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S100A1 AND CALMODULIN REGULATION OF RYANODINE RECEPTOR IN STRIATED MUSCLE

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Summary

The release of Ca^{2+} ions from the sarcoplasmic reticulum through ryanodine receptor calcium release channels represents the critical step linking electrical excitation to muscular contraction in the heart and skeletal muscle (excitation-contraction coupling). Two small Ca^{2+} binding proteins, S100A1 and calmodulin, have been demonstrated to bind and regulate ryanodine receptor activity *in vitro*. This review focuses on recent work that has revealed new information about the endogenous roles of S100A1 and calmodulin in regulating skeletal muscle excitation-contraction coupling. S100A1 and calmodulin bind to an overlapping domain on the ryanodine receptor type 1 to tune the Ca^{2+} release process, and thereby regulate skeletal muscle function. We also discuss past, current and future work surrounding the regulation of ryanodine receptors by calmodulin and S100A1 in both cardiac and skeletal muscle, and the implications for excitation-contraction coupling.

1. Introduction

Excitation-contraction (EC) coupling is the process by which membrane depolarization triggers sarcoplasmic reticulum (SR) Ca^{2+} release and subsequent muscle contraction. In skeletal muscle, membrane depolarization alters the molecular interaction between dihydropyridine receptors (DHPRs) in transverse (t) tubule membranes and the ryanodine receptor Ca^{2+} release channels (RyR1) in the adjacent junctional SR membranes, triggering rapid RyR1 activation and Ca^{2+} release into the cytosol [1–5]. Both activation and deactivation of Ca^{2+} release in skeletal muscle are under tight control of the voltage-dependent conformational changes in the DHPR, and the resulting change in molecular interaction between DHPRs and RyR1s. EC coupling in cardiac muscle uses different isoforms of the DHPR and RyR, which are coupled by Ca^{2+} ions that enter the myocyte via DHPR Ca^{2+} channels and activate nearby RyR2 Ca^{2+} release channels through Ca^{2+}-induced Ca^{2+} release (CICR) (Reviewed in [6]).

Despite the tight inter-molecular regulation of skeletal muscle SR Ca^{2+} release, numerous intracellular modulators interact directly or indirectly with RyR1 to fine tune Ca^{2+} release.

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RyR1 is a massive 560 kD homotetramer, with approximately 4/5^{ths} of the channel exposed to the cytoplasm and the remaining 1/5th located in the transmembrane or luminal SR [7]. This large cytoplasmic face is the target of several small molecule regulators, such as ATP, Ca^{2+}, and Mg^{2+} [8], and is the putative DHPR interacting site [9, 10]. Multiple endogenous proteins have also been proposed to regulate RyR1 function at the cytoplasmic face, including FKBP12 [11], calmodulin (CaM) [12], and S100A1 [13]. In cardiac muscle the same regulators serve to modulate RyR2 activity [14–16]. In recent years, work from our group and collaborators has provided significant insight into the regulation of RyR1 by CaM and S100A1, primarily through the use of genetically engineered mouse models. This review will focus on the emerging picture of how CaM and S100A1 compete for binding RyR1 to regulate Ca^{2+} release in skeletal muscle. We will also discuss the possibilities and implications for similar interactions between CaM and S100A1 with RyR2 in cardiac muscle.

2. Calmodulin and S100A1: small Ca^{2+} binding proteins that bind and regulate RyR1

Calmodulin is a small (~17 kD), ubiquitous, highly conserved EF-hand containing Ca^{2+} binding protein. Roughly 50% of CaM localizes to cellular membranes, while the other half resides in the cytosol and nucleus [17]. CaM binds four Ca^{2+} ions cooperatively, and undergoes a Ca^{2+}-dependent conformation change that increases its affinity for target proteins [17]. CaM binds directly to RyR1 and is well documented to regulate activity of isolated RyR1 channels *in vitro*. EM and FRET based studies show CaM binds to the large cytoplasmic face of RyR1 ([18, 19], and single channel measurements demonstrated that at low [Ca^{2+}] (100nM) Ca^{2+} free (ie, apo-CaM) increases the activity of RyR1, while at higher Ca^{2+} concentrations (≥1μM) Ca-CaM inhibits channel activity several fold [20]. Further studies complemented these findings, leading to the classification of apo-CaM as a weak agonist of RyR1 and Ca-CaM as a stronger inhibitor of the channel [21, 22]. CaM binds to one site per RyR1 subunit (4 per tetramer), and protects RyR1 from proteolytic cleavage at amino acid residues 3630 and 3637. Combined with sequence analysis for CaM binding motifs, the CaM binding domain (CaM BD) has been identified as residues 3614–3643 of rabbit RyR1 [23] on the cytoplasmic face of each RyR1 subunit [24]. Rabbit RyR1 3614–3643 corresponds to mouse RyR1 3615–3644; in the rabbit sequence residue numbers are shifted one residue lower for RyR1 and one residue higher for RyR2 compared to the mouse sequence, which should be kept in mind when comparing reports in the literature.

More recently, CaM has been shown to interact with RyR1 in a more complex manner. The C-terminal lobe of CaM binds to the previously classified CaM BD of RyR1, while the N-terminal lobe may interact with a second site non-contiguous with the primary sequence [25]. The N-terminal lobe has been proposed to bind between residues 1975 and 1999 of the RyR1 monomer adjacent to the C-terminal binding site. Disulfide bonds between these sites supports proximity between the two domains, which could explain CaM binding non-contiguously to this site of intersubunit interaction [26]. Upon binding Ca^{2+}, CaM has been shown to shift in its binding site [18, 19]. Zhang et al. (2003) proposed that this shift mediates the switch between apo-CaM activating RyR1 to Ca-CaM inhibiting the channel. Furthermore, Rodney et al. (2005) proposed that apo-CaM binding the CaM BD may sensitize RyR1 to activation by disrupting intersubunit interactions, while Ca-CaM may produce an inhibitory effect by stabilizing intersubunit interactions and promoting a closed configuration of the channel.

Although CaM binding to RyR1 and modulation of RyR1 activity *in vitro* has been thoroughly demonstrated [20, 22, 27], *in situ* analysis of CaM's role in EC coupling with functionally coupled DHPR-RyR1 has been limited. O'Connell et al. (2002) showed that

myotubes expressing RyR1 with mutations to the CaM BD showed minimal alterations in EC coupling [28]. However, until recently no studies had evaluated endogenous CaM's *in vivo* role in adult skeletal muscle, leaving a void between *in vitro* analysis of CaM's effect on RyR1 and an analysis of its physiologic contribution to EC coupling.

2.1. S100A1

The S100 family of proteins are Ca^{2+} binding proteins that appeared relatively late in evolution and are only expressed in vertebrates. One of the first of the now more than 20 family members characterized was S100A1, originally referred to as S100α [29, 30]. S100A1 is a small (21kD), dimeric Ca^{2+} binding protein that, like CaM, contains 4 EF-hand Ca^{2+} binding domains in its dimerized form [31, 32]. The first EF hand is a "pseudo" EF hand, as it binds Ca^{2+} with lower affinity (100–500 μM) than the second, canonical EF hand, which binds Ca^{2+} in the standard range (1–50 μM) [31, 32]. This relatively low Ca^{2+} affinity of S100A1 can be greatly strengthened by glutathionylation [33] as well as the presence of a target interacting protein [34, 35], allowing S100A1 to sense nanomolar intracellular Ca^{2+} concentrations *in vivo*. Ca^{2+} affinity is a key determinant in the function of S100A1, since like CaM it has no intrinsic enzymatic activity, but instead interacts with target proteins though a Ca^{2+}-dependent mechanism to elicit biological responses [29, 36]. Upon binding Ca^{2+}, two of the EF-hands undergo dramatic conformational changes, exposing a hydrophobic binding pocket that is thought to be the target protein interacting region [32, 37].

Although S100 family members share considerable sequence homology, they are expressed in tissue specific patterns. S100A1 is the most highly expressed family member in striated muscle, and also exhibits expression in other organ systems such as the brain, kidney, and spleen [30, 38, 39]. In striated muscle, S100A1 demonstrates highest expression in cardiac muscle, followed by slow twitch and fast twitch skeletal muscle, respectively [40, 41]. In fast twitch skeletal muscle, estimates of S100A1 protein concentration range from 0.5 – 15 μM [40, 42, 43]. The effective concentration of S100A1 locally available for the modulation of RyR1 is likely higher, however, as S100A1 has been shown, both at an ultrastructural level and with immunofluorescence, to localize to A band/I band junctions and SR membranes [44–46], where RyR1 Ca^{2+} release occurs in skeletal muscle.

While a considerable body of knowledge has amassed about S100A1's role in cardiac muscle (see below and review by [47]), research on this protein in skeletal muscle has lagged behind. Early experiments showed that S100A1 enhanced RyR1 Ca^{2+} release in SR terminal cisternae preparations [48]. These authors presciently noted that despite belonging to the same superfamily of EF-hand Ca^{2+} binding proteins, CaM and S100A1 appeared to have contrasting effects on the activation of RyR1-mediated Ca^{2+} release, a notion we have explored in detail in recent years and will focus on in this review. S100A1 has also been shown to bind purified RyR1 by affinity chromatography (with mid-nanomolar affinity, similar to CaM), and to potentiate the open probability of RyR1 reconstituted in a lipid bilayer [13]. Furthermore, exogenous S100A1 increases caffeine-evoked force transients in skinned skeletal muscle fibers [41]. These *in vitro* studies laid mechanistic groundwork for our understanding of S100A1 regulation of RyR1 Ca^{2+} release; however, they did not explore physiologic voltage-gated Ca^{2+} release in a functionally intact skeletal muscle system.

2.2. Calmodulin and S100A1 bind to an overlapping conserved region of the ryanodine receptor Ca^{2+} release channel

It is not uncommon for S100 proteins to bind similar structural motifs as CaM [49, 50]. Sequence scanning of mouse RyR1 showed that residues 3617–3628, which begin 2 residues

into the N-terminus of the CaM BD, very closely match the S100 consensus binding sequence (Fig. 1), closer than any other sequence in the entirety of RyR1. S100A1 and CaM were shown to bind with similar affinities to a peptide generated from this region of RyR1 [46]. The solution structure of S100A1 bound to this peptide of RyR1 has since been solved using NMR spectroscopy [35], and the crystal structure of CaM bound to the CaM BD is also available [51]. Of note, the S100A1 binding domain of RyR1 (mouse residues 3617–3628) is perfectly conserved in the corresponding sequence of RyR2 (mouse residues 3582–3593; Fig. 1; see below). Furthermore, there are only three residue differences between RyR1 and RyR2 over the entire CaM binding domain.

Competition assays using isolated SR vesicle preparations were used to evaluate whether CaM and S100A1 compete for binding the CaM BD of the full length RyR1. CaM-linked beads were mixed with intact SR vesicles expressing full length RyR1, allowing CaM to bind to the CaM BD of RyR1 in the SR vesicles. Addition of S100A1 displaced the RyR1-containing vesicles from the CaM beads in a dose dependent fashion [46]. Importantly, S100A1 could displace the RyR1-containing vesicles from the CaM-linked beads at 100 nM Ca^{2+}, suggesting that S100A1 binds RyR1 at $[Ca^{2+}]_i$ inherent to resting conditions in skeletal muscle fibers. The reverse experiment using S100A1-linked beads showed that addition of CaM also fully displaced S100A1 from RyR1 [35]. As both CaM and S100A1 fully displace one another from the CaM BD, CaM and S100A1 likely share one major, high affinity binding site on each RyR1 monomer (or 4 per tetrameric RyR1 channel), although there are additional sites of interaction [13, 20, 52]. In addition, the N-terminal CaM BD peptide also fully displaced the SR vesicles from S100A1-linked beads, further suggesting that S100A1 interaction with RyR1 occurs through this domain [35]. We will thus refer to this site of presumed competitive binding of CaM or S100A1 to RyR1 as the CaM/S100A1 BD for the remainder of this review.

3. *S100A1*$^{-/-}$ and *RyR1*$^{D/D}$ mice: studying the endogenous regulation of RyR1 by S100A1 and CaM

As detailed above, there is considerable evidence that exogenous S100A1 and CaM both bind RyR1 and regulate channel activity *in vitro*. To complement and advance these studies, our group has evaluated the *in situ* roles of endogenous S100A1 and CaM through the use of two genetic mouse models: 1) *S100A1*$^{-/-}$ mice [46]; 2) transgenic mice expressing a specific point mutation to the CaM/S100A1 BD of RyR1 (RyR1-L3625D, *RyR1*$^{D/D}$ mice) [53]. This mutation has previously been demonstrated to impair CaM binding and regulation of RyR1 *in vitro* [22], and we recently showed that the mutation also abolishes the *in vitro* binding of S100A1 to RyR1 [53] (Fig. 1). Single channel studies performed in isolated membrane fractions from *RyR1*$^{D/D}$ mice demonstrated that this point mutation impairs apo-CaM activation of RyR1 and Ca-CaM inhibition of the channel. Importantly, the large activating effect S100A1 had on single channels isolated from control mice was fully eliminated in channels isolated from *RyR1*$^{D/D}$ mice [53]. This finding provides strong support that S100A1 primarily regulates RyR1 through the CaM/S100A1 BD. *RyR1*$^{D/D}$ mice thus provide the unique advantage of eliminating non-specific effects that arise from global protein ablation, allowing a focused investigation on regulation of the CaM/S100A1 BD of RyR1. Additionally, when analyzed in parallel to results from *S100A1*$^{-/-}$ mice, the study of *RyR1*$^{D/D}$ mice has allowed us to begin to isolate the specific roles of CaM and S100A1 in the regulation of RyR1 Ca^{2+} release.

3.1. S100A1 potentiates SR Ca^{2+} release and twitch force in skeletal muscle

To study the endogenous roles of CaM and S100A1 we studied EC coupling in enzymatically dissociated flexor digitorum brevis (FDB) skeletal muscle fibers [54] isolated

from *S100A1*$^{-/-}$ and *RyR1*$^{D/D}$ mice. When stimulated by a brief field stimulus to initiate a single action potential (AP) [55], *S100A1*$^{-/-}$ fibers demonstrate a ~30% reduction in the amplitude of cytosolic Ca^{2+} transients when compared to fibers from wild-type (WT) littermates [46]. Similarly, Ca^{2+} transients are suppressed at all test membrane potentials in voltage-clamped *S100A1*$^{-/-}$ fibers [56]. This reduction in Ca^{2+} transient amplitude is independent of changes in either resting cytosolic [Ca^{2+}] [46] or releasable SR Ca^{2+} stores [55]. Additionally, there is no change in the waveform of the propagated AP (Fig. 2A) [55], or the RyR1-activating component of DHPR charge movement (Qβ) in *S100A1*$^{-/-}$ fibers (Fig. 2B) [57], suggesting a direct effect on RyR1 (Fig. 2). When S100A1 protein expression is restored in *S100A1*$^{-/-}$ fibers by adenoviral delivery of S100A1, Ca^{2+} transient amplitude is restored back to the WT level, suggesting that the depressed Ca^{2+} transients in *S100A1*$^{-/-}$ fibers did not result from a compensatory response to genetic manipulation [46]. A Ca^{2+} removal model [58] was used to calculate the time course of SR Ca^{2+} release flux underlying the AP-evoked Ca^{2+} transients in *S100A1*$^{-/-}$ fibers. Using this model the peak Ca^{2+} release flux during a single AP was found to be reduced ~30% in *S100A1*$^{-/-}$ fibers. This depressed Ca^{2+} release on a single cell level translates to weaker force production of the whole muscle *in vivo*; the isometric force generated by the tibialis anterior (TA) muscles of anesthetized *S100A1*$^{-/-}$ animals is reduced ~25% when stimulated with a single AP [55].

SR Ca^{2+} release is also suppressed in *RyR1*$^{D/D}$ fibers when compared to littermate controls. Both indo-1 and fluo-4 recordings of Ca^{2+} transients are similarly reduced in amplitude in *RyR1*$^{D/D}$ and *S100A1*$^{-/-}$ fibers stimulated by a single AP [46, 53]. Furthermore, there is a 41% average decrease in peak SR Ca^{2+} release flux during voltage clamp depolarization of *RyR1*$^{D/D}$ fibers [53], a remarkably similar suppression to the 42% decrease seen in *S100A1*$^{-/-}$ fibers under identical conditions [56]. As the elimination of S100A1 (*S100A1*$^{-/-}$) and mutation to the CaM/S100A1 BD (*RyR1*$^{D/D}$) cause a virtually identical suppression of Ca^{2+} release, we hypothesize that the elimination of the S100A1 activating effects on RyR1 may fully account for the suppressed Ca^{2+} release in *RyR1*$^{D/D}$ fibers stimulated with a single AP (Fig. 3A). Consistent with this proposal, *in vivo* TA twitch force elicited by a single AP is reduced 25% in *RyR1*$^{D/D}$ muscle, the same degree of suppression seen in *S100A1*$^{-/-}$ muscle [53].

3.2. CaM contributes to the inactivation of SR Ca^{2+} release during repetitive skeletal muscle stimulation

Based on the above rationale, disrupting just CaM binding to the CaM/S100A1 BD is hypothesized to have little effect on Ca^{2+} release elicited by a single AP from rest. However, during high frequency (100Hz) trains of APs (a more physiologic firing pattern of motor units in small mammals [59]), a clear role for CaM regulation of RyR1 Ca^{2+} release becomes apparent. Although the amplitude of the Ca^{2+} transient in response to a single AP is depressed in *RyR1*$^{D/D}$ fibers, during a 100Hz train of stimuli the Ca^{2+} transient amplitude (relative to the amplitude due to the initial pulse) increases more and more with each stimulus when compared to WT counterparts (i.e., there is a greater relative “summation” of Ca^{2+} transients in *RyR1*$^{D/D}$ fibers, as seen in Fig. 3B [53]). The time course of SR Ca^{2+} release flux during repetitive stimulation suggests that the greater summation of Ca^{2+} transients is a result of slowed inactivation of release flux in *RyR1*$^{D/D}$ fibers. This is in stark contrast to *S100A1*$^{-/-}$ fibers, where there is *less* summation of the Ca^{2+} transients and *greater* inactivation of Ca^{2+} release flux during repetitive stimulation (Fig. 3B; [55]). These finding are supported by voltage clamp studies, where a prolonged “shoulder” of Ca^{2+} release flux is evident during sustained depolarization of *RyR1*$^{D/D}$ fibers, again suggesting slowed inactivation of release flux [53]. Importantly, this shoulder is absent in *S100A1*$^{-/-}$ fibers [56].

Taken together these results suggest that as cytosolic [Ca^{2+}] increases during repetitive or prolonged fiber depolarization, endogenous Ca-CaM contributes to the inactivation of Ca^{2+} release. This is consistent with earlier *in vitro* studies (see above). Impaired Ca-CaM inactivation accounts for the greater relative summation of Ca^{2+} transients and slowed inactivation of release flux in fibers with a mutated CaM/S100A1 BD (*RyR1*$^{D/D}$). As *S100A1*$^{-/-}$ fibers demonstrate the opposite effects (reduced summation, greater inactivation), this may be attributed to increased Ca-CaM-dependent inactivation in these fibers, as Ca-CaM is free to bind and inactivate RyR1 more readily in the absence of its endogenous competitor, S100A1 (Fig. 3B). Consistent with this model, TA muscles of anesthetized *RyR1*$^{D/D}$ mice demonstrate greater summation of force during tetanic stimulation (attributable to impaired Ca-CaM inactivation of Ca^{2+} release). *S100A1*$^{-/-}$ muscle, on the other hand, shows reduced force summation (greater Ca-CaM-dependent inactivation), and during prolonged tetanic stimulation *S100A1*$^{-/-}$ muscle fatigues more rapidly than WT muscle. This suggests that un-inhibited Ca-CaM inactivation of Ca^{2+} release is negatively affecting muscular performance in these animals [55]. One possible reservation concerning the preceding interpretation is that the two genetically engineered mouse lines that we have examined, *S100A1*$^{-/-}$ and *RyR1*$^{D/D}$ are derived from slightly different mouse strains. Whether this influences the observed effects of these genetic manipulations remains to be determined.

3.3. Reaction schemes for activation and inactivation of WT, *S100A1*$^{-/-}$ and *RyR1*$^{D/D}$ skeletal muscle fibers

Figure 4 presents our hypothesized reaction schemes for SR Ca^{2+} channel gating in skeletal muscle from wild type (WT; Fig. 4A), *S100A1*$^{-/-}$ (Fig. 4B) and *RyR1*$^{D/D}$ (Fig. 4C) mice. In these schemes, the TT voltage sensor, RyR1, S100A1 and CaM are represented by the symbols V, R, S and C, respectively, and V* represents the voltage sensor in its voltage activated state. In WT fibers (Fig. 4A) in the resting state (left), the CaM/S100A1 BD is assumed to be either unoccupied (left foreground) or to bind S100A1 (left, background) at the CaM/S100A1 BD. Since *S100A1*$^{-/-}$ has the same effect as *RyR1*$^{D/D}$ on initial Ca^{2+} release (see above), Ca^{2+}-free calmodulin (i.e., apo-CaM) is assumed not to bind significantly to RyR1 in resting muscle fibers. Because interaction of RyR1 with the TT voltage sensor in resting mammalian fibers suppresses Ca^{2+} spark activity [60–62], we represent both the resting and activated state of the voltage sensor as interacting with RyR1, but this interaction has opposite effects on RyR1 depending on whether the voltage sensor is in its resting (V, blue) or activated (V*, red) state.

Muscle fiber depolarization (red) during an action potential or by voltage clamp activates the TT voltage sensor, which in turn opens the SR Ca^{2+} release channel (red) with or without bound S100A1 (middle; background or foreground, respectively), but the release is less in the absence of S100A1, as shown by the comparison of release in fibers from WT and *S100A1*$^{-/-}$ mice (above). For simplicity, the likely multi-state sequence for voltage sensor activation [58, 63] is here represented by a single voltage dependent transition, either with or without bound S100A1. The activated RyR1 channels release Ca^{2+} ions into the myoplasm (the elevation in myoplasmic [Ca^{2+}] is indicated by green bolts), resulting in Ca^{2+} dependent inactivation of the RyR1 channels by two different binding mechanisms (middle, right; green). First, Ca^{2+} ions can bind directly to Ca^{2+} regulatory inhibitory sites on RyR1 and inactivate the channel independently of whether or not S100A1 is bound at the CaM/S100A1 BD (right; background or foreground, respectively). Alternatively, Ca^{2+} ions can bind to calmodulin, and Ca-CaM can then bind to the CaM/S100A1 BD of RyR1 and also contribute to channel inactivation providing that S100A1 is ***not*** occupying that site (right, foreground). The scheme in Fig 4A assumes that inactivation by direct binding of Ca^{2+} ions to RyR1 is mediated by locally elevated [Ca^{2+}] in the immediate vicinity of open channels,

whereas inactivation by CaCaM binding to RyR1 is mediated by global pool cytosolic Ca^{2+} from both channel types driving Ca^{2+} binding to CaM followed by Ca-CaM binding to RyR1. Alternative scenarios for effects of global and local Ca^{2+} may also be possible.

In *S100A1*$^{-/-}$ fibers (Fig. 4B), there is no S100A1 present so any states in the WT reaction scheme with S100A1 bound (background of Scheme A) cannot be attained and are thus missing from Scheme B. In this case there is only a single resting state of RyR1, and after voltage dependent activation, all channels can undergo inactivation by either Ca^{2+} binding directly to RyR1 (upper right) or by Ca-CaM binding to the otherwise unoccupied CaM/S100A1 BD (lower right). In the *RyR1*$^{D/D}$ fibers (Fig. 4C), neither S100A1 nor Ca-CaM can bind to the CaM/S100A1 BD, so there is only a single voltage activated state and a single inactivated state in which Ca^{2+} binds directly to RyR1.

3.4. Limitations of our interpretations

There are several caveats regarding Fig. 4. First, Fig. 4 represents a state diagram, with no indication of molecular mechanism for the functional properties of any of the indicated states. Second, it is highly simplified. For example, each RyR1 channel is a homo tetramer, with one binding site per monomer or four sites per tetrameric channel, yet we represent binding only to a single site. This works for independent binding, but would require modification for cooperative binding or if the functional effect is not proportional to fractional occupancy. Third, RyR1 is also modulated by binding of a number of other ligands (e.g., ATP, Mg^{2+}, FKBP12, homer protein and others). However, if the contributions of each of these ligands is the same in WT, *S100A1*$^{-/-}$ and *RyR1*$^{D/D}$ fibers, then they can simply be considered as constant contributors to the overall effectiveness of voltage dependent channel activation, and thus not require explicit inclusion in the reaction schemes comparing WT, *S100A1*$^{-/-}$ and *RyR1*$^{D/D}$ fibers. Finally, the lack of apo-CaM binding to the resting channels was deduced assuming that all effects on SR Ca^{2+} release seen in S100A1$^{-/-}$ fibers were due solely to elimination of S100A1 binding to the CaM/S100A1 BD, which we concluded from the rationale presented in preceding sections.

4. Regulation of cardiac muscle by CaM and S100A1

Activation of cardiac myocytes involves voltage dependent activation of DHPR voltage sensors and opening of SR RyR Ca^{2+} release channels, but the isoforms of both molecules are different in cardiac (CaV1.2 or α1c; RyR2) and skeletal (CaV1.1 or α1s; RyR1) muscle. Whereas DHPR-RyR1 communication in skeletal muscle is via direct molecular communication (above), in cardiac myocytes voltage sensor activation causes opening of L-type calcium channels in the sarcolemma and transverse tubules, and the resulting Ca^{2+} influx triggers SR Ca^{2+} release via RyR2 Ca^{2+} release channels, which in cardiac muscle are activated by CICR [64].

Intensive research over the last decade has provided clues about the mechanisms involved in control of cardiac contractility by CaM and S100A1. A multitude of studies have shown that both CaM and S100A1 can interact with dozens of different proteins, enzymes and structural proteins critical for cardiac performance. CaM regulates RyR2, CaV1.2, SERCA2a-phospholamban complex, IP3R2 (type-2 inositol trisphosphate Ca^{2+} release channel), and phosphorylase kinase (reviewed in : [65, 66]. S100A1 also modulates RyR2, CaV1.2, SERCA2a-phospholamban complex, the mitochondrial ATP synthase, and binds to the myofilament protein titin (reviewed in [47]). These CaM and S100A1 target proteins represent important regulators of cardiac EC coupling, Ca^{2+} and energy homeostasis. Here, we will focus on the actions of CaM and S100A1 on the RyR2-mediated Ca^{2+} release process (for reviews regarding molecular, structural, and disease related details of RyR2s and their modulation by CaM, S100A1 and other regulators see: [47, 65, 67–70]).

4.1. CaM modulates cardiac RyR2

When studied using isolated SR vesicles or purified RyRs incorporated into planar lipid bilayers, modulation of RyR2 activity by CaM is unique when compared to modulation of RyR1 and RyR3. At nanomolar free [Ca^{2+}], apo-CaM has an inhibitory effect on RyR2 channel activity, whereas apo-CaM potentiates RyR1 and RyR3 channel activity. At micromolar free [Ca^{2+}], Ca-CaM inhibits all RyR isoforms in isolated preparations [67].

Mutagenesis studies showed that apo-CaM and Ca-CaM inhibit RyR2 via preferential binding to a high-affinity binding domain comprised of amino acid residues 3580–3609 of mouse RyR2 that is conserved among the RyR isoforms (Figure 1; [15]). CaM shifts the Ca^{2+}-dependence of RyR2 activation to higher Ca^{2+} concentrations and hence decreases RyR2 opening at all Ca^{2+} concentrations [15, 71]. It has been proposed that CaM promotes RyR2 channel closing following SR Ca^{2+} release during EC coupling [72]. In support of this hypothesis, neonatal cardiomyocytes isolated from mice engineered with a disrupted RyR2 CaM BD (RyR2-W3586A/L3590D/F3602A) show impaired CaM regulation of RyR2 and have prolonged and abnormal SR Ca^{2+} release, which leads to cardiac hypertrophy and early death [73]. Our results in skeletal muscle fibers (above) with a disrupted RyR1 CaM/S100A1 BD indicate that Ca-CaM has a less pronounced inhibitory effect on RyR1, which manifests during repetitive AP stimulation (above), whereas Ca-CaM has a stronger inhibitory effect on RyR2 which is evident during a single AP [73]. These differences between RyR1 and RyR2 inhibition by Ca-CaM might arise from different mechanisms (i.e., differential Ca-CaM binding affinities at the CaM BD, variable CaM-CaM BD interaction dwell times, fluctuations of local [CaM], etc.). It is also noteworthy that three point mutations to the CaM BD were required to block CaM inhibition of RyR2 while only a single mutation was needed for RyR1. It is not teleologically surprising that Ca-CaM inhibition may play a more dramatic role in cardiac EC coupling, as the lack of tight voltage sensor control of Ca^{2+} release in heart cells may allow greater regulation of CICR by modulatory proteins such as CaM. Regardless of the mechanisms at play, these contrasting differences between RyR1 and RyR2 inhibition by CaM demand further research.

4.2. S100A1 effects on cardiac RyR2

S100A1 is predominantly expressed in the heart [38]. In ventricular cardiomyocytes, as in the case of skeletal FDB fibers, S100A1 mostly displays a striated-like pattern and is also localized at both the junctional and longitudinal SR, myofilaments, intercalated discs, and mitochondria [74, 75].

Several studies have identified the functional impact of S100A1 on intracellular Ca^{2+} homeostasis and have found that S100A1 increases diastolic and systolic performance via augmented SR Ca^{2+} reuptake and by increasing the gain of CICR, respectively [16, 74, 76–79]. S100A1 reduces [3H]-ryanodine binding to RyR2-enriched SR vesicles at low (nanomolar) [Ca^{2+}] [74, 80], however, at high (micromolar) [Ca^{2+}], S100A1 enhances [3H]-ryanodine binding to RyR2 [74]. Other evidence in support of the inhibitory role of S100A1 on RyR2 at low $[Ca^{2+}]_i$ comes from a study showing that S100A1 can reduce Ca^{2+} spark frequency in quiescent cardiomyocytes [78] and SR leak in cardiac SR vesicle preparations [74, 79], supporting the idea that S100A1 promotes diastolic RyR2 closure during cardiomyocyte relaxation. On the other hand, experiments in voltage-clamped cardiomyocytes and SR vesicle preparations [8, 77, 79, 80] demonstrated that S100A1 increased SR Ca^{2+} release, suggesting that S100A1 can augment RyR2 opening during systole. Interestingly, the intracellular actions of S100A1 were independent of sarcolemmal Ca^{2+} fluxes (i.e., S100A1 did not affect Ca^{2+} entry via L-type Ca^{2+} channels or via the Na-Ca^{2+} exchanger [80]. However, other studies have shown that exogenous S100A1 enhances L-type Ca^{2+} current in both neonatal ventricular myocytes [81] and in neuronal cultures

[82], but in contrast, chronic ablation of S100A1 leads to enhanced L-type Ca^{2+} influx in adult myocytes [83]. These contrasting findings, coupled with the multitude of roles proposed for S100A1 in cardiac EC coupling, has created the need for further experimentation to define the precise contribution of S100A1 to EC coupling in the young, mature, and failing heart.

4.3. Do S100A1 and CaM also compete for binding to the CaM BD of RyR2 in heart cells?

As with many of its other binding partners, S100A1 binding to RyR2 is Ca^{2+} dependent, and amino acid residues 75–94 within the α-helical S100A1 C-terminal domain are a crucial interface for interaction [41]. Recent collaborative studies of our group revealed that S100A1 binds to and competes with CaM at the CaM/S100A1 BD of RyR1 in skeletal muscle [35, 46]. Given that this region is highly conserved between RyR1 and RyR2 [15] (Fig. 1), we hypothesize that S100A1 and CaM may compete for binding to the CaM BD of RyR2 as they do for RyR1 in skeletal muscle. However, the S100A1 interaction site(s) as well as binding stoichiometries with the macromolecular RyR2 complex remain to be characterized.

5. Do CaM and/or S100A1 bind to more than one site at RyRs?

The preceding sections provide evidence that both CaM and S100A1 are ubiquitous calcium sensors that can fine-tune the Ca^{2+} sensitivity of RyRs by binding to an overlapping region exposed to the cytoplasm. This modulation is RyR isoform specific and depends on the concentration of free cytosolic Ca^{2+}. The differential effects of CaM and S100A1 binding on RyR activity could also be explained by binding interactions at more than one CaM/S100A1 binding site. While we have presented evidence above suggesting one major, high affinity, overlapping binding site shared by CaM and S100A1, S100A1 ligand overlays on a panel of RyR1 fusion proteins have identified at least three distinct S100A1 binding domains with different binding affinities for the cytoplasmic portion of RyR1 [13]. Recent observations employing isothermal titration calorimetry has determined the thermodynamic parameters of CaM binding to three distinct regions in skeletal (RyR1) and cardiac (RyR2) channels [52]. It is clear that one goal of future research will be to establish the functional consequences of CaM and/or S100A1 binding to these distinct regions in RyR1 and RyR2 channels.

6. What other factors affect CaM or S100A1 binding to RyRs?

Recently, reduced affinity for CaM binding to RyR2 with PKA phosphorylation was found in a catecholaminergic polymorphic ventricular tachycardia (CPVT)-associated mouse model (Arg2474Ser), resulting in spontaneous local Ca^{2+} release events leading to lethal arrythmias [84]. Modified CaM binding affinity for RyR1 or RyR2 may occur when the RyR channel or CaM itself is modulated by other endogenous regulators (i.e., FKBP12, homer protein, PKA, others). As S100A1's affinity to bind both Ca^{2+} and target protein is regulated by diverse processes [33, 34], similar effects are expected for S100A1 binding to both RyR1 and RyR2 upon convergent modulation. This scenario of complex RyR meta-modulation by CaM/S100A1 and other important signaling molecules is largely unexplored and warrants further research, particularly in the context of altered protein expression profiles and activity in heart disease and muscular fatigue.

7. Concluding remarks

Our results are consistent with a simplified model of S100A1 and CaM regulation of RyR1 Ca^{2+} release in skeletal muscle. Under resting conditions, the CaM/S100A1 binding domain of RyR1 is predominantly regulated by S100A1, which potentiates SR Ca^{2+} release and force production when the muscle is stimulated (Fig. 3A). As $[Ca^{2+}]_i$ becomes elevated

during maintained stimulation, Ca-CaM binding becomes more dominant, displacing S100A1 from RyR1 and promoting channel inactivation (Fig. 3B). In this manner CaM and S100A1 compete to fine tune SR Ca^{2+} release during normal muscle activation. Disrupting this system leads to decreased force production and impaired muscular performance. However, many questions remain. How is this competitive balance affected by other cellular regulatory mechanisms, or during muscular exercise, fatigue and disease? For instance, does muscle activity or muscle chronic paralysis regulate the expression levels of CaM/S100A1? What are the molecular details of CaM/S100A1 interaction with RyR1 and RyR2, and how do these interactions exert their effect on channel activity? Does the differential expression of S100A1 and CaM among fast and slow fiber types confer some specificity to Ca^{2+} release to meet the varied demands of these muscle types? Clearly, more work is needed to address these important issues.

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S100 consensus binding sequence			K	-	L	-	W	-	-	I	L																			
RyR1 CaM/S100A1 BD (mouse 3615-3644	K	S	K *	K	A	V	W	H	K	L	L	S	K	Q *	R	R	R	A	V	V	A	C	F	R	M	T	P	L	Y	N
RyR2 CaM(S100A1?) BD (mouse 3580-3609)	R	S	K *	K	A	V	W	H	K	L	L	S	K	Q *	R	K	R	A	V	V	A	C	F	R	M	A	P	L	Y	N

Fig. 1. S100A1 and calmodulin compete for binding to an overlapping region of RyR1

The S100 consensus binding sequence is identified in RyR1 residues 3617–3625. This sequence lies in the N-terminus of the previously characterized calmodulin binding domain (CaM BD) of RyR1 (3615–3644). A 12 amino acid peptide containing this sequence (residues within the asterisks) is perfectly conserved between RyR1 and RyR2 and has a similar affinity for S100A1 and Ca-CaM [46]. Mutation of L3625D (red) abrogates both CaM and S100A1 binding to RyR1. This mutation impairs S100A1 activation of SR Ca^{2+} release and Ca-CaM inactivation of SR Ca^{2+} release [53]. We therefore refer to this region as the CaM/S100A1 binding domain. The CaM BD is highly conserved between RyR1 and RyR2 (red shading marks differing residues). Mutations of W3586A/L3590D/F3602A (green) abrogate CaM from binding and inactivating RyR2 in heart cells [73]. It is currently unknown whether S100A1 regulates Ca^{2+} release in the heart through interaction with this domain. The residue numbers used here correspond to mouse RyR1 and RyR2. Note that for the rabbit, residue numbers are shifted one residue lower for RyR1 and one residue higher for RyR2 compared to the mouse sequence.

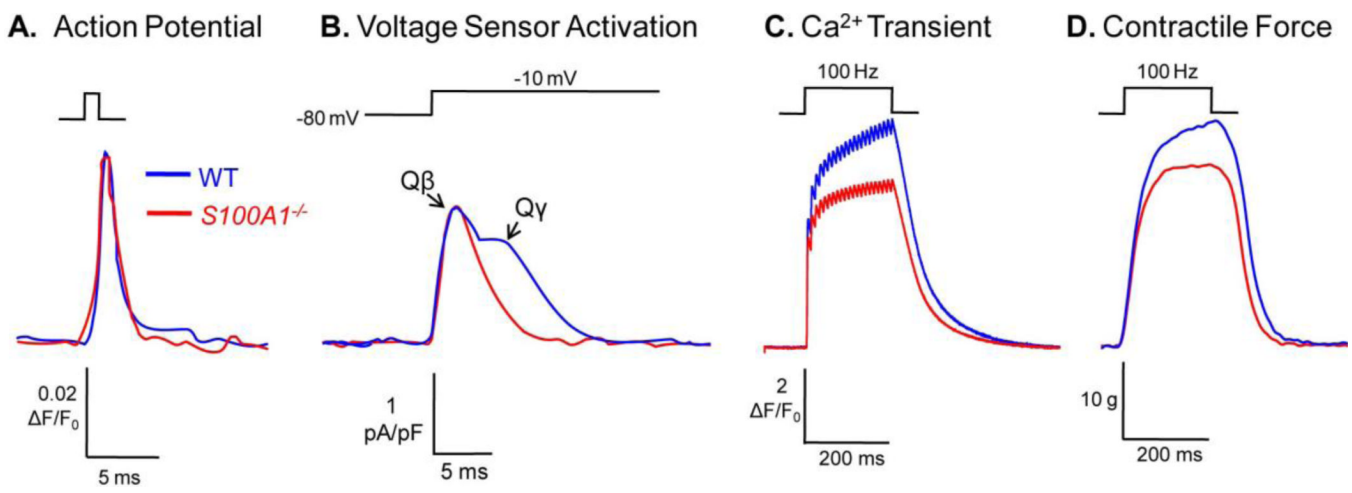

Fig. 2. S100A1 modulation of skeletal muscle EC coupling

A) Di-8 ANEPPS recordings of action potentials elicited by field stimulation of WT (blue) and *S100A1*$^{-/-}$ (red) fibers. Genetic ablation of S100A1 has no effect on the propagated AP in the t-system of skeletal muscle fibers [55]. **B)** DHPR intramembrane charge movement currents elicited by a voltage clamp depolarization to −10 mV of WT and *S100A1*$^{-/-}$ fibers. Ablation of S100A1 has no effect on the RyR1-activating component of DHPR charge movement (Qβ), but does blunt a secondary component of DHPR charge movement (Qγ) that is a consequence of optimal SR Ca^{2+} release [57]. **C)** Fluo-4 recordings of Ca^{2+} transients elicited by 100 Hz field stimulation of WT and S100A1$^{-/-}$ fibers. The Ca^{2+} transient is depressed in *S100A1*$^{-/-}$ fibers, and demonstrates less summation during the train of stimuli [46, 55]. **D)** Tetanic force generated by the tibialis anterior of anesthetized WT and S100A1$^{-/-}$ animals in response to 100 Hz stimulation. Maximal force is suppressed in S100A1$^{-/-}$ muscle [55].

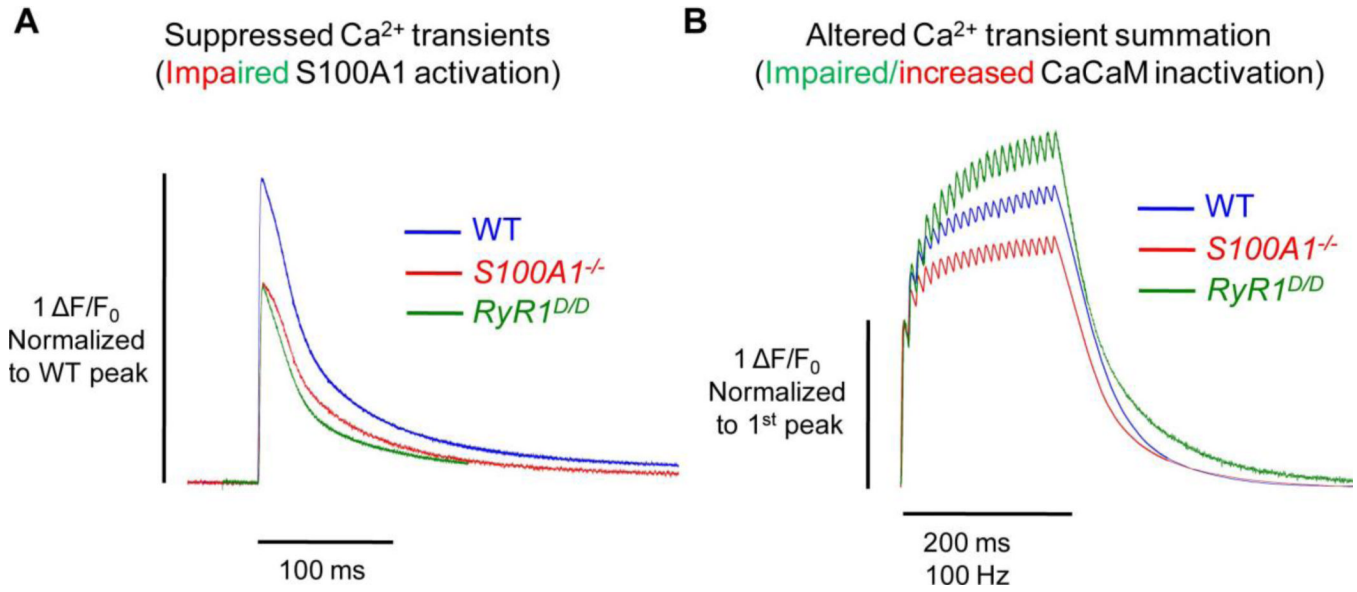

Fig. 3. S100A1 and CaM differentially regulate RyR1 Ca^{2+} release

A) In response to a single action potential, the peak amplitude of the Ca^{2+} transient is similarly suppressed in the absence of S100A1 (*S100A1*$^{-/-}$, red) and in fibers with a mutated CaM/S100A1 binding domain of RyR1 (*RyR1*$^{D/D}$, green). This suggests that under resting conditions, S100A1 may predominantly occupy the CaM/S100A1 BD and potentiate Ca^{2+} release upon muscle activation. **B)** Fibers with a mutated CaM/S100A1 BD demonstrate greater relative summation of Ca^{2+} transients during repetitive activation. Conversely, fibers lacking S100A1 show less summation. This suggests that upon a rise in $[Ca^{2+}]_i$ during prolonged muscle activation, Ca-CaM may predominantly occupy the CaM/S100A1 BD and inactivate some portion of Ca^{2+} release. This inactivation is impaired in *RyR1*$^{D/D}$ fibers, and enhanced in S100A1$^{-/-}$ fibers, as they lack CaM's endogenous competitor. All data traces represent average data from [46, 53, 55].

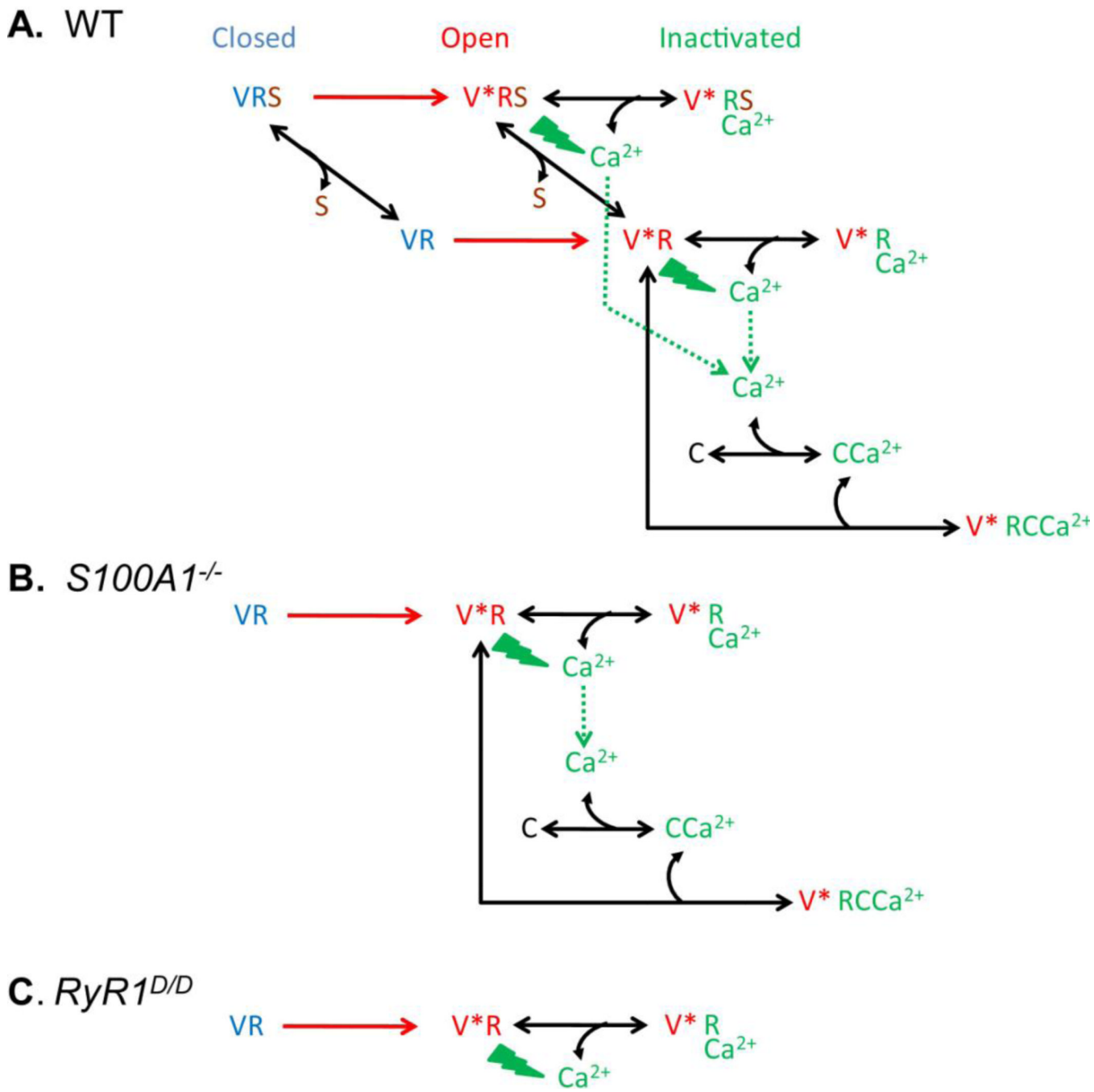

Fig.4. Reaction schemes for activation and inactivation of RyR1 Ca^{2+} release channels in skeletal muscle fibers from wild type (A), *S100A1*$^{-/-}$ (B) and *RyR1*$^{D/D}$ (C) mice

V, R, S and C represent the TT voltage sensor, the SR ryanodine receptor/Ca^{2+} release channel, S100A1 and calmodulin, with V* representing the voltage activated state of V. Channels move sequentially left to right from the resting (blue) to the activated (red) to the inactivated state (green) due to voltage activation of V (red) to Ca^{2+}-dependent inactivated states (green). In **A**, S100A1 occupied states are depicted in the background, and S100A1 free states are in the foreground. See text (3.3, 3.4) for further details.