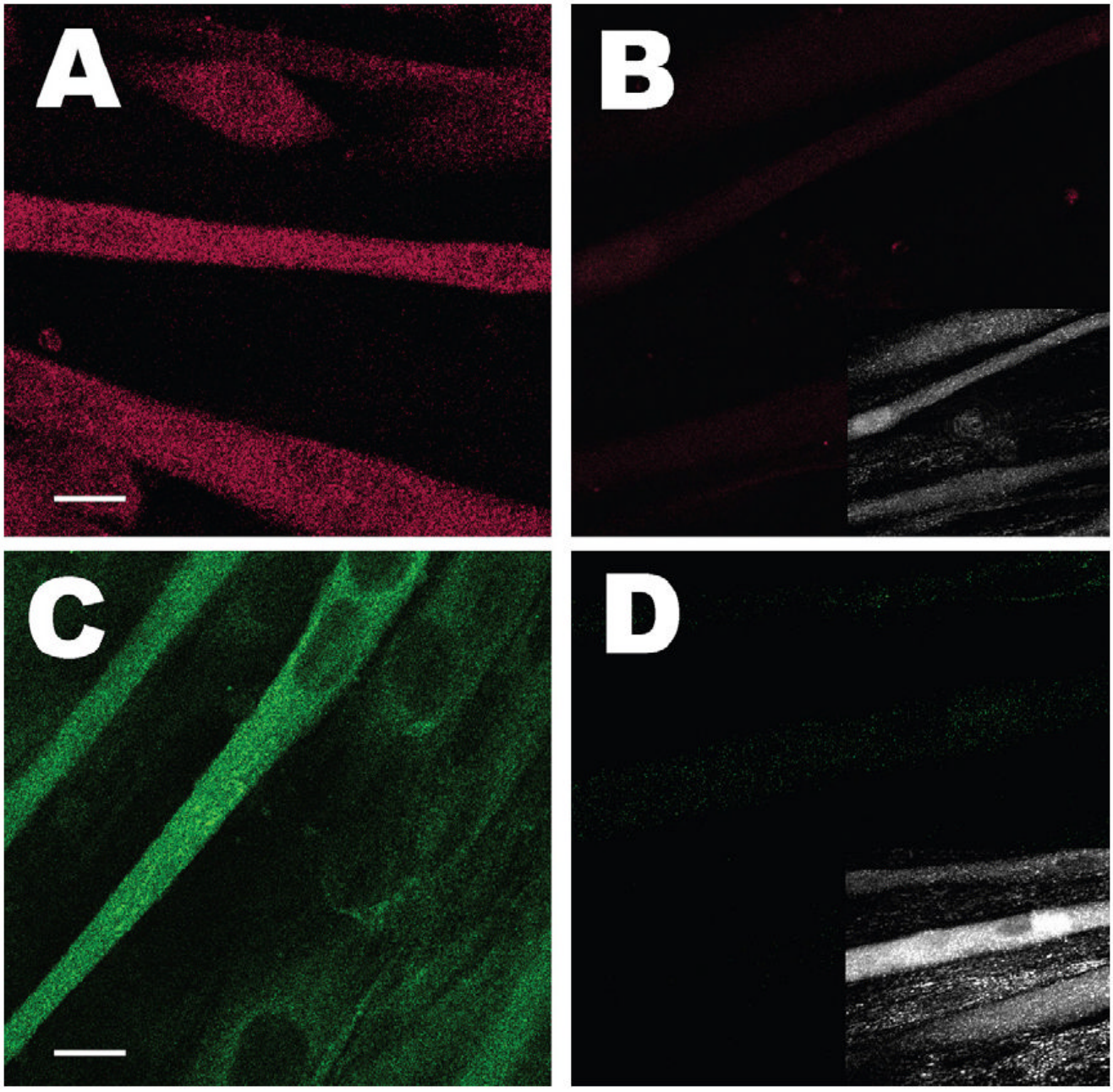

Figure 2.

Efficacies of knocking down junctin and triadin in C_2C_{12} myotubes. Eight-day-old control (A and C) or double knockdown (B and D) myotubes were probed with rabbit anti-junctin polyclonal antibody (A and B) or mouse anti-triadin monoclonal antibody (C and D) followed by the corresponding Alexa 647-conjugated goat-anti-rabbit or Alexa 488-conjugated goat-anti-mouse secondary antibody. The efficacies of the triadin siRNA and junctin siRNA are indicated by the nearly complete elimination of fluorescence in the double knockdown myotubes. Insets in B and D show pictures of corresponding double knockdown myotubes. Scale bars in (A) and (C): 10 μm.

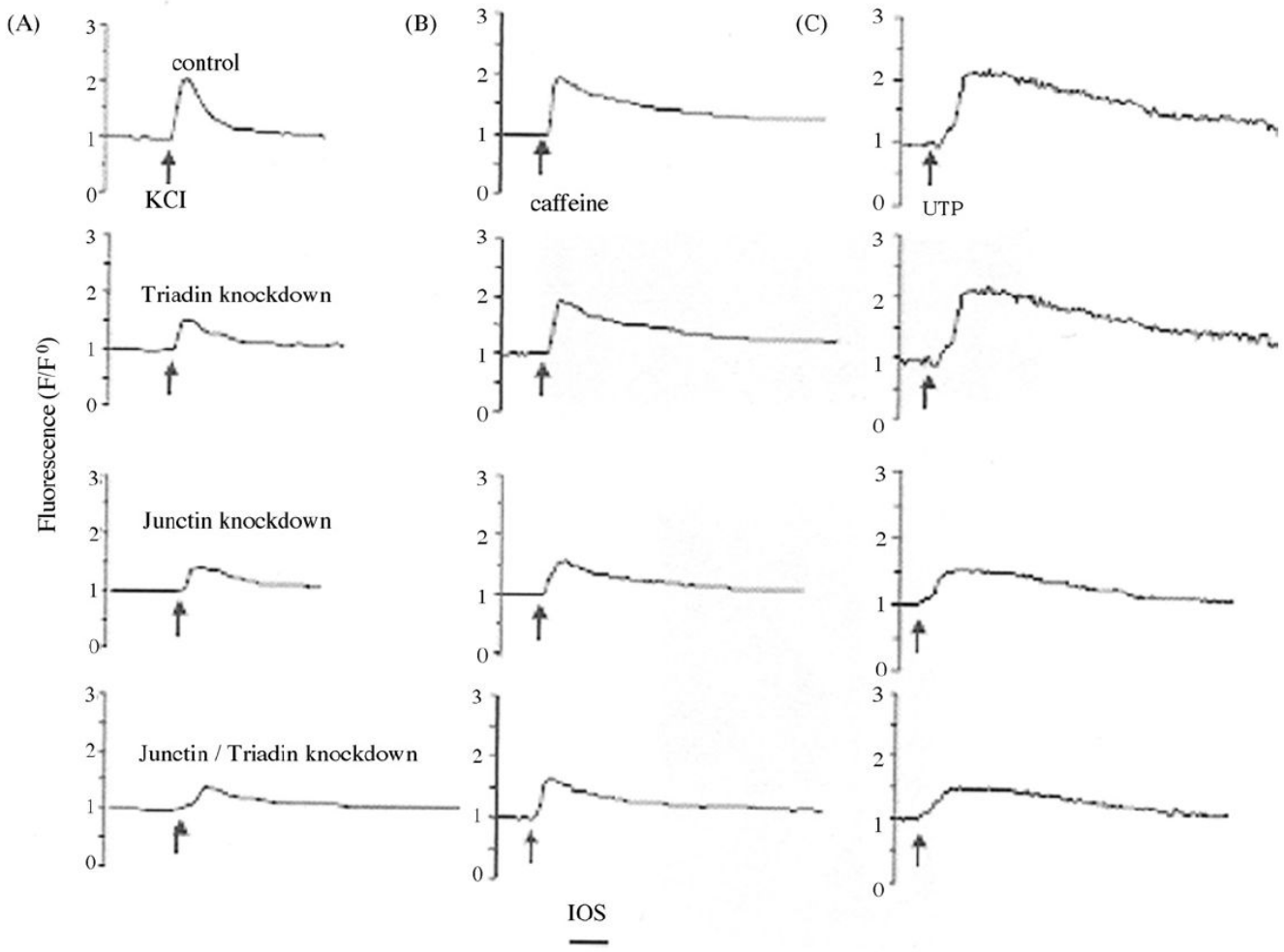

Figure 3.

Effects of junctin and triadin knockdown on stored Ca^{2+} release in 8-day-old C_2C_{12} myotubes. Depolarization (10 mM KCl) (A), caffeine (10 mM) (B), and UTP (400 μM) (C) induced Ca^{2+} release were determined in Ca^{2+}-free KRH bath solutions as changes of Fluo-4 fluorescence (F/F_0) in C_2C_{12} myotubes infected with rAAV-control (top traces), rAAV-triadin (second traces), rAAV-junctin (third traces), and rAAV-junctin/triadin (bottom traces).

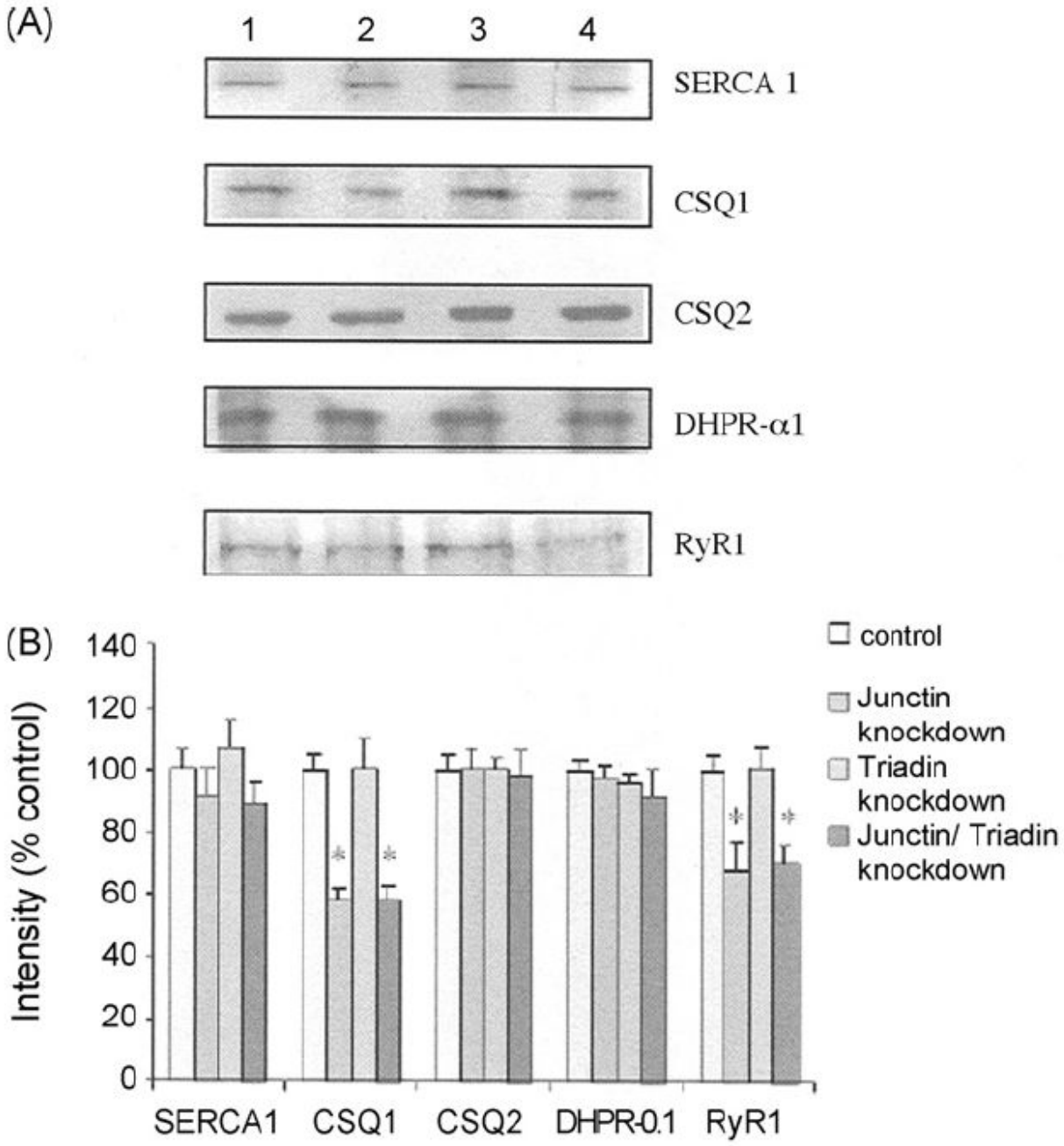

Figure 4.

Immunoblot analysis of C_2C_{12} myotubes. (A) Protein levels in C_2C_{12} myotubes infected with rAAV-control (lane 1), rAAV-junctin (lane 2), rAAV-triadin (lane 3), and both rAAV-junctin and rAAV-triadin (lane 4). Immuoblots were performed on 8-day-old C_2C_{12} myotube lysates. (B) Normalized intensities of protein bands. Data are the mean ± S.E. of three experiments. *$p < 0.05$ compared to controls.

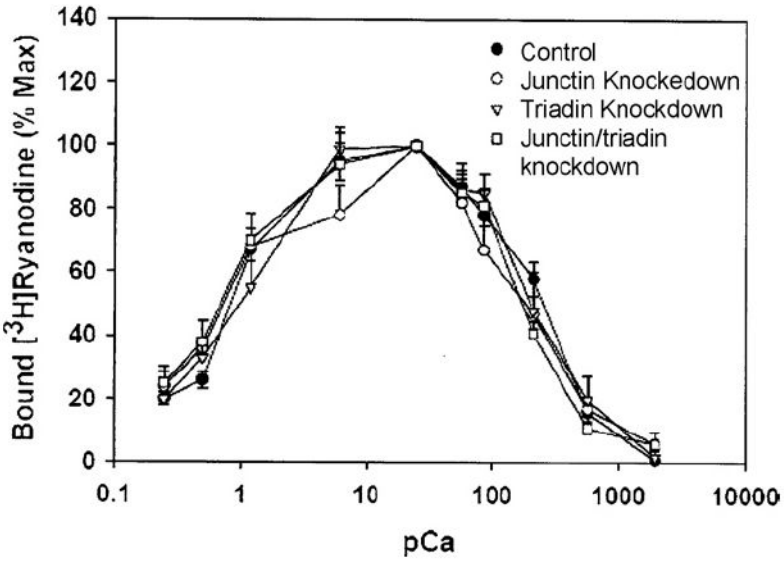

Figure 5.

Ca^{2+} dependence of [^{3}H]ryanodine binding of 8-day-old junctin and triadin knockdown C_2C_{12} myotubes. Specific [^{3}H]ryanodine binding was determined in 250 mM KCl, 20 mM imidazole, pH 7.0, media containing 2.5 nM [^{3}H]ryanodine and the indicated concentrations of free Ca^{2+}. Data are the mean ± S.E. of three to four experiments.

Table 1

Effects of junctin/triadin knockdown on stored Ca^{2+} release in C_2C_{12} myotubes

Addition	Control	Junctin knockdown	Triadin knockdown	Junctin/triadin knockdown
10 mM KCl	1.98 ± 0.25	1.61 ± 0.18*	1.49 ± 0.13*	1.27 ± 0.08*
0.4 mM 4-chloro-*m*-cresol	1.88 ± 0.23	1.65 ± 0.17*	2.05 ± 0.14	1.60 ± 0.11*
10 mM caffeine	1.83 ± 0.17	1.65 ± 0.14*	1.88 ± 0.24	1.69 ± 0.18*
1 μM thapsigargin	1.86 ± 0.17	1.64 ± 0.16*	1.93 ± 0.24	1.63 ± 0.14*
0.4 mM UTP	2.07 ± 0.29	1.46 ± 0.08*	1.90 ± 0.16	1.48 ± 0.07*

Peak values of fluorescence increases (*F*/*F0*) in C_2C_{12} myotubes were determined in Fluo-4 loaded cells in Ca^{2+}-free KRH buffer. Data are the mean ± S.E. of 8–17 experiments.

* $p < 0.05$ compared to control.

Table 2

Quantitative RT-PCR of mRNAs from junctin and triadin knockdown myotubes.

MRNA	Control	Junctin knockdown	Triadin knockdown	Junctin/triadin knockdown
RyR1	100	131 ± 14	99 ± 7	103 ± 6
RyR3	100	126 ± 12	109 ± 11	124 ± 13
DHPR-α1	100	82 ± 23	107 ± 26	72 ± 22
CSQ1	100	137 ± 29	68 ± 26	76 ± 23
CSQ2	100	130 ± 22	105 ± 11	116 ± 16
SERCA1	100	109 ± 10	97 ± 31	94 ± 19

RT-PCR analyses were performed using total RNA isolated from 8-day-old myotubes. Relative levels of gene expression as a percentage of control were determined for each gene with β-actin as reference. Data are the mean ± S.E. of 3–12 samples.

Table 3

B_{max} values of [^{3}H]ryanodine binding and $^{45}Ca^{2+}$uptake rates of membranes isolated from control and junctin/triadin knockdown C_2C_{12} myotubes

Sample	B_{max} of [^{3}H]ryanodine binding (% of control)	$^{45}Ca^{2+}$ uptake rate (% of control)
Control	100	100
Junctin knockdown	63 ± 9*	89 ± 12
Triadin knockdown	113 ± 54	107 ± 16
Junctin/triadin knockdown	77 ± 32	92 ± 7

The total number of [^{3}H]ryanodine binding sites (B_{max}) and $^{45}Ca^{2+}$ uptake rates of crude membrane fractions were determined as described in Materials and methods. Data are mean ± S.E. of six experiments.

* $p < 0.05$ compared to control.