

JUNCTIONAL MEMBRANE STRUCTURE AND STORE OPERATED CALCIUM ENTRY IN MUSCLE CELLS

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1. ABSTRACT

The store-operated Ca^{2+} channel (SOC) located on the plasma membrane (PM) mediates capacitative entry of extracellular Ca^{2+} following depletion of intracellular Ca^{2+} stores in the endoplasmic or sarcoplasmic reticulum (ER/SR). It plays important roles in a variety of cell signaling processes, including proliferation, apoptosis, gene regulation and motility. In skeletal muscle, the L-type Ca^{2+} channel on the surface membrane has slow kinetics of activation in response to voltage stimulation, and therefore does not support entry of extracellular Ca^{2+} . Recent studies have provided functional evidence for the existence of SOC in muscle cells. Severe dysfunction of SOC is identified in muscle cells lacking either ryanodine receptors located on the SR membrane, or mitsugumin 29 - a membrane protein located in the triad junction of skeletal muscle. These results indicate that SOC activation requires an intact interaction between PM and SR, and is linked to conformational changes of ryanodine receptors. The cumulative entry of Ca^{2+} through SOC not only provides the mechanism for refilling of intracellular Ca^{2+} stores, but may also add to the Ca^{2+} needed for muscle contraction under conditions of intensive exercise and fatigue. The proper coupling of PM with ER/SR, in the triad junction in skeletal muscle or dyad junction in cardiac muscle, is essential not only for the membrane excitation-induced intracellular Ca^{2+} release but also for the store depletion-initiated capacitative Ca^{2+} entry.

2. INTRODUCTION

Ca^{2+} ions are important second messengers in many signal transduction pathways. They participate in essentially every cellular process, ranging from gene regulation and protein trafficking, to cell differentiation and apoptosis, to membrane excitability and cell motility. In general, there are two sources of this signaling ion in the cell: channels in the plasma membrane (PM) that open to allow external Ca^{2+} to flow into the cytoplasm, and internal stores in the form of endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) that release Ca^{2+} into the cytosol. The effective coupling of extracellular Ca^{2+} entry and intracellular Ca^{2+} release requires an intimate communication between PM and ER/SR.

In many excitable cells, entry of extracellular Ca^{2+} can be achieved via activation of voltage-gated Ca^{2+} channels by membrane depolarization. This initial Ca^{2+} signal is further amplified as the entering Ca^{2+} triggers additional mobilization of intracellular Ca^{2+} stores through activation of IP3 receptor or ryanodine receptor (RyR) channels, a process known as Ca^{2+} -induced Ca^{2+} release (CICR) (5, 19, 90). In most non-excitabile cells, the initial Ca^{2+} signal usually originates from release of Ca^{2+} from internal stores via activation of IP3 receptors located on the ER. The entry of extracellular Ca^{2+} into non-excitabile cells can be triggered by either direct activation through receptor molecules located on the PM or indirect activation by a

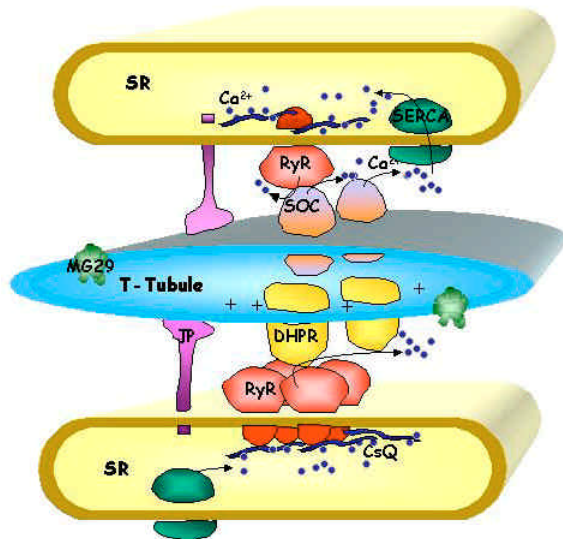


Figure 1. Molecular components of excitation-contraction coupling in muscle cells. A close contact of T-tubule and SR membrane is essential for the coupling of extracellular Ca^{2+} entry and intracellular Ca^{2+} release. The DHPR located on the T-tubule membrane functions as an L-type Ca^{2+} channel, as well as the voltage sensor of the plasma membrane. The RyR located on the SR membrane functions as the Ca^{2+} release channel. Junctophilins (JP) are proteins located in the SR membrane, and presumably provide the link of SR with plasma membrane for the formation of the triad junction. MG29 is a synaptophysin-family member protein located on the T-tubule membrane. The store-operated Ca^{2+} channel (SOC) resides on the T-tubule membrane that is in the close proximity of the RyR. Calsequestrin (CSQ) is a Ca^{2+} binding protein located in the SR lumen. SERCA Ca^{2+} pump is responsible for maintaining the high Ca^{2+} load in the SR membrane.

variety of second messengers, in a process generally referred to as the receptor-operated Ca^{2+} entry (ROCE) pathway (28). Another commonly observed mechanism of regulated Ca^{2+} entry in non-excitable cells is a process known as the capacitative Ca^{2+} entry or store-operated Ca^{2+} entry (SOCE) (91). In this mechanism, the depletion of intracellular stores due to activation of IP_3 receptor or RyR or other Ca^{2+} -releasing signals activates a signaling pathway leading to the opening of PM-located store-operated Ca^{2+} channels.

In recent years, the SOCE pathway has received great interest not only because of its unusual nature as retrograde signaling, but also due to its wide occurrence in both excitable and non-excitable cells and its possible role in physiological and pathophysiological situations (9, 20, 27, 38, 81, 134). More recently, the capacitative Ca^{2+} entry has been identified in skeletal and cardiac muscle cells, and its physiological functions are being recognized (34, 60, 80, 118).

2.1. Calcium movements in muscle cells – intracellular release and extracellular entry

Coupling of electrical excitation across the PM to

intracellular Ca^{2+} release from the SR and subsequent muscle contraction (E-C coupling) occurs in the junction between the transverse-tubular (T-tubule) invagination of PM and the terminal cisternae of SR in a structure known as the “triad junction”, where two major protein components are located: the dihydropyridine receptors (DHPR), which function as voltage sensors of the T-tubule membrane, and the RyRs, which function as the Ca^{2+} release channels in the SR (6, 7, 8, 22, 33, 68, 77) (see Figure 1). In skeletal muscle, the triad junction provides the structural framework for the interaction between DHPR and RyR that mediates direct signal transduction of voltage-induced Ca^{2+} release (VICR). The operation of VICR in skeletal muscle appears to involve a direct physical interaction between DHPR and RyR, without requiring the transmembrane movement of extracellular Ca^{2+} (95, 96, 99). In the heart, however, depolarization of PM initiates rapid Ca^{2+} influx through activation of the DHPR/L-type Ca^{2+} channel, which triggers opening of the RyR/ Ca^{2+} release channel via CICR (18, 66, 124). Peripheral coupling between PM and SR in the form of dyad junction mediates efficient communication from DHPR to RyR in the cardiac muscle.

The DHPR/L-type Ca^{2+} channel located in the T-tubules of skeletal muscle has slow activation kinetics (23, 29, 67), and does not support Ca^{2+} influx under normal physiological conditions. Therefore, the twitch force in skeletal muscle is triggered mainly by the acute release of Ca^{2+} from the SR, primarily via VICR, and secondarily amplified by CICR through activation of neighboring RyRs not directly coupled to DHPR (96, 99). The internal Ca^{2+} stores located in the SR of muscle cells and ER of other cells have a limited capacity for Ca^{2+} storage. As a result, cells possess the capability of SOCE as a means of renewing depleted intracellular Ca^{2+} . Although evidence indicates that physical docking of ER with PM is involved in the activation of SOC, the molecules and/or signals that couple ER/SR Ca^{2+} depletion to opening of SOC remain largely unknown (82, 92, 93).

2.2. Transient receptor potential proteins (TRP) and store-operated Ca^{2+} channel

The initial leads to identify the molecular basis of SOCE were provided by the study of phototransduction in *Drosophila melanogaster*. Work in *Drosophila* photoreceptors showed that mutations in a protein encoded by the *trp* gene were associated with a defect in Ca^{2+} entry following light stimulation (70, 87, 128). Expression of TRP in a baculovirus system produced Ca^{2+} currents when intracellular Ca^{2+} stores were depleted with thapsigargin (TG), a result indicative of a role of TRP in mediating SOCE (85, 120). To date, seven mammalian homologues bearing 35-43% identity to the *Drosophila* TRP have been cloned and termed TRP-canonical or TRPC1-7 (9, 11, 14, 78, 79, 138, 139). A common characteristic of all TRPC proteins is that they form Ca^{2+} -permeable nonselective cation channels. Functional studies using various heterologous expression systems have provided evidence supporting the role of TRPCs in SOCE and/or ROCE. First, all TRPCs mediate Ca^{2+} entry in response to phospholipase C (PLC) stimulation by receptor agonists

Store-Operated Ca^{2+} channel in muscle cells

(9). Second, TRPC1, 2, 4 and 5 are activated by TG (64, 88, 89, 121, 126). Third, activities of endogenous SOCs were inhibited by antisense TRPC1 oligonucleotides, by expression of antisense RNA of TRPCs (64, 86, 138), or by treatment with antibodies against TRPC1 or TRPC2 (130). Fourth, expression of the amino-terminus of TRPC3 inhibited the endogenous SOCE in human endothelium in a dominant-negative manner (41). Finally, TRPC4 knockout mice showed reduced vasorelaxation in association with diminished store-operated Ca^{2+} currents in aortic endothelium (35).

The conundrum is that TG does not always activate TRPC-based channels. In most cells, the channels formed by TRPC3, 6, and 7 appear to be largely insensitive to TG but respond very well to the stimulation of PLC (10, 78, 137). However, in chicken DT40 cells, heterologous expression of human TRPC3 seem to lead to TG-stimulated Ba^{2+} influx (122). TRPC4 and TRPC5 have been shown to be store-operated channels by some research groups (86, 126), but not by others (78, 98, 104). The discrepancies may reflect the complexity of SOCs both in subunit composition and in functional regulation. Native SOCs may contain four non-identical TRPC subunits or TRPC plus other unidentified subunits (54). Channels formed due to over-expression of a single TRPC type could have anomalous compositions, leading to abnormal single channel conductance, ion selectivity, pharmacological profiles, and sensitivity to some essential regulatory elements (93). In the case of *Drosophila* TRP-like and TRP γ , a SOC was formed when both were expressed together, but not when each TRP was expressed individually (129, 131). Thus, it remains a challenge to find the “right” mix of TRPC proteins to make a channel with the same properties as the Ca^{2+} release-activated Ca^{2+} (CRAC) channel, the best characterized SOC (46, 81, 140).

Channel-like proteins distantly related to TRPCs have also been cloned. They are now collectively called members of the TRP superfamily and are further classified according to their sequence homology into four subfamilies: TRPV, TRPM, TRPP, and TRPML (20, 71). Some of them have been suggested to participate in the formation of SOCs. For example, TRPV6 (CaT1) was shown to be similar to the CRAC channel when expressed in HEK 293 cells (135). However, a more recent study argues that it has a quite distinct pore property from the CRAC channel (123). Members of the TRPV subfamily form heat-activated channels (capsaicin receptor, TRPV1, and TRPV2) (16,17), stretch-inactivated or osmolarity-regulated channels (OTRPC4 or TRPV4) (103, 108, 127), a growth factor-regulated channel (GRC, the same sequence as TRPV2) (52), and Ca^{2+} transporting epithelial channels (ECaC or CaT: TRPV5 and TRPV6) (45, 83, 84). A number of TRP proteins are implicated in etiology of human diseases, such as autosomal dominant polycystic kidney disease (PC2 or TRPP1) (117), metastatic melanoma (melastatin, or TRPM1) (44), and mucopolidosis type IV (mucolipin or TRPML1) (3, 106).

2.3. Activation of store-operated Ca^{2+} channel – cross talk between cell surface and intracellular membranes

The activation mechanism of SOCs is an unresolved problem. Three major hypotheses have been

proposed. First, a small diffusible factor may be responsible for activating SOCs. A Ca^{2+} influx factor seems to be present in the acid extracts of activated Jurkat cells and platelets, or a Ca^{2+} store-depleted yeast mutant strain (24, 94, 116), but its identity has not been determined. Second, IP_3 Rs may directly bind to the SOCs and activate them through a conformational coupling mechanism (48), in a manner similar to the activation of skeletal muscle RyR by the DHPR in E-C coupling (95, 96, 99). Third, insertion of preformed channel-containing vesicles in the PM in a fashion similar to the secretion of neurotransmitters may be required for the activation of SOCs (133).

During the past few years, increasing evidence has accumulated supporting the conformational coupling hypothesis, owing mainly to studies conducted on TRPC channels. Functional coupling by IP_3 Rs was first shown for TRPC3 expressed in HEK293 cells (55) and then for a native SOC found in A431 epithelium (53). These were followed by the demonstration that IP_3 Rs and TRPC proteins coimmunoprecipitated in several systems (10, 56, 65, 97) and finally the identification of IP_3 R-TRPC binding domains (10, 115, 136). Additional evidence also suggests that RyRs could substitute for the IP_3 Rs to activate TRPC3 and native SOCs (57, 58). Moreover, the binding of IP_3 Rs to TRPCs is inhibited by calmodulin, which prevents the channels from being spontaneously active, and inactivates the channels after they are activated (115, 136). Therefore, Ca^{2+} release channels, IP_3 Rs and RyRs, and calmodulin appear to control the gating of SOCs through direct interaction with TRPC proteins.

3. JUNCTOPHILINS AND MITSUGUMINS – LINKERS OF JUNCTIONAL STRUCTURE BETWEEN CELL SURFACE AND INTRACELLULAR MEMBRANES

Perhaps the best studied example of Ca^{2+} signaling is that defined by VICR in skeletal muscle. Research over the past 30 years or so has defined certain key molecular components involved in E-C coupling (see Figure 1), and has also begun to elucidate the machinery underlying the signal transduction step from activation of DHPR to activation of RyR (77). A central focus in current E-C coupling research is to understand the molecular and structural components that define the close T-tubule/SR interaction, and to study the roles of accessory proteins that may interact with DHPR or RyR to modulate the VICR, CICR, and SOCE.

In addition to DHPRs and RyRs, other proteins of the T-tubule/SR junction also play critical roles in muscle E-C coupling. Transgenic mice that lack expression of either DHPRs or RyRs still form seemingly normal triad junctions (32, 47), indicating that structural components other than the DHPR/RyR interaction are needed for a close apposition of T-tubules and SR membranes. Chinese hamster ovary (CHO) cells transfected with both DHPR and RyR cDNAs showed neither Ca^{2+} release in response to membrane depolarization, nor close association between the PM and ER (105, 107, 109). Thus, the DHPR/RyR

interaction is neither necessary nor sufficient for the formation of triad and dyad junctions. Although several transmembrane proteins with no established physiological roles have been identified as components of triad junctions (42, 50), none appears to be a candidate molecule for mediating the physiological coupling of the junctional complex. Furthermore, the gap size between T-tubule and SR was reduced when the triad lacked RyRs (from ~12 nm in normal muscle to ~7 nm in *ryr1(-/-)ryr3(-/-)* muscle) (47), suggesting an elastic property of the proposed bridge between the two junctional membranes.

In an attempt to identify structural components supportive of triad junction-like membrane arrangements, Takeshima and colleagues have used a combination of monoclonal antibody immunocytochemistry and cDNA library screening techniques, and identified a group of novel membrane proteins termed mitsugumins and junctophilins that in cardiac and skeletal muscle are exclusively localized to the triad and dyad junctions, respectively (75, 76, 113, 114).

3.1. Mitsugumin 29 and transverse tubule structure in skeletal muscle

Mitsugumin29 (MG29) is a synaptophysin-family member protein, with a molecular weight of 29 kDa, localized specifically in the triad junction of skeletal muscle, and to a lesser extent also present in the tubular membranes of the kidney (113). In mature skeletal muscle, MG29 is present predominantly on the T-tubule membrane (12, 59). Similar to other synaptophysin family proteins, MG29 forms an oligomeric structure - a homohexamer (13), that may be essential for the function of MG29 in muscle development and contraction.

To examine the physiological function of MG29, Nishi *et al* have created a mutant mouse with targeted disruption of the *mg29* gene (75). Abnormalities of membrane ultrastructure around the triad junction were detected in skeletal muscle from the *mg29(-/-)* mice: the T-tubules were swollen and sometimes missing from the A-I junction, and the SR networks were poorly formed with vacuolated and fragmented structures, leading to misalignment of triad junctions. In the *mg29(-/-)* muscle, apparently normal tetanus tension was observed, whereas twitch tension was significantly reduced. Interestingly, the mutant muscle showed faster decrease of twitch tension under Ca^{2+} -free conditions compared with the control muscle (75). The morphological and functional abnormalities of the mutant muscle indicate that MG29 is essential for both refinement of the membrane structure and effective E-C coupling in skeletal muscle.

3.2. Junctophilin type 1 (JP1) and triad junction in skeletal muscle

A family of membrane proteins named junctophilins (JP) was identified as the major protein components at junctional membrane complexes in excitable cells (114). Three subtypes of JP have been identified, JP1, JP2 and JP3, which exhibit tissue specific distribution. JP1 is predominantly expressed in skeletal muscle, JP2 is distributed in both skeletal and heart muscles, and JP3 is

abundantly expressed in the brain and testis. The lung and stomach, tissues containing smooth muscle, showed weak hybridization signals with the JP2-specific probe, suggesting expression of JP2 in smooth muscle cells as well (114).

The primary amino acid sequence of JP proteins revealed a unique secondary structure with a large cytoplasmic region and a carboxyl-terminal transmembrane segment spanning the SR/ER. The cytoplasmic region of JP contains repeated motifs of 14 amino acid residues termed “membrane occupation and recognition nexus” or MORN motifs, and exhibit selective binding affinity to the plasma membrane. The MORN motif is a novel protein-folding module shared by functionally different proteins and probably having a specific physiological role.

Electron microscopy revealed junctional complexes between the ER and PM in cells expressing JP1 (114). The average gap size between PM and ER in the junctional structure was ~7.6 nm. When a soluble form of JP1 lacking the carboxy-terminal transmembrane segment was expressed, immunolabeling was detected specifically at the PM, but lacked ER-PM junction. This demonstrates a specific binding affinity of the cytoplasmic domain of JP1 for the PM. The JP1 mediated junctional complexes showed structural characteristics similar to those of the peripheral coupling detected commonly in muscle cell types. The gap between T-tubule and SR in the triad junction from normal muscle is ~12 nm, whereas the mutant muscle lacking RyR has a gap size of ~7 nm. This suggests that RyR likely restricts the gap size. Since the “foot” structure of RyR is absent in heterologous cells expressing JP1, the gap size of 7.6 nm probably correspond to that of the mutant triad junction lacking RyR. Together, these studies suggest that the JP family proteins, by their nature of specific anchorage to the SR/ER membrane and selective interaction with the PM, may play essential roles in the formation of junctional membrane complexes.

To examine the physiological role of JP1 in skeletal muscle, Ito *et al* (49) have generated mutant mice lacking JP1. The JP1 knockout mice showed no milk suckling and died shortly after birth. Ultrastructural analysis demonstrated that triad junctions were reduced in number, and that the SR was often structurally abnormal in the skeletal muscles of the mutant mice. The mutant muscle developed less contractile force evoked by low-frequency electrical stimuli and showed abnormal sensitivities to extracellular Ca^{2+} . These data indicate that JP1 contributes to the construction of triad junctions and is essential for the efficiency of signal conversion during E-C coupling in skeletal muscle.

3.3. Junctophilin type 2 (JP2) and dyad junction in cardiac muscle

JP2 is abundantly expressed in the heart. It appears to be essential for dyad junction formation because disruption of its expression in mice produces embryonic lethality, as a result of defective junctional membrane coupling and unsynchronized intracellular Ca^{2+} transients (114). To survey functional abnormalities in the *jp2(-/-)*

mice, the hearts from E9.5 embryos were subjected to Ca^{2+} -imaging analysis using an intracellular Ca^{2+} indicator. In wild-type hearts, all of the myocytes showed spontaneous and synchronized Ca^{2+} transients, and the transients disappeared in a Ca^{2+} -free bathing solution, because cardiac E-C coupling requires Ca^{2+} influx via DHPR. However, in mutant hearts from the *jp2*(-/-) embryos, a large number of myocytes showed irregular Ca^{2+} transients that were not synchronized with heartbeats and occurred randomly. The random transients were even retained in the Ca^{2+} -free bathing solution, albeit with reduced frequency. Therefore, the abnormal Ca^{2+} transients in the mutant hearts are primarily independent of Ca^{2+} influx through DHPR.

Deficiency of peripheral coupling, observed in the mutant myocytes prior to cardiac arrest, supports the notion that JP proteins contribute to the formation of junctional membrane complexes in various cell types. Generation of the junctional structure may require at least two processes: first the SR and PM must approach each other, and then the JP proteins anchor the two membranes together in a stable complex. A few peripheral couplings retained in the *jp2*(-/-) myocytes might correspond to an unstable form of the junctional membrane complexes that is juncophilin-independent. Alternatively, other JP subtypes might contribute to the formation of the peripheral coupling in the *jp2*(-/-) myocytes, i.e. low levels of JP1 expression in the adult hearts were suggested by Northern blot analysis.

The deficient junctional membrane structure and abnormal intracellular Ca^{2+} transients observed in cardiac myocytes of the JP2 knockout mice resemble some of the functional changes associated with diseased human heart, i.e., widening of the dyadic membrane cleft and reduced efficiency of intracellular Ca^{2+} release measured in congestive heart failure (CHF) (40, 43). Although the pathogenesis of CHF is probably multi-factorial, alterations in E-C coupling are a central finding in all animal models of CHF and in failing human heart. Presumably, mutations or altered expression of mitsugumins might induce human diseases by affecting the Ca^{2+} signaling of excitable cells.

4. RYANODINE RECEPTOR-MEDIATED ACTIVATION OF STORE-OPERATED Ca^{2+} CHANNEL

Conformation coupling between IP_3 receptors and SOC is analogous to E-C coupling between DHPRs and RyRs in skeletal muscle. A retrograde interaction between RyR and DHPR also exists in skeletal muscle, as the absence of RyR hinders the function of DHPR (30, 73). Of the three known RyR isoforms, RyR1, expressed in skeletal muscle, is the only one capable of interacting with DHPR. RyR2, the cardiac isoform, is not capable of coupling to DHPR (74, 112). RyR3, widely expressed in both excitable and nonexcitable cells and whose main function appear to be limited to CICR, is not known to interact directly with a DHPR (21, 31, 102, 125, 132).

Growing evidence suggests that Ca^{2+} stores of many nonmuscle cells, including neurons (2), neuroendocrine (4), lymphocytes (39, 100) and epithelial

cells (62) express RyRs. Thus, pharmacological agents such as caffeine and the second messenger cADPR (61) can release Ca^{2+} from internal stores of these cells. Furthermore, Ca^{2+} release by activation of RyRs in nonmuscle cells activates SOCE in a manner similar to Ca^{2+} release from the IP_3 -sensitive stores (4, 119). The functional coupling between RyR and SOC, if it exists, is likely to be influenced by the structural interaction between PM and SR. In the case of muscle cells, those proteins participate in the formation of dyad and triad junction, e.g. mitsugumins and juncophilins may modulate the interaction between RyR and SOC and the overall Ca^{2+} signaling process.

4.1. Interaction between TRP and ryanodine receptor

Using co-expression and reconstitution studies, Kiselyov *et al.* (57) provided direct evidence supporting a conformational coupling between RyR and SOC in non-excitable cells. Ca^{2+} release from RyR-sensitive stores was shown to activate TRPC3 channels in electrophysiological measurements. And the RyR-TRPC3 complex can be demonstrated in co-immunoprecipitation studies. Furthermore, the coupling appears to be specific for RyR1, since RyR2 did not appear to interact with TRPC3 and to gate the channel. The data show that regulation of SOC or ROC by RyRs is not unique to skeletal muscle. Thus, the gating of PM Ca^{2+} channels by intracellular Ca^{2+} release channels may be viewed as a general and widespread paradigm in Ca^{2+} signaling.

4.2. Dysfunction of SOC in muscle cells lacking RyR1 and RyR3

The first direct demonstration of SOCE in skeletal muscle was provided by Kurebayashi and Ogawa (60). They used muscle fibers isolated from the extensor digitorum longus (EDL) of adult mice, and depleted the SR Ca^{2+} stores by repetitive treatments with high- K^+ solutions in combination with inhibitors of the SERCA Ca^{2+} pump. The SOCE in skeletal muscle was sensitive to blockade by Ni^{2+} , resistant to nifedipine, and suppressed by plasma membrane depolarization. This SOCE pathway is sufficient to refill the depleted SR Ca^{2+} store within several minutes in skeletal muscle fibers. The Mn^{2+} influx through SOC measured by quenching of Fura-2 fluorescence was observed only when the SR was severely depleted of Ca^{2+} . The voltage-dependence of SOCE in skeletal muscle exhibited inward rectification, which was similar to the CRAC current described in other preparations (46).

To test the putative regulation of SOC by RyR in muscle cells, we used primary cultured myotubes derived from neonates of the *ryr1*(-/-)*ryr3*(-/-) and *ryr1*(+/-)*ryr3*(-/-) mice (110, 111). It is known that deletion of either RyR1 or RyR3 does not disrupt the triad junction structure (47). Therefore, the *ryr1*(-/-)*ryr3*(-/-) and *ryr1*(+/-)*ryr3*(-/-) mice offer unique models to examine the potential regulation of SOC by RyR1, the main isoform of RyR present in skeletal muscle. The *ryr1*(+/-)*ryr3*(-/-) myotubes responded to caffeine stimulation with rapid release of Ca^{2+} from SR (Figure 2). SOCE was observed upon addition of 2 mM $(\text{Ca}^{2+})_o$. Simultaneous application of caffeine and ryanodine, ligands that induce permanent opening of the RyR/ Ca^{2+} release channel, resulted in SR Ca^{2+} release similar to

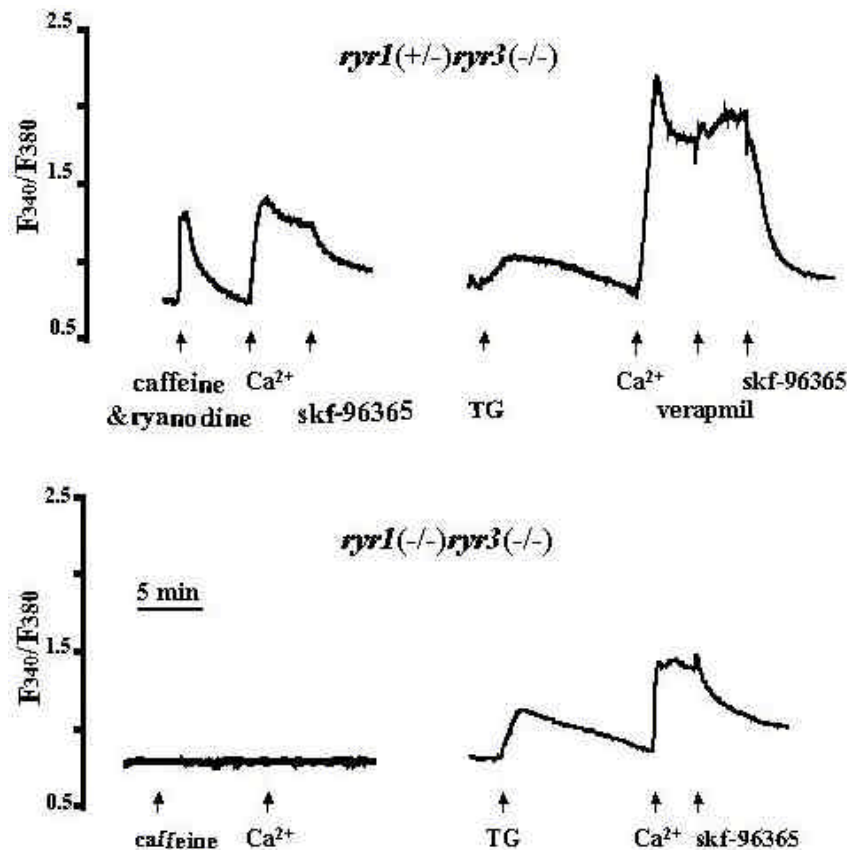


Figure 2. Ryanodine receptor-mediated activation of store-operated Ca^{2+} channel in skeletal muscle. In *ryr1(+/-)ryr3(-/-)* myotubes, inhibition of the SERCA Ca^{2+} pump by 10 μM thapsigargin (TG), or activation of RyR/ Ca^{2+} release channel by caffeine and ryanodine resulted in depletion of SR Ca^{2+} , and triggered store-operated Ca^{2+} entry (SOCE) after addition of 2 mM $(\text{Ca}^{2+})_o$. A large component of SOCE was sensitive to blockade by 20 μM SKF-96365 but not by 50 μM verapamil. In *ryr1(-/-)ryr3(-/-)* cells, caffeine could not induce Ca^{2+} release from SR due to the lack of RyR1 and RyR3. The amplitude of SOCE in *ryr1(-/-)ryr3(-/-)* myotubes was significantly smaller than that in *ryr1(+/-)ryr3(-/-)* myotubes, when the SR Ca^{2+} store was completely depleted with TG.

caffeine alone, but the extent of SOCE was significantly larger. TG, a potent inhibitor of the SR Ca^{2+} pump, produced sustained depletion of Ca^{2+} from SR and induced even greater activation of SOCE in *ryr1(+/-)ryr3(-/-)* cells. Near complete inhibition of SOCE in *ryr1(+/-)ryr3(-/-)* cells was observed upon addition of 20 μM SKF 96365, a known blocker of SOC (63, 69). The *ryr1(-/-)ryr3(-/-)* myotube, lacking both *ryr1* and *ryr3*, failed to respond to caffeine or ryanodine, but contained TG-sensitive SR Ca^{2+} store similar to that present in *ryr1(+/-)ryr3(-/-)* cells. SOC activity in *ryr1(-/-)ryr3(-/-)* cells, however, was significantly smaller than that in *ryr1(+/-)ryr3(-/-)* cells. After depletion of SR Ca^{2+} with thapsigargin, *ryr1(-/-)ryr3(-/-)* cells exhibited a residual component of SOCE (~40% of that present in *ryr1(+/-)ryr3(-/-)* cells), which was partially sensitive to inhibition by SK&F 96365 (Figure 2).

Our data provide direct evidence for a RyR-coupled activation of SOC in skeletal muscle. Based on the different degree of SOCE triggered by caffeine, caffeine and ryanodine, or TG in *ryr1(+/-)ryr3(-/-)* cells, we conclude that SOCs in skeletal muscle can function in a

graded manner depending on the SR Ca^{2+} content or the conformation of RyR (80).

5. STORE-OPERATED Ca^{2+} ENTRY AND MUSCLE FATIGUE

5.1. Involvement of extracellular Ca^{2+} entry in muscle fatigue

Fatigue is an important functional property of skeletal muscle, and is defined as a reversible decrease in the isometric contractile force in response to an increase in the frequency or duration of stimulation (1). Optimal muscle performance revolves around the maintenance of intracellular Ca^{2+} homeostasis, such that inadequate Ca^{2+} release from the SR would lead to reduced force output observed in muscle fatigue. The decline in intracellular Ca^{2+} release could result from improper coupling between DHPR and RyR, reduction of the SR Ca^{2+} content, direct modification of RyR function, or dysfunction of SOCE.

Experiments with intact muscle fiber show that the size of the Ca^{2+} store declines during fatigue and

Store-Operated Ca^{2+} channel in muscle cells

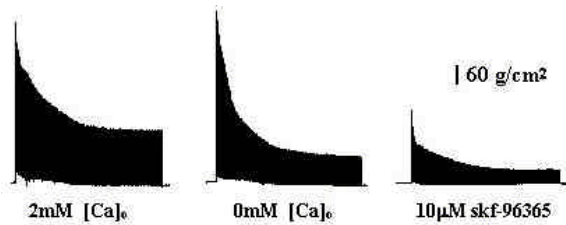


Figure 3. Involvement of extracellular Ca^{2+} entry in muscle fatigue. Differential fatigability of soleus muscles were observed with tetanic stimulation at a frequency that produced maximum force output (80 Hz), either in the presence of 2 mM $(\text{Ca}^{2+})_o$ (left panel), 0 Ca^{2+} plus 0.5 mM EGTA (middle panel), or 2 mM $(\text{Ca}^{2+})_o$ plus 10 μM SKF-96365 (right panel). The degree of fatigue was significantly higher with the removal of Ca^{2+} from the extracellular solution, or after inhibition of the SOCE by SKF-96365. See refs 72 and 80 for more details.

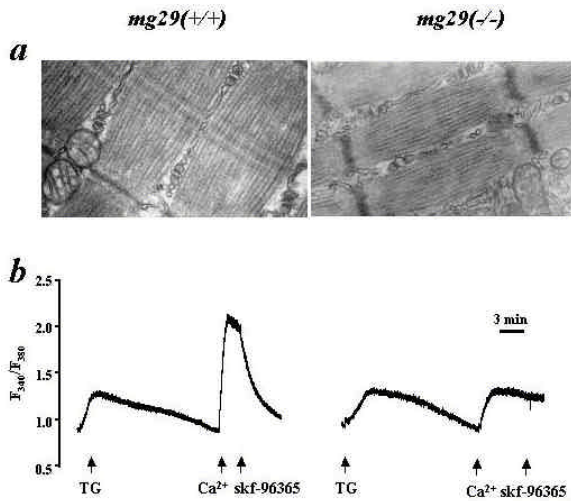


Figure 4. Dysfunction of store-operated Ca^{2+} channel in muscle cells lacking MG29. *a*. Abnormal membrane structure in skeletal muscle from *mg29(-/-)* mice. Electron micrographs revealed swollen T-tubules and vacuolated SR networks in *mg29(-/-)* muscle (right panel) compared with the wild type control or *mg29(+/-)* muscle (left panel). *b*. SOCE in individual skeletal muscle myotubes was significantly lower in *mg29(-/-)* cells, compared with that in *mg29(+/-)* cells. A large component of SOCE in *mg29(+/-)* cells was sensitive to blockade by SKF-96365 (20 μM), but not by verapamil

recovers upon rest (1). With skinned muscle fiber preparation, it was shown that increased intracellular phosphate can lead to reduction of Ca^{2+} released from the SR (36, 37). Skeletal muscles which lack the enzyme creatine kinase, do not exhibit the usual rise of phosphate during fatigue and, under these conditions, the decline of Ca^{2+} release is absent or delayed (25, 26). It has been suggested that the rise in myoplasmic phosphate can lead to Ca^{2+} -phosphate precipitation within the SR, and the resultant reduction in free Ca^{2+} within the SR would contribute to the reduced Ca^{2+} release during fatigue (1, 51).

It is well known that $(\text{Ca}^{2+})_o$ is essential for priming of the DHPR/voltage sensor in the active state (15), but the demonstration of a functional role for extracellular Ca^{2+} influx in skeletal muscle E-C coupling has eluded detection despite being well studied. Although the twitch force initiated by a single electrical stimulation does not depend on the movement of extracellular Ca^{2+} , the fatigue pattern of isolated muscle fibers shows a clear dependence on extracellular Ca^{2+} (Figure 3). Changing the bath solution from 2 mM $(\text{Ca}^{2+})_o$ to 0 $(\text{Ca}^{2+})_o$ significantly increased the fatigability of the muscle, as indicated by the reduction in the sustained force output at the end of the tetanic stimulation. The reduction of SR Ca^{2+} stores associated with muscle fatigue can trigger the activation of SOCE, which could play a role in the overall Ca^{2+} handling properties of skeletal muscle. By blocking the SOCE pathway with SKF-96365, one observes a significant change in the fatigue behavior of skeletal muscle (Figure 3).

5.2. Dysfunction of SOC and muscle fatigue in *mg29(-/-)* muscle

Unlike the normal skeletal muscle whose E-C coupling machinery does not rely on the entry of extracellular Ca^{2+} , skeletal muscle from the mutant *mg29(-/-)* mice exhibits clear dependence on extracellular Ca^{2+} for contraction (75, 80). As MG29 is involved in the coupling between T-tubule and SR, presumably the expression of MG29 will have functional impact on the RyR-mediated activation of SOC in muscle cells.

A distinct phenotype of the *mg29(-/-)* mice is their failure to complete an endurance test, i.e., their skeletal muscles exhibit increased susceptibility to fatigue. Nagaraj *et al* (72) showed that muscle fibers isolated from the *mg29(-/-)* not only fatigued to a greater extent, but also recovered significantly less than the wild type muscles. Following fatigue, the mutant EDL and soleus muscles produced lower twitch forces than the wild-type muscles. Additionally, fatiguing produced a rightward shift in the force-frequency relationship in the mutant mice compared with the wild type controls.

Quantitative measurements of intracellular $(\text{Ca}^{2+})_i$ with Fura-2 fluorescent indicator in individual myotubes derived from different mutant mice enabled us to identify a defective regulation of SOC in *mg29(-/-)* cells, and begin to define the mechanism of SOC operation in skeletal muscle (80). As shown in Figure 4, treatment of cells with TG in 0 $(\text{Ca}^{2+})_o$ solution induced measurable SOCE in both control and *mg29(-/-)* cells after addition of 2 mM $(\text{Ca}^{2+})_o$. Note that the extent of SOCE in *mg29(-/-)* cells is less than 40% of that seen in control cells. SKF-96365, a known blocker of SOC, blocked this SOCE in control cells by >90%. In contrast, the SKF-sensitive component of CCE is severely compromised in *mg29(-/-)* cells. Further studies show that the *mg29(+/-)* and *mg29(-/-)* muscles showed comparable responses to fatigue stimulation at 0 $(\text{Ca}^{2+})_o$ and after inhibition of SOC with SKF-96365. This suggests that the increased susceptibility to fatigue stimulation is likely a consequence of dysfunction of SOC in *mg29(-/-)* muscle cells.

6. PERSPECTIVE – EMERGING QUESTIONS OF SOC FUNCTION IN Ca^{2+} HOMEOSTASIS OF MUSCLE CELLS

In recent years, much research has focused on the mechanism linking depletion of intracellular Ca^{2+} stores to the activation of plasma membrane Ca^{2+} channels. Most of the studies were performed with non-excitable cells. There is considerable evidence for a conformational coupling mechanism by which depletion of intracellular Ca^{2+} stores induces a conformational change in IP_3 receptor or RyR, which interacts with and activates a plasma membrane Ca^{2+} channel. A close association between T-tubules of PM and terminal cisternae of SR not only determines the spatial coupling between DHPR and Ca^{2+} release channel, but also fine tunes the efficacy of Ca^{2+} signaling in muscle cells. It remains to be studied how the JP proteins affect the retrograde Ca^{2+} signaling in muscle and other excitable cells.

At present, the TRPs remain the most popular candidate protein for SOC. There is evidence that channels formed by TRP proteins can be gated by Ca^{2+} store depletion, although this is not consistent in all studies. However, it seems clear that none of the TRPs so far studied shows the properties required for I_{CRAC} . The key missing property is Ca^{2+} selectivity, which has not been observed for any TRPC family member. It is highly possible that the molecular entity determining SOC may consist of a macromolecular complex that spans the cell surface and intracellular membranes, i.e. a multimeric structure.

In summary, significant progresses have been made in elucidating the physiological roles of SOCE and ROCE. In particular, in the process of cell proliferation, apoptosis and smooth muscle contraction, the role of SOCE has been well documented. In striated muscle cells, we now know that SOC participates in muscle fatigue. We do not yet know how alteration of SOC could affect long term muscle development and gene adaptation. Identification of the molecular components of SOC remains the key task for future studies.

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