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SYMPOSIUM REVIEW

Checking your SOCCs and feet: the molecular mechanisms of Ca^{2+} entry in skeletal muscle

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It has long been known that skeletal muscle contraction persists in the absence of extracellular Ca^{2+}. Nevertheless, recent evidence indicates that multiple distinct Ca^{2+} entry pathways exist in skeletal muscle: one active at negative potentials that requires store depletion (store-operated calcium entry or SOCE) and a second that is independent of store depletion and is activated by depolarization (excitation-coupled calcium entry or ECCE). This review highlights recent findings regarding the molecular identity, subcellular localization, and inter-relationship between SOCE and ECCE in skeletal muscle. The respective roles of ryanodine receptors (RyRs), dihydropyridine receptors (DHPRs), inositol-1,4,5-trisphosphate receptors (IP_3Rs), canonical transient receptor potential channels (TRPCs), STIM1 Ca^{2+} sensor proteins, and Orai1 Ca^{2+} permeable channels in mediating SOCE and ECCE in skeletal muscle are discussed. Differences between SOCE and ECCE in skeletal muscle with Ca^{2+} entry mechanisms in non-excitable cells are also reviewed. Finally, potential physiological roles for SOCE and ECCE in skeletal muscle development and function, as well as other currently unanswered questions and controversies in the field are also considered.

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Skeletal muscle contraction and relaxation are controlled by the precise temporal delivery and removal of myoplasmic Ca^{2+}. During excitation–contraction (EC) coupling in skeletal muscle, an action potential initiated at the neuromuscular junction rapidly propagates down the surface and transverse tubule (t-tubule) membranes and induces voltage-driven conformational changes in the t-tubule dihydropyridine receptor (DHPR) or voltage sensor that triggers the opening of ryanodine receptor (RYR1) channels to release Ca^{2+} stored in the terminal cisternae of the sarcoplasmic reticulum (SR) (Melzer *et al.* 1995). Muscle relaxation results from termination of release following membrane repolarization and subsequent resequestration of myoplasmic Ca^{2+} back into the SR via sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps. As a result, skeletal muscle contraction is orchestrated by highly coordinated mechanisms that control SR Ca^{2+} release and reuptake (Melzer *et al.* 1995).

Ever since the original work of Armstrong *et al.* (1972) over three decades ago, it has become widely appreciated that twitch contractions of skeletal muscle persist in the complete absence of extracellular calcium. Subsequent work revealed that activation of SR Ca^{2+} release during skeletal muscle EC coupling arises from a direct mechanical 'outside-in' conformational coupling mechanism resulting from voltage-driven changes in DHPR voltage sensors that are transmitted to RyR1 Ca^{2+} release channels in the SR (Melzer *et al.* 1995). Importantly, unlike cardiac muscle, the DHPR–RyR1 conformational coupling mechanism in skeletal muscle is completely independent of the entry of extracellular Ca^{2+}. Specifically, depolarization-induced SR Ca^{2+} release in muscle persists following removal of external Ca^{2+} (Armstrong *et al.* 1972) or blockade of Ca^{2+} influx with either diltiazem (Gonzalez-Serratos *et al.* 1982) or Cd^{2+} and La^{3+} (Nakai *et al.* 1996), and is unaffected by a mutation in the DHPR pore that abolishes Ca^{2+} permeation though the channel (Dirksen & Beam, 1999).

While clearly serving the essential function as the voltage sensor for mechanical activation of

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RyR1-mediated Ca^{2+} release during EC coupling, the skeletal muscle DHPR is also a slowly activating voltage-gated L-type Ca^{2+} channel (L-channel). Indeed, mechanical coupling between the DHPR and RyR1 in skeletal muscle represents a bi-directional interaction as both the DHPR controls RyR1-mediated SR Ca^{2+} release (orthograde coupling) and RyR1 channels in turn influence the Ca^{2+} conducting properties of the DHPR (retrograde coupling) (Nakai *et al.* 1996). However, as noted above, a physiological role for Ca^{2+} entry through the DHPR channel pore has largely been dismissed as Ca^{2+} entry is not required for skeletal muscle twitch contraction and the kinetics of L-channel activation (~50–100 ms) is too slow to permit significant Ca^{2+} influx during a brief (2–5 ms) skeletal muscle action potential.

In spite of the well-accepted mechanism of bi-directional mechanical DHPR–RyR1 communication during skeletal muscle EC coupling, the recent identification of two apparently distinct trans-sarcolemmal Ca^{2+} entry mechanisms in skeletal muscle has served to reinvigorate the field by potentially challenging previously accepted paradigms. Kurebayashi & Ogawa (2001) identified a store-operated Ca^{2+} entry (SOCE) mechanism in adult rat EDL muscle fibres capable of replenishing previously depleted SR Ca^{2+} stores ~2 min following the reintroduction of extracellular Ca^{2+}. Three years later, Cherednichenko *et al.* (2004) identified a store-independent Ca^{2+} entry pathway that is activated be repetitive or prolonged depolarization (excitation-coupled Ca^{2+} entry or ECCE). The discovery of these two distinct Ca^{2+} entry pathways stimulated subsequent work designed to elucidate their respective molecular components and activation mechanisms. In addition, the identification of the SOCE and ECCE pathways in skeletal muscle also served to renew efforts to determine the potential role(s) of trans-sarcolemmal Ca^{2+} flux in skeletal muscle in ensuring store repletion (Kurebayashi & Ogawa, 2001), limiting fatigue resistance (Pan *et al.* 2002), stimulating NFAT transactivation (Rosenberg *et al.* 2004; Stiber *et al.* 2008), promoting muscle differentiation (Darbellay *et al.* 2009), and modifying muscle disease (Vandebrouck *et al.* 2002; Wang *et al.* 2005; Cherednichenko *et al.* 2008). This review provides a critical evaluation of recent work directed at elucidating the molecular identity, subcellular localization, and inter-relationship between SOCE and ECCE in skeletal muscle. In addition, several unanswered questions, gaps in knowledge, and areas of ongoing controversy are also discussed in the hope of stimulating further debate and future potential lines of investigation.

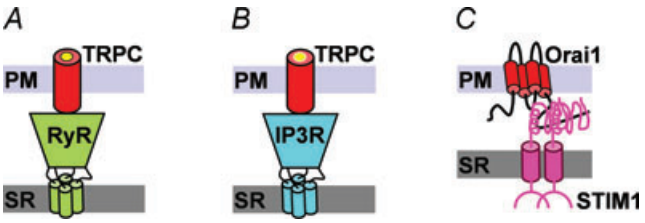

Figure 1. Proposed molecular models for SOCE in skeletal muscle
A, conformational coupling between the ryanodine receptor (RyR) and canonical transient receptor potential (TRPC) channels. *B*, conformational coupling between inositol-1,4,5-trisphosphate (IP_3) receptors and TRPC channels. *C*, conformational coupling between ER/SR stromal interaction molecule 1 (STIM1) Ca^{2+} sensor proteins and Ca^{2+} permeable Orai1 channels. For clarity, only one Orai1 subunit is shown.

Proposed molecular models for SOCE in skeletal muscle

The identification of SOCE in adult mammalian muscle by Kurebayashi & Ogawa (2001) stimulated a series of studies designed to identify the physiological role and molecular components of SOCE in skeletal muscle. Prior results in non-muscle cells demonstrated that activation of store-operated Ca^{2+} channels (SOCCs) occurs following Ca^{2+} release from both IP_3-sensitive (Kiselyov *et al.* 1998) and RyR-sensitive (Bennett *et al.* 1998) stores. Extracellular Ca^{2+} entry through SOCCs in HEK293 cells activated following caffeine-induced Ca^{2+} release is significantly increased following transient expression of RyR1 (Tong *et al.* 1999). Subsequently, Kiselyov *et al.* (2000) concluded that store-dependent human conical transient receptor potential type 3 (hTRPC3) channels are activated by conformational coupling to RyR channels. Together, these results suggested that SOCE in skeletal muscle might also be mediated by conformational coupling between RyR1 and TRPC channels in the t-tubule membrane (Fig. 1*A*). Indeed, the magnitude of SOCE is reduced in skeletal myotubes derived from RyR1/RyR3 deficient mice (Pan *et al.* 2002). Subsequent studies identified a potential role for conformational coupling between RyR1 and TRPC channels in skeletal muscle (Vandebrouck *et al.* 2002; Rosenberg *et al.* 2004) and that azumolene, an analog of dantrolene that binds RyR1, inhibits a component of SOCE that is coupled to RyR1 activation (Zhao *et al.* 2006). However, as a SOCE in myotubes persists even in the absence of RyRs (Pan *et al.* 2002; Lyfenko & Dirksen, 2008), the ryanodine receptor is clearly not a required or essential component of the SOCE pathway in muscle.

In an elegant study conducted in toad skeletal muscle fibres, Launikonis *et al.* (2003) provided evidence for conformational coupling between IP_3 receptors and store-operated Ca^{2+} entry channels located in the T-tubule membrane (Fig. 1*B*). In this study, SOCE was quantified following store depletion by assessing a reduction in the fluorescence of a low affinity Ca^{2+} dye (Fluo-5N) which was trapped within the t-tubule system following mechanical skinning. Importantly, activation of SOCCs in this assay was markedly reduced following

inhibition of IP_3 receptors with heparin, a potent IP_3 receptor competitive antagonist, which could also be reversed by IP_3 (Launikonis *et al.* 2003). A similar conformational coupling between IP_3 receptors and SOCCs (presumably TRPC3 channels) was subsequently suggested to be activated in skeletal myotubes during testosterone-induced Ca^{2+} oscillations (Estrada *et al.* 2005). However, the role of IP_3 receptors in activation of SOCE remains uncertain given the relatively low level of IP_3R expression and preferential localization to the nuclear envelope rather than the SR terminal cisternae. Similarly, TRPC3 channels are unlikely to function as the SOCC in muscle since SOCE is unaffected by either TRPC3 knockdown (Lee *et al.* 2006) or transient expression of dominant negative TRPC3 or TRPC6 constructs (data not shown).

The demonstration over the past several years that stromal interaction molecule 1 (STIM1) is an ER Ca^{2+} sensor protein (Roos *et al.* 2005) that orchestrates store-dependent activation of Ca^{2+} selective Orai1 channels (Feske *et al.* 2006; Vig *et al.* 2006) in the plasma membrane has single-handedly transformed the field by enabling detailed molecular and mechanistic investigations of SOCE for the first time. These studies revealed that store depletion triggers STIM1 oligimerization, translocation to ER contact sites with the plasma membrane, and the subsequent interaction, clustering and activation of transmembrane Ca^{2+} influx through Orai1-containing channels (see below for details). Several observations provide strong evidence that a similar STIM1/Orai1 activation mechanism also underlies SOCE in skeletal muscle. First of all, both STIM1 and Orai1 are expressed at high levels in skeletal muscle and deficiency of either protein in mice results in the loss of SOCE and the development of myopathy (Stiber *et al.* 2008; Vig *et al.* 2008). Second, severe combined immunodeficiency (SCID) patients possessing a loss-of-function Orai1 mutation (R91W) also present with a significant skeletal muscle myopathy (Feske *et al.* 2006). Finally, we demonstrated using both Ca^{2+} entry and Mn^{2+} quench assays that SOCE in skeletal muscle myotubes is abolished following either STIM1 knockdown or expression of dominant negative Orai1 (E106Q), and markedly reduced following expression of a Ca^{2+} permeation defective Orai1 mutant (E190Q) (Lyfenko & Dirksen, 2008). Together, these studies provide compelling evidence that a similar STIM1/Orai1 mechanism that underlies SOCE in T-lymphocytes is also operative in skeletal muscle (Fig. 1*C*).

CRAC activation via STIM1–Orai1 conformational coupling in T-lymphocytes

Following its initial landmark description by Putney (1986), remarkably limited progress was made in defining the molecular mechanism for activation of SOCE (or 'capacitative Ca^{2+} entry', as originally termed) over the next 20 years. However, the recent identification of STIM1 as the ER Ca^{2+} sensor and Orai1 as the Ca^{2+} release activated Ca^{2+} (CRAC) channel in T-lymphocytes provided the molecular tools required to begin to unravel this long-standing mystery.

There are two human STIM homologues (STIM1 and STIM2), both of which contain a single transmembrane (TM) domain with an N-terminal luminal domain and a C-terminal cytosolic domain (Fig. 2*A*). While STIM1 is predominantly localized within the ER, it has also been reported to be expressed at lower levels in the plasma membrane (Zhang *et al.* 2005). The N-terminal region of STIM1/2 contains single Ca^{2+} binding EF hand (k_d $\sim$400 μM; Stathopulos *et al.* 2006) and sterile-α-motif (SAM) domains. With Ca^{2+} bound, the EF hand and SAM domains are folded together in a conformation that inhibits STIM aggregation (Stathopulos *et al.* 2006). Ca^{2+} dissociation from the EF hand domain results in a conformational change in the protein that unfolds the EF hand and SAM domains, thus permitting STIM aggregation (Stathopulos *et al.* 2006, 2008). The larger C-terminal region of STIM1 contains two adjacent coiled-coil (c-c) domains within an ezrin–radixin moesin (ERM) domain, as well as serine-proline (S/P)- and lysine-rich (KKK) domains. A similar structure is also observed for STIM2 except the S/P domain is replaced with a histidine-proline-glutamate-rich (P/H/E) domain. There are three human Orai homologues (Orai1–3), each exhibiting four conserved transmembrane domains, as well as cytosolic N- and C-termini (Fig. 2*B*). Orai1 also contains an N-terminal proline-arginine-rich (PR) domain, a glyosylation site between TM2 and TM3, and a C-terminal coiled-coil protein interaction domain that is required for interaction with STIM1, formation of ORAI1–STIM1 puncta and channel activation (Muik *et al.* 2009). While debate persists with regard to whether the CRAC channel at rest exists as a dimer (Penna *et al.* 2008) or tetramer (Ji *et al.* 2008), the activated CRAC channel is a functional tetramer of four Orai1 subunits (Mignen *et al.* 2008) in which conserved glutamate residues in TM1 (E106) and TM3 (E190) are required for Ca^{2+} permeation through the channel (Prakriya *et al.* 2006).

The discoveries of STIM1 as the ER Ca^{2+} sensor and Orai1 as the Ca^{2+} permeable CRAC channel has stimulated a rapidly evolving and remarkably detailed elucidation of the molecular mechanism for CRAC channel activation in non-excitable cells (Fig. 3*A*). Under resting conditions, when intracellular stores are replete with Ca^{2+}, STIM1 proteins are diffusely distributed throughout the ER as STIM1 monomers are stabilized by Ca^{2+} binding to the EF hand motif. Store depletion results in Ca^{2+} unloading from the EF hand and subsequent oligomerization of STIM1 (Stathopulos *et al.* 2006; Liou

et al. 2007; Muik *et al.* 2009). STIM1 oligomers then aggregate and redistribute into discrete puncta located in ER junctions 10–25 nm from the plasma membrane (Wu *et al.* 2006). Association with the plasma membrane within these puncta appears to involve an interaction between membrane phospholipids and the C-terminal polybasic domains of oligomerized STIM1 (Liou *et al.* 2007; Park *et al.* 2009). Orai1 proteins are then recruited into STIM1-containing puncta (Luik *et al.* 2006; Wu *et al.* 2006), where CRAC channel activation results from an interaction between a highly conserved C-terminal domain of STIM1 (termed the CRAC activation domain, CAD (Park *et al.* 2009) or STIM1 Orai activating region, SOAR (Yuan *et al.* 2009)) and the C-terminal coiled-coil domain of Orai1 (Muik *et al.* 2009). An incompletely resolved issue is the number of STIM1 proteins required for CRAC channel activation, though indirect evidence suggests that two to four STIM1 proteins are required for CRAC channel activation (Ji *et al.* 2008; Luik *et al.* 2008). The proposed role for CRAC activation resulting from a Ca^{2+} influx factor (CIF) released during store depletion (Bolotina, 2008) is inconsistent with the observations that both rapamycin-induced heterodimerization of STIM1 oligomers (Luik *et al.* 2008) and intracellular application CAD/SOAR peptides are sufficient alone to activate CRAC channels (Park *et al.* 2009) even in the absence of store depletion. Together, these results provide strong support for an 'inside-out' STIM1–Orai1 conformational coupling mechanism for CRAC channel activation.

SOCE activation via STIM1–Orai1 conformational coupling in skeletal muscle?

The elucidation of STIM1–Orai1 conformational coupling as the mechanism for CRAC channel activation in T-lymphocytes and demonstration that STIM1 and Orai1 are required for SOCE in skeletal muscle (Stiber *et al.* 2008; Lyfenko & Dirksen, 2008) suggest that SOCE in muscle also involves conformational STIM1–Orai1 coupling. In support of this idea, both STIM1 (Stiber *et al.* 2008) and Orai1 (Vig *et al.* 2008) are expressed at high levels in skeletal muscle and STIM1 is pre-localized to SR junctions with the surface membrane in myotubes and in the SR terminal cisternae in adult skeletal muscle (Stiber *et al.* 2008). Thus, in contrast to T-lymphocytes and other non-excitable cells, STIM1 in skeletal muscle is pre-segregated to junctional sites of potential SOCE under resting conditions (i.e. with a full SR Ca^{2+} store). Important questions to be addressed in future work will be to determine the mechanism(s) responsible for the pre-clustering of STIM1 to SR-sarcolemmal junctions in resting muscle and whether junctional STIM1 proteins exist as monomers or oligomers, and/or are already located in a preformed complex with Orai1.

STIM1 oligomerization and redistribution into puncta at ER junctions with the plasma membrane in T-lymphocytes results in a significant ~1 min time delay between store depletion and CRAC channel activation. However, the presence of extensive pre-formed SR

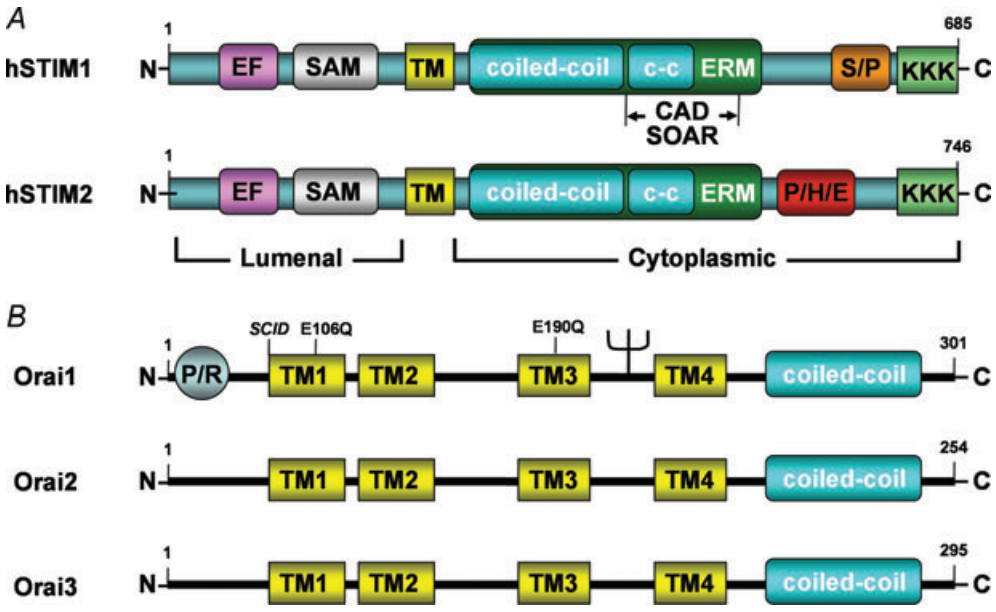

Figure 2. Structural features of human STIM and Orai proteins
A, structural features of human STIM proteins. EF, Ca^{2+} binding EF hand domain; SAM, sterile-α-motif; TM, transmembrane domain; c-c, coiled coil domain; ERM, ezrin–radixin moesin domain; S/P serine-proline-rich domain; KKK, lysine-rich domain; CAD, channel activation domain; SOAR, STIM1–Orai activation region; P/H/E, proline-histidine-glutimate-rich domain. *B*, structural features of human Orai proteins. P/R, proline-arginine-rich domain; TM, transmembrane domain; c-c, coiled coil domain; SCID, severe combined immunodeficiency.

contacts with the t-tubule system (i.e. triads) containing pre-localized STIM1 and Orai1 proteins could provide an ideal system for rapid and local activation of SOCE in skeletal muscle. Indeed, Launikonis & Rios (2007) found that SOCE across the t-tubule membrane is graded in nature and activated <1 s following store depletion. Two hypothetical mechanisms to account for such rapid and local activation of SOCE in skeletal muscle are illustrated in Fig. 3*B* and *C*. In resting muscle with a full complement SR Ca^{2+} store (Fig. 3*B*, upper), STIM1 monomers are shown to be pre-localized within the triad junction, directly across from inactive Orai1 channels. Given the high local concentration of STIM1 and Orai1 in the junction, STIM1 oligomer formation and subsequent SOCE channel activation would occur relatively rapidly following Ca^{2+} dissociation from the EF hand of STIM1 during depletion of Ca^{2+} within the terminal cisternae (Fig. 3*B*, lower). Alternatively, preferential localization of STIM1 proteins to the SR terminal cisternae in resting muscle might reflect pre-association of STIM1 to the C-terminal region of inactive Orai1 channels in the adjacent t-tubule membrane (Fig. 3*C*, upper). This possibility is supported by the recent demonstration that the CAD domain of STIM1 can directly bind to Orai1 (Park *et al.* 2009) and the fact that the likelihood of Orai1 binding to STIM1 will be greatly increased when the local concentration of the two interacting domains is increased. For the model presented in Fig. 3*C*, extremely rapid activation of SOCE would result from a direct conformational change in the preformed STIM1–Orai1 interaction following Ca^{2+} unloading from the STIM1 EF hand during local store depletion (Fig. 3*C*, lower). Though highly speculative, the provocative mechanism proposed in Fig. 3*C* would enable an exquisitely fast means for activating SOCCs in muscle as it would bypass time delays required for STIM1 oligomerization, redistribution and Orai1 recruitment/binding observed in non-excitable cells. Such an efficient and high fidelity 'inside-out' STIM1–Orai1 conformational coupling mechanism for SOCE activation in muscle might be required to keep pace with the rapid Ca^{2+} release dynamics characteristic of 'outside-in' DHPR-RyR1 conformational coupling that dictates Ca^{2+} release during muscle excitation.

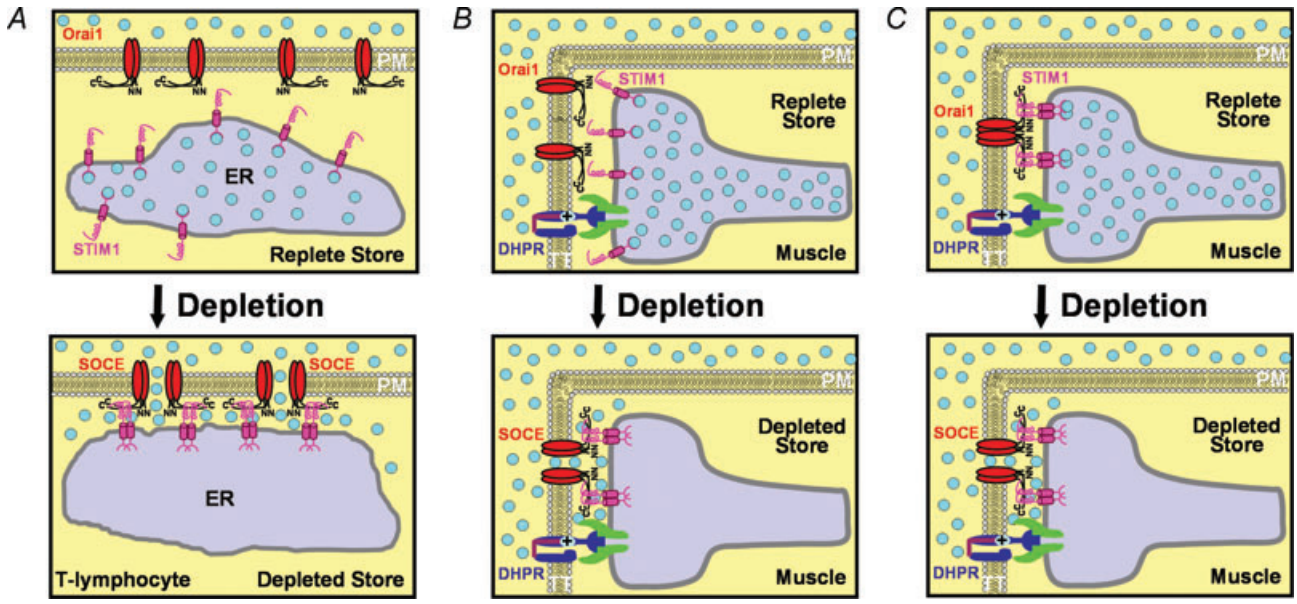

Figure 3. Proposed models for activation of SOCE in T-lymphocytes and skeletal muscle
A, model for activation of SOCE in T-lymphocytes. At rest (upper), Ca^{2+}-bound STIM1 proteins are diffusely distributed in the ER and Orai1 proteins are randomly located in the plasma membrane (shown as non-functional dimer; Penna *et al.* 2008). Following store depletion (lower), Ca^{2+} unbinding from the STIM1 EF hand results in STIM1 oligomerization and redistribution into discrete puncta under the plasma membrane. Orai1 channels are recruited into these puncta and conformational coupling between STIM1 oligomers and tetrameric Orai1 channels results in activation of SOCE. *B*, proposed model for rapid activation of SOCE in skeletal muscle. At rest (upper), Ca^{2+}-bound STIM1 proteins are pre-localized to the SR terminal cisternae and Orai1 proteins are located in the t-tubule membrane. Following store depletion (lower), Ca^{2+} unbinding from the STIM1 EF hand results in STIM1 oligomerization, recruitment of nearby Orai1 subunit, and conformational activation of SOCE. *C*, alternate proposed model for ultra-rapid activation of SOCE in skeletal muscle. At rest (upper), Ca^{2+}-bound STIM1 proteins in the SR terminal cisternae are pre-bound to inactive tetrameric Orai1 channels located in the t-tubule membrane. Following store depletion (lower), Ca^{2+} unbinding from the STIM1 EF hand results in an immediate conformational change in the STIM1–Orai1 interaction that rapidly activates SOCE.

Molecular identity of the ECCE pathway in skeletal muscle

In 2004, Pessah and colleagues identified a novel mechanism for depolarization-induced Ca^{2+} influx in skeletal muscle myotubes (Cherednichenko *et al.* 2004). This trans-sarcolemmal Ca^{2+} influx pathway was termed 'excitation-coupled Ca^{2+} entry' (ECCE) because it is activated by KCl depolarization and high frequency stimulation and requires functional DHPR–RyR1 coupling (ECCE is absent in myotubes derived from RyR1-null and α_{1S}-null mice). This study also concluded that ECCE was not mediated by Ca^{2+} permeation through the DHPR L-type Ca^{2+} channel pore during depolarization because ECCE activity (assessed from the maximum rate of KCl-induced Mn^{2+} quench of fura-2 fluorescence) was normal in α_{1S}-null myotubes expressing a Ca^{2+} impermeable α_{1S} pore mutant (SkEIIIK; (Dirksen *et al.* 1999)). ECCE was subsequently shown to also be operable in adult skeletal muscle fibres (Cherednichenko *et al.* 2008), potentiated by ryanodine (Cherednichenko *et al.* 2004, 2008), insensitive to 1 μM nifedipine (Yang *et al.* 2007), abolished by siRNA-mediated knockdown of the DHPR α_2-δ subunit (Gach *et al.* 2008), inhibited by La^{3+}, Gd^{3+}, SKF-96356, 2-APB and dantrolene (Hurne *et al.* 2005; Yang *et al.* 2007; Cherednichenko *et al.* 2008), and augmented by mutations in RyR1 that cause malignant hyperthermia (Yang *et al.* 2007; Cherednichenko *et al.* 2008).

Several lines of evidence demonstrate that Ca^{2+} influx through the ECCE pathway is fundamentally distinct from store-operated Ca^{2+} entry. First of all, activation of the ECCE pathway does not require store depletion (Cherednichenko *et al.* 2004; Hurne *et al.* 2005). Second, both DHPR and RyR1 are required for ECCE, but not for SOCE (Cherednichenko *et al.* 2004; Lyfenko & Dirksen, 2008). Third, while depolarization activates ECCE, the strong inwardly rectifying voltage dependence of the SOCC current (Stiber *et al.* 2008) results in depolarization inhibiting Ca^{2+} entry through this mechanism (Kurebayashi & Ogawa, 2001). However, conceivably ECCE could either reflect DHPR–RyR1 conformational coupling activating a distinct Ca^{2+} permeation pathway or providing a unique store-independent mechanism for opening Ca^{2+} permeable Orai1 channels. Such a scenario is plausible considering the fact that both the SOCE (STIM1–Orai1) and EC coupling (DHPR–RyR1) machineries co-localize within pre-formed SR–sarcolemmal junctions. To distinguish between these two possibilities, we assessed effects on ECCE of molecular interventions that abolish SOCE. We found that ECCE was unaffected by both siRNA-mediated STIM1 knockdown and expression of dominant-negative Orai1 (E106Q), interventions sufficient to eliminate SOCE (Lyfenko & Dirksen, 2008). Together, these results demonstrate that store-operated and excitation-coupled Ca^{2+} entry arise from two distinct molecular pathways of Ca^{2+} influx across the t-tubule membrane (Fig. 4).

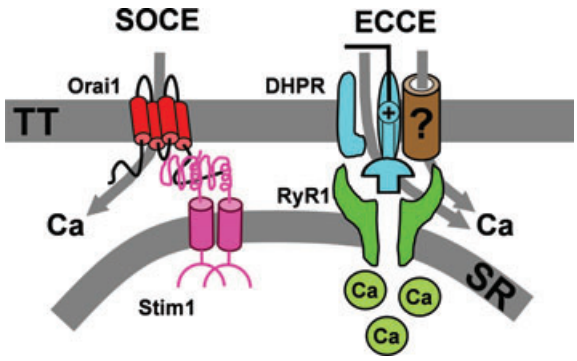

Figure 4. SOCE and ECCE muscle are determined by distinct molecular complexes within SR–sarcolemmal junctions
Left, SOCE involves 'inside-out' conformational coupling between SR STIM1 Ca^{2+} sensor proteins and tetrameric Ca^{2+} permeable Orai1 channels located in the t-tubule membrane. For clarity, only one Orai1 subunit is shown. Right, ECCE involves 'outside-in' conformational coupling between the DHPR voltage sensor and the SR Ca^{2+} release channel. The Ca^{2+} permeation pathway for ECCE involves Ca^{2+} flux through the L-type Ca^{2+} channel pore and/or an unidentified associated channel. Figure modified with permission from Lyfenko & Dirksen (2008).

Identification of the ECCE permeation pathway

The identity of the Ca^{2+} permeation pathway(s) for ECCE is still being debated. Cherednichenko *et al.* (2004) proposed that DHPR–RyR1 conformational coupling activated an undefined Ca^{2+} permeable channel. As noted above, this report excluded the potential role of Ca^{2+} permeation through the DHPR pore since Mn^{2+} flux in α_{1S}-null myotubes was fully restored following expression of SkEIIIK. ECCE also does not involve Ca^{2+} influx through either Orai1 (Lyfenko & Dirksen, 2008) or TRPC3 (Lee *et al.* 2006) channels. However, the contribution of the L-type Ca^{2+} channel conductance to ECCE was recently re-evaluated by Bannister *et al.* (2009). This study found that L-type Ca^{2+} channels in myotubes exhibits a similar pharmacological profile as that shown for ECCE, with inhibition by La^{3+}, Gd^{3+}, SKF-96356, 2-APB and high concentrations of nifedipine. Significant L-type Ca^{2+} current was also observed under conditions designed to mimic KCl-induced ECCE activation (~10 s depolarization to −10 mV in 2 mM extracellular Ca^{2+}). Finally, the study also demonstrated Mn^{2+} permeation through the L-type Ca^{2+} channel pore and that depolarization-induced Ca^{2+} entry is not detectable in SkEIIIK-expressing α_{1S}-null myotubes. Together, these results indicate that Ca^{2+} permeation through the L-type Ca^{2+} channel pore may indeed

represent an important contribution to Ca^{2+} entry via the ECCE pathway (Bannister *et al.* 2009).

A major discrepancy in previous studies is the observation that KCl-mediated Mn^{2+} flux was normal (Cherednichenko *et al.* 2004) but Ca^{2+} entry absent (Bannister *et al.* 2009) following SkEIIIK expression in α_{1S}-null myotubes. Bannister *et al.* (2009) suggested the difference between the two studies could be explained by KCl depolarization driving the SkEIIIK channel into a high P_O mode that restores divalent ion permeability to levels sufficient to resolve Mn^{2+} quench, but not Ca^{2+} influx. Indeed, strong depolarization in the presence of Bay K 8644 produced a transient inward tail current during repolarization (Bannister *et al.* 2009). However, neither the identity of the charge carrier (e.g. Ca^{2+}, Cl^-, TEA^+, H^+) during the inward tail current nor the degree to which SkEIIIK divalent permeation is promoted by KCl application protocols typically used to activate ECCE were determined. Thus, the role of KCl-induced potentiation of Mn^{2+} flux through SkEIIIK channels for the normal rate of fura-2 quench reported by Cherednichenko *et al.* (2004) remains unclear.

Additional discrepancies with prior published results are also inconsistent with the notion that ECCE-mediated Ca^{2+} influx arises solely from Ca^{2+} permeation through the DHPR pore. First, ECCE is abolished following knockdown of the DHPR α_2-δ subunit, in spite of the fact that the L-type Ca^{2+} current conductance is unaffected and the kinetics of channel activation/inactivation only marginally altered (Gach *et al.* 2008). Second, ECCE activity is inhibited ~70% by dantrolene (10 μM) and augmented >6-fold by ryanodine (250 μM) (Cherednichenko *et al.* 2004, 2008), in spite of the fact that these agents also do not significantly alter maximal L-type Ca^{2+} channel conductance and only marginally shift the voltage dependence of channel activation (Balog & Gallant, 1999; Szentesi *et al.* 2001; Bannister *et al.* 2009). Third, ECCE is activated during high frequency electrical stimulation (Cherednichenko *et al.* 2004, 2008; Hurne *et al.* 2005), a condition seemingly less likely than prolonged KCl depolarization to activate Ca^{2+} entry through slowly activating skeletal muscle L-type Ca^{2+} channels. Fourth, mutation of a conserved cysteine residue in RyR1 (C4958S) enhanced ECCE (by slowing deactivation), in spite of the fact that L-type Ca^{2+} current magnitude was reduced ~60% without a change in channel inactivation kinetics (Hurne *et al.* 2005). Finally, ECCE is increased ~2-fold by mutations in RyR1 that result in MH (Yang *et al.* 2007; Cherednichenko *et al.* 2008), while these mutations do not markedly alter the magnitude and voltage dependence of the L-type Ca^{2+} channel conductance (Dirksen & Avila, 2004). In spite of these apparent discrepancies, quantitative comparisons between effects of interventions on L-type Ca^{2+} currents and ECCE flux can be misleading as even minor alterations in L-channel activity may result in significant changes in integrated divalent ion influx recorded during prolonged KCl depolarization. Nevertheless, while Ca^{2+} permeation through the L-type Ca^{2+} channel pore may contribute to Ca^{2+} influx through the ECCE pathway, additional factors and/or auxiliary influx pathways may also be involved. Clearly, additional work is required in order to reach a more comprehensive understanding of the permeation pathway that underlies ECCE.

Conclusions and future perspectives

In summary, SOCE in skeletal muscle involves store depletion triggering rapid conformational coupling between STIM1 luminal Ca^{2+} sensor proteins located in the SR and calcium permeable Orai1 channels present in the transverse (t)-tubule membrane. Unlike T-lymphocytes and other non-excitable cells, STIM1 and Orai1 proteins pre-localize within the triad junction in skeletal muscle under resting conditions, thus permitting extremely fast and efficient trans-sarcolemmal Ca^{2+} influx during store depletion. Prolonged and repetitive depolarization activates ECCE via conformational coupling between DHPR voltage sensor proteins in the t-tubule membrane and RyR1 Ca^{2+} release channels in the SR terminal cisternae. The store-operated and excitation-coupled Ca^{2+} entry pathways reflect two distinct molecular channel complexes within the triad junction that enable trans-sarcolemmal Ca^{2+} entry across a wide range of transmembrane voltages. SOCE is rapidly activated by 'inside-out' STIM1–Orai1 conformational coupling during store depletion that provides a mechanism for trans-sarcolemmal Ca^{2+} influx at negative voltages. On the other hand, ECCE is activated by 'outside-in' DHPR–RyR1 conformational coupling that permits trans-sarcolemmal Ca^{2+} influx at depolarized potentials.

A number of important unresolved issues surrounding SOCE and ECCE in skeletal muscle remain to be addressed. With regard to SOCE, future work will need to determine the mechanism responsible for targeting STIM1 to the SR terminal cisternae in resting muscle, as well as the molecular mechanism for rapid and graded activation. In addition, it will also be important to determine if other potential store-operated Ca^{2+} influx mechanisms (e.g. STIM1 coupling to TRPC or Orai1/TRPC channels) are also operable in skeletal muscle. Finally, functional roles of STIM2 and Orai2/3, as well as other potential functions for STIM1 (e.g. in ER/SR stress, activation of unfolded protein response) will also require further investigation. With regard to ECCE, the relative contribution of Ca^{2+} permeation through the α_{1S} pore and/or via another associated channel certainly merits additional attention. However, the most important long-term goal of future work will be to determine the respective physiological roles

of SOCE and ECCE in muscle development (Stiber *et al*. 2008), fatigue (Pan *et al*. 2002; Zhao *et al*. 2005), aging (Zhao *et al*. 2008), and as modifiers of muscle disease (Vandebrouck *et al*. 2002; Zhao *et al*. 2006; Yang *et al*. 2007; Cherednichenko *et al*. 2008).

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