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A Retrograde Signal from Calsequestrin for the Regulation of Store-operated Ca^{2+} Entry in Skeletal Muscle*

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Calsequestrin (CSQ) is a high capacity Ca^{2+}-binding protein present in the lumen of sarcoplasmic reticulum (SR) in striated muscle cells and has been shown to regulate the ryanodine receptor Ca^{2+} release channel activity through interaction with other proteins present in the SR. Here we show that overexpression of wild-type CSQ or a CSQ mutant lacking the junction binding region (amino acids 86–191; Δ*junc*-CSQ) in mouse skeletal C2C12 myotube enhanced caffeine- and voltage-induced Ca^{2+} release by increasing the Ca^{2+} load in SR, whereas overexpression of a mutant CSQ lacking a Ca^{2+} binding, aspartate-rich domain (amino acids 352–367; Δ*asp*-CSQ) showed the opposite effects. Depletion of SR Ca^{2+} by thapsigargin initiated store-operated Ca^{2+} entry (SOCE) in C2C12 myotubes. A large component of SOCE was inhibited by overexpression of wild-type CSQ or Δ*junc*-CSQ, whereas myotubes transfected with Δ*asp*-CSQ exhibited normal function of SOCE. These results indicate that the aspartate-rich segment of CSQ, under conditions of overexpression, can sustain structural interactions that interfere with the SOCE mechanism. Such retrograde activation mechanisms are possibly taking place at the junctional site of the SR.

Calsequestrin (CSQ)[1] is a sarcoplasmic reticulum (SR) resident protein in muscle cells whose primary known function is to buffer Ca^{2+} in the lumen of SR. It binds Ca^{2+} with high capacity (40–50 Ca^{2+}/CSQ) and moderate affinity (K_d ~1 mM) (1). Recent studies have shown, however, that CSQ participates in the active Ca^{2+} release process from SR not simply by being a passive Ca^{2+} storage protein but also by actively modulating the function of the ryanodine receptor (RyR), the primary SR Ca^{2+} release channel involved in excitation-contraction coupling (2–6). The carboxyl terminus of CSQ contains an aspartate-rich region (amino acids 354–367) (7, 8), which functions as a major Ca^{2+} binding motif (9) and also interacts with triadin or junctin, proteins of the SR membrane complexed to RyR with unclear roles in the operation of excitation-contraction coupling. A different region of CSQ (amino acids 86–191) has been suggested previously to bind to junctin and triadin (6, 17). The functional significance of these CSQ regions in muscle Ca^{2+} signaling has not been examined.

The internal Ca^{2+} store of muscle cells, located in the SR, has a limited capacity; it must be replenished regularly through the entry of Ca^{2+} from the external environment. Depletion of SR Ca^{2+} stores, following activation of RyR or other Ca^{2+} release mechanisms, triggers Ca^{2+} entry from the external environment through a process known as capacitative Ca^{2+} entry via activation of store-operated Ca^{2+} channels (SOC) located in the cell surface membrane (10, 13, 14). Research into the molecular and cellular function of store-operated Ca^{2+} entry (SOCE) has been carried out primarily in non-excitable cells (*i.e.* lymphocytes, mast cells, etc.) and to some extent in smooth muscle cells (11, 12). Recently, Kurebayashi and Ogawa (13) presented the first functional evidence for the existence of SOC in skeletal muscle. We have extended their observations and shown that activation of SOC in skeletal muscle is coupled to retrograde signaling via conformational changes in the RyR (14).

In this study, we test the hypothesis that the RyR might receive information on the state of SR Ca^{2+} depletion via a direct retrograde signal from CSQ and thereby modulate both RyR-mediated Ca^{2+} release and RyR-mediated SOCE. We found that overexpression of CSQ not only enhances active Ca^{2+} release through the RyR but also suppresses SOCE. Deletion of the Ca^{2+} binding, aspartate-rich region of CSQ in these overexpression experiments resulted in reversal of the suppression of SOCE by wt-CSQ. Our data suggest that modulation of the RyR complex by CSQ from the luminal side of the SR could play a major role in regulating Ca^{2+} homeostasis in muscle cells and begin to define regions of CSQ that differentially interact with the RyR complex.

EXPERIMENTAL PROCEDURES

Cell Culture—C2C12 myoblasts derived from mouse skeletal muscle were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, as described by Shin *et al.* (15). Differentiation of myoblasts into myotubes was induced by changing the culture medium to DMEM supplemented with 2% horse serum (HS) and 1% penicillin/streptomycin. Experiments were performed on C2C12 myotubes expressing RyR, *i.e.* from the fifth day of culture in HS-DMEM,

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[1] The abbreviations used are: CSQ, calsequestrin; SOCE, store-operated Ca^{2+} entry; RyR, ryanodine receptor; SOC, store-operated Ca^{2+} channel; SR, sarcoplasmic reticulum; Tg, thapsigargin; wt, wild-type; DMEM, Dulbecco's modified Eagle's medium; HS, horse serum; HA, hemagglutinin; pCMS, promoter CMV IE, MCS, SV; GFP, green fluorescent protein; EGFP, enhanced GFP; CHO, Chinese hamster ovary; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis (acetoxy-methyl ester); ER, endoplasmic reticulum.

when it was possible to select myotubes having mature skeletal-type excitation-contraction coupling.

Cloning and Gene Transfection—The wt-CSQ cDNA from rabbit skeletal muscle and two deletion mutants of CSQ, Δ*junc*-CSQ and Δ*asp*-CSQ, were originally cloned into the pCDNA-HA 3.1 vector. For functional studies with C2C12 cells, the CSQ cDNAs were subcloned from pcDNA3.1-HA to pCMS-EGFP to create pCMS-EGFP(wt-CSQ), pCMS-EGFP(Δ*asp*-CSQ), and pCMS-EGFP(Δ*junc*-CSQ). The pCMS-EGFP plasmid contains two separate promoters that drive the transcription of green fluorescent protein (GFP, under the SV40 promoter) and the gene of interest (*i.e.* wt-CSQ or its mutants, under the CMV promoter) (16), thereby providing a convenient way of selecting transfected cells using GFP fluorescence. pCMS-EGFP vector alone or vector containing wt-CSQ, Δ*asp*-CSQ, or Δ*junc*-CSQ cDNAs were transfected into proliferating myoblasts using LipofectAMINE plus™ reagent according to the manufacturer's instructions. The culture medium was changed to HS-DMEM to allow differentiation of myoblasts into myotubes 12 h after transfection.

Immunocytochemistry—Five days after culturing in HS-DMEM medium, the C2C12 myotubes growing on coverslips and transfected with pcDNA3.1-HA plasmids containing wt-CSQ, Δ*asp*-CSQ, or Δ*junc*-CSQ were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) for 5 min. The cells were then incubated for 30 min with primary monoclonal antibody against HA or polyclonal antibody against skeletal CSQ for detecting exogenous HA-CSQ fusion proteins or endogenous CSQ protein. The cells were washed four times with 0.1% Triton X-100, followed by incubation with rhodamine-conjugated secondary antibody for 30 min in phosphate-buffered saline containing 1% bovine serum albumin. For detection of endogenous RyR, the cells were incubated for 30 min with primary polyclonal antibody against RyR and treated with fluorescein-conjugated secondary antibody. The coverslips were then mounted with 90% glycerol and 0.1% *O*-phenylenediamine in phosphate-buffered saline. Immunofluorescence was analyzed under a Leica DMRBE microscope (Heidelberg, Germany) equipped with a ×100 objective and filters for epifluorescence. Wild-type and CSQ mutant protein expression was demonstrated by Western blot following transient transfection in Chinese hamster ovary (CHO) cells, rather than C2C12 cells, because of the low efficiency of transfection in the latter (see Fig. 1*b*). The expressed CSQ protein was probed with polyclonal anti-CSQ antibody. The protein-antibody complex was blotted with a horseradish peroxidase-linked secondary antibody, and the signal was detected on Eastman Kodak Co. films using a chemiluminescent kit (Pierce, Rockford, IL).

Single Cell Ca^{2+} Measurement—The detailed procedure has been described elsewhere (15). Briefly, C2C12 myotubes were loaded with Fura-2/AM fluorescent Ca^{2+} indicator. Individual myotubes expressing exogenous CSQ were selected by the presence of GFP fluorescence, as described above. The changes in intracellular Ca^{2+} in single live cells was measured following exposure to 10 mM caffeine or 1 μM thapsigargin (Tg), with no $[Ca^{2+}]_o$ present in the bath solution (Ca^{2+}-free balanced salt solution containing 140 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.2, 0.5 mM EGTA).

Mn^{2+} Quenching Assay of Store-operated Ca^{2+} Entry—The detailed procedure has been described elsewhere (14). Briefly, to measure Mn^{2+} influx rate through SOC, 0.5 mM Mn^{2+} was added to the extracellular medium after Tg-induced SR Ca^{2+} depletion with or without the buffering of cytosolic Ca^{2+} by 50 μM BAPTA-AM. The Mn^{2+} quenching of Fura-2 fluorescence was measured at the Ca^{2+}-independent wavelength of Fura-2 excitation (360 nm). The decay of Fura-2 fluorescence upon Mn^{2+} addition was expressed as percent decrease in Fura-2 fluorescence per unit time (initial fluorescence = 100%).

Statistical Analysis—Values are means ± S.E. Significance was determined by Student's *t* test or analysis of variance. A value of $p < 0.05$ was used as criterion for statistical significance.

RESULTS

Localization of Exogenous Wild-type and Mutant CSQ in SR of C2C12 Cells—CSQ contains a putative junctin-binding region (amino acids 86–191; *junc*), as well as the Ca^{2+}-binding aspartate-rich region (amino acids 354–367; *asp*) (7, 17). To examine the function of *junc* and *asp* regions of CSQ, two deletion mutants, Δ*junc*-CSQ and Δ*asp*-CSQ, were generated using the PCR-based method for expression and functional studies in C2C12 cells (9). To distinguish the subcellular distribution of endogenous CSQ from expressed exogenous CSQ, wt and the CSQ mutants were expressed as HA-CSQ fusion proteins in differentiated C2C12 myotubes. Subcellular localization of HA-tagged proteins was performed by immunostaining with monoclonal antibody against HA. These experiment revealed a perinuclear distribution of HA-Δ*asp*-CSQ expressed in C2C12 myotubes (Fig. 1*b*), in a pattern that is indistinguishable from that of endogenous CSQ present in the SR detected by polyclonal anti-CSQ (Fig. 1*a*). The subcellular distributions of HA-wt-CSQ and HA-Δ*junc*-CSQ were similar to that of endogenous CSQ, indicating that both exogenously expressed proteins were also localized to the SR (data not shown). This was further confirmed by co-localization studies with polyclonal anti-RyR, as shown in the *lower panels* of Fig. 1, *a* and *b*. Clearly, the patterns of RyR distribution are virtually identical to those of wt and mutant CSQ expressed in C2C12 cells.

To confirm that the translational products of the various CSQ cDNAs were indeed CSQ, Western blots were performed on SDS-PAGE separated proteins derived from CHO cells transiently transfected with the wt-CSQ, Δ*asp*-CSQ, and Δ*junc*-

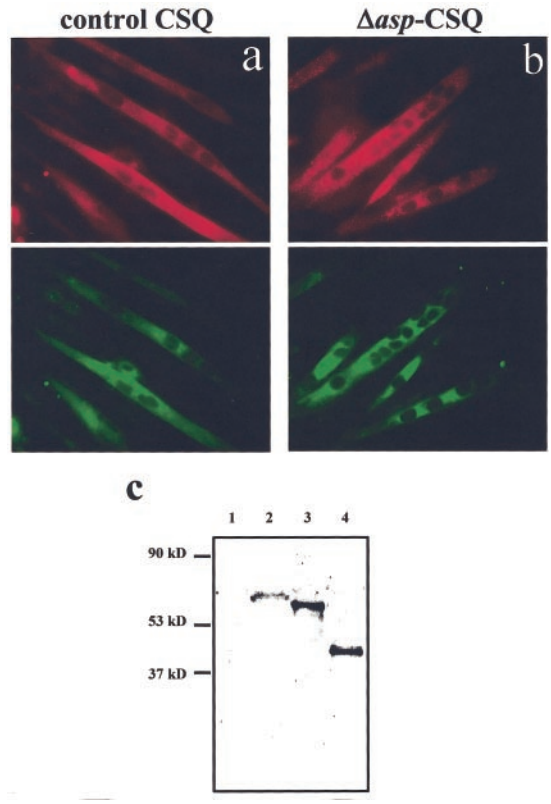

FIG. 1. **Localization of CSQ mutants overexpressed in SR of C2C12 myotubes.** *a* and *b*, Co-localization of endogenous CSQ and exogenous HA-Δ*asp*-CSQ expressed in C2C12 myotubes. *Upper panels*, monoclonal anti-HA antibody (*b*) or polyclonal anti-CSQ antibody (*a*) was used to label CSQ proteins. Their subcellular localization was visualized by red fluorescence of rhodamine-conjugated secondary antibody. *Lower panels*, endogenous RyR was labeled with polyclonal anti-RyR1 antibody and visualized by the green fluorescence of fluorescein-conjugated secondary antibody. The similar patterns of subcellular distribution observed with RyR and HA-Δ*asp*-CSQ expressed in C2C12 cells indicate the SR localization of exogenously expressed CSQ. Exogenously expressed HA-wt-CSQ, HA-Δ*junc*-CSQ, also showed the similar patterns to that of endogenously expressed CSQ (*a*) (data not shown). *c*, Western blot of CSQ expressed in CHO cells. The various CSQ cDNAs were introduced into CHO cells using LipofectAMINE. Total cell lysates (30 μg, 12 h after gene transfection) were separated on a 10% SDS-polyacrylamide gel and subjected to Western blot analysis with polyclonal anti-CSQ. *Lane 1*, mock-transfected cells; *lane 2*, cells transfected with wt-CSQ; *lane 3*, cells transfected with Δ*asp*-CSQ; *lane 4*, cells transfected with Δ*junc*-CSQ.

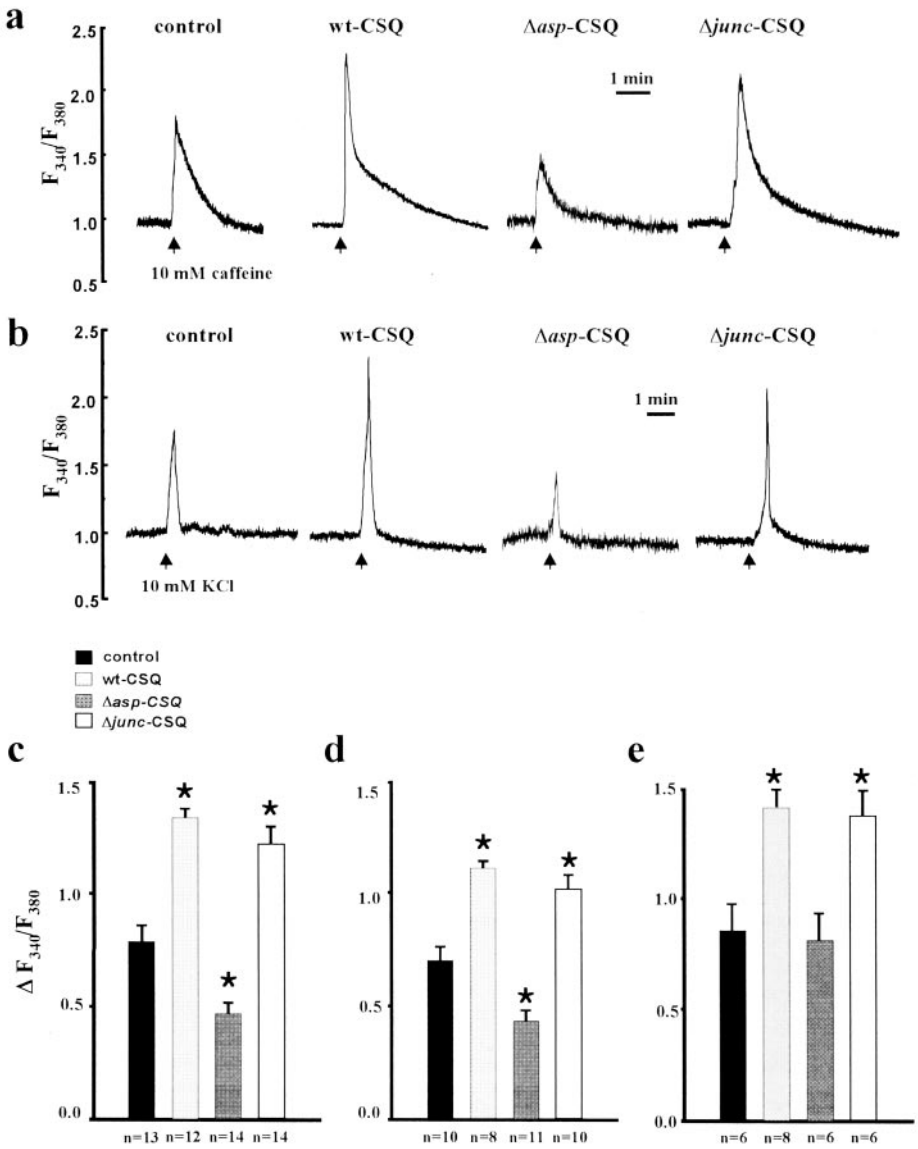

FIG. 2. **Intracellular Ca^{2+} release in C2C12 myotubes transfected with wt-CSQ, Δ*asp*–CSQ, and Δ*junc*-CSQ.** *a*, C2C12 myotubes transfected with pCMS-EGFP vector containing cDNA for wt-CSQ, Δ*asp*-CSQ, or Δ*junc*-CSQ were loaded with 5 μM Fura-2-AM. Myotubes showing green fluorescence on the fifth day of differentiation were used for intracellular Ca^{2+} measurements in a bath solution containing no $[Ca^{2+}]_o$. Addition of 10 mM caffeine induced Ca^{2+} release detected as the changes in the Fura-2 fluorescence ratio at excitation wavelengths of 340 and 380 nm (F_{340}/F_{380}). *b*, depolarization of the cell surface membrane via addition of 10 mM KCl to the bath solution initiates voltage-induced Ca^{2+} release in all cell preparations. Averaged data from multiple experiments are presented in *c* with caffeine-induced Ca^{2+} release and in *d* with voltage-induced Ca^{2+} release. *e*, A23187 was used to release the total intracellular Ca^{2+} store in individual C2C12 myotubes bathed in no Ca^{2+} balanced salt solution, and the released Ca^{2+} was assayed by changes in Fura-2 fluorescence. The data are presented as mean ± S.E. from multiple experiments (*n*, number of cells).

CSQ cDNAs. With LipofectAMINE-mediated gene transfection, CHO cells have higher transfection efficiency than C2C12 cells (30–60% for CHO *versus* 3–6% for C2C12), making it easier to detect expressed CSQ proteins. As shown in Fig. 1*c*, proteins of the predicted molecular masses are identified by anti-CSQ antibody. As with the C2C12 cells, immunostaining studies of these CHO cells also indicated that the expressed CSQ proteins were localized in the ER (not shown).

Differential Effects of wt-CSQ and Δasp-CSQ on Intracellular Ca^{2+} Release in Skeletal Muscle—Insertion of the various CSQ cDNAs into another eukaryotic expression vector, pCMS-EGFP, enabled selection of significantly transfected C2C12 cells using GFP fluorescence. The pCMS-EGFP plasmid expresses 1:1 ratio of GFP and CSQ under the control of two independent promoters (15, 16). Individual C2C12 myotubes exhibiting similar levels of GFP fluorescence, and therefore most likely similar level of exogenous CSQ proteins, were selected for functional studies with caffeine-induced Ca^{2+} release measurements. As shown in Fig. 2*a*, application of 10 mM caffeine resulted in Ca^{2+} release from SR in myotubes transfected with GFP alone (control). The peak amplitude of caffeine-induced Ca^{2+} release in myotubes transfected with wt-CSQ was ~1.7-fold higher than cells transfected with GFP alone ($\Delta F_{340}/F_{380}$ = 0.80 ± 0.02, *n* = 13, GFP; 1.38 ± 0.03, *n* = 11, wt-CSQ) (Fig. 2*c*). In contrast, expression of Δ*asp*-CSQ in C2C12 cells significantly reduced caffeine-induced Ca^{2+} release (0.41 ± 0.03, *n* = 14). Myotubes transfected with Δ*junc*-CSQ, on the other hand, showed similar enhancement of the amplitude of the caffeine-induced Ca^{2+} release transient (1.23 ± 0.03, *n* = 14) as wt-CSQ (Fig. 2*c*).

A simple explanation for the enhancement of caffeine-induced Ca^{2+} release in C2C12 myotubes overexpressing wt-CSQ is that this phenomenon likely reflects release from a concomitantly increased SR Ca^{2+} store. To determine whether the SR Ca^{2+} store was indeed increased, we treated cells with A23187, a Ca^{2+} ionophore that will release the entire intracellular Ca^{2+} store and allow its quantitation (18). As shown in Fig. 2*e*, the A23187-releasable Ca^{2+} pool was significantly larger in myotubes transfected with wt-CSQ and Δ*junc*-CSQ than those transfected with GFP alone (0.87 ± 0.12, *n* = 6, GFP; 1.43 ± 0.13, *n* = 8, wt-CSQ; 1.35 ± 0.11, *n* = 6, Δ*junc*-CSQ), whereas cells transfected with Δ*asp*-CSQ contained an A23187-releasable Ca^{2+} pool that was statistically identical to GFP controls (0.83 ± 0.15, *n* = 6) (Fig. 2*e*).

We then endeavored to determine whether the effects of transfected CSQ proteins on depolarization-induced Ca^{2+} release would parallel the results seen with caffeine-induced Ca^{2+} release. Changing the extracellular KCl concentration

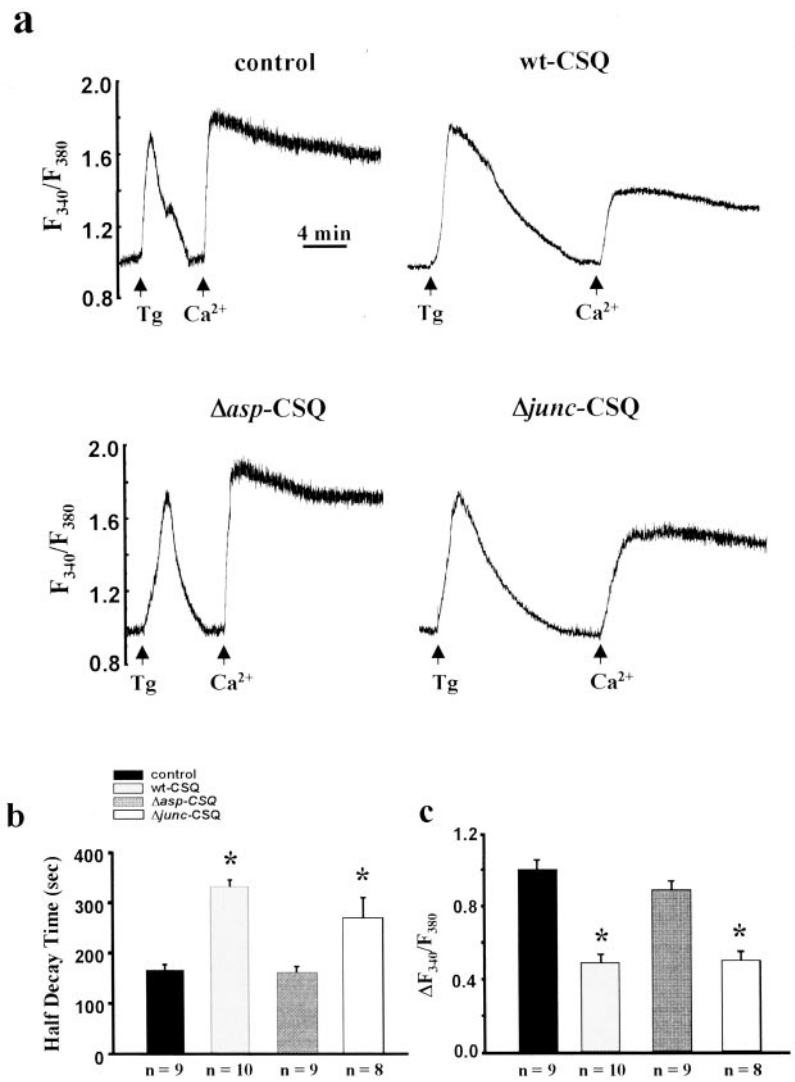

FIG. 3. **Altered Ca^{2+} transients in C2C12 cells transfected with wt-CSQ, Δ*asp*-CSQ, and Δ*junc*-CSQ.** *a*, passive movements of Ca^{2+} across the SR membrane were induced by an application of 1 μM Tg, a SR Ca^{2+}-ATPase inhibitor, with no Ca^{2+} balanced salt solution in the external solution. After Tg-induced depletion of SR Ca^{2+} stores, an addition of 2 mM Ca^{2+} in bath solution induced SOCE. Changes of intracellular Ca^{2+} in myotubes transfected with GFP alone (*control*), wt-CSQ, Δ*asp*-CSQ, or Δ*junc*-CSQ were monitored. Each trace is a representative of eight to ten independent experiments. *b*, the $t_{1/2}$ of Tg-induced Ca^{2+} efflux from SR are summarized as means ± S.E. *c*, the relative SOCE values after Tg-induced SR Ca^{2+} depletion are shown as means ± S.E.

from 2.8 to 10 mM led to depolarization of the cell surface membrane and induced the release of Ca^{2+} from the SR in C2C12 cells. As shown in Fig. 2, *b* and *d*, the peak amplitude of depolarization-induced Ca^{2+} release in cells overexpressing wt-CSQ and Δ*junc*-CSQ was again ~1.4–1.6-fold higher than that of GFP controls (0.74 ± 0.01, n = 10, GFP; 1.20 ± 0.03, n = 8, wt-CSQ; 1.02 ± 0.01, n = 10, Δ*junc*-CSQ), whereas overexpression of Δ*asp*-CSQ led to a significantly decreased depolarization-induced Ca^{2+} release (0.46 ± 0.03, n = 11). These results parallel the aggregate caffeine-induced Ca^{2+} release data shown in Fig. 2*c*. These data suggest that Δ*asp*-CSQ may either directly suppress RyR channel activity or reduce the efficiency of signal transduction from the dihydropyridine receptor to the RyR. Theoretically, Δ*asp*-CSQ could suppress SR Ca^{2+} release by reducing the Ca^{2+} buffering capacity of the SR. The aggregate Ca^{2+} store data presented in Fig. 2*e*, however, demonstrate that the Ca^{2+} store of the GFP control cells and the Δ*asp*-CSQ cells are equivalent.

Overexpression of functional CSQ, wt or mutant, in C2C12 cells should inevitably increase the Ca^{2+} buffering capacity of the SR and thereby alter the duration of passive Ca^{2+} movement across the SR membrane through as yet undescribed leak pathways. The kinetics of decay of this passive myoplasmic Ca^{2+} signal reflects a competition between continuing Ca^{2+} leak from the SR store and the removal of myoplasmic Ca^{2+} to the external environment by various plasma membrane-based mechanisms. One would expect that cells containing an elevated SR Ca^{2+} store would have a longer kinetic decay of the myoplasmic Ca^{2+} signal. To test this possibility, Tg, a potent inhibitor of SR Ca^{2+}-ATPase (19), was used to block the Ca^{2+} uptake function of the SR membrane, allowing for depletion of the luminal Ca^{2+} store via SR Ca^{2+} leak pathways. Our results, shown in Fig. 3*A*, demonstrate that the peak amplitudes of Tg-induced increases in myoplasmic $[Ca^{2+}]_i$ were comparable among the GFP control and those overexpressing wt-CSQ, Δ*asp*-CSQ, and Δ*junc*-CSQ. As predicted, however, the decay phase of Ca^{2+} transients in myotubes overexpressing wt-CSQ or Δ*junc*-CSQ, shown in Fig. 2*e* to contain greater Ca^{2+} stores than control or Δ*asp*-CSQ cells, were significantly longer ($t_{1/2}$ = 167 ± 11 s, n = 9, GFP; 332 ± 11 s, n = 10, wt-CSQ; 295 ± 28 s, n = 8, Δ*junc*-CSQ) (Fig. 3*b*). Importantly, the decay pattern of Tg-induced Ca^{2+} transients in cells overexpressing Δ*asp*-CSQ was similar to the GFP control ($t_{1/2}$ = 161 ± 8 s, n = 9). These results suggest that removal of the *asp*-rich region significantly reduces Ca^{2+} buffering capacity of endogenous CSQ, or to the exclusion of endogenous CSQ, does not participate in Ca^{2+} buffering of the SR. These results are consistent with our previous finding that the *asp*-rich region contains a major Ca^{2+} binding motif (9).

Inhibition of Store-operated Ca^{2+} Entry in Skeletal Muscle by wt-CSQ—We have shown recently (14) that depletion of SR Ca^{2+} storage leads to activation of SOCE in skeletal muscle. The activation of SOC in skeletal muscle appears to be coupled to conformational changes of RyR. Because our data above

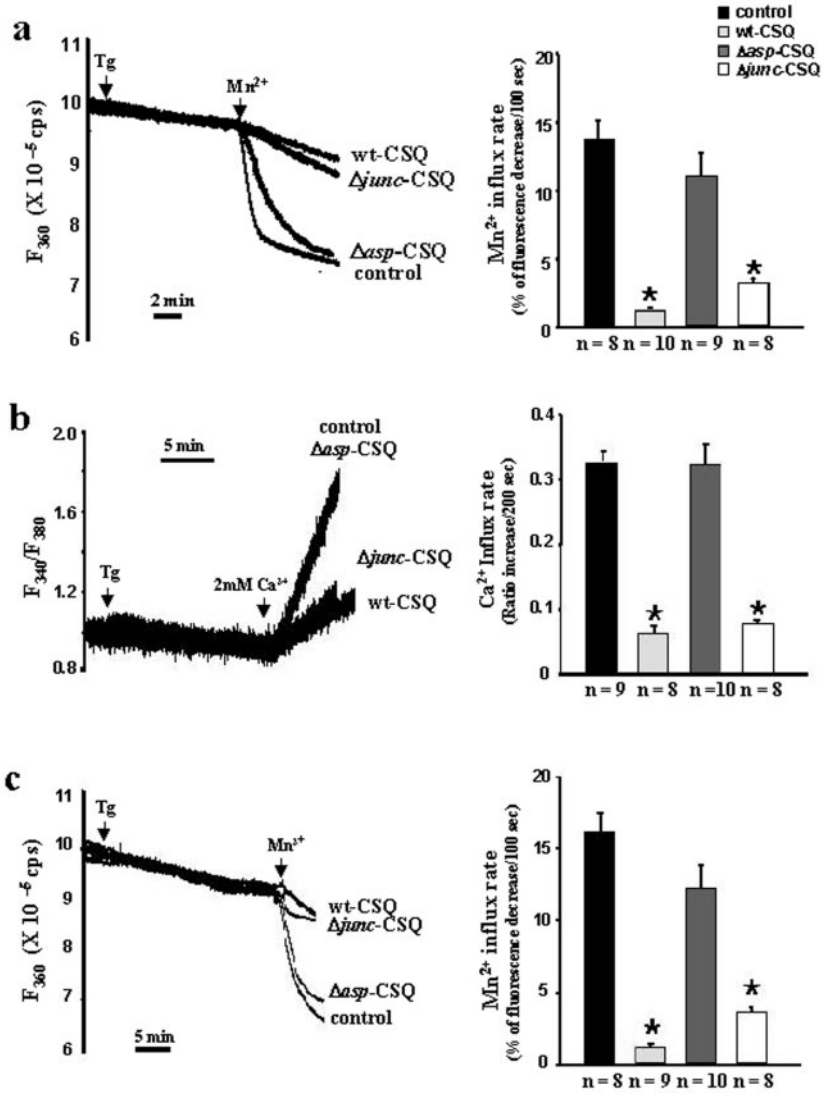

FIG. 4. **Inhibition of store-operated Ca^{2+} entry into C2C12 cells by CSQ.** *a*, the quenching of Fura-2 fluorescence by 0.5 mM Mn^{2+} entry after Tg-induced SR Ca^{2+} depletion was monitored in control, wt-CSQ, Δ*asp*-CSQ, and Δ*junc*-CSQ transfected myotubes, respectively (*left panel*). Fluorescence was measured with excitation wavelength of 360 nm and emission wavelength of 510 nm in an arbitrary unit, count per second (*cps*). Changes in the rate of Fura-2 quenching by Mn^{2+} were expressed as percent of fluorescence decrease per 100 s and averaged from multiple experiments (*right panel*). *b*, effects of BAPTA on intracellular Ca^{2+} movement in C2C12 cells. Ca^{2+} measurements were performed after preincubation of the myotubes with 50 μM BAPTA-AM for 30 min. Addition of 1 μM Tg did not cause detectable changes in Fura-2 fluorescence (F_{340}/F_{380}) in all four cell preparations. 15 min after addition of Tg, 2 mM Ca^{2+} was added to the bath solution, and rates of F_{340}/F_{380} increase were observed (*left panel*). Averaged data from multiple experiments are presented in the *right panel*. *c*, similar to studies shown in *a*, the Mn^{2+} quenching measurement of Fura-2 was performed in C2C12 myotubes after 30 min of preincubation with 50 μM BAPTA-AM (*left panel*). Data from multiple experiments were presented as means ± S.E. (*right panel*).

suggest that the Ca^{2+} store, as determined by the functional Ca^{2+} binding capacity of CSQ, determines the degree of Ca^{2+} release via the RyR, we asked whether this Ca^{2+} store could affect SOCE in this system. Sustained treatment of C2C12 myotubes with 1 μM Tg in Ca^{2+}-free medium resulted in complete depletion of SR Ca^{2+}. Addition of 2 mM Ca^{2+} to the bath solution after the myoplasmic Ca^{2+} signal had returned to baseline triggered SOCE in these Ca^{2+}-depleted cells (Fig. 3*a*). The degree of SOCE in myotubes transfected with Δ*asp*-CSQ was similar to GFP control cells (Fig. 3, *a* and *c*). Strikingly, overexpression of wt-CSQ and Δ*junc*-CSQ in the presence of a Tg-depleted SR Ca^{2+} store resulted in significant inhibition of SOCE (1.03 ± 0.07, $n = 9$, GFP; 0.51 ± 0.04, $n = 10$, wt-CSQ; 0.56 ± 0.06, $n = 8$, Δ*junc*-CSQ) (Fig. 3*c*).

The total SOCE measured in these experiments is likely the result of a summation of competing processes, SR Ca^{2+} uptake and release and surface membrane Ca^{2+} extrusion and influx. To isolate the measurement of SOC-mediated Ca^{2+} influx, we used the technique of Mn^{2+} quenching of the Fura-2 fluorescence (14). Mn^{2+} is known to be able to permeate into cells via SOC but is impervious to surface membrane extrusion processes or SR uptake by Ca^{2+} pumps. Hence, Mn^{2+} fluorescence quenching represents a measurement of unidirectional Ca^{2+} flux into cells via SOC. Under resting conditions (*i.e.* cells with an intact SR Ca^{2+} pool), no detectable Mn^{2+} quenching of Fura-2 was observed (not shown). Myotubes with Tg-depleted SR Ca^{2+} stores in a Ca^{2+}-free medium exhibited rapid quenching of Fura-2 fluorescence upon addition of 0.5 mM Mn^{2+} to the bath solution (Fig. 4*a*). Surprisingly, cells overexpressing wt-CSQ and Δ*junc*-CSQ displayed significant reduction in the rate of Fura-2 fluorescence quenching even with a depleted SR Ca^{2+} store. On average, ~10-fold reduction in Mn^{2+} influx rate was observed in cells overexpressing wt-CSQ and Δ*junc*-CSQ compared with control. Consistent with the results shown in Fig. 3, overexpression of Δ*asp*-CSQ did not appear to affect the rate of Mn^{2+} influx in C2C12 cells (Fig. 4*a*). If the presence of exogenous CSQ is merely to increase the Ca^{2+} load of the SR, then the complete depletion of this load should give equivalent activation of SOCE and resultant Mn^{2+} fluorescence quenching in all four of the cell preparations. Our results imply (*a*) that CSQ itself initiates a signal to SOCs, and (*b*) that the *asp*-rich region of the protein is likely involved in this signal transmission process.

Lack of Effect of BAPTA on SOCE in Skeletal Muscle—Studies from other investigators (33) suggest that gating of SOC is sensitive to the local level of $[Ca^{2+}]_i$. To test the role of $[Ca^{2+}]_i$ on the CSQ-mediated changes in the activation of SOCE in skeletal muscle, C2C12 cells were equilibrated with 50 μM BAPTA-AM, a concentration sufficient to buffer the changes in $[Ca^{2+}]_i$ because of passive Ca^{2+} movement across

the SR membrane, as indicated by the complete lack of Tg-induced changes in Fura-2 signal (Fig. 4*b*). Fifteen min after the addition of Tg, changing the bath solution from no $[Ca^{2+}]$ to 2 mM $[Ca^{2+}]$ resulted in measurable increases in the Fura-2 signal, indicating significant Ca^{2+} influx across the cell surface membrane (Fig. 4*b*). Myotubes transfected with wt-CSQ and Δ*junc*-CSQ showed slower Ca^{2+} entry than GFP control and Δ*asp*-CSQ-transfected myotubes. Direct Mn^{2+} quenching studies of Tg-induced Ca^{2+}-depleted and BAPTA-buffered myotubes confirmed that the changes in Fura-2 fluorescence above were because of activation of SOCE (Fig. 4*c*). Here, a striking reduction in the rate of Mn^{2+} quenching was observed in myotubes overexpressing wt-CSQ and Δ*junc*-CSQ, but not Δ*asp*-CSQ, with 50 μM BAPTA-AM present in the cytosol. These results indicate that buffering of $[Ca^{2+}]_i$ does not interfere with function of SOC in skeletal muscle and that the wt-CSQ-mediated inhibition of SOCE in C2C12 cells is unlikely to correlate with any changes in myoplasmic $[Ca^{2+}]_i$.

DISCUSSION

Until recently, CSQ has been thought of as the SR Ca^{2+}-binding protein whose function is simply to sequester Ca^{2+} in the vicinity of the RyR/Ca^{2+} release channel, to maintain a store for this ion, and to facilitate its rapid release during excitation-contraction coupling in muscle cells (2–6). We have shown here that overexpression of wt-CSQ enhances both caffeine- and voltage-induced Ca^{2+} release in skeletal muscle myotubes that are associated with an increased Ca^{2+} store in the SR. A profound reduction of SOCE was observed in cells overexpressing wt-CSQ or Δ*junc*-CSQ, but not Δ*asp*-CSQ, in cells with depleted SR Ca^{2+} stores and whose myoplasmic Ca^{2+} concentrations were buffered with BAPTA. Thus, the SR Ca^{2+} store is necessary for RyR-dependent Ca^{2+} release, but Ca^{2+} store *per se* is not the sole signal that regulates SOCE. Rather, CSQ adds a proximal signal in the regulation of SOCE in muscle cells. Our data suggests that the *asp*-rich region of CSQ is essential for retrograde signaling in both RyR-mediated Ca^{2+} release and regulation of SOCE in skeletal muscle.

The enhancement of caffeine-induced Ca^{2+} release by wt-CSQ in C2C12 myotubes is similar to that seen in cardiomyocytes isolated from transgenic mice overexpressing CSQ (20, 21). In those studies, caffeine-induced Ca^{2+} release was increased by ~10-fold in the CSQ transgenic mouse, paralleling the ~10-fold overexpression of CSQ in the heart. Because Δ*asp*-CSQ did not change the total SR Ca^{2+} store, the negative effect of this mutant on caffeine- and voltage-induced Ca^{2+} release in skeletal muscle may reflect a reduced activity of RyR or its interaction with accessory proteins or reduced local Ca^{2+} in the vicinity of RyR. Our data suggest that the *asp*-rich region of CSQ may regulate the proper functioning of the RyR, either by directly interacting with this channel or affecting other partners in the RyR/Ca^{2+} release channel complex. Others have suggested that proper formation of a quaternary molecular complex among CSQ, triadin, junctin, and RyR plays a critical role in the active Ca^{2+} release process across the SR membrane (6, 20). Indeed, both the carboxyl-terminal-containing *asp*-rich and amino-terminal regions of CSQ have been suggested as necessary for forming this quaternary SR Ca^{2+} release complex (8, 22, 23). Our previous studies have shown that the *asp*-rich region of CSQ binds Ca^{2+}, and this region is also involved in interaction with triadin (9). Thus, it is possible that overexpression of Δ*asp*-CSQ may alter the conformation of the quaternary complex and therefore cause inhibition of the RyR channel function.

A surprising and critical observation of the present study is that overexpression of wt-CSQ inhibits the function of SOCE in skeletal muscle. The CSQ-mediated inhibition of SOCE appears to involve the *asp*-rich region of CSQ, because the inhibitory effect was only observed with wt-CSQ and Δ*junc*-CSQ but not with Δ*asp*-CSQ. Our studies provide the first direct evidence for regulation of SOCE, a cell surface membrane function, through the luminal side of the SR membrane. Previous studies with other cell types have suggested that the physical docking of the ER or SR with the cell surface membrane is involved in the activation of SOC, presumably through contact interaction between SOC and protein components in the ER or SR (*e.g.* the inositol 1,4,5-trisphosphate receptor or RyR) (10, 24–26). Alternatively, the release of as yet undefined diffusible second messenger(s) from the intracellular organelle into the cytosol has been proposed to serve as an activator of SOC in response to depletion of intracellular Ca^{2+} stores (27, 28). Our recent studies with primary cultured skeletal muscle cells derived from different genetically engineered mouse models suggest that activation of SOC can be achieved in a graded fashion, depending on the filling state of the intracellular Ca^{2+} stores and/or the conformational changes of RyR (14). Although the gene(s) responsible for SOC has yet to be identified, and the exact nature of signal transduction involved in the activation of SOC remains largely unknown, our data indicate that the aspartate-rich segment of calsequestrin, under conditions of overexpression, can sustain structural interactions that interfere with the SOCE mechanism. These interactions are possibly taking place at the junctional site of the SR. Previous studies suggested that cardiomyocytes overexpressing CSQ showed abnormal enlarged junctional SR structure in triad junction, resulting in alteration of calcium signaling in muscle cells (20, 21). It will be interesting, therefore, to see how the absence of CSQ in a knock-out model would affect the function of SOC in skeletal muscle.

The presence of exogenously expressed CSQ in the SR lumen adds extra Ca^{2+} buffering capacity and increases the driving force for Ca^{2+} movement across the SR membrane. Our experiments with Tg-induced SR Ca^{2+} store depletion and myoplasmic BAPTA Ca^{2+} buffering have ruled out the possibility that the reduction of SOCE seen with overexpression of wt-CSQ and Δ*junc*-CSQ results from an incomplete depletion of SR Ca^{2+} stores or because of potential changes in myoplasmic $[Ca^{2+}]_i$ (29, 30). A previous study (19) in mouse fibroblast cells showed that overexpression of calreticulin, a major Ca^{2+}-binding protein in the ER lumen of non-muscle cells, also inhibited SOCE through a mechanism that is independent of its Ca^{2+} binding properties. Examination of the primary amino acid sequence of calreticulin reveals that, similar to CSQ, it too contains a highly negatively charged region at its carboxyl terminus. We speculate that the conservation of this negatively charged region of the carboxyl terminus of both of these SR/ER Ca^{2+}-binding proteins supports a significant functional role for the protein. We further suggest that this region in calreticulin will be involved in regulating SOCE in non-muscle cells.

Similar to the retrograde interaction between the inositol 1,4,5-trisphosphate receptor and SOC in non-excitable cells (10, 11, 30), a retrograde RyR-dihydropyridine receptor interaction exists in the skeletal muscle, as revealed by reduced dihydropyridine receptor function in RyR knock-outs (31, 32). Cumulative evidence also suggests that the conformational state of the RyR can regulate the function of SOC (10, 14). Our data reported here provide additional evidence for a tight link between Ca^{2+} homeostasis in SR and Ca^{2+} permeability in the cell surface membrane. Overexpression of CSQ in skeletal muscle not only affects caffeine- and voltage-induced Ca^{2+} release but also regulates SOCE. The aspartate residues located in the carboxyl terminus of CSQ not only constitute binding pockets for Ca^{2+} but also can regulate the function of the surface

membrane-located, store-operated Ca^{2+} channel, likely via retrograde interaction with the junctional protein complex in the SR.

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MEMBRANE TRANSPORT STRUCTURE FUNCTION AND BIOGENESIS:

A Retrograde Signal from Calsequestrin for the Regulation of Store-operated Ca^{2+} Entry in Skeletal Muscle

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