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

Received for publication, September 4, 2002, and in revised form, October 30, 2002
Published, JBC Papers in Press, November 4, 2002, DOI 10.1074/jbc.M209045200

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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; Δjun-CSQ) in mouse skeletal C2C12 myotubes 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; Δasp-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 Δjun-CSQ, whereas myotubes transfected with Δasp-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) 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 (SOC) 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). In skeletal 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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* This work was supported in part by grants from the Korea Ministry of Science and Technology (Critical Technology 21, 00-J-LF-01-B-54), Korea Science and Engineering Foundation (Basic Research Program 1999-1-20700-002-5), and the Brain Korea 21 Project (to D. H. K.) and by National Institutes of Health Grants R01-AG15556, R01-HL69000, and R01-CAP95379 (to J. M.) and R01-AR045509 (to J. P.). 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.

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† The abbreviations used are: CSQ, calsequestrin; SOC, 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 tetrais (acectoxy-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, Δjunc-CSQ and Δasp-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 pcCMV-EGFP to create pcCMV-EGFP(wt-CSQ), pcCMV-EGFP(Δasp-CSQ), and pcCMV-EGFP(Δjunc-CSQ). The pcCMV-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. pcCMV-EGFP vector alone or vector containing wt-CSQ, Δjunc-CSQ, or Δasp-CSQ cDNAs were transfected into proliferating myotubes using LipofectAMINE plus™ reagent according to the manufacturer’s instructions. The culture medium was changed to HS-DMEM to allow differentiation of myotubes 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, Δasp-CSQ, or Δjunc-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, 13 mM NaH2PO4, 2 mM KH2PO4, 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 fluorescence-conjugated secondary antibody. The coverslips were then mounted with 90% glycerol and 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 transfusion in Chinese hamster ovary (CHO) cells, rather than C2C12 cells, because of the low efficiency of transfection in the latter (see Fig. 1b). The expressed CSQ protein was probed with polyclonal anti-CSQ antibody. The protein-antibody complex was blotted with a horseradish peroxidase-conjugated secondary antibody, and the signal was detected on Eastman microscale films using a chemiluminescent kit (Pierce, Rockford, IL).

Single Cell Ca2+ Measurement—The detailed procedure has been described elsewhere (15). Briefly, C2C12 myotubes were loaded with Fura-2/AM fluorescent Ca2+ indicator. Individual myotubes expressing exogenous CSQ were selected by the presence of GFP fluorescence, as described above. The changes in intracellular Ca2+ in single live cells was measured using Fura-2 fluorescence following exposure to 10 mM caffeine or 1 μM thapsigargin (Tg), with no [Ca2+]i, present in the bath solution (Ca2+-free balanced salt solution containing 140 mM NaCl, 2.8 mM KCl, 2 mM MgCl2, 10 mM HEPES, pH 7.2, 0.5 mM EGTA).

Mn2+-Quenching Assay of Store-operated Ca2+ Entry—The detailed procedure has been described elsewhere (14). Briefly, to measure Mn2+ influx rate through SOCE, 0.5 mM Mn2+ was added to the extracellular medium after Tg-induced SR Ca2+ depletion with or without the buffering of cytosolic Ca2+ by 50 μM BAPTA-AM. The Mn2+ quenching of Fura-2 fluorescence was measured at the Ca2+-independent wavelength of Fura-2 excitation (380 nm). The decay of Fura-2 fluorescence upon Mn2+ 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 Ca2+-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, Δjunc-CSQ and Δasp-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 experiments revealed a perinuclear distribution of HA-Δasp-CSQ expressed in C2C12 myotubes (Fig. 1b), in a pattern that is indistinguishable from that of endogenous CSQ present in the SR detected by polyclonal anti-CSQ (Fig. 1a). The subcellular distributions of HA-wt-CSQ and HA-Δjunc-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. 1b. 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, Δasp-CSQ, and Δjunc-CSQ cDNAs. The expressed wild-type and CSQ mutants were detected by Western blot analysis with polyclonal anti-CSQ antibody. The expressed proteins were also localized to the SR (data not shown). The detailed procedure has been described elsewhere (15). Briefly, C2C12 myotubes were loaded with Fura-2/AM fluorescent Ca2+ indicator. Individual myotubes expressing exogenous CSQ were selected by the presence of GFP fluorescence, as described above. The changes in intracellular Ca2+ in single live cells was measured using Fura-2 fluorescence following exposure to 10 mM caffeine or 1 μM thapsigargin (Tg), with no [Ca2+]i, present in the bath solution (Ca2+-free balanced salt solution containing 140 mM NaCl, 2.8 mM KCl, 2 mM MgCl2, 10 mM HEPES, pH 7.2, 0.5 mM EGTA).

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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. 1c, 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 Ca²⁺ 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 Ca²⁺ release measurements. As shown in Fig. 2a, application of 10 mM caffeine resulted in Ca²⁺ release from SR in myotubes transfected with GFP alone (control). The peak amplitude of caffeine-induced Ca²⁺ release in myotubes transfected with wt-CSQ was 1.7-fold higher than cells transfected with GFP alone (ΔF<sub>junc</sub>/F<sub>junc</sub> = 0.80 ± 0.02, n = 13, GFP; 1.38 ± 0.03, n = 11, wt-CSQ) (Fig. 2c). In contrast, expression of Δasp-CSQ in C2C12 cells significantly reduced caffeine-induced Ca²⁺ release (0.41 ± 0.03, n = 14). Myotubes transfected with Δjunc-CSQ, on the other hand, showed similar enhancement of the amplitude of the caffeine-induced Ca²⁺ release transient (1.23 ± 0.03, n = 14) as wt-CSQ (Fig. 2c).

A simple explanation for the enhancement of caffeine-induced Ca²⁺ release in C2C12 myotubes overexpressing wt-CSQ is that this phenomenon likely reflects release from a concomitantly increased SR Ca²⁺ store. To determine whether the SR Ca²⁺ store was indeed increased, we treated cells with A23187, a Ca²⁺ ionophore that will release the entire intracellular Ca²⁺ store and allow its quantitation (18). As shown in Fig. 2d, the A23187-releasable Ca²⁺ pool was significantly larger in myotubes transfected with wt-CSQ and Δjunc-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, Δjunc-CSQ), whereas cells transfected with Δasp-CSQ contained an A23187-releasable Ca²⁺ pool that was statistically identical to GFP controls (0.83 ± 0.15, n = 6) (Fig. 2e).

We then endeavored to determine whether the effects of transfected CSQ proteins on depolarization-induced Ca²⁺ release would parallel the results seen with caffeine-induced Ca²⁺ release. Changing the extracellular KCl concentration...
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, a, and d, the peak amplitude of depolarization-induced Ca$^{2+}$ release in cells overexpressing wt-CSQ and Δasp-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, Δjunc-CSQ), whereas overexpression of Δasp-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. 2c. These data suggest that Δasp-CSQ may either directly suppress RyR channel activity or reduce the efficiency of signal transduction from the dihydropyridine receptor to the RyR. Theoretically, Δasp-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. 2c, however, demonstrate that the Ca$^{2+}$ store of the GFP control cells and the Δasp-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 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. 3A, demonstrate that the peak amplitudes of Tg-induced increases in myoplasmic [Ca$^{2+}$], were comparable among the GFP control and those overexpressing wt-CSQ, Δasp-CSQ, and Δjunc-CSQ. As predicted, however, the decay phase of Ca$^{2+}$ transients in myotubes overexpressing wt-CSQ or Δjunc-CSQ, shown in Fig. 2e to contain greater Ca$^{2+}$ stores than control or Δasp-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, Δjunc-CSQ) (Fig. 3b). Importantly, the decay pattern of Tg-induced Ca$^{2+}$ transients in cells overexpressing Δasp-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
suggest that the Ca²⁺ store, as determined by the functional Ca²⁺ binding capacity of CSQ, determines the degree of Ca²⁺ release via the RyR, we asked whether this Ca²⁺ store could affect SOCE in this system. Sustained treatment of C2C12 myotubes with 1 μM Tg in Ca²⁺-free medium resulted in complete depletion of SR Ca²⁺. Addition of 2 mM Ca²⁺ to the bath solution after the myoplasmic Ca²⁺ signal had returned to baseline triggered SOCE in these Ca²⁺-depleted cells (Fig. 3a). The degree of SOCE in myotubes transfected with Δasp-CSQ was similar to GFP control cells (Fig. 3, a and c). Strikingly, overexpression of wt-CSQ and Δjunc-CSQ in the presence of a Tg-depleted SR Ca²⁺ 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, Δjunc-CSQ) (Fig. 3c).

The total SOCE measured in these experiments is likely the result of a summation of competing processes, SR Ca²⁺ uptake and release and surface membrane Ca²⁺ extrusion and influx. To isolate the measurement of SOC-mediated Ca²⁺ influx, we used the technique of Mn²⁺ quenching of the Fura-2 fluorescence (14). Mn²⁺ is known to be able to permeate into cells via SOCl and is impervious to surface membrane extrusion processes or SR uptake by Ca²⁺ pumps. Hence, Mn²⁺ fluorescence quenching represents a measurement of unidirectional Ca²⁺ flux into cells via SOC. Under resting conditions (i.e., cells with an intact SR Ca²⁺ pool), no detectable Mn²⁺ quenching of Fura-2 was observed (not shown). Myotubes with Tg-depleted SR Ca²⁺ stores in a Ca²⁺-free medium exhibited rapid quenching of Fura-2 fluorescence upon addition of 0.5 mM Mn²⁺ to the bath solution (Fig. 4a). Surprisingly, cells overexpressing wt-CSQ and Δjunc-CSQ displayed significant reduction in the rate of Fura-2 fluorescence quenching even with a depleted SR Ca²⁺ store. On average, ~10-fold reduction in Mn²⁺ influx rate was observed in cells overexpressing wt-CSQ and Δjunc-CSQ compared with control. Consistent with the results shown in Fig. 3, overexpression of Δasp-CSQ did not appear to affect the rate of Mn²⁺ influx in C2C12 cells (Fig. 4a). If the presence of exogenous CSQ is merely to increase the Ca²⁺ load of the SR, then the complete depletion of this load should give equivalent activation of SOCE and resultant Mn²⁺ 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 [Ca²⁺]. To test the role of [Ca²⁺], 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 [Ca²⁺], because of passive Ca²⁺ movement across...
the SR membrane, as indicated by the complete lack of Tg-induced changes in Fura-2 signal (Fig. 4b). 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. 4b). Myotubes transfected with wt-CSQ and Δjunc-CSQ showed slower Ca\(^{2+}\) entry than GFP control and Δasp-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. 4c). Here, a striking reduction in the rate of Mn\(^{2+}\) quenching was observed in myotubes overexpressing wt-CSQ and Δjunc-CSQ, but not Δasp-CSQ, with 50 μM BAPTA-AM present in the cytosol. These results indicate that buffering of [Ca\(^{2+}\)], 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\(^{2+}\)].

**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 Δjunc-CSQ, but not Δasp-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 SOC. 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 SOC 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 Δasp-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 Δasp-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 SOC 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 Δjunc-CSQ but not with Δasp-CSQ. Our studies provide the first direct evidence for regulation of SOC, 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 buffering have ruled out the possibility that the reduction of SOCE seen with overexpression of wt-CSQ and Δjunc-CSQ results from an incomplete depletion of SR Ca\(^{2+}\) stores or because of potential changes in myoplasmic [Ca\(^{2+}\)].

(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.

Acknowledgments—We thank Dr. Kevin P. Campbell for providing cDNA encoding the rabbit CSQ and Dr. Woo Jin Park for providing polyclonal anti-CSQ antibody. We also thank Mr. Chun Shik Park for invaluable technical help in immunocytochemistry.

REFERENCES

1. Fliegel, L., Ohnishi, M., Carpenter, M. R., Khanna, V. K., Reithmeier, R. A., and MacLennan, D. H. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1167–1171
2. Szegedi, C., Sarkozy, S., Herzog, A., Jona, I., and Varsanyi, M. (1999) Biochem. J. 337, 19–22
3. Donoos, P., Beltran, M., and Hidalgo, C. (1996) Biochemistry 35, 13419–13425
4. Guo, W., and Campbell, K. P. (1995) J. Biol. Chem. 270, 9027–9030
5. Jones, L. R., Zhang, L., Sanborn, K., Jorgensen, A. O., and Kelley, J. (1995) J. Biol. Chem. 270, 30787–30796
6. Zhang, L., Kelley, J., Schmeisser, G., Kovayashi, Y. M., and Jones, L. R. (1997) J. Biol. Chem. 272, 23389–23397
7. Yano, K., and Zarrain-Herzberg, A. (1994) Mol. Cell. Biochem. 135, 61–70
8. Wang, S., Trumble, W. R., Liao, H., Wesson, C. R., Dunker, K., and Kang, C. H. (1998) Nat. Struc. Biol. 5, 476–483
9. Shin, D. W., Ma, J., and Kim, D. H. (2000) FEBS Lett. 486, 178–182
10. Kiselyov, K. I., Shin, D. M., Wang, Y., Pessah, I. N., Allen, P. D., and Muallem, S. (2000) Mol. Cell. 6, 421–431
11. Broad, L. M., Braun, F. J., Lievremont, J. P., Bird, G. S., Kurusaki, T., and Putney, J. W. Jr. (2001) J. Biol. Chem. 276, 15945–15952
12. Ng, L. C., and Gurses, A. M. (2001) Circ. Res. 89, 923–929
13. Kurebayashi, N., and Ogawa, Y. (2001) J. Physiol. 533, 185–199
14. Pan, Z., Yang, D., Nagaraj, R. Y., Nosek, T. A., Nishi, M., Takeshima, H., Cheng, H., and Ma, J. (2002) Nat. Cell Biol. 4, 378–383
15. Shin, D. W., Pan, Z., Bandypadhyay, A., Bhat, M. B., Kim, D. H., and Ma, J. (2002) Biophys. J. 83, 2539–2549
16. Pan, Z., Damron, D., Nieminen, A. L., Bhat, M. B., and Ma, J. (2000) J. Biol. Chem. 275, 19978–19984
17. Collins, J. H., Tarcsafalvi, A., and Ikemoto, N. (1990) Biochem. Biophys. Res. Commun. 167, 189–193
18. Pizzo, P., Fasolato, C., and Porzian, T. (1997) J. Cell Biol. 136, 355–366
19. Mery, L., Mesaeli, N., Michalak, M., Opas, M., Lew, D. P., and Krause, K. H. (1996) J. Biol. Chem. 271, 9332–9339
20. Jones, L. R., Suzuki, Y. J., Wang, W., Kovayashi, Y. M., Ramesh, V., Franzini-Armstrong, C., Cleemann, L., and Morad, M. (1998) J. Clin. Invest. 101, 1385–1393
21. Sato, Y., Ferguson, D. G., Sako, H., Dorn, G. W., II, Kadambi, V. J., Yatani, A., Huit, B. D., Walsh, R. A., and Kranias, E. (1998) J. Biol. Chem. 273, 28470–28477
22. Maguire, P. B., Briggs, F. N., Lennon, N. J., and Ohlendieck, K. (1997) Biochem. Biophys. Res. Commun. 240, 721–727
23. Gatti, G., Trifari, S., Mesaeli, N., Parker, J. M. R., Michalak, M., and Meldolesi, J. (2001) J. Cell Biol. 154, 525–534
24. Ma, H. T., Patterson, R. L., van Rossum, D. B., Birnbaumer, L., Mikoshiba, K., and Gill, D. L. (2000) Science 286, 1647–1651
25. Kiselyov, K., Migney, G. A., Zhu, M. X., and Muallem, S. (1999) Mol. Cell 4, 423–429
26. Bennett, D. L., Boutman, M. D., Berridge, M. J., and Cheek, T. R. (1998) Biochem. J. 329, 349–357
27. Kim, H. Y., and Hanley, M. R. (1999) Mol. Cells 9, 326–332
28. Trepakova, E. S., Coutura, P., Hunton, D. L., Marchase, R. B., Cohen, R. A., and Bolotina, V. M. (2000) J. Biol. Chem. 275, 26158–26163
29. Madge, L., Marshall, I. C. B., and Taylor, C. W. (1997) J. Physiol. 498, 351–369
30. Putney, J. W., Jr., Broad, L. M., Braun, F. J., Lievremont, J. P., and Bird, G. S. (2001) J. Cell Sci. 114, 2223–2229
31. Nakai, J., Dirksen, R. T., Nguyen, H. T., Pessah, I. N., Beeam, K. G., and Allen, P. D. (1996) Nature 380, 72–75
32. Franzini-Armstrong, C., and Protasi, F. (1997) Physiol. Rev. 77, 699–729
33. Pierro, L., and Parekh, A. B. (1999) J. Membr. Biol. 168, 9–17

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