Functional Coupling between TRPC3 and RyR1 Regulates the Expressions of Key Triadic Proteins*

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We have shown that TRPC3 (transient receptor potential channel canonical type 3) is sharply up-regulated during the early part of myotube differentiation and remains elevated in mature myotubes compared with myoblasts. To examine its functional roles in muscle, TRPC3 was “knocked down” in mouse primary skeletal myoblasts using retroviral–delivered small interference RNAs and single cell cloning. TRPC3 knockdown myoblasts (97.6 ± 1.9% reduction in mRNA) were differentiated into myotubes (TRPC3 KD) and subjected to functional and biochemical assays. By measuring rates of Mn2+ influx with Fura-2 and Ca2+ transients with Fluo-4, we found that neither excitation-coupled Ca2+ entry nor thapsigargin-induced store-operated Ca2+ entry was significantly altered in TRPC3 KD, indicating that expression of TRPC3 is not required for engaging either Ca2+ entry mechanism. In Ca2+ imaging experiments, the gain of excitation-contraction coupling and the amplitude of the Ca2+ release seen after direct RyR1 activation with caffeine was significantly reduced in TRPC3 KD. The decreased gain appears to be due to a decrease in RyR1 Ca2+ release channel activity, because sarcoplasmic reticulum (SR) Ca2+ content was not different between TRPC3 KD and wild-type myotubes. Immunoblot analysis demonstrated that TRPC1, calsequestrin, triadin, and junctophilin 1 were up-regulated (1.46 ± 1.91, 1.42 ± 0.08, 2.99 ± 0.32, and 1.91 ± 0.26-fold, respectively) in TRPC3 KD. Based on these data, we conclude that expression of TRPC3 is tightly regulated during muscle cell differentiation and propose that functional interaction between TRPC3 and RyR1 may regulate the gain of SR Ca2+ release independent of SR Ca2+ load.

TRPCs are one family of transient receptor potential (TRP) cation channels. This entire group of channels has been predicted to consist of six transmembrane segment channels that allow the entry of Ca2+ and Na+ into the cell (1–3). One of the TRPC subtypes, TRPC3, is highly expressed in the brain, skeletal muscle, and cardiac muscle (4–6). It has been reported that TRPC3-mediated Ca2+ entry can be induced by two different mechanisms: 1) direct activation by binding of exogenous organic molecules or endogenous metabolites such as diacylglycerol analogues (7), and 2) phospholipase C-mediated activation via phospholipase C-coupled receptor. The latter mechanism can be subdivided into two different submechanisms. One submechanism is store-operated Ca2+ entry (SOCE) (8–10) (for example, when Ca2+ store depletion through IP3R by phospholipase C pathway triggers TRPC3 activation). The second submechanism is receptor-operated Ca2+ entry (11–13) (for example, in B lymphocytes, when TRPC3 is activated by physical coupling with phospholipase C γ1 and is responsible for the secondary intracellular Ca2+ entry after B-cell receptor activation) (14).

Physiological evidence has been demonstrated for the involvement of TRPC3 in many processes. For example, TRPC3 activation via phospholipase C γ1/IP3R pathway is essential for brain-derived neurotrophic factor-dependent growth cone guidance in cerebellar granule neurons (15). In arterial smooth muscle cells, TRPC3 is activated by purinergic receptors, and then Ca2+ influx through TRPC3 induces depolarization and vasoconstriction (16). Using heterologous expression studies in HEK 293 cells, it has been shown that TRPC3 physically interacts with cytosolic Ca2+ signaling proteins such as IP3R, where it competes with calmodulin for the same binding site on IP3R (17). In addition, it has been reported that TRPC3 interacts with ryanodine receptors (RyRs) (18, 19); however, the functional roles of TRPC3 in skeletal muscle have not been well addressed.

RyRs function as Ca2+ release channels in the sarcoplasmic reticulum (SR) and are essential proteins for excitation-contraction (EC) coupling in striated muscles (20, 21). RyRs have been shown to have bidirectional communication with a second essential protein in EC coupling, the dihydropyridine receptor (DHPR), which acts as both a sarcolemmal L-type voltage-gated Ca2+ channel (20) and the mechanical trigger for RyR activation (21). During EC coupling, as a result of membrane depolarization, Ca2+ influx through DHPR or conformational changes of DHPR via its gating charge movement activates RyR2 in cardiac muscle or RyR1 in skeletal muscle respectively. The Ca2+ stored in SR is then released through RyRs into the cytosol to cause muscle contraction. Recently, functional coupling between RyR and TRPC1 (22) or TRPC3 (18) has been shown in heterologous expression systems such as Chinese hamster ovary and HEK cells. In the case of TRPC1, it has been proposed that the functional interaction between RyR1 and TRPC1 is a physical interaction with an undefined region in the cytoplasmic foot region of RyR1 (22). Furthermore, involvement of TRPCs has been reported in the abnormal calcium influx observed in muscle disease models such as dystrophic mouse skeletal muscle fibers (6). Therefore, although in skeletal muscle it is possible for EC coupling to occur in the absence of extracellular Ca2+ (21), the possible involvement of extracellular Ca2+ influx and the interactions of RyR1 with Ca2+ ion-conduct-
ing channel proteins in sarcolemma (other than the DHPR) during EC coupling still remain to be elucidated. In the current study, we have defined the role of TRPC3 during EC coupling and muscle contraction in primary mouse skeletal muscle myoblasts/myotubes in which TRPC3 mRNA and protein expression was reduced (>97% and >94%, respectively) by using small interference RNA.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fetal bovine serum, cell culture media, trypsin, t-glutamine, penicillin/streptomycin, and basic fibroblast growth factor were obtained from Invitrogen. Caffeine, KCl, ryanodine, thapsigargin, cyclopiazonic acid, and heat-inactivated horse serum were obtained from Sigma-Aldrich. Monoclonal anti-RyR1 antibody (34C) was provided by Drs. J. Airey and J. Sutko (Developmental Studies Hybridoma Bank, Iowa City, IA). Anti-TRPC3 antibody was obtained from Alomone Labs (Jerusalem, Israel). Anti-DHPR, sarcoplasmic reticulum calcium ATPase (SERCA), triadin, and calsequestrin antibodies were obtained from Affinity BioReagents (Golden, CO). Anti-junctophilin 1 antibody was a kind gift from Dr. Jianjie Ma. Anti-calmodulin, FKBP12, and ATPase (SERCA) antibodies were obtained from Drs. J. Airey and J. Sutko (Developmental Studies Hybridoma Bank, Iowa City, IA). Anti-DHPR, sarcoplasmic reticulum calcium ATPase (SERCA), triadin, and calsequestrin antibodies were obtained from Affinity BioReagents (Golden, CO). Anti-junctophilin 1 antibody was a kind gift from Dr. Jianjie Ma. Anti-calmodulin, FKBP12, and ATPase (SERCA), triadin, and calsequestrin antibodies were obtained from Developmental Studies Hybridoma Bank, Iowa City, IA. Anti-TRPC3 antibody was obtained from Alomone Labs (Jerusalem, Israel). Anti-calmodulin, FKBP12, and ATPase (SERCA) antibodies were obtained from Affinity BioReagents (Golden, CO). Anti-junctophilin 1 antibody was a kind gift from Dr. Jianjie Ma. Anti-calmodulin, FKBP12, and ATPase (SERCA), triadin, and calsequestrin antibodies were obtained from Developmental Studies Hybridoma Bank, Iowa City, IA.

**Cell Cultures**—The method used to derive primary wild-type myoblasts from mouse skeletal muscle was previously described (23–25). After ~48 h in growth medium, myoblasts were replated either on 10-cm dishes coated with collagen to prepare cell lysates or on 96-well plates coated with collagen for Ca2+ imaging and Mn2+ quench experiments. When cells reached ~70% confluence, growth medium was replaced with differentiation medium (containing 5% heat-inactivated horse serum and no growth factors) and placed into an 18% CO2 incubator to induce differentiation. HEK293 cells were grown at 37 °C in a 5% CO2 incubator in high glucose Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA).

**Creation of TRPC3 Knockdown Primary Myoblasts**—To knock down the mRNA of TRPC3 in primary myoblasts, vectors expressing short hairpin RNA sequences were used. First, two different sequences were selected using a program from Dharmacon siDESIGN center (Dharmacon, Chicago, IL) based on the cDNA sequence of TRPC3 (GenBank accession number NM_019510). BLAST searches confirmed that the selected oligonucleotide sequences did not possess homology to any other genes. Each of two 19-nucleotide sequences (Fig. 1A, Sequences I and II) was inserted into a retroviral vector (pSIREN-RetroQ, Clontech Laboratories, Mountain View, CA). Retroviral particles were packaged by transfecting each short hairpin RNA-expressing vector into HEK293-based packaging cells with FuGENE transfection reagent, and the harvested supernatant was filtered with 0.2 μm non-pyrogenic disc filters (Pall Corporation, Ann Arbor, MI). The filter-through (TRPC3 KD retroviruses I and II) was stored at 70 °C before use. Primary mouse skeletal muscle myoblasts were incubated for 3 h with both TRPC3 KD retroviruses I and II and polybrene (8 μg/ml) on 10-cm dishes coated with collagen to prepare cell lysates or on 96-well plates coated with collagen for Ca2+ imaging and Mn2+ quench experiments. When cells reached ~70% confluence, growth medium was replaced with differentiation medium (containing 5% heat-inactivated horse serum and no growth factors) and placed into an 18% CO2 incubator to induce differentiation. HEK293 cells were grown at 37 °C in a 5% CO2 incubator in high glucose Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin.

**TRPC3 Knockdown Primary Myoblasts**—To knock down the mRNA of TRPC3 in primary myoblasts, vectors expressing short hairpin RNA sequences were used. First, two different sequences were selected using a program from Dharmacon siDESIGN center (Dharmacon, Chicago, IL) based on the cDNA sequence of TRPC3 (GenBank accession number NM_019510). BLAST searches confirmed that the selected oligonucleotide sequences did not possess homology to any other genes. Each of two 19-nucleotide sequences (Fig. 1A, Sequences I and II) was inserted into a retroviral vector (pSIREN-RetroQ, Clontech Laboratories, Mountain View, CA). Retroviral particles were packaged by transfecting each short hairpin RNA-expressing vector into HEK293-based packaging cells with FuGENE transfection reagent, and the harvested supernatant was filtered with 0.2 μm non-pyrogenic disc filters (Pall Corporation, Ann Arbor, MI). The filter-through (TRPC3 KD retroviruses I and II) was stored at 70 °C before use. Primary mouse skeletal muscle myoblasts were incubated for 3 h with both TRPC3 KD retroviruses I and II and polybrene (8 μg/ml) on 10-cm dishes coated with collagen to prepare cell lysates or on 96-well plates coated with collagen for Ca2+ imaging and Mn2+ quench experiments. When cells reached ~70% confluence, growth medium was replaced with differentiation medium (containing 5% heat-inactivated horse serum and no growth factors) and placed into an 18% CO2 incubator to induce differentiation. HEK293 cells were grown at 37 °C in a 5% CO2 incubator in high glucose Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin.

**Clone Numbers 1–5**—To knock down the mRNA of TRPC3, each palindromic double array of 19 base pairs (indicated by dots in Sequences I and II) that were complementary sequences to parts of TRPC3 mRNA was inserted to a type of retroviral vector using 5’-BamHI and 3’-EcoRI sites. Vector map was adapted from the web site of Clontech Laboratories (Clontech Laboratories). P_Cam, Pu6, and P_vir PGK, human U6, and SV40 promoters; Puro’ or Amp’, puromycin or ampicillin resistance; 5’-LTR CMV/MSV, mouse cytomegalovirus type I and sarcoma virus hybrid promoter; 3’-LTR, 3’-MoMuLV LTR with poly(A) region; SV40 ori and ColE1 ori, replication initiation sites; Ψ, extended packaging signal; B, real-time PCR analysis of cDNA that was prepared from total mRNA isolated from each myotube clone. Clone numbers 1–5 showed a >90% reduction in the mRNA level of TRPC3 compared with that of wild-type myotubes. Clone number 1–2 (TRPC3 KD) showed the most dramatic decrease (>97%) and was used for all further studies. The data are the mean ± S.E. of two duplicated independent experiments. C, solubilized cell lysate from TRPC3 KD myotubes was subjected to immunoblot analysis with anti-TRPC3 antibody. The expression of TRPC3 in TRPC3 KD myotubes was reduced >94%. The data are the mean ± S.E. of three independent experiments.

**Duplicate.** The reaction conditions were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplification and detection were performed with the ABI PRISM Sequence Detection System (Applied Biosystems, Foster City, CA).

**Ca2+ Imaging and Mn2+ Quench Experiments**—Ca2+ transients (cytosolic Ca2+ amount) and Mn2+ influx were measured as described.
Functional Coupling between TRPC3 and RyR1

FIGURE 2. Decreased cytosolic Ca\(^{2+}\) transients associated with EC coupling in TRPC3 KD myotubes. A, to induce EC coupling, 60 mM KCl was treated to wild-type (black) or TRPC3 KD (gray) myotubes loaded with Fluo-4. TRPC3 KD myotubes showed significantly decreased cytosolic Ca\(^{2+}\) transient during EC coupling (left), suggesting a decreased gain of EC coupling. A representative normalized trace of TRPC3 KD myotubes compared with that of wild-type myotubes is shown on the right. Inactivation slopes were fitted to a linear equation and represented by dotted lines. TRPC3 KD myotubes showed faster inactivation than wild-type myotubes. Histograms are shown for normalized peak amplitude of TRPC3 KD myotubes to that of wild-type myotubes (B) or normalized residual amplitude at 20 s after the peak (vertical dotted line in panel A, left) to its peak amplitude (C). The residual amplitude in TRPC3 KD myotubes was significantly smaller than that of wild-type myotubes. D, SR depletion by treatment with 10 \(\mu\)M cyclopiazonic acid. Depletion traces of wild-type and TRPC3 KD myotubes were colored in black and gray, respectively. E, depletion peak amplitude of TRPC3 KD myotubes was normalized by that of wild-type myotubes. The data are the mean ± S.E. of 115 (Wild type) or 202 (TRPC3 KD) independent experiments. For normalized peak amplitude in panels B and E, peak amplitude of wild-type myotubes was set to 1. For normalized residual amplitude in panel C, each peak amplitude of wild-type or TRPC3 KD myotubes was set to 1. *, significant difference compared with wild type (\(p<0.05\)). **, significant difference compared with its peak amplitude (\(p<0.05\)).

Statistical Analysis—Results are given as means ± S.E. with the number of experiments mentioned in the figure legends. Significance of the differences was analyzed by the paired or unpaired \(t\)-test (GraphPad InStat, v2.04). Differences were considered to be significant when \(p<0.05\). Graphs were prepared using Origin v7.

RESULTS

Small Interference RNA Knock Down of TRPC3 Expression—Total mRNA preparations of fourteen different TRPC3 knockdown clones were subjected to RT-PCR and then real-time PCR to examine TRPC3 mRNA levels (Fig. 1B). The most effective clone was clone number 2–21 (with 97.6 ± 1.9% reduction in the mRNA level). Immunoblot analysis with solubilized cell lysate from clone number 2–21 myotubes showed 94.6 ± 0.4% reduction in the protein level (Fig. 1C). This clone was named TRPC3 KD and used for all further functional and biochemical experiments.

Functional Consequence of TRPC3 Knock Down—We examined cytosolic Ca\(^{2+}\) transients in response to depolarization and direct RyR1 activation in TRPC3 KD myotubes loaded with Fluo-4. Wild-type myotubes were used as a control. To mimic the membrane depolarization of muscle, 60 mM KCl was applied to myotubes for 30 s (Fig. 2A, left). Interestingly, TRPC3 KD myotubes had a 40% decrease in peak amplitude of depolarization-induced Ca\(^{2+}\) release compared with wild-type myotubes (Fig. 2B, normalized peak amplitude of TRPC3 KD myotubes was 0.61 ± 0.05 when the peak amplitude of wild-type myotubes was set

previously (25–27). Briefly, differentiated primary wild-type and TRPC3 KD myotubes were loaded with Fluo-4 for Ca\(^{2+}\) imaging and Fura-2 for Mn\(^{2+}\) quench experiments (500 \(\mu\)M MnCl\(_2\)). Dye-loaded myotubes were imaged with an intensified CCD camera with a ×40 objective. Caffeine, KCl, cyclopiazonic acid, ryanodine, and thapsigargin were dissolved in imaging buffer or Me\(^2\)SO (<0.1%) and applied to myotubes by a 16-channel perfusion pipette (AutoMate Scientific, Berkley, CA). To maintain osmolarity, in the case of KCl (60 mM) application the concentration of Na\(^{+}\) was lowered and the chloride product was maintained.

Co-immunoprecipitation—Co-immunoprecipitation was done as previously described (28). Briefly, myotubes were homogenized and incubated with a lysis buffer (1% Triton X-100, 10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Na\(_3\)VO\(_4\), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 20 \(\mu\)g/ml of aprotinin, 12.5 \(\mu\)g/ml of leupeptin, and 20 \(\mu\)g/ml of pepstatin A) to prepare the cell lysate. For triad, free Ca\(^{2+}\) concentration of the lysis buffer was set at 100 \(\mu\)M by adding 5 mM CaCl\(_2\). The cell lysate was then quickly centrifuged to remove insolubilized masses, and the protein concentration of the supernatant was determined. Total protein (500 \(\mu\)g) of each diluted lysate (4×) with Triton X-100-free lysis buffer was incubated with 25 \(\mu\)l of anti-RyR1 antibody overnight at 4 °C, followed by further incubation with protein G-Sepharose beads for 4 h. Beads were washed three times with the lysis buffer to remove non-specifically bound proteins. The immune complexes were treated with SDS-sample buffer and subjected to SDS-PAGE and immunoblot analysis.
FIGURE 3. Decreased RyR1 activity in TRPC3 KD myotubes. A, wild-type (black) or TRPC3 KD (gray) myotubes loaded with Fluo-4 were exposed to 40 mM caffeine. The peak amplitude of the caffeine response was decreased in TRPC3 KD myotubes (left). A representative normalized trace of TRPC3 KD myotubes compared with that of wild-type myotubes is shown on the right. Inactivation slopes were fitted to linear equation and represented by a dotted line. There was no significant change in inactivation slopes between wild-type and TRPC3 KD myotubes. The histogram for normalized peak amplitudes of TRPC3 KD myotubes by that of wild-type myotubes (B) or normalized residual amplitude at 20 s after the peak (indicated as a vertical dotted line in panel A, left) to its peak amplitude (G) is shown. There was also no significant change in normalized residual amplitudes of wild-type and TRPC3 KD myotubes. The data are the mean ± S.E. of 115 (Wild type) or 202 (TRPC3 KD) independent experiments. For normalized peak amplitude in panel B, peak amplitude of wild-type myotubes was set to 1. For normalized residual amplitude in panel C, each peak amplitude of wild-type or TRPC3 KD myotubes was set to 1. *, significant difference compared with wild-type ($p < 0.05$). **, significant difference compared with its peak amplitude ($p < 0.05$).

The second hypothesis was that TRPC3 could be the yet to be identified excitation-coupling calcium entry (ECCE) channel (29) and that the decrease in gain and decreased residual ratio after KCl application was secondary to a decrease in depolarization-induced Ca$^{2+}$ entry. We tested this hypothesis using Mn$^{2+}$ quench experiments in myotubes loaded with Fura-2 treated with (Fig. 4A) or without (supplemental Fig. S1) 500 μM Ryanodine for 30 min to block RyR1 activity. 60 mM KCl was applied to myotubes to mimic membrane depolarization in the presence of extracellular Mn$^{2+}$, and the rate of Mn$^{2+}$ quench at the Fura-2 isosbestic point was determined. We found that the initial rate of ECCE in TRPC3 KD myotubes was not significantly different from that of wild-type myotubes (3.30 ± 0.35 (TRPC3 KD) versus 3.44 ± 0.44 (Wild type)), suggesting that TRPC3 is not the ECCE-mediating channel and does not interact with the ECCE-mediating protein(s) or play a role in the three-way communication among RyR1, the DHPR, and the ECCE channel.

Because measurement of SR stores with cyclopiazonic acid (or thapsigargin) is difficult to quantitate, we determined whether the near abolishment of TRPC3 could interfere with SOCE. This was tested in myotubes loaded with Fura-4 (Fig. 4B) in which SR Ca$^{2+}$ stores had been previously depleted by the application of 200 nM thapsigargin in the absence of extracellular Ca$^{2+}$. After total store depletion was confirmed by the lack of any response to KCl or caffeine, 2 mM Ca$^{2+}$ was applied to the bath and the initial rate of Ca$^{2+}$ entry was determined. The initial rate of SOCE was not different in TRPC3 KD myotubes compared with that of wild-type myotubes (2.60 ± 0.45 (TRPC3 KD) versus 3.16 ± 0.52 (Wild type)). Although the maximal amplitude of the SOCE transient was reduced in TRPC3 KD myotubes (0.92 ± 0.06 (TRPC3 KD) versus 1.15 ± 0.11 (Wild type)), this difference was not statistically significant ($p = 0.09$) (Fig. 4C).

The Effects of TRPC3 Knock Down on Muscle Protein Expression—To examine expression profiles of the 3 other TRPCs expressed in muscle and 10 triadic proteins involved in EC coupling in TRPC3 KD myotubes, solubilized cell lysates from wild-type and TRPC3 KD myotubes were subjected to immunoblot analysis (Fig. 5). The results of this analysis showed that TRPC1 was up-regulated (1.46 ± 1.91-fold) in response to the knock down of TRPC3, whereas the expression of TRPC4 and TRPC6 were unchanged (Fig. 5A). Of the 10 triadic proteins examined,
7 showed no change in expression (Fig. 5B). However triadin, juncto-
philin 1 (JP1), and calsequestrin (CSQ) had significant increases in basal
expression levels (2.99 ± 0.32, 1.91 ± 0.26, and 1.42 ± 0.08-fold, respectively) associated with the 94% reduction of TRPC3 expression.

Interestingly, like TRPC3, all three proteins are known to interact with
RyR1 and have been shown to regulate RyR1 activity (20, 21, 30).

**Protein-Protein Interactions Determined by Co-immunoprecipi-
tation—**
Co-immunoprecipitation experiments were performed with
34C (anti-RyR1 antibody) to examine whether there was any change in
the amount of proteins interacting with RyR1 associated with the knock
down of TRPC3 (Fig. 5C). As expected, co-immunoprecipitation
of TRPC3 with RyR1 was absent in TRPC3 KD myotubes (data not shown).
Of the three triadic proteins whose expression was up-regulated, we
found that there was an increased complex formation of RyR1 with
etriadin (3.26 ± 0.59-fold) and JP1 (2.02 ± 0.20-fold), but not CSQ, in
the solubilized TRPC3 KD myotube lysates.

**DISCUSSION**

In this study, TRPC3 knock down in skeletal muscle myotubes was
accomplished using a retroviral system to stably deliver small interfer-
ence RNA. One clone (TRPC3 KD, clone number 2–21) had a >97% reduction in mRNA levels and >94% reduction in protein levels (Fig. 1).

Myotubes from this clone were subjected to Ca
2 decoration and Mn
2 quench experiments to examine the functional consequences of TRPC3
knock down in muscle cells. These were followed by biochemical assays to
reveal underlying mechanisms of the functional changes.

TRPC3 KD myotubes had a significantly decreased gain of EC cou-
ing, which is reflected as a decreased cytosolic Ca
2 transient during
EC coupling (Fig. 2). In accordance with the fact that the major Ca
2 source for muscle contraction is Ca
2 release through RyR1 as a result of
a physical interaction between RyR1 and the DHPR during skeletal
EC coupling (21), RyR1 activity in TRPC3 KD myotubes was signifi-
cantly reduced (Fig. 3). Because we ruled out store depletion as the cause
for this reduction by showing that the SR Ca
2 content is the same in
wild-type and TRPC3 KD myotubes (Fig. 2, D and E) and that the initial
rate of SOCE is unchanged (Fig. 4), it is likely that the reduced cytosolic
Ca
2 transient during EC coupling in TRPC3 KD myotubes resulted from
a direct reduction of RyR1 activity. This possibility is supported by the
fact that in addition to a decreased gain of EC coupling, the knock
down of TRPC3 is also associated with a decrease in the gain of Ca
2 release in response to direct activation of RyR1 by caffeine (Fig. 3). Two
other mechanistic possibilities that link the reduced EC coupling and
caffeine-induced release with our protein expression data (Fig. 5) are: 1) by increasing triadin and CSQ, it is very possible that their increased
expression affects how tightly Ca
2 is bound to the complex immedi-
ately ready for Ca
2 release at the junctional endoplasmic reticulum
store or, more likely, 2) the coupling efficiency of RyR1 activation to
mobilize the Ca
2 immediately surrounding luminal space is diminished.
These interpretations are warranted given that both proteins have
been implicated in coupling RyR1 conformation to Ca
2 release from the
lumen (31–34).

One of our original hypotheses was that TRPC3 was the EC channel,
because TRPC3 could be co-immunoprecipitated with RyR1 (18) and
the newly discovered EC current could be blocked by 2-APB, SKP96365,
and La
3+, all of which are known to block TRPC3 current (29). This hypothesis was unequivocally proven incorrect as the initial
rate of ECCE of TRPC3 KD myotubes was not significantly different
from that of wild type (Fig. 4A) (1.23 ± 0.15 (TRPC3 KD) versus 1.30 ±
0.08 (wild type)). In addition, these results showed that the decreased
gain in EC coupling was not secondary to a decreased ECCE current.

A possible connection between the reduced RyR1 activity and the
absence of TRPC3 is the up-regulation of triadin and subsequent direct
inhibition of RyR1 function caused by the increase in the amount of
triadin complexed with RyR1 (Fig. 5, B and C). Triadin has been previ-
ously suggested to be an direct inhibitory protein of RyR1 activity (35),
and overexpression of the primary skeletal 95-kDa isoform of triadin,
Trisk 95, resulted in the almost complete abolition of skeletal-type
depolarization-induced Ca
2 release in the absence of extracellular Ca
2 and a significant decrease in gain in the presence of extracellular
Ca
2 (36). This is in accordance with our hypothesis. However, arguing
against this hypothesis is the fact that in the Trisk 95-overexpressing
cells, caffeine-induced Ca
2 release was not different from control.

Another interesting protein that shows increased complex formation
with RyR1 in TRPC3 KD myotubes is JP1. JP1 has been shown to facil-

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**FIGURE 4. Unchanged ECCE and SOCE in TRPC3 KD myotubes.** A, to measure Ca
2 influx during excitation (ECCE), Mn
2 influx by the treatment of 60 mM KCl was measured in
myotube loaded with Fura-2 with 500 μM ryanodine for 30 min to block RyR1 activity. There was no significant difference in initial rates of influx between wild-type and TRPC3 KD myotubes. The data are the mean ± S.E. of 36 (Wild type) or 14 (TRPC3 KD) independent experiments. B, to measure Ca
2 influx after SR depletion (SOCE), SR of wild-type and TRPC3 KD myotubes was depleted of Ca
2 by treatment with 200 nM thapsigargin in the absence of extracellular Ca
2. When the cell would no longer respond to a caffeine or KCl depolarization stimulus, 2 mM Ca
2 was applied to extracellular side (batch), and the rate of Ca
2 entry was determined from the slope of the cytoplasmic Ca
2 transient. There was no significant difference in initial rates of influx between wild-type and TRPC3 KD myotubes; although there was a trend toward a smaller maximal amplitude in
TRPC3 KD myotubes, this difference was not significant (p < 0.09) (C). The data are the mean ± S.E. of 20 (Wild type) or 17 (TRPC3 KD) independent experiments. Traces from wild-type and TRPC3 KD myotubes are black and gray, respectively. The initial rate of influx was fitted to linear equation and represented by dotted lines.
Functional Coupling between TRPC3 and RyR1

A

![Image](https://example.com/image1.png)

B

![Image](https://example.com/image2.png)

C

![Image](https://example.com/image3.png)

**FIGURE 5.** Expression level of three other TRPC isoforms and EC-coupling proteins in TRPC3 KD myotubes. A, solubilized cell lysate from TRPC3 KD myotubes was subjected to immunoblot analysis to examine the expression level of three other TRPC isoforms (TRPC1, TRPC4, and TRPC6) expressed in muscle myotubes. There were no changes in TRPC4 and TRPC6. However, expression level of TRPC1 was significantly increased. B, expression levels of 10 EC-coupling proteins were also examined with α-tubulin as a control. Expression levels of triadin, JP1, and CSQ were significantly increased. C, the three proteins showing increased expression in panel A were subjected to co-immunoprecipitation with RyR1 using anti-RyR1 antibody (34C). More triadin and JP1 than CSQ were co-immunoprecipitated with RyR1 in TRPC3 KD myotubes. *, significant difference compared with wild type (p < 0.05). The data are the mean ± S.E. of three independent experiments.

The hypothesis that there is a functional interaction between TRPC3 and RyR1 is supported by the observation that expression of TRPC3 was enhanced by the expression of RyR1 in dyspedic IB5 (lacking RyR1) myotubes (supplemental Fig. S2, upper left). Regulation of TRPC3 expression by RyR1 was also confirmed with wild-type (RyR1−/−) and RyR1-deficient (RyR1−/−) primary myotubes (supplemental Fig. S2, right and histogram), indicating that this regulation is not dependent on the origin of the cells.

Interestingly, although the expression of two of the three other TRPCs expressed in skeletal muscle, TRPC4 and TRPC6, is unchanged in TRPC3 KD myotubes, there is an increased expression level of TRPC1 (Fig. 5A). Considering recent reports about SOCE through heteromeric TRPC channels composed of endogenous TRPC1, TRPC3, and TRPC7 in HEK293 cells (38) and the proven tandem action of TRPC1 and TRPC3 to mediate SOCE in a rat hippocampal neuronal cell line (39), it is very likely that TRPC1 and TRPC3 are partners in making heteromeric TRPC channels in muscle. Thus it is likely that the increase in the expression of TRPC1 found here is sufficient to compensate for
the absence of TRPC3 when the rate of depletion-activated SOCE is measured and prevents the conclusion that TRPC3 plays no role in SOCE in wild-type myocytes (Fig. 4, B and C).

Studies with neurons have revealed that TRPC3 is highly expressed in the rat hippocampal cell line and mediates the specific SOCE required for its differentiation, but not that associated with proliferation (39). The studies conclude that TRPC3 activity is essential for the guidance of nerve growth cone in rat pontine neurons (15). In the same context, we found that during differentiation of primary wild-type myoblasts to myotubes, expression level of TRPC3 peaked just after starting differentiation (day 1) and then gradually decreased (Ref. 19 and supplemental Fig. S3). However, the fully differentiated myotubes on day five still expressed more TRPC3 than growing myoblasts (day zero) (2.85 ± 0.21-fold). Thus, in the case of mouse skeletal muscle, regulated TRPC3 expression is associated with muscle cell differentiation, although its role is not yet known.

In conclusion, in this study we examined roles of TRPC3 in mouse skeletal muscle. We found that there appears to be a direct specific functional interaction between RyR1 and TRPC3 that, when interrupted, lowers the gain of the RyR1 activity without any depletion of the Ca2+ store. It is also possible that, either instead or in addition to this direct interaction, the up-regulation of triadin and JPI associated with the absence of TRPC3 may play a role in down-regulating RyR1 activity. Our data clearly show that TRPC3 is not the ECCE-associated protein, and because of an associated up-regulation of the expression of TRPC1, which is known to be a partner in forming heteromeric SOCE channels in other tissues, it was not possible to see any difference in SOCE function associated with the knock down of TRPC3.

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