Knocking Down Type 2 but Not Type 1 Calsequestrin Reduces Calcium Sequestration and Release in C2C12 Skeletal Muscle Myotubes*

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We examined the roles of type 1 and type 2 calsequestrins (CSQ1 and CSQ2) in stored Ca2+ release of C2C12 skeletal muscle myotubes. Transduction of C2C12 myoblasts with CSQ1 or CSQ2 small interfering RNAs effectively reduced the expression of targeted CSQ protein to near undetectable levels. As compared with control infected or CSQ1 knockdown myotubes, CSQ2 and CSQ1/CSQ2 knockdown myotubes had significantly reduced stored Ca2+ release evoked by activators of intracellular Ca2+ release channel/ryanodine receptor (10 mM caffeine, 200 μM 4-chloro-m-cresol, or 10 mM KCl). Thus, CSQ1 is not essential for effective stored Ca2+ release in C2C12 myotubes despite our in vitro studies suggesting that CSQ1 may enhance ryanodine receptor channel activity. To determine the basis of the reduced stored Ca2+ release in CSQ2 knockdown myotubes, we performed immunoblot analyses and found a significant reduction in both sarco/endoplasmic reticulum Ca2+-ATPase and skeletal muscle ryanodine receptor proteins in CSQ2 and CSQ1/CSQ2 knockdown myotubes. Moreover, these knockdown myotubes exhibited reduced Ca2+ uptake and reduced stored Ca2+ release by UTP (400 μM) that activates a different family of intracellular Ca2+ release channels (inositol 1,4,5-trisphosphate receptors). Taken together, our data suggest that knocking down CSQ2, but not CSQ1, leads to reduced Ca2+ storage and release in C2C12 myotubes.

Calcium release from intracellular stores is a key step in a wide variety of cellular processes (1–3). In striated muscles, the release of stored Ca2+ from the sarcoplasmic reticulum (SR)2 into cytosol by intracellular Ca2+ release channels known as ryanodine receptors (RyRs) initiates muscle contraction. Subsequently, the sequestration of cytosolic Ca2+ back into the SR by the sarcoplasmic/endoplasmic Ca2+ ATPase (SERCA) leads to muscle relaxation (3). In addition to RyRs and SERCA, a number of SR proteins also play a significant role in Ca2+ storage and release and therefore may be important in striated muscle function. In this regard, the high-capacity, low-affinity Ca2+-binding protein calsequestrin (CSQ) has drawn increased attention. Although CSQ is thought to increase Ca2+ storage capacity of SR in striated muscles (4–6), more recent studies have suggested that CSQ may also affect stored Ca2+ release by modulating RyR channel activities directly (7–9) or indirectly (10–12). Murine muscles express two CSQ isoforms that share ~60% amino acid sequence homology (13). Crystal structure analysis has shown that the two CSQ isoforms form linear polymers as the Ca2+ concentration is increased (14). Type 1 CSQ (CSQ1) is expressed in fast-twitch skeletal muscle and is concentrated in the terminal cisternae of SR (15) where it interacts with triadin, an RyR-associated protein (8, 16). Consistent with its role in Ca2+ storage, overexpression of CSQ1 in skeletal muscle-derived C2C12 myotubes increased Ca2+ store size (17). In addition, CSQ1 has also been shown to modulate skeletal muscle RyR (RyR1) channel activity directly (7–9) as well as through RyR-associated proteins such as triadin and junction (11, 18). Preliminary studies with a knock-out mouse model, null for CSQ1, have suggested that CSQ1 is an important determinant of skeletal muscle triad (SR-T-tubule) structure (19). Slow-twitch skeletal muscle co-express CSQ1 and type 2 CSQ (CSQ2) (20–22). In cardiac myocytes, CSQ2 forms a large multiprotein complex that includes the cardiac RyR (RyR2), triadin, and junctin (23). Overexpression of CSQ2 in cardiac myocytes increased SR Ca2+ levels as well as impaired SR Ca2+ release (24–26). A deficiency in CSQ2 (27) and a CSQ missense mutation (D307H) (28) both caused catecholamine-induced polymorphic ventricular tachycardia in humans. The importance of CSQ2 in skeletal muscle, however, is not well understood.

To better understand the roles of CSQ1 and CSQ2 in stored Ca2+ release of skeletal muscle, we used siRNAs to knockdown CSQ1 and/or CSQ2 in 8-day-old C2C12 myotubes that otherwise robustly expressed both CSQ isoforms. After confirming the efficacies as well as specificities of our gene-silencing approach, RyR channel activities in the presence or absence of each CSQ isoform were examined using [1H]ryanodine binding and single channel recordings. In addition, stored Ca2+ release in different experimental groups of C2C12 myotubes evoked by activators of RyRs was assessed in the absence of extracellular Ca2+. Although our in vitro studies suggest that CSQ1 may enhance RyR channel activity, CSQ1 knockdown myotubes unexpectedly had preserved stored Ca2+ release evoked by activators of RyRs. In contrast, CSQ2 and CSQ1/CSQ2 knockdown myotubes had significantly reduced stored Ca2+ release to the same RyR activators. Our subsequent data suggest that CSQ2, but not CSQ1, maintains type 1 SERCA (SERCA1) protein expression and Ca2+ sequestration activity in C2C12 myotubes. Knocking down CSQ2 therefore leads to reduced Ca2+ storage and thus reduced stored Ca2+ release in these cells. In addition, a deficiency in CSQ2 may lead to reduced RyR1 expression in C2C12 myotubes thus further compromising their stored Ca2+ release. A preliminary report of this work has been presented in abstract form (29).

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2 The abbreviations used are: SR, sarcoplasmic reticulum; RyR, ryanodine receptor; RyR1, type 1 RyR; RyR2, type 2 RyR; RyR3, type 3 RyR; CSQ1, type 1 calsequestrin; CSQ2, type 2 calsequestrin; SERCA1, type 1 sarco/endoplasmic reticulum Ca2+-ATPase; siRNA, small interfering RNA; RAVV, recombinant adenovirus-associated virus; iP3, inositol 1,4,5-trisphosphate receptor; KRH, Krebs-Ringer-Henseleit; PIPES, 1,4-piperazinediethanesulfonic acid; DHPR, dihydropyridine receptor.
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4 This work was supported by National Institutes of Health Grants HL 73051 and AR18687. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
5 The abbreviations used are: SR, sarcoplasmic reticulum; RyR, ryanodine receptor; RyR1, type 1 RyR; RyR2, type 2 RyR; RyR3, type 3 RyR; CSQ1, type 1 calsequestrin; CSQ2, type 2 calsequestrin; SERCA1, type 1 sarco/endoplasmic reticulum Ca2+-ATPase; siRNA, small interfering RNA; RAVV, recombinant adenovirus-associated virus; iP3, inositol 1,4,5-trisphosphate receptor; KRH, Krebs-Ringer-Henseleit; PIPES, 1,4-piperazinediethanesulfonic acid; DHPR, dihydropyridine receptor.
EXPERIMENTAL PROCEDURES

Materials—Immature C57Bl/6 skeletal muscle cells (myoblasts) derived from normal adult C57Bl/6 mouse leg muscles were purchased from ATCC (Manassas, VA). Double strand recombinant adenoviruses (rAAV) vector was generously provided by Dr. Douglas McCarty (University of North Carolina, Chapel Hill). Fura-2-AM was purchased from Molecular Probes (Eugene, OR) and Invitrogen-2 was obtained from TEF LABS (Austin, TX). Goat polyclonal anti-IP3, R2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), mouse monoclonal anti-IP3, R3 from BD Biosciences, and anti-junctin from ProSci Incorporated (San Diego, CA). Mouse monoclonal anti-RyR1/ RyR2 IgG (34Cl clone) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa). A rabbit polyclonal IgG raised against a 13-amino acid region specific for RyR3 and a C-terminal cys- teine (KKRRGQKVEKPEC) was prepared as described previously (30). All other primary antibodies were purchased from Affinity Bioreagents (Golden, CO). [3H]Ry njANine was obtained from PerkinElmer Life Sciences. Phospholipids were from Avanti Polar Lipids (Alabaster, AL). All other chemicals were obtained from Sigma unless specified otherwise.

Cell Culture—C57Bl/6 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 and cultured for 48 h to reach 100% confluence (defined as day 0 myotubes). To induce myogenic differentiation, the growth medium was then changed to differentiation medium (Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum and 1× antibiotics/antimycotics) on day 0. The differentiation process was ended every subsequent day.

Construction of Vector, Packaging, and Purification of rAAV—The oligonucleotides encoding the specific siRNA for each CSQ isoform were inserted into pSilencer-1.0 vector (Ambion, Austin TX) downstream of the U6 promoter using Apal and EcoRI sites. U6 promoter-driven expression cassettes were inserted into rAAV vector (pTR-U1α-RFP-U6) using the KpnI and NotI sites. The pTR-U1α-RFP-U6 vector contains red fluorescent protein and was kindly provided by Dr. Douglas McCarty (University of North Carolina, Chapel Hill). The resulting vectors are termed rAAV-CSQ1 and rAAV-CSQ2. The sequences of oligonucleotides encoding the CSQ1 and CSQ2 siRNA were 5′-CTGAA-GAGACACGTTTATTTACCAGAGATAAACGCTGTCTTC- AG-3′ and 5′-CAGTGGGATGCTGTTAATTTAAGAGATTTTAC- CAGTACCCTCAGT-3′. BLAST searches confirmed that the selected oligonucleotide sequences were not homologous to any other genes. A control sequence of 5′-TTCCTCGAGAACGTCAGTTCAAGAGA- ACGTGGACAAGCTTGCAGATAAACTTTAATTTTCGAG-3′ was used to construct rAAV-control as a negative 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 (31, 32).

Recombinant AAV Transduction—For rAAV transduction, C57Bl/6 myoblasts were seeded at a concentration of 6 × 10⁵ cells per 10-cm plate or 1 × 10⁷ per well in 6-well plates. About 24 h after seeding, myoblasts were infected with rAAV carrying the CSQ-siRNA silencing cassette at 1 × 10⁴ particles/cell and transferred into differentiation medium 24 h after infection. The differentiation medium was changed everyday subsequently.

Immunoblot and Immunofluorescence Analyses—Unless otherwise indicated, 8-day-old C57Bl/6 myotubes grown on 6-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 μg of lysate was separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with antibodies for CSQ1, CSQ2, triadin 95, junctin, α-DHPR, RyRs, IP3Rs, and SERCA1. Western blots were developed using 3,3’- diaminobenzidine or enhanced chemiluminescence and quantified using Kodak Digital Science ID Image Analysis Software. For immunofluorescence analysis, 8-day-old C57Bl/6 myotubes fixed with 3% paraformaldehyde were permeabilized with buffer containing 20 mM HEPES, pH 7.4, 0.3 μM sucrose, 50 mM NaCl, 3 mM MgCl₂, and 0.5% Triton X-100, and incubated with anti-CSQ1 or anti-CSQ2 antibody, followed by incubation with Alexa Fluor 488-labeled goat anti-mouse or goat anti-rabbit antibody. Images were analyzed by confocal microscopy.

Preparation of Membrane Fractions—C57Bl/6 myotubes grown on 10-cm tissue culture dishes were washed twice with 3 ml of ice-cold phosphate-buffered saline containing 5 mM EDTA and harvested in phosphate-buffered saline containing 5 mM EDTA and protease inhibitors by removal from the plates by scraping. Cells were collected by centrifugation, resuspended in the above solution without EDTA, again pelleted, and stored at −80 °C. A crude membrane fraction was obtained by homogenizing the cells in 0.15 M KCl, 20 mM imidazole, pH 7, solution containing 0.3 μM sucrose, 0.1 mM EDTA, 1 mM glutathione (oxidized), and protease inhibitors with the use of a Tekmar homogenizer (6 s at 13,500 rpm). Cell homogenates were centrifuged for 30 min at 100,000 × g, pellets were resuspended in 0.15 M KCl, 20 mM imidazole, pH 7, solution containing 0.3 μM sucrose and protease inhibitors, and stored at −135 °C. Proteoliposomes containing purified rabbit skeletal muscle RyR1 ion channels were prepared in the presence of protease inhibitors (33).

Purification of Calsequestrins—Rabbit skeletal muscle (6) and canine cardiac muscle (34) CSQs were purified as described.

Stored Ca²⁺ Release—Stored Ca²⁺ release was determined using the fluorescent Ca²⁺ indicator dye Fluor 4-AM. 8-Day-old C57Bl/6 myotubes grown on glass coverslips were washed three times with phosphate-buffered saline and loaded with 5 μM Fluor 4-AM for 1 h at 37 °C in Krebs-Ringer-Henseleit (KRH) buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 6 mM glucose, 1.2 mM MgCl₂, 2 mM CaCl₂, and 25 mM HEPES, pH 7.4). After loading, cells were rinsed with KRH buffer to remove non-hydrolyzed fluorophore and kept in KRH buffer for 30 min to complete de-esterification. Individual cells in Ca²⁺-free KRH buffer (2 mM CaCl₂ was replaced by 0.5 mM EGTA) were defined as regions of interest, and average fluorescence was measured by using the program ImageMaster (Photon Technology International, Lawrenceville, NJ). Resting calcium levels were monitored with Fura-2. Cells were loaded with 5 μM Fura 2-AM and excited alternatively at 340 and 380 nm. The fluorescence emission was collected at 510 nm. Resting intracellular calcium concentrations were calculated using the equation: [Ca²⁺]ᵣ = Kᵣ(F₃₈₀max/F₃₄₀min)[(R – Rₖᵣ)/(Rₚₑ₅₉0 – Rₘᵣᵣ)] – Rₘᵣᵣ. A dissociation constant (Kᵣ) of 224 nM (35, 36) was used for the binding of calcium to Fura-2 at 37 °C. Rₘᵣᵣ, and Rₖᵣᵣ, were determined in each experimental group by the consecutive addition of 10 μM ionomycin and 50 mM EGTA.

Restoring Ca²⁺ Release in CSQ Knockdown Myotubes—To determine whether CSQ2 is specifically responsible for the decreased stored Ca²⁺ release of C57Bl/6 myotubes treated with CSQ2 siRNA, we performed additional experiments. We first amplified CSQ2 cDNAs from a mouse skeletal muscle cDNA library (Clontech) by PCR and then constructed the CSQ2 cDNAs into PcIneo vector using NheI and NotI sites. 3 μg of PcIneo-CSQ2 plasmid DNA, 1 μg of pTR-U1α-RFP vector (as a reporter), and 10 nm chemically synthesized siRNA specific for CSQ2 (see above targeting sequence) (Qiagen, Valencia, CA) were then mixed in 10 μl of Lipofectamine 2000 (Invitrogen) and co-transfected into C57Bl/6 myoblasts in each well of the 6-well plate. In two other groups of

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C2C12 myoblasts, PcIneo-CSQ2 was replaced by the same amount of PcIneo vector in one group and CSQ2 siRNA was omitted in the other group. The transfection efficiency was about 5%. C2C12 myoblasts were then allowed to differentiate over 8 days and myotubes with red fluorescence were examined for caffeine-induced stored Ca^{2+} release as described above.

[^3]H]Ryanodine Binding—Unless otherwise indicated, crude membrane fractions prepared from C2C12 cells were incubated with 2.5 nM[^3]H]ryanodine in 20 mM imidazole, pH 7.0, 250 mM KCl, 150 mM sucrose, 1 mM glutathione (oxidized), 20 mM leupeptin, and 200 mM Pefabloc, and the indicated free Ca^{2+} concentrations. Nonspecific binding was determined using a 1000-fold excess of unlabeled ryanodine. After 20 h at 24 °C, aliquots of the samples were diluted with 8.5 volumes of ice-cold water and placed on Whatman GF/B filters preincubated with 2% polyethyleneimine in water. Filters were washed with 3 x 5 ml of ice-cold 0.1 M KCl, 1 mM K-PIPES, pH 7.0. Radioactivity remaining on the filters was determined by liquid scintillation counting to obtain bound[^3]H]ryanodine.

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 mM leupeptin, 200 mM Pefabloc, and 100 mM Ca^{2+}. Specific[^3]H]ryanodine binding was determined as described above.

[^45]Ca^{2+} Uptake—ATP-dependent[^45]Ca^{2+} uptake by C2C12 membranes 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 (37)), 10 mM ruthe- nium red (to inhibit RyRs (38)), 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.

Single Channel Recordings—Single channel measurements were performed using Mueller-Rudin-type planar lipid bilayers containing a 5:3:2 mixture of bovine brain phosphatidylethanolamine, phosphatidyl- serine, and phosphatidicholine (25 mg of total phospholipid/ml of n-decane) (39). SR vesicles of 8-day-old C2C12 myotubes were added to the cis (SR cytosolic side) chamber of a bilayer apparatus and fused in the presence of an osmotic gradient (250 mM cis KCl, 20 mM trans KCl in 20 mM K-HEPES, pH 7.4, with 2 μM Ca^{2+}). Amounts of membranes were adjusted to obtain single channel activities not more than 30 min after their addition. After appearance of channel activity, trans (SR luminal) KCl concentration was increased to 250 mM to prevent further fusion of membranes. The trans side of the bilayer was defined as ground. The large cytosolic regulatory region of the channels faced the cis chamber in a majority (>98%) of the recordings (40). Purified canine cardiac and rabbit skeletal muscle CSQs were added to the trans chamber. Electrical signals were filtered at 2 kHz, digitized at 10 kHz, and analyzed as described (40).

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 (41). Free Ca^{2+} concentrations of ≥1 μM 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

Efficiencies and Specificities of Knocking Down CSQ1 and CSQ2 in C2C12 Myotubes—C2C12 myoblasts were infected with rAAV vectors containing the sequences for red fluorescent protein, U6 promoter (ptrs-U1a-RFP-U6), and CSQ1 or CSQ2 specific siRNA. These C2C12 myoblasts were then allowed to differentiate into multinucleated myotubes over 8 days (Fig. 1) as described under “Experimental Procedures.” In addition, C2C12 myoblasts were doubly infected to knockdown both CSQ isoforms or were infected with rAAV vector that contained a control oligonucleotide sequence. Transduction efficiencies among these four groups of C2C12 myotubes were all near 100% as judged by the red fluorescent protein expression of the myotubes. As shown Fig. 1, A–D, every myotube expressed the red fluorescent protein when examined using confocal microscopy.

In our preliminary studies, we had found that C2C12 myotubes robustly expressed both CSQ isoforms after 6 or more days of culture in
CSQ Knockdown in C2C12 Myotubes

FIGURE 2. Immunoblot analyses of C2C12 myotubes. CSQ1 and CSQ2 levels in C2C12 myotubes infected with rAAV-CSQ1 (A), rAAV-CSQ2 (B), or rAAV-CSQ1/rAAV-CSQ2 (C) were examined using immunoblot technologies. Lane 1, C2C12 myotubes infected with rAAV-control. Lanes 2–4, three independent groups of 8-day-old myotubes infected with rAAV-CSQ. D, time course of CSQ expression in control, rAAV-CSQ1-, or rAAV-CSQ2-infected C2C12 myotubes. Lanes 1, 2, and 3 are control C2C12 myotubes at days 1, 3, and 6. Lanes 2, 4, and 6 are days 1, 3, and 6 C2C12 myotubes infected by rAAV-CSQ2 (upper row) and rAAV-CSQ1 (lower row). E, immunoblots were performed on 8-day-old C2C12 myotube lysate. Lane 1, C2C12 myotubes infected with rAAV-control. Lanes 2–4, C2C12 myotubes infected with rAAV-CSQ1, rAAV-CSQ2, and rAAV-CSQ1/rAAV-CSQ2, respectively.

differentiation medium (e.g. Fig. 2D). The cellular distribution of CSQ isoforms in 8-day-old myotubes was then examined after labeling these cells with anti-CSQ1 or anti-CSQ2 antibody and followed by Alexa 488-conjugated secondary antibodies. As shown in Fig. 1, E and F, the two CSQ isoforms had a similar cellular distribution in 8-day-old (control) myotubes. Transductions with rAAV-CSQ1 and rAAV-CSQ2 reduced the corresponding CSQ isoform to nearly undetectable levels in 8-day-old C2C12 myotubes. Representative results are shown in Fig. 1, G and H (background green fluorescence is barely noticeable).

The efficacies of knocking down the two CSQ isoforms in C2C12 myotubes were confirmed by immunoblot analyses. As with our immunofluorescence studies, CSQ1 and CSQ2 proteins (both had an apparent molecular mass of ~60 kDa on gels) in myotubes were reduced to undetectable levels by their corresponding siRNAs (Fig. 2, A–C). Also notable were the specificities of CSQ siRNAs. That is, the level of CSQ2 protein was not affected in C2C12 myotubes infected with rAAV-CSQ1 and similarly the level of CSQ1 protein was not affected in C2C12 myotubes infected with rAAV-CSQ2 (Fig. 2, A and B). These results clearly show that infecting C2C12 myoblasts with rAAV encoding CSQ1- or CSQ2-specific siRNA sequence results in the specific knockdown of corresponding CSQ protein in C2C12 myotubes. These results also validated the isoform specificities of anti-CSQ antibodies used in our immunoblot and immunofluorescence studies. Furthermore, these results indicate that knocking down one particular CSQ isoform does not lead to a compensatory overexpression of the other isoform.

The expression levels of CSQ1 and CSQ2 proteins in different stages of C2C12 myotubes were investigated using a combination of immunoblot and protein densitometry analyses. Immunoblot studies revealed that CSQ1 and CSQ2 were absent in 1-day-old myotubes (Fig. 2D, lane 1). CSQ2 was first detected in 3-day-old myotubes (lane 3) but by day 6 both CSQ isoforms were easily detectable (lane 5). The expression level of CSQ1 was not altered in 3- or 6-day-old C2C12 cells infected with rAAV-CSQ2 (Fig. 2D, upper panel, compare lanes 3 and 4, and 5 and 6). Conversely, transduction with rAAV-CSQ1 had no noticeable effect on CSQ2 levels in 3- and 6-day-old C2C12 myotubes (Fig. 2D, lower panel).

We were able to estimate the relative expression of CSQ isoforms in 8-day-old myotubes because our knockdown method is highly efficacious and knocking down one CSQ isoform does not lead to a compensatory overexpression of the other isoform (Fig. 2A–D). Densitometry analyses of the Coomassie Blue-stained gels showed that, as compared with rAAV-control infection, rAAV-CSQ1 infection reduced the CSQ protein band by 61 ± 4% in 8-day-old myotubes (not shown). In comparison, rAAV-CSQ2 transduction reduced the CSQ protein band by 31 ± 5% (n = 3). These data suggest that 8-day-old C2C12 myotubes expressed CSQ1 and CSQ2 at a protein ratio of ~2:1 despite the apparent higher signals of CSQ2 bands in immunoblots (Fig. 2D).

Regulation of RyR by CSQ—To investigate how the two CSQ isoforms may regulate channel activities of RyR1 and RyR3 (8-day-old C2C12 myotubes expressed both isoforms, Ref. 42, see also below) thereby affecting stored Ca2+ release in skeletal myotubes, we performed single RyR channel measurements with membranes isolated from 8-day-old C2C12 myotubes doubly infected with rAAV-CSQ1 and rAAV-CSQ2 and therefore devoid of both CSQ1 and CSQ2 (Fig. 2C). The regulatory effects of each CSQ isoform as reflected by the changes in RyR channel open probabilities (Pₒ) were then determined by adding exogenous purified rabbit skeletal muscle CSQ1 to the trans (SR luminal) side of lipid bilayer setup. Because the purified rabbit CSQ1 preparation also contained CSQ2 as a minor component as determined by immunoblot analysis, we also performed a limited number of experiments with purified canine calsequestrin that contained only CSQ2. Similarly, we studied membranes isolated from rAVV control-infected myotubes as comparison. To establish well defined cytoplasmic Ca2+ concentrations, [K+Ca2+]cytosol rather than Ca2+ was used as the current carrier (43). Fig. 3A shows a representative single channel recording study that investigated the effects of 4 μM purified rabbit skeletal muscle CSQ on RyR1 Pₒ, Baseline Pₒ was recorded with 2 μM free Ca2+ and 1 mM ATP in the cis (cytosolic) side of bilayer chamber. Adding 1 mM Ca2+ to the trans (SR luminal) chamber increased Pₒ from both CSQ1/CSQ2 depleted and control membranes. The subsequent addition of 4 μM CSQ to the trans chamber, however, increased Pₒ from CSQ-knockdown but not from control membranes. Table 1 summarizes the averaged Pₒ values of RyRs isolated from control and CSQ1/CSQ2 knockdown myotubes without and with 1 mM luminal Ca2+ and after the addition of 2–4 μM CSQ. The effect of CSQ was dependent on the luminal concentration of Ca2+. Adding CSQ in the presence of a low trans Ca2+ concentration (2 μM Ca2+) had no effect on Pₒ from CSQ knockdown membranes but Pₒ was significantly enhanced by the subsequent addition of 1 mM Ca2+ (Fig. 3B, Table 1).

CSQ was less effective in enhancing channel activities when only CSQ1 isoform was knocked down. In the presence of 1 mM luminal Ca2+, addition of 2 μM CSQ1 increased CSQ1-depleted RyR channel activities 1.09 ± 0.03-fold (n = 9, p < 0.05) as compared with 1.75 ± 0.18-fold increase of Pₒ values of RyRs isolated from myotubes devoid of both CSQ1 and CSQ2 isoforms. The modest effect of CSQ1 on CSQ1-depleted channels suggests that CSQ2 likely remained with these channels and impeded access of CSQ1.

The regulatory effect of CSQ on RyRs appears to be dependent on other SR proteins such as triadin and junctin (18). Our single channel recording studies of purified rabbit skeletal muscle RyR1 indicated that 4 μM rabbit CSQ had no noticeable enhancing effect on RyR1 Pₒ, even in the presence of 1 mM luminal Ca2+ (data not shown).
In single channel recording studies, CSQ2 purified from canine cardiac muscle had a more modest enhancing effect on RyR channel activities as compared with purified rabbit skeletal muscle CSQ. In the presence of 1 mM luminal Ca$^{2+}$, adding 2 μM CSQ2 caused a small (but significant) increase in CSQ1/CSQ2-depleted RyR single channel activities ($P_o = 0.24 \pm 0.05$ versus $0.29 \pm 0.06$, $n = 7$, $p < 0.05$).

The effects of rabbit CSQ on RyR channel activities were examined further. Fig. 4 shows that a fairly wide range of CSQ concentrations (0.4–4 μM) was effective in increasing channel open probability by 1.5–2-fold. A higher CSQ concentration of 12 μM returned $P_o$ to near control levels. Control measurements showed that this decrease was not because of a change in luminal Ca$^{2+}$ concentration. Kinetic analysis showed that CSQ (in the presence of 1 mM luminal Ca$^{2+}$) increased $P_o$ by raising the number of single channel events without significantly changing the mean open times (Table 2). Taken together, the results of our single channel measurements suggest that rabbit CSQ (which contains CSQ1 as the major component), and to a smaller extent canine CSQ2, enhance C2C12 myotube RyR channel activities in the presence of 1 mM luminal Ca$^{2+}$ by increasing the transition rates from the closed to open states.

We also tested the possibility that CSQ knockdown affected the Ca$^{2+}$ dependence of RyR activities isolated from CSQ knockdown and control myotubes, using the RyR-specific probe ryanodine (3, 44). Fig. 5 shows that knockdown of CSQ1, CSQ2, and both isoforms did not significantly alter the bimodal Ca$^{2+}$ dependence of RyR activity.

**TABLE 1**

Effects of SR luminal Ca$^{2+}$ and purified rabbit skeletal muscle CSQ on RyR single channel activities from control and CSQ1/CSQ2 knockdown myotubes

SR luminal skeletal muscle CSQ concentration was 2–4 μM. Data are the mean ± S.E. of number of experiments indicated in parentheses. Included in these are multiple channel recordings (3 of 9 and 2 of 5 in control; 3 of 9 and 3 of 4 in CSQ1/CSQ2 knockdown).

<table>
<thead>
<tr>
<th>Additions</th>
<th>Control</th>
<th>CSQ1/CSQ2 knockdown</th>
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<tr>
<td>2 μM Ca$^{2+}$ cis + 1 mM ATP cis +1 mM Ca$^{2+}$ trans</td>
<td>0.12 ± 0.03(9)</td>
<td>0.17 ± 0.02(9)</td>
</tr>
<tr>
<td>+1 mM Ca$^{2+}$ trans + CSQ trans</td>
<td>0.25 ± 0.06(4)</td>
<td>0.24 ± 0.04(4)</td>
</tr>
<tr>
<td>2 μM Ca$^{2+}$ cis + 1 mM ATP cis + CSQ trans</td>
<td>0.16 ± 0.05(5)</td>
<td>0.21 ± 0.03(4)</td>
</tr>
<tr>
<td>+ CSQ trans</td>
<td>0.14 ± 0.02</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>+ CSQ trans + 1 mM Ca$^{2+}$ trans</td>
<td>0.26 ± 0.04(5)</td>
<td>0.35 ± 0.03(6)</td>
</tr>
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</table>

*p < 0.05 versus 2 μM Ca$^{2+}$ cis + 1 mM ATP cis.

*p < 0.05 versus 1 mM trans Ca$^{2+}$.

*p < 0.05 versus 2–4 μM CSQ trans.
CSQ Knockdown in C2C12 Myotubes

FIGURE 4. Channel activities of RyRs as a function of CSQ concentration. Single channel activities were determined as described in the legend to Fig. 3 in the presence of the indicated concentrations of purified rabbit skeletal muscle CSQ and 1 mM trans (SR lumenal) Ca\(^{2+}\). Data are the mean \pm S.E. of four to nine experiments. *, p < 0.05 compared with control (without CSQ).

TABLE 2
Effects of SR luminal Ca\(^{2+}\) and CSQ on kinetic parameters of single channel recordings
SR luminal rabbit skeletal muscle calsequestrin (CSQ) concentration was 2–4 \(\mu\)M. Average normalized parameters (in the absence of 1 mM Ca\(^{2+}\) + CSQ) at 2 \(\mu\)M Ca\(^{2+}\) cis + 1 mM ATP cis were for control and CSQ1/CSQ2-depleted RyRs, respectively: \(F_p = 0.13 \pm 0.05\) and 0.15 \pm 0.04, Events/min = 7,763 \pm 858 and 10,114 \pm 2,284, \(T_o = 1.00 \pm 0.33\) and 0.94 \pm 0.14 ms, \(T_c = 7.64 \pm 1.17\) and 6.67 \pm 1.26 ms. Channel parameters were calculated from 6 recordings each that contained a single channel activity.

<table>
<thead>
<tr>
<th>Additions</th>
<th>(P_o)</th>
<th>(T_o)</th>
<th>(T_c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 (\mu)M Ca(^{2+}) cis + 1 mM ATP cis</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>+ 1 mM Ca(^{2+}) trans</td>
<td>243 \pm 38(^a)</td>
<td>221 \pm 34(^a)</td>
<td>92 \pm 9</td>
</tr>
<tr>
<td>+1 mM Ca(^{2+}) trans + CSQ trans</td>
<td>256 \pm 34(^a)</td>
<td>262 \pm 40(^a)</td>
<td>90 \pm 7</td>
</tr>
<tr>
<td>CSQ1/CSQ2 knockdown</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 (\mu)M Ca(^{2+}) cis + 1 mM ATP cis</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>+ 1 mM Ca(^{2+}) trans</td>
<td>154 \pm 19(^a)</td>
<td>220 \pm 41(^a)</td>
<td>82 \pm 17</td>
</tr>
<tr>
<td>+1 mM Ca(^{2+}) trans + CSQ trans</td>
<td>277 \pm 33(^a,b)</td>
<td>296 \pm 49(a)</td>
<td>116 \pm 33</td>
</tr>
</tbody>
</table>

\(^a\) p < 0.05 compared with 2 \(\mu\)M Ca\(^{2+}\) cis + 1 mM ATP cis.

\(^b\) p < 0.05 compared with 1 mM Ca\(^{2+}\) trans addition.

FIGURE 5. Ca\(^{2+}\) dependence of \[^{3}H\]ryanodine binding to control and CSQ-knockdown C2C12 myotubes. Specific \[^{3}H\]ryanodine binding was determined in 250 mM KCl, 20 mM imidazole, pH 7.0, media containing 2.5 mM \[^{3}H\]ryanodine and the indicated concentrations of free Ca\(^{2+}\). Data are mean \pm S.E. of three to four experiments.

Stored Ca\(^{2+}\) Release in CSQ Knockdown Myotubes Provoked by Activators of Intracellular Ca\(^{2+}\) Release Channels—Because our single channel recording studies suggest that both CSQ1 and CSQ2 may enhance stored Ca\(^{2+}\) release by RyRs in C2C12 myotubes, we then tried to correlate these results with the effects of CSQ knockdown on stored Ca\(^{2+}\) release in intact myotubes using the fluorescent Ca\(^{2+}\) indicator Fluo-4 in the absence of extracellular Ca\(^{2+}\). In initial experiments using the fluorescent Ca\(^{2+}\) indicator Fura-2, we determined that there was no significant difference in the resting cytosolic Ca\(^{2+}\) concentration of control and each of the three CSQ-knockdown myoblast groups (data not shown). Depolarization-induced, RyR-mediated stored Ca\(^{2+}\) release in 8-day-old myotubes was provoked by 10 mM KCl. Stored Ca\(^{2+}\) release, as reflected by the Fluo-4 fluorescence over baseline fluorescence ratio \((F_r/F_o)\), was significantly smaller in CSQ2 and CSQ1/CSQ2 knockdown myotubes (Fig. 6A) as compared with the control myotubes. The peak \(F_r/F_o\) ratio was 3.2 \pm 0.1 for the control group, 2.4 \pm 0.2 for the CSQ2 knockdown group, and 2.2 \pm 0.2 for the CSQ1/CSQ2 knockdown group (Table 3). In contrast, the peak \(F_r/F_o\) in CSQ1 knockdown myotubes induced by 10 mM KCl was comparable with the control myotubes. To confirm that RyR-mediated stored Ca\(^{2+}\) release is reduced in CSQ2 knockdown myotubes, we also treated the four groups of C2C12 myotubes with caffeine (which activates RyR1 and RyR3 independent of the DHPR) or 4-chloro-m-cresol (which activates RyR1 but not RyR3 (45)) (the 8-day-old C2C12 myotubes expressed both RyR1 and RyR3 (Fig. 2E)). Indeed, the Ca\(^{2+}\) responses of 8-day-old C2C12 myotubes to 10 mM caffeine or 200 \(\mu\)M 4-chloro-m-cresol in the absence of extracellular Ca\(^{2+}\) were also significantly decreased in CSQ2 and CSQ1/CSQ2 but not in CSQ1 knockdown myotubes (Fig. 6B and C, Table 3).

To determine whether the lack of CSQ2 is specifically responsible for the reduced stored Ca\(^{2+}\) release of C2C12 myotubes treated with CSQ2 siRNA,
C2C12 myoblasts were treated with the same CSQ2 siRNA but synthesized chemically with or without co-transfection with a plasmid containing the sequence of CSQ2. Another group of myotubes was used as control (see "Experimental Procedures"). In these experiments, the peak fluorescences ($F/F_0$) were 3.0 ± 0.1 ($n = 7$) for control myotubes and 2.1 ± 0.1 ($n = 8$) for myotubes treated with CSQ2 siRNA ($p < 0.05$). We found that the marked reduction in caffeine-induced stored Ca$^{2+}$ release caused by CSQ2 siRNA is partially restored in myotubes co-transfected with CSQ2 siRNA and a plasmid containing the sequence of CSQ2 ($F/F_0 = 2.7 ± 0.1$, $n = 6$, $p < 0.05$ as compared with CSQ2 siRNA myotubes). These results suggest that the reduction in stored Ca$^{2+}$ release in CSQ2 siRNA-treated myotubes was specific with regard to CSQ2 knockdown.

![FIGURE 6. Effects of CSQ knockdown on stored Ca$^{2+}$ release in C2C12 myotubes. Depolarization (10 mM KCl addition) (A), caffeine (10 mM) (B), and 4-chloro-m-cresol (200 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 C2C12 myotubes infected with rAAV-control (top traces), rAAV-CSQ1 (second traces), rAAV-CSQ2 (third traces), and rAAV-CSQ1/rAAV-CSQ2 (bottom traces).]

### TABLE 3

**Effects of CSQ knockdown on stored Ca$^{2+}$ release in C2C12 myotubes**

The peak values of fluorescence increases ($F/F_0$) in C2C12 myotubes were determined in Fluo 4-loaded cells in Ca$^{2+}$-free KRH buffer. Data are the mean ± S.E. of 8–16 experiments.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Control</th>
<th>CSQ1 knockdown</th>
<th>CSQ2 knockdown</th>
<th>CSQ1/CSQ2 knockdown</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM KCl</td>
<td>3.2 ± 0.1</td>
<td>3.1 ± 0.2</td>
<td>2.4 ± 0.2$^a$</td>
<td>2.2 ± 0.2$^a$</td>
</tr>
<tr>
<td>10 mM Caffeine</td>
<td>3.1 ± 0.1</td>
<td>2.9 ± 0.2</td>
<td>2.5 ± 0.3$^a$</td>
<td>2.1 ± 0.2$^a$</td>
</tr>
<tr>
<td>200 μM 4-Chloro-m-cresol</td>
<td>2.9 ± 0.1</td>
<td>2.9 ± 0.2</td>
<td>2.3 ± 0.1$^a$</td>
<td>2.2 ± 0.2$^a$</td>
</tr>
<tr>
<td>1 μM Thapsigargin</td>
<td>3.0 ± 0.1</td>
<td>3.0 ± 0.2</td>
<td>2.3 ± 0.1$^a$</td>
<td>2.0 ± 0.1$^a$</td>
</tr>
<tr>
<td>400 μM UTP</td>
<td>2.9 ± 0.2</td>
<td>2.9 ± 0.3</td>
<td>2.2 ± 0.2$^a$</td>
<td>2.3 ± 0.1$^a$</td>
</tr>
</tbody>
</table>

$^a$ $p < 0.05$ compared to control.
The above results suggest that CSQ2 knockdown may impair Ca\textsuperscript{2+} storage in C\textsubscript{2}C\textsubscript{12} myotubes thereby leading to reduced Ca\textsuperscript{2+} release. This possibility was further explored using pharmacological agents that empty Ca\textsuperscript{2+} stores in these myotubes but not through RyRs. In initial studies, we found that, in the absence of extracellular Ca\textsuperscript{2+}, neither adding UTP (an activator of IP\textsubscript{3}Rs, 400 μM) following caffeine nor adding caffeine following UTP elicited an additional increase in Fluo-4 fluorescence in control and CSQ1 knockdown myotubes (not shown), suggesting that the two intracellular Ca\textsuperscript{2+} channel families release stored Ca\textsuperscript{2+} from the same stores. The effects of CSQ knockdown on IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release were then determined by adding 400 μM UTP to the four groups of C\textsubscript{2}C\textsubscript{12} myotubes in Ca\textsuperscript{2+}-free KRH bath solutions. UTP elicited significantly less Ca\textsuperscript{2+} release in rAVV-CSQ2 and rAVV-CSQ1/rAVV-CSQ2-infected C\textsubscript{2}C\textsubscript{12} myotubes as compared with rAVV-control and rAVV-CSQ1-infected myotubes (Table 3). Consistently, in the absence of extracellular Ca\textsuperscript{2+}, thapsigargin (which empties intracellular Ca\textsuperscript{2+} stores by inhibiting SERCA1) also elicited significantly less Ca\textsuperscript{2+} responses in rAVV-CSQ2 and in rAVV-CSQ1/rAVV-CSQ2-infected C\textsubscript{2}C\textsubscript{12} myotubes (Table 3). We conclude that knocking down CSQ2 reduces SR Ca\textsuperscript{2+} stores and Ca\textsuperscript{2+} release in 8-day-old C\textsubscript{2}C\textsubscript{12} myotubes.

**Immunoblot Analyses of C\textsubscript{2}C\textsubscript{12} Myotube Ca\textsuperscript{2+} Channels and Transporters**—Because our Ca\textsuperscript{2+} transient measurements unexpectedly showed that CSQ2, but not CSQ1, is important for effective stored Ca\textsuperscript{2+} release in C\textsubscript{2}C\textsubscript{12} myotubes, we then undertook comprehensive immunoblot analyses to determine how knocking down each of the two CSQ isoforms may alter the expression of Ca\textsuperscript{2+} channels and transporters that may affect stored Ca\textsuperscript{2+} release in C\textsubscript{2}C\textsubscript{12} myotubes. The immunoblot analyses showed that the combined RyR (RyR1 and RyR3) protein levels in CSQ2 and CSQ1/CSQ2 knockdown myotubes were decreased by 27 ± 5 and 30 ± 6% (n = 3, p < 0.05), respectively, as compared with control and CSQ1 knockdown myotubes (Fig. 2E) (n = 3). SERCA1 protein levels were reduced by 50 ± 8 and 57 ± 7% (n = 3, p < 0.05) in CSQ2 and CSQ1/CSQ2 knockdown myotubes, respectively (Fig. 2E), as compared with the other two groups. In contrast, no differences in RyR3, IP\textsubscript{3}R 1–3, triadin, junction, or the α1 subunit of L-type Ca\textsuperscript{2+} channel (α1-DHPR) protein expression were detected among the four groups of C\textsubscript{2}C\textsubscript{12} myotubes, as compared with the actin control.

The decreased protein expression of RyRs was also supported by a ligand binding assay, using the RyR-specific probe ryanodine. *B* \textsubscript{max} values of [\textsuperscript{3}H]ryanodine binding were similar in CSQ1 knockdown and control myotubes (Table 4). CSQ2 and CSQ1/CSQ2 knockdown myotubes had reduced *B* \textsubscript{max} values of [\textsuperscript{3}H]ryanodine binding (0.21 and 0.24 pmol/mg of protein, respectively, versus control value of 0.35 pmol/mg of protein), which suggests a lower RyR protein expression as compared with control myotubes (Table 4). Most of this decrease could be accounted for by RyR1 (Fig. 2E).

**DISCUSSION**

In fast-twitch skeletal muscle CSQ2 is highly expressed during the fetal stage but then is gradually replaced by CSQ1 (20, 46). In slow-twitch skeletal muscle the two CSQ isoforms are co-expressed in adult animals (22, 47). X-ray crystallographic analyses have indicated that the two CSQ isoforms have almost identical crystal structures and form polymers as the Ca\textsuperscript{2+} concentration in the SR increases. Whether the two CSQ isoforms perform redundant or specific functions in skeletal muscle remains poorly understood. Herein we described an efficacious and specific knockdown method that allowed us to differentiate the roles of the two CSQ isoforms in stored Ca\textsuperscript{2+} release of skeletal muscle cell line C\textsubscript{2}C\textsubscript{12} myotubes. CSQ proteins in skeletal muscle have a half-life of ~5 days (49) and thus are difficult to knockdown once expressed. Accordingly, we injected C\textsubscript{2}C\textsubscript{12} myoblasts with rAVV vectors containing CSQ siRNA sequences before these cells mature into CSQ-expressing myotubes. Both CSQ proteins were absent or barely detectable in 1-day-old (control) myotubes but became easily detectable 3 (CSQ2) to 6 (CSQ1) days after forming myotubes. A similar time course of CSQ mRNA expression in maturing C\textsubscript{2}C\textsubscript{12} myotubes had been reported previously (46). Therefore, the high efficacies of CSQ knockdowns in our study are probably in part due to the timing of CSQ siRNAs delivery. Moreover, we found that knocking down one CSQ isoform did not decrease the expression nor caused a compensatory overexpression of the other isoform in C\textsubscript{2}C\textsubscript{12} myotubes. The high efficacies and specificities of our knockdown approach thus enable us to attribute the observed functional changes of C\textsubscript{2}C\textsubscript{12} myotubes to a specific CSQ knockdown rather than to the nonspecific effects of rAAV infection.

*C* \textsubscript{2}C\textsubscript{12} myotubes with knockdown of both CSQ isoforms provided membranes that contained RyR1 and RyR3 and associated proteins (triadin and junction) but depleted of CSQ proteins. Accordingly, we measured the single channel activities of skeletal RyRs in these membranes before and after adding individual CSQ isoform exogenously as previous reports have suggested that in striated muscles CSQ forms a tight multiprotein complex with RyRs and modulates the Ca\textsuperscript{2+} release of RyRs indirectly via a linkage protein, triadin. We found that both CSQ isoforms isolated from rabbit skeletal muscle (which is largely CSQ1) and CSQ isolated from canine heart (CSQ2) individually enhanced RyR channel opening probabilities in the presence of high luminal Ca\textsuperscript{2+} concentrations (1 mM), although the effect of CSQ2 was much smaller but nevertheless statistically significant (adding 2 μM CSQ2 increased *P* \textsubscript{o} from 0.24 ± 0.05 to 0.29 ± 0.06, *p* < 0.05). In contrast, adding rabbit skeletal muscle CSQ had no effect on the channel activities of RyRs in membranes isolated from control C\textsubscript{2}C\textsubscript{12} myotubes under identical conditions. These data indeed support the notion that CSQ proteins form a tight

### Knocking Down CSQ2 but Not CSQ1 Reduces Ca\textsuperscript{2+} Uptake by the SR—To determine whether the decrease in SERCA1 protein expression among CSQ2 knockdown myotubes may also explain their reduced stored Ca\textsuperscript{2+} release, SERCA1 activities in the four different groups of myotubes were determined by measuring the 45Ca\textsuperscript{2+} uptake rates of isolated membrane preparations. In agreement with the reduced SERCA1 protein levels (Fig. 2E), 45Ca\textsuperscript{2+} uptake rates of membranes isolated from CSQ2 and CSQ1/CSQ2 knockdown myotubes were significantly lower than those of controls and CSQ1 knockdown myotubes (7.8 and 8.4 versus 18.6 and 16.5 nmol/mg protein/min, respectively) (Table 4). Thus, CSQ2 knockdown may impair Ca\textsuperscript{2+} storage in C\textsubscript{2}C\textsubscript{12} myotubes. In combination with the data presented in Table 3, we conclude that knocking down CSQ2 reduces cytosolic Ca\textsuperscript{2+} sequestration and thereby may reduce Ca\textsuperscript{2+} stores and Ca\textsuperscript{2+} release in 8-day-old C\textsubscript{2}C\textsubscript{12} myotubes.

### TABLE 4

<table>
<thead>
<tr>
<th>Sample</th>
<th><em>B</em> \textsubscript{max} of [\textsuperscript{3}H]ryanodine binding</th>
<th>45Ca\textsuperscript{2+} uptake rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/mg protein</td>
<td>nmol/mg protein/min</td>
</tr>
<tr>
<td>Control</td>
<td>0.35 ± 0.04</td>
<td>18.6 ± 3.5</td>
</tr>
<tr>
<td>CSQ1 knockdown</td>
<td>0.35 ± 0.04</td>
<td>16.5 ± 3.4</td>
</tr>
<tr>
<td>CSQ2 knockdown</td>
<td>0.21 ± 0.03*</td>
<td>7.8 ± 2.3*</td>
</tr>
<tr>
<td>CSQ1/CSQ2 knockdown</td>
<td>0.24 ± 0.02*</td>
<td>8.4 ± 2.1*</td>
</tr>
</tbody>
</table>

* *p* < 0.05 compared to control.

Knocking Down CSQ2 but Not CSQ1 Reduces Ca\textsuperscript{2+} Uptake by the SR—To determine whether the decrease in SERCA1 protein expression among CSQ2 knockdown myotubes may also explain their reduced stored Ca\textsuperscript{2+} release, SERCA1 activities in the four different groups of myotubes were determined by measuring the 45Ca\textsuperscript{2+} uptake rates of isolated membrane preparations. In agreement with the reduced SERCA1 protein levels (Fig. 2E), 45Ca\textsuperscript{2+} uptake rates of membranes isolated from CSQ2 and CSQ1/CSQ2 knockdown myotubes were significantly lower than those of controls and CSQ1 knockdown myotubes (7.8 and 8.4 versus 18.6 and 16.5 nmol/mg protein/min, respectively) (Table 4). Thus, CSQ2 knockdown may impair Ca\textsuperscript{2+} storage in C\textsubscript{2}C\textsubscript{12} myotubes. In combination with the data presented in Table 3, we conclude that knocking down CSQ2 reduces cytosolic Ca\textsuperscript{2+} sequestration and thereby may reduce Ca\textsuperscript{2+} stores and Ca\textsuperscript{2+} release in 8-day-old C\textsubscript{2}C\textsubscript{12} myotubes.
protein complex with skeletal RyRs that rendered these channels from control C2C12 myotubes insensitive to exogenously added CSQ. Moreover, because exogenously added CSQ proteins had no channel modulating effect on purified skeletal muscle RyRs, our data also support the notion that CSQ proteins (especially CSQ1) may indirectly facilitate stored Ca\textsuperscript{2+} release by skeletal RyRs via linkage protein(s) (50, 51).

It is therefore unexpected that we consistently found that knocking down CSQ2, but not CSQ1, reduced stored Ca\textsuperscript{2+} release in C2C12 myotubes especially that in 8-day-old C2C12 myotubes accounts for only one-third of CSQ proteins by densitometry analysis. We observed the reduction of stored Ca\textsuperscript{2+} release in CSQ2 knockdown (and double CSQ knockdown) myotubes caused by all three RyR activators that act on different skeletal RyR sites. It is therefore unlikely that knocking down CSQ2 reduces skeletal RyR Ca\textsuperscript{2+} release via specific allosteric mechanisms, such as disrupting the functional linkage between the DHPR and RyR1 required for activation of RyR1 by depolarization (KCl) (52). Moreover, we observed a similar reduction in stored Ca\textsuperscript{2+} release in CSQ2 knockdown myotubes induced by an activator of IP\textsubscript{3}Rs (400 \textmu M UTP) (48) and by thapsigargin (1 \textmu M), an inhibitor of SERCA (Table 3). Taken together, these data suggest that knocking down CSQ2 leads to a reduction in Ca\textsuperscript{2+} storage in C2C12 myotubes thereby leading to a general reduction in stored Ca\textsuperscript{2+} release.

In agreement with Beard et al. (18), luminal CSQ did not affect single, purified single RyR1 channel activities or channels from control myotubes, presumably because CSQ remained with control channels. However, we found that RyR channels from CSQ1/CSQ2 knockdown C2C12 myotubes were activated by purified skeletal and cardiac muscle CSQ, whereas CSQ inhibited the CSQ-depleted RyR1 channel complex (11). The reason for this discrepancy is not clear but could arise from different preparations and experimental conditions used by Beard et al. (11) and us. One difference was that skeletal SR vesicles predominately contain RyR1 (18), whereas C2C12 myotubes express RyR1 and RyR3. However, we observed an inactivation of CSQ-depleted channel activities by the purified CSQs in only 1 (of 18) single channel recording, which suggests that both RyR1 and RyR3 were likely activated by CSQ. There are also certain limitations in using high salt concentrations or knocking down CSQs. The use of high salt and Ca\textsuperscript{2+} concentrations might have resulted in an irreversible rearrangement of the RyR1 protein complex, an incomplete removal of CSQ, or loss of other proteins. CSQ knockdown may affect another yet to be identified protein involved in RyR and CSQ complex interaction, which could have resulted in the lower channel activities (P\textsubscript{o} values) recorded with control membranes compared with membranes devoid of CSQ1/CSQ2. Whether CSQ modulates RyR activity directly or via triadin and junctin or an unidentified associated protein remains an open question.

Our immunoblot analyses suggest that the reduction in Ca\textsuperscript{2+} store size in CSQ2 knockdown myotubes is at least in part due to a reduction of expression of SERCA1 protein. The expression of SERCA1 protein in CSQ2 and CSQ1/CSQ2 knockdown myotubes is ~50% of the control and the CSQ1 knockdown myotubes. This reduction in SERCA1 had a large functional significance, as the Ca\textsuperscript{2+} uptake rate by SR vesicles prepared from CSQ2 or CSQ1/CSQ2 knockdown myotubes was also decreased by ~50%. At this point, the reduction of SERCA1 protein expression in CSQ2 knockdown myotubes remains unexplained. As the expression of other SR proteins, such as junctin, triadin, and RyR3, is mostly preserved in CSQ2 knockdown myotubes, it is unlikely that knocking down CSQ2 caused a general retardation of C2C12, myotube maturation. However, it is notable that RyR1 expression appears to be decreased in CSQ2 knockdown myotubes. The reduction of RyR1 protein in CSQ2 knockdown myotubes is supported by a significant decrease in \textsuperscript{3}Hryanodine binding of membranes isolated from CSQ2 knockdown myotubes. The etiology and the functional significance of this reduction in RyR1 protein expression are currently unknown. To determine whether knocking down CSQ2 reduces SERCA1 and RyR1 protein expression in C2C12 myotubes at the mRNA level, we are currently undertaking comprehensive quantitative real-time reverse transcriptase-PCR analyses of the four experimental C2C12 myotube groups.

Effectively knocking down CSQ1 and CSQ2 in our study leads to a few unexpected findings that challenge the traditional views of the roles of these proteins in skeletal muscle. In particular, knocking down CSQ1 had no noticeable effects on Ca\textsuperscript{2+} storage and stored Ca\textsuperscript{2+} release in C2C12 myotubes despite extensive prior data suggesting the roles of CSQ1 in buffering Ca\textsuperscript{2+} in the SR and in modulating RyR channel activities (4–9). In this study we have ruled out that CSQ1 knockdown leads to a compensatory overexpression of the other CSQ isoform thereby masking the functional roles of CSQ1 in stored Ca\textsuperscript{2+} release in C2C12 myotubes. The next step in defining the functional roles of CSQ1 and CSQ2 would be the deletion of the two CSQ genes in skeletal muscle of mice and then study these animals for functional alterations in skeletal muscle. As a deficiency in CSQ2 protein may lead to fatal cardiac arrhythmia in young children (27), selective knock-out of CSQ2 in the skeletal muscle of mice may be necessary.

Acknowledgments—We thank Dr. Barbara Muller-Borer and Wanglei Du for assistance with immunofluorescence studies.

REFERENCES

Knocking Down Type 2 but Not Type 1 Calsequestrin Reduces Calcium Sequestration and Release in C2C12 Skeletal Muscle Myotubes

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