Calsequestrin (CASQ1) rescues function and structure of calcium release units in skeletal muscles of CASQ1-null mice

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Am J Physiol Cell Physiol 302: C575–C586, 2012. First published November 2, 2011; doi:10.1152/ajpcell.00119.2011.—Amplitude of Ca2+ transients, ultrastructure of Ca2+ release units, and molecular composition of sarcoplasmic reticulum (SR) are altered in fast-twitch skeletal muscles of calsequestrin-1 (CASQ1)-null mice. To determine whether such changes are directly caused by CASQ1 ablation or are instead the result of adaptive mechanisms, here we assessed ability of CASQ1 in rescuing the null phenotype. In vivo reintroduction of CASQ1 was carried out by cDNA electro transfer in flexor digitorum brevis muscle of the mouse. Exogenous CASQ1 was found to be correctly targeted to the junctional SR (jSR), as judged by electron microscopy; peak amplitudes of Ca2+ transients increased as judged by immunofluorescence and confocal microscopy; terminal cisternae found to be correctly targeted to the junctional SR (jSR), as judged by reintroduction of CASQ1 was carried out by cDNA electro transfer in flexor digitorum brevis muscle of the mouse. Exogenous CASQ1 was found to be correctly targeted to the junctional SR (jSR), as judged by electron microscopy; peak amplitudes of Ca2+ transients increased as judged by immunofluorescence and confocal microscopy; terminal cisternae (TC) lumen was filled with electron dense material and its width was significantly increased, as judged by electron microscopy; peak amplitude of Ca2+ transients was significantly increased compared with null muscle fibers transfected only with green fluorescent protein (control); and finally, transfected fibers were able to sustain cytosolic Ca2+ concentration during prolonged tetanic stimulation. Only the expression of TC proteins, such as calsequestrin 2, sarcalumenin, and triadin, was not rescued as judged by Western blot. Thus our results support the view that CASQ1 plays a key role in both Ca2+ homeostasis and TC structure.

numbers of mitochondria and density of RYR1 were also reported (35). In addition, CASQ1-null mice (mostly males) displayed a high risk of spontaneous sudden death and underwent lethal episodes of a syndrome resembling both human malignant hyperthermia and environmental heat stroke when exposed to heat or halogenated anesthetics (8, 39).

Although extensive work has been done in CASQ1-null mice, it still remains to be determined to which extent the complex CASQ1-null phenotype is either directly caused by CASQ1 ablation or by compensatory mechanisms that mask CASQ1 functions. To this aim, the present study assesses whether acute reintroduction of CASQ1 can rescue some features of the CASQ1-null phenotype.

MATERIALS AND METHODS

CASQ1-Null and Wild-Type Mice

Experiments were carried out in 4-mo-old CASQ1-null and wild-type (WT) C57 BL/6 female mice. CASQ1-null mice were obtained as previously described (35). All experimental protocols have been approved by Institutional Ethical Committees of the Universities of Padova and of Chieti. Mice were kept in accredited animal facilities and killed by anaesthetic euthanasia.

Plasmids and cDNAs

Enhanced green fluorescent protein (eGFP) the bicistronic mammalian expression vector, pCMS-eGFP (BD Biosciences, Clontech Laboratories, San Jose): the first box is a multi-cloning site (MCS) downstream of the immediate early promoter of cytomegalovirus (PCMVIE) that allows expression of the gene of interest; the second box contains the SV40 strong promoter upstream of the eGFP suitable as a transfection marker. eGFP, a red-shifted variant of WTGFP, is compatible with fura-2 excitation and emission spectra and was used as indicator of transfection in Ca2+ transient recordings. For CASQ1-eGFP, the murine CASQ1 cDNA was cloned in the MCS of pCMS-eGFP; the resulting vector, pCMS-eGFP-CASQ1, was kindly provided by Dr. E. Rios (Rush University, Chicago, IL). Amplification and electroporation grade purification of plasmidic DNA were performed using the Maxiprep purification kit (Qiagen Sciences, Germantown, MD) according to the manufacturer’s protocols.

In Vivo Electroporation

Flexor digitorum brevis (FDB) was chosen as the experimental model because there are established and reliable protocols for both in vivo electroporation of the muscle and enzymatic dissociation of single muscle fibers (41). Electroporation of posterior pad muscles was performed according to DiFranco et al. (11) with modifications: mice were deeply anesthetized using 4% isoflurane in O2 with an approved gas anesthetic machine. Anesthesia was maintained using a rodent face mask; injection of 5 μl of iataluronidase was followed 1 h later by injection of 10 μl (10 μg) of plasmidic DNA for each foot. After 10 min, electroporation was performed with an Electro Square Porator ECM 830 (20 pulses of 100 V/cm, 20 ms). Mice were killed 7 days after electroporation. Transfected muscles were referred to as WT + eGFP (WT FDB muscle transfected with pCMS-eGFP, mock), CASQ1-null + eGFP (CASQ1-null FDB muscle transfected with pCMS-eGFP, control), and CASQ1-null + CASQ1 (CASQ1-null FDB muscle transfected with pCMS-eGFP-CASQ1).

Preparation of Total Homogenates and of Single Fibers Pools

Total homogenates from FDB muscles. Seven days after transfection, FDB muscles from control and CASQ1-null mice were dissected, homogenized for 3 min in 3% SDS (wt/vol), 1 mM EGTA, boiled for 5 min, and centrifuged at 900 g for 15 min. Protein concentration of supernatants, referred to as total homogenates, was determined according to (26).

Single fibers pools. After dissociation of FDB muscles, 10 or 20 fibers were collected under the dissection microscope (at ×60) from individual FDB muscles and stored in 20 μl of Laemmli’s solubilization buffer (21). Silver staining of total homogenate from WT and CASQ1-null muscles was performed according to Morrissey (31).

Western Blot Analysis of Total Homogenates and of Single Fibers Pools

Each sample. For each sample, 15–50 μg of protein were loaded on 7.5–10% SDS-polyacrylamide gels depending on the protein being investigated: 50 μg for dihydropyridine receptor (DHPR), SERCA1, TRs, and calreticulin; 25 μg for CASQ2; 15 μg for BiP; 40 μg for JCT; and 30 μg for sarcalumenin and GP 53. The following dilutions and primary antibodies were used: monoclonal anti SERCA1 (1: 5,000), polyclonal anti both CASQ1/CASQ2 (1:1,000), monoclonal anti CASQ1 (1:1,000), monoclonal anti-TR (1:1,000), monoclonal anti BiP (1:500), monoclonal anti calreticulin (1:1,100), and monoclonal anti α1-subunit of DHPR1 (1:500) were all from Affinity Bioreagents (Affinity Bioreagents, Golden, CO); and monoclonal anti-sarcalumenin and 53 kDa glycoprotein (1:200) and monoclonal anti JCT (1:200) were from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary anti-mouse or anti-rabbit (1:5,000) alkaline phosphatase-conjugated antibodies were from Sigma (Milan, Italy).

Single fiber pools. For pools of single fibers, SDS-PAGE was carried out on 10% polyacrylamide gels and Western blot was performed using monoclonal antibodies against CASQ1 (dilution 1:800), TRs (dilution 1:800), and fast myosin heavy chain (1:1,000; Sigma). Secondary anti-mouse or anti-rabbit (1:5,000) alkaline phosphatase-conjugated antibodies were from Sigma (Milan, Italy). The immunodecorated bands were visualized by a ready-to-use, precipitating substrate system for alkaline phosphatase (BCIP/NBT liquid substrate system; Sigma).

Densitometric Analysis and Image Processing

Images were obtained with an HP Scanjet scanner and Adobe Photoshop CS2 version 9.0. Densitometry was performed with Scion Image Software without modification of the images (raw images) to quantify protein band intensities. Normalization of the signal was obtained relative to that of actin stained by Ponceau red the immunological signal referable to TR of each pool of fibers was divided by the signal obtained by actin stained by Ponceau red for the very same pool of fibers (internal normalization; see Fig. 5C). This ratio was obtained for each pool and t-test was performed between two groups: control (fibers transfected with pCMS-eGFP) and CASQ1 expressing (fibers transfected with pCMS-eGFP-CASQ1). In the case of CASQ2, to correlate densitometric signals to protein content, calibration curves were generated as follows: increasing amounts of total homogenate of WT FDB (ranging between 10 and 30 μg) were loaded on the same gel and processed at the same time. A calibration curve was obtained for each blot and extrapolation was made to the relative protein quantity (see also Ref. 30). For display purposes, blots of JCT (see Fig. 1) and of TR 95 and 51 (see Fig. 5) were modified by Adobe Photoshop linear tools for brightness and contrast.

Immunofluorescence Microscopy

Single fibers from FDB were obtained 7 days after electroporation, plated on laminin coated coverslips for 48 h, and then fixed with 4% (wt/vol) formalin Sigma. Samples were permeabilized with 0.1% (vol/vol) Triton X-100 and 15% (vol/vol) goat serum in PBS for 1 h and incubated overnight with primary antibodies at 4°C without Triton X-100. Both monoclonal anti-CASQ1 antibodies and polyclonal anti-CASQ1/CASQ2 antibodies were diluted 1:500 in PBS. After three 20-min washes, samples were incubated for 2 h at room
temperature with secondary rhodamine goat anti-mouse and/or goat anti-rabbit antibodies (1:500; Jackson ImmunoResearch Laboratories, Lexington, KY). Specimens were observed in either a fluorescence microscope (Leica DMLB; Leica Microsystems, Mannheim, Germany) or a confocal microscope Leica TCS-SP2.

Preparation of Samples for Electron Microscopy and Data Analysis

FDB muscles were dissected, fixed, and prepared for electron microscopy (EM) as described in (34). Ultrathin sections were cut in a Leica Ultracut R microtome (Leica Microsystem) using a Diatome diamond knife (Diatome CH-2501; Biel, Switzerland). After being stained in 4% uranyl acetate and lead citrate, sections were examined in a FP 505 Morgagni Series 268D electron microscope (FEI, Brno, Czech Republic) at 60 kV equipped with a Megaview III digital camera and AnalySIS software (Olympus Soft Imaging Solutions).

Intracellular Ca\(^{2+}\) Measurements

Single FDB fibers were isolated by a modified collagenase/protease method as described previously (10). There was no difference in fibers yield between transfected and nontransfected muscles. Forty-eight hours after dissociation, fibers were incubated with 5 \(\mu\)M fura-2 acetoxyethyl ester (fura-2 AM; Invitrogen, San Giuliano Milanese, Italy) in 25 mM NaCl, 5 mM KCl, 1 mM MgSO\(_4\), 1 mM KH\(_2\)PO\(_4\), 5.5 mM glucose, 1 mM CaCl\(_2\), 20 mM HEPES, and 1% BSA pH7.4 (incubation buffer) for 30 min at 37°C. After fura-2 loading, fibers were washed twice for 10 min in the incubation buffer without BSA. A minimum of 30 min was allowed for fura-2 deesterification before the fibers were imaged. Intracellular Ca\(^{2+}\) transients were measured at 25°C using a dual-beam excitation fluorescence photometry setup (IonOptix, Milton, MA). Single muscle fibers were stimulated with two distinct frequency protocols and routinely five fibers were examined in each Petri dish: 1) after 10 min of steady-state pacing at 0.5 Hz, 10 Ca\(^{2+}\) transients were recorded from each fiber; and 2) a train of stimulation (60 Hz for 2 s) was delivered with a recovery time of 5 min between registrations. Ca\(^{2+}\) transients were analyzed using IonWizard software designed by IonOptix. Peak amplitude was calculated subtracting basal fluorescence values from peak values.

Statistical Analysis

Data were expressed as means ± SE. Student’s t-test was used for comparisons between CASQ1-null and WT data. One-way ANOVA test was used when three groups were compared using Prism GraphPad. Statistical significance was set at \(P < 0.05\).

RESULTS

Expression of SR and T-Tubule Markers in FDB Muscles

Quantitative Western blot analysis of total homogenates of FDB muscles from CASQ1-null and WT mice was performed to assess the expression of proteins involved in Ca\(^{2+}\) handling and SR structure. In Fig. 1A, controls for gel loading and electrotransfer are shown; the comparison between 3 \(\mu\)g crude homogenates from WT and CASQ1-null muscles did not reveal any appreciable difference in protein pattern. The main band at 42 kDa represents actin, as identified by anti-actin antibody, and was used as the loading control in Fig. 1B.

Fig. 1. Western blot analysis of flexor digitorum brevis (FDB) muscles from calsequestrin-1 (CASQ1)-null and wild-type (WT) mice. A. left lanes: silver staining of crude homogenates from WT and CASQ1-null FDB muscles and no appreciable differences in protein pattern. Arrow indicates position of CASQ in WT crude homogenates. A, right lanes: protein pattern of crude homogenates from WT and CASQ1-null muscles. In samples from CASQ1-null mice, CASQ2, triadin (TR) 95, sarcoplasmic-endoplasmic reticulum calcium ATPase 1 (SERCA1); and sarcalumenin (Srl) were significantly decreased compared with WT (* \(P < 0.001\), unpaired two-tailed t-test). DHPR, dihydropyridine receptor; JCT, junctin; BiP, immunoglobulin binding protein; CRL, calreticulin.
Representative immunoblots and densitometric analysis are shown in Fig. 1, B and C, respectively. CASQ2, TR 95, sarcalumenin, and SERCA1 were significantly reduced in FDB from CASQ1-null muscles (P < 0.05), whereas no significant changes were detected for DHPR, TR 51, JCT, glycoprotein 53, calreticulin, and BiP. Based on calibration curves (see MATERIALS AND METHODS for details), mean values of the CASQ2 signals in arbitrary units, 16.82 ± 1.52 and 77.55 ± 4.93 in CASQ1-null and WT FDB, respectively, were converted to protein equivalents (WT 22.42, CASQ1-null 12.42), i.e., CASQ2 was reduced by 50% in FDB muscles from CASQ1-null mice. Thus, SR proteins endowed with Ca²⁺ binding properties and potential substitutes for CASQ1 were found either not changed, e.g., calreticulin, or decreased, e.g., CASQ2 and sarcalumenin.

Structural features of jSR-T-Tubule Junctions and CRUs in FDB Muscle Fibers

Figure 2 shows the ultrastructural differences between CRUs of WT and CASQ1-null fibers from FDB muscles. Changes affecting CRUs of CASQ1-null FDB muscles were qualitatively identical to those previously described in EDL muscles (35), the main difference being the reduction in size of the SR terminal cisternae. The average width of SR terminal cisternae was calculated in triads and in multiple CRUs (only present in CASQ1-null fibers) by measuring the distance between the opposing membranes of TCs, as marked by dashed lines in Fig. 2, B, E, and F, and in Fig. 3. Ablation of CASQ1 resulted in dramatic shrinkage of the SR lumen: 25.0 ± 4.5 vs. 62.4 ± 10.5 nm in CASQ1-null and WT fibers, respectively (Fig. 3). Measurements of TC width were variable. However, distribution graphs (Fig. 3, bottom) showed that only 15 out of 716 TCs presented a lumen width ≤40 nm in WT fibers, whereas only 18 out of 487 measurements were ≥40 nm in CASQ1-null fibers. Additional morphological changes were detected in CRUs of CASQ1-null fibers, as follows: 1) Several CRUs displayed multiple rows of RYRs (see arrows in Fig. 2F), in CASQ1-null fibers, whereas junctions between T-tubules and jSR (or couplons) were usually in the form of triads (arrows in Fig. 2A) and usually contained two rows of RYRs (arrows in Fig. 2, B and C), in WT fibers. 2) Several CASQ1-null fibers contained multilayered junctions (larger arrows in Fig. 2 D). Multilayered CRUs (Fig. 2F) were also found in EDL muscles (35), although the percentage of fibers presenting multilayered junctions was significantly lower in FDB compared with EDL muscles (35 vs. 70%). 3) Several CRUs in CASQ1-null fibers

![Image of calcium release units (CRUs) in FDB muscles from WT and CASQ1-null mice.](http://ajpcell.physiology.org/)

**Fig. 2.** Morphology of calcium release units (CRUs) in FDB muscles from WT and CASQ1-null mice. A–C: in WT samples, CRUs were 1) positioned on both sides of the Z line (A, arrows), 2) usually formed by 3 elements (B and C, triads), and 3) transversely oriented. Ryanodine receptors (RYRs) formed 2 rows along the junctional face of terminal cisternae (TC; B and C, arrows), which presented a wide lumen (dashed lines in B). D–F: in CASQ1-null samples, CRUs were abnormal (D, arrows) because 1) often longitudinally oriented (D, empty arrow), 2) frequently composed of multiple layers (D and F, large arrows), and 3) contained multiple rows of RYRs decorating the junctional membane of TC (F, arrows), which presented a reduced lumen (distance between dashed lines in E and F; cfr. Ref. 35). Bars: A and D = 0.2 μm; B, C, E, and F = 0.1 μm. SR, sarcoplasmic reticulum.
were longitudinally oriented (empty arrow in Fig. 2D), a feature which is rare in adult WT fibers.

Cytosolic Ca\(^{2+}\) Transients Induced by Electrical Stimulation in CASQ1-null FDB Muscle Fibers

The ratiometric probe fura-2 was used to determine the free [Ca\(^{2+}\)]. At rest, [Ca\(^{2+}\)] levels were similar in WT and CASQ1-null muscle fibers (data not shown), whereas the transient induced by a single pulse showed a lower amplitude in muscle fibers lacking CASQ1: mean peak amplitude values were 0.6934 ± 0.0653 and 0.5129 ± 0.0342, respectively (see also Ref. 35). Moreover CASQ1-null muscle fibers were unable to sustain high and steady levels of cytosolic [Ca\(^{2+}\)] during prolonged tetanic stimulations (60 Hz for 2 sec) whereas WT fibers could, as shown in Fig. 4A. This behavior is similar to that observed in CASQ2/CASQ1-null FDB muscle fibers (5). The decline of [Ca\(^{2+}\)] was quantified measuring the area below each tracing and measuring the peak value at the first pulse (t0) and at the last pulse of the train (t2). As reported in Fig. 4B, the area was significantly smaller in CASQ1-null muscle fibers, compared with WT. In CASQ1-null fibers, the amplitude of the last response (t2) was significantly lower than also Ref. 35). Moreover CASQ1-null muscle fibers were unable to sustain high and steady levels of cytosolic [Ca\(^{2+}\)] during prolonged tetanic stimulations (60 Hz for 2 sec) whereas WT fibers could, as shown in Fig. 4A. This behavior is similar to that observed in CASQ2/CASQ1-null FDB muscle fibers (5). The decline of [Ca\(^{2+}\)] was quantified measuring the area below each tracing and measuring the peak value at the first pulse (t0) and at the last pulse of the train (t2). As reported in Fig. 4B, the area was significantly smaller in CASQ1-null muscle fibers, compared with WT. In CASQ1-null fibers, the amplitude of the last response (t2) was significantly lower than

![Graphs](http://ajpcell.physiology.org/ by 10.220.33.4 on November 4, 2016)
Reconstitution of the CASQ1 Complement: Correct Localization of CASQ1 Despite Persisting Low Levels of TR

Acute reexpression of CASQ1 in FDB muscles of CASQ1-null mice was achieved by cDNA electrotransfer. Seven days after electroporation with either pCMS-eGFP-CASQ1 or pCMS-eGFP, FDB muscles were enzymatically dissociated and single fibers were plated. All fibers reexpressing CASQ1 were readily detected under the dissection microscope because of the green fluorescence, and ranged between 30 and 80% of the total plated fibers, i.e., the efficiency of transfection, dissociation, and plating was variable. The correlation between eGFP expression and CASQ1 signal was, by and large, only qualitative: in fact, the eGFP expression did not show significant positive correlation with the CASQ1 amount in total homogenates. The CASQ1 content was measured in green single fibers and in total homogenates by densitometry of Western blot, estimated to be variable in single fibers and determined to be, on average, 61% of that of WT in total homogenates (not shown), i.e., there was a sizeable although incomplete reconstitution of the total CASQ1 complement.

To determine whether reexpression of CASQ1 could rescue expression of TR, we used Western blot analysis in pools of 20 single green fibers, dissociated from FDB muscles expressing either eGFP alone (control) or both eGFP and CASQ1. Representative Western blots of such pools obtained by either control or CASQ1-transfected muscles are shown in Fig. 5, A and B. Transfected CASQ1-null fibers expressed CASQ1 (A) in variable amounts compared with WT fibers, whereas fibers transfected with empty vector did not express CASQ1. In Fig. 5, A and B, variability of myosin and actin among the pools was visible. This indicates that the dimension of the fibers in each pool was not constant and that normalization of the TR signal was necessary to compare its expression between control and CASQ1-expressing pools. The TR signal was expressed as percentage of the actin signal of the same pool that was considered 100%. Three pools of CASQ1-null + eGFP and three pools of CASQ1-null + CASQ1 were compared with the t-test. The mean values and SE were plotted in the bar graph of Fig. 5C. Densitometric analysis (Fig. 5C) indicated that expression of TR 95 and TR 51 was not significantly changed, despite the presence of large amounts of exogenous CASQ1. Moreover, changes in expression of other SR proteins were evaluated in total FDB homogenates because of lower concentration of relevant proteins. Western blot with antibodies specific for either CASQ2, BiP, calreticulin, sarcalumenin, glicoprotein 53, or JCT showed that the expression of none of these proteins was significantly altered by acute expression of CASQ1 (data not shown).

The correct localization of exogenous CASQ1 was assessed by immunofluorescence and confocal microscopy. Following electroporation with pCMS-eGFP-CASQ1, dissociation, and plating, single fibers were analyzed by immunofluorescence with anti-CASQ1 antibodies. Confocal analysis showed that expression of eGFP was variable. Almost all fibers expressing eGFP were also immunolabeled with anti-CASQ1 antibodies, whereas all fibers negative for eGFP were also negative for anti-CASQ1 antibodies. In eGFP-positive fibers (Fig. 6, E and F), the well-known, characteristic double rows of anti-CASQ-positive foci at the A-I interface, distributed in the whole fiber (Fig. 6F, inset) that identify exogenous CASQ1, were detectable. Since the immunostaining pattern was identical to that of WT fibers (Fig. 6B), these results provide evidence that exogenous CASQ1 was correctly targeted to TC. As a negative control, such a regular immunostaining pattern was absent in CASQ1-null fibers transfected with eGFP alone (Fig. 6D).

Content and Width of TC are restored by Expression of Exogenous CASQ1 in FDB Muscle Fibers

Qualitative and quantitative EM analysis of CRUs in FDB fibers after electroporation with either pCMS-eGFP-CASQ1 or pCMS-eGFP is reported in Fig. 7 and Fig. 3. Seven days after electroporation, eGFP fluorescence was detectable in all mus-
cles after dissection but disappeared at the end of the fixation procedure for EM and was, thus, not useful to select transfected fibers in EM experiments. Moreover, expression of CASQ1 following electroporation with pCMS-eGFP-CASQ1 was variable, as shown above (Fig. 5, A and B) and Western blot analysis could not be performed in fibers fixed for EM. Following electroporation with pCMS-eGFP-CASQ1, the most striking change was the appearance of clusters of fibers in which the size of the TC lumen was increased. In FDB electroporated with pCMS-eGFP-CASQ1, three groups of fibers were identified based on morphological appearance of TC: group 1: fibers displaying enlarged TCs (see Fig. 7B, A-C of Fig. 3); group 2: fibers with flat TCs, virtually identical to those in CASQ1-null muscles (see Fig. 7C); and group 3: fibers in which TCs were overswollen (see Fig. 7, D and E). In three different FDB muscles electroporated with pCMS-eGFP-CASQ1, 44 out 130 fibers (34% on average) displayed enlarged TC size (group 1) and were classified as transfected fibers. Such fibers always contained many (even if not all) triads, CRUs with dilated SR (such as that in Fig. 7B, dashed lines), which appeared filled with an electron dense matrix and were virtually identical to those of WT fibers (Fig. 2, B and C). Fibers containing no triads (or multilayered CRUs) with dilated TC (group 2) were classified as not transfected, i.e., their CRUs (Fig. 7C) were identical to those of CASQ1-null fibers (compare with Fig. 2, E and F). Finally, very few fibers (<5%) with overswollen TC were classified as overtransfected (group 3). In three control FDB muscles electroporated with pCMS-eGFP, only fibers containing flat TCs (Figs. 3D and 7F), virtually identical to those in CASQ1-null muscles, were found. Therefore, electroporation with pCMS-eGFP-CASQ1 restored the TC content in a group of fibers classified as

Fig. 6. Localization of exogenous CASQ1 in single FDB fibers from CASQ1-null mice after in vivo transfection. Three fibers representative of the three groups described in MATERIALS AND METHODS are shown. A, B, C, and D: WT (A and B) and a CASQ1-null fiber (C and D) electroporated with pCMS-eGFP immunostained with primary anti-CASQ1 antibody and secondary rhodamin conjugated antibody. A and C: fibers imaged with laser beam settings suitable for eGFP (green) fluorescence. B and D: rhodamine (red) fluorescence referable to endogenous CASQ1 (note absence of the signal in D). E and F: CASQ1-null fiber transfected with pCMS-eGFP-CASQ1 and immunostained as before, the image was obtained with the same laser settings as B and F. Double-row, cross striation visible in B and F was taken as indication of correct targeting of exogenous CASQ1 to CRUs. Note that each row represents the triads aligned at the A-I interface, and in some area 4 dots belonging to the same sarcomere are resolved (arrows in inset). Bar = 12.5 μm. F, inset: area at higher magnification (×4).
transfected. The width of TC lumen was measured in transfected fibers and in control fibers electroporated with pCMS-eGFP (Fig. 3). All CRUs (both triads and multiple junctions) were measured: the average width of TC in transfected fibers was $66.3 \pm 10.2$ nm (see Fig. 3), a value significantly different ($P < 0.001$) from that of CASQ1-null fibers ($25.0 \pm 4.5$ nm), but very similar to that of WT fibers ($62.4 \pm 10.5$ nm). Distribution graphs (Fig. 3, bottom) show that in transfected fibers 285 out of 302 TC displayed a lumen width $>40$ nm (see dashed lines). Measurements of TC width and relative distribution graphs of control fibers (Fig. 3, column IV) show that the average TC width was $27.5 \pm 5.9$ nm, with 330 out of 368 TC being $<40$ nm, i.e., virtually identical to those of CASQ1-null muscles. Very few TCs present a lumen width of $\sim 40$ nm further confirming that electroporation per se and expression of eGFP alone did not increase TC size. Finally, transfection with pCMS-eGFP-CASQ1 yielded very few fibers with overswollen TCs (Fig. 7, D and E). These fibers classified as overtransfected are comparable to cardiac myocytes overexpressing CASQ2, which causes large swelling of SR terminal cisternae (19). Junctional SR of swollen TCs is entirely filled with an electron dense matrix (see asterisks in Fig. 7, whose structure is referable to CASQ network (cfr. 19). Not transfected and overtransfected fibers were not included in measurements of TC width, as reported in Fig. 3, column III.

Longitudinally oriented and multilayered CRUs are not commonly found in adult WT skeletal muscle fibers but are frequently found both in CASQ1-null fibers (see Fig. 2) and in transfected fibers. Quantitative analysis, although, showed that frequency of multiple junctions was decreased: the number of multiple junctions/100 $\mu$m$^2$ dropped from 5.51 $\pm$ 1.03 in CASQ1-null fibers to 1.68 $\pm$ 0.6 in transfected fibers.

Cytosolic [Ca$^{2+}$] and Amplitude of Electrically Induced Ca$^{2+}$ Transients Are Restored by Expression of Exogenous CASQ1

To assess whether CASQ1 reexpression was able to restore SR function, fura-2 signals were recorded in three groups of single fibers: 1) WT fibers transfected with eGFP (mock), 2) CASQ1-null fibers transfected with eGFP (control), and 3) CASQ1-null fibers transfected with CASQ1 + eGFP. The experimental protocol included mock-transfected WT fibers to avoid artifacts of transfection per se on intracellular Ca$^{2+}$ kinetics. Figure 8A shows that the resting basal level of fura-2, indicative of basal Ca$^{2+}$ concentration was virtually identical at 25°C in fibers obtained from CASQ1-null mice transfected with either CASQ1 or eGFP, and in WT fibers. In contrast, the peak amplitude of the Ca$^{2+}$ transient induced by a single electrical pulse showed a significant increase upon CASQ1 reexpression (Fig. 8A). Mean values of fluorescence ratio (amplitude) were as follows: WT + eGFP: 0.635 $\pm$ 0.043, CASQ1-null + eGFP: 0.466 $\pm$ 0.024, CASQ1-null + CASQ1: 0.566 $\pm$ 0.031. Moreover, the peak mean value of CASQ1-expressing fibers was not significantly different ($P = 0.36512$) from that of mock-transfected WT fibers, further suggesting that exogenous CASQ1 was able to rescue peak amplitude to values comparable to those of WT fibers.

Comparison of the Ca$^{2+}$ transients induced by high frequency (60 Hz) stimulation trains showed that, upon transfection of CASQ1, the decline of fura-2 signals during the stimulation, typically observed in CASQ1-null fibers (see Fig. 4), was significantly reduced (Fig. 8, B and C). CASQ1-transfected muscle fibers of CASQ1-null mice were able to sustain high [Ca$^{2+}$], during tetanus, whereas eGFP-transfected fibers (control) were not (Fig. 8B). The global cytoplasmic Ca$^{2+}$ was also significantly
increased upon CASQ1 reexpression, as judged by the area increase under the tracings. Responses to the first (t0) and last (t2) stimuli of the train were also increased upon CASQ1 reexpression, compared with control fibers (Fig. 8C).

**DISCUSSION**

The aim of the present study was to assess the rescue of the complex CASQ1-null phenotype attempted by acute in vivo reintroduction of exogenous CASQ1. Figure 9 summarizes the main results of the present study. Assessment of the rescued phenotype, 7 days after conspicuous expression of CASQ1 (up to 61% of the WT complement), was carried out at the molecular, functional, and ultrastructural levels. The functional rescue, as judged by Ca$^{2+}$ release and storage properties, was virtually complete, the ultrastructural changes were substantially, even if not completely, reversed, whereas the expression of SR proteins, including CASQ1 molecular partners such as TR and intraluminal Ca$^{2+}$ binding proteins, was not significantly modified.

**Phenotype of CASQ1-Null muscle Fibers of FDB: Functional, Ultrastructural, and Molecular Characteristics**

In agreement with previously published observations on CASQ1-null fibers, average amplitude of the cytosolic free Ca$^{2+}$ induced by low frequency stimulation was reduced and basal resting cytosolic Ca$^{2+}$ was unchanged. Interestingly,
CASQ1-null FDB fibers were unable to sustain high level of cytosolic Ca\(^{2+}\) during a tetanus (Fig. 4) in keeping with analogous results obtained in fibers lacking both CASQ1 and 2 (5, 36). This represents a likely indication that, in CASQ1-null FDB fibers, the amount of Ca\(^{2+}\) releasable is reduced in relation to a decreased content of SR and that contribution of CASQ2 is not relevant. The functional impact on Ca\(^{2+}\) homeostasis might derive from either CASQ1 absence and/or adaptive molecular and structural alterations.

Structurally, FDB fibers of CASQ1-null mice exhibited modifications intermediate between those previously observed in soleus and EDL (35). For example, multilayered junctions, mostly found in fast glycolytic fibers, which are abundant in EDL (70%) but scarce in soleus, were present in FDB but in lower percentage (35%). Since FDB has a fiber type composition intermediate between EDL and soleus (4, 9, 17), an intermediate phenotype can be expected in FDB fibers. The reasons for the different impact of CASQ1 ablation in relation to fiber type composition are not known; plausible explanatory hypotheses contemplate the complete lack of CASQ2 in these fibers; different content of cytoplasmic Ca\(^{2+}\) binding proteins such as parvalbumin, different molecular ratios of CASQ1, SERCA, and RYR1; and specific kinetics of Ca\(^{2+}\) fluxes in slow-twitch vs. fast-twitch fibers (32, 42).

In total homogenates from FDB, expression of some SR proteins (CASQ2, TR, sarcalumenin) was severely reduced in CASQ1-null mice, whereas expression of JCT was not significantly increased (Fig. 1). Franzini-Armstrong (13) recently reviewed the issue of the jSR ultrastructure in muscle fibers and are unable to sustain prolonged Ca\(^{2+}\) release during a tetanic stimulation. TT, transverse-tubule.

CASQ1 Controls Morphology of SR Terminal Cisternae

Acute expression of CASQ1 in CASQ1-null FDB was attained by cDNA electrophoretic transfer of pCMS-eGFP-CASQ1: timing of expression (7 days) appears adequate for reversion of many, even if not all, ultrastructural and functional changes (7). Expression of exogenous CASQ1 in CASQ1-null fibers results in correct protein targeting to jSR, in proximity of CRUs. This is proved by two independent sets of data: immunofluorescence showing correct positioning of CASQ1-positive foci (Fig. 6) and EM revealing accumulation of an electron dense matrix in the lumen of TC, whose size increases significantly (Figs. 3 and 7). Since control fibers electroporated with pCMS-eGFP did not show any sign of morphology rescue, the plausible interpretation is that exogenous expression of CASQ1 restored electron dense content of TC and enlarged TC lumen and that the overall process is specifically CASQ1 dependent. Since the TC width of transfected fibers was similar to that of WT fibers, our interpretation is that exogenous expression of CASQ1 restored TC width. Intracellular targeting and concentration of CASQ1 to jSR, enlargement of TC lumen, and sizeable reduction of multilayered junctions occurred without rescue of TR content and without major alterations in SR protein composition (Fig. 5). These findings imply that morphology of TC is finely controlled by the amount of CASQ1, supported also by the fact that overexpression of CASQ1 results in overwelling of TC (Fig. 7, D and E), an effect identical to that described in cardiomyocytes overexpressing CASQ2 (19). Moreover, these findings indirectly support the oligomerization model for CASQ1 segregation to TC, regardless of TR, one of the so-called anchoring proteins (33, 29).

The quantitative analysis of density of abnormal, multilayered junctions indicates that, upon expression of CASQ1, there was a trend for regression and suggests that CASQ1 also plays a direct role in plasticity of the entire sarcotubular system and, therefore, could be involved in pathological conditions, such as denervation, and in knock-out murine models of ER/SR resident proteins (49, 34). Fast formation and regression of multiple CRUs have been observed after denervation and reinher-
vation in rats, respectively (49). Since restoration of normal CRUs was incomplete, it would be interesting to assess whether longer times of expression were needed to restore completely the morphology of CRUs. Moreover, it is not known whether there is a direct correlation between the number of remaining abnormal triads and the amount of reexpressed CASQ1.

Longitudinal CRUs are still present after CASQ1 reintroduction. In this respect, it is known that, during normal, postnatal muscle development, TC orientation is completed much later than the attainment of the adult CASQ1 complement (16, 51). Moreover, in TR-null mice, where oblique or longitudinally oriented triplets are described, JCT is also downregulated: thus, TR and JCT are possible candidates for the control of CRUs orientation. Among JCT and the different TR isoforms, our data suggest that TR 95, decreased in CASQ1-null mice (Fig. 1) and not rescued upon CASQ1 reintroduction (Fig. 5), might play such a role. Interestingly, TR 51 is not downregulated in CASQ1-null mice, in agreement with a different role of TR 51 previously suggested yet still elusive (27, 43).

**CASQ1 Controls Ca\(^{2+}\) Homeostasis**

The recovery of Ca\(^{2+}\) storage and release properties after CASQ1 reintroduction was virtually complete (Fig. 8). In FDB from CASQ1-null muscles, detailed investigations of the SR protein profile (Fig. 1) revealed marked decrease of sarcalumenin, SERCA1, CASQ2, and TR 95, all proteins reported to modulate SR refilling. Consequently, the inability to store Ca\(^{2+}\) within the SR might be due to the combination of reduced intraluminal Ca\(^{2+}\) buffer capacity (absence of CASQ1 and deficiency of sarcalumenin), slowed Ca\(^{2+}\) uptake rate (deficiency of SERCA1), and decreased Ca\(^{2+}\) uptake capacity (rapid inactivation of SERCA1 by high intraluminal Ca\(^{2+}\) concentration). However, since SERCA1, sarcalumenin, CASQ2 and TR 95 content did not increase significantly in CASQ1-transfected muscles, the recovery of Ca\(^{2+}\) storage recorded in our experiments (Fig. 8) was directly determined only by accumulation of exogenous CASQ1 in TC, which increases SR Ca\(^{2+}\) capacity and facilitates Ca\(^{2+}\) store refilling via SERCA1. These results support the view that CASQ1 alone is sufficient to provide the amount of calcium required for a prolonged tetanic contraction.

**Conclusions**

This study defines some of the functional and structural roles of CASQ1 in vivo. Reconstitution of the CASQ1 complement has been successfully accomplished for the first time in FDB muscle fibers of CASQ1-null mice. Restoration of the Ca\(^{2+}\) storage capacity allows CASQ1-transfected muscle fibers to effectively cope with either single electrical pulses or prolonged high frequency train of pulses, preventing SR depletion. This is unambiguous evidence that the ability to store Ca\(^{2+}\) is directly dependent on CASQ1 and that proper coordination of Ca\(^{2+}\) reuptake and Ca\(^{2+}\) release also requires CASQ1. In addition, our data also confirm the crucial role of CASQ1 in determining shape and size of TC in skeletal muscle fibers.

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**REFERENCES**


13. C585RESCUE OF FUNCTION/STRUCTURE IN CASQ1-NULL FIBERS


