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REVIEW

Bridging the myoplasmic gap II: more recent advances in skeletal muscle excitation–contraction coupling

Roger A. Bannister*

ABSTRACT

In skeletal muscle, excitation–contraction (EC) coupling relies on the transmission of an intermolecular signal from the voltage-sensing regions of the L-type Ca^{2+} channel ($Ca_V1.1$) in the plasma membrane to the channel pore of the type 1 ryanodine receptor (RyR1) nearly 10 nm away in the membrane of the sarcoplasmic reticulum (SR). Even though the roles of $Ca_V1.1$ and RyR1 as voltage sensor and SR Ca^{2+} release channel, respectively, have been established for nearly 25 years, the mechanism underlying communication between these two channels remains undefined. In the course of this article, I will review current viewpoints on this topic with particular emphasis on recent studies.

KEY WORDS: 1,4-dihydropyridine receptor, DHPR, $Ca_V1.1$, α_1S, L-type, Excitation–contraction coupling, Skeletal muscle

Introduction

Eight years ago, I summarized the depth of knowledge concerning the basic mechanism of excitation–contraction (EC) coupling in skeletal muscle in an article entitled 'Bridging the myoplasmic gap: recent advances in skeletal muscle excitation–contraction coupling' (Bannister, 2007). At the time, the progression of the field was slowing, as informative advances were becoming infrequent and increasingly incremental after the explosion of work in the decade following the identification of the L-type Ca^{2+} channel $Ca_V1.1$ as the voltage sensor and the type 1 ryanodine receptor (RyR1) as the SR Ca^{2+} release channel. Investigators had nearly exhausted strategies tailored for use in myotubes cultured from knock-out mice and in isolated *in vitro* systems (e.g. lipid bilayer recordings). Given the limitations of those experimental systems, the overall complexity of the problem and the relatively small number of laboratories in pursuit of its resolution, the slow rate of progress at the time was understandable. However, the field has been given a reviving jolt by some innovative new approaches in the last couple of years. In light of these new gains, I will update my past review here in the aptly titled, 'Bridging the myoplasmic gap II: more recent advances in skeletal muscle excitation–contraction coupling'.

As noted above, nearly all investigators familiar with the topic agree on the roles of $Ca_V1.1$ and RyR1 as EC coupling voltage sensor and SR Ca^{2+} release channel, respectively (Tanabe et al., 1988; Nakai et al., 1996). I would also venture to say that there is consensus amongst investigators in the field that the intermolecular communication between these two channels that supports EC coupling occurs without a major contribution from Ca^{2+} flux into the myoplasm via the conventional ion conduction pore housed in the principal α_{1S} subunit of the $Ca_V1.1$ heteromultimer (Armstrong et al., 1972; Tanabe et al., 1988; Dirksen and Beam, 1999). In this regard, the mechanism of EC coupling in skeletal muscle is fundamentally different from that in cardiac muscle, which requires Ca^{2+} flux via the $Ca_V1.2$ (α_{1C}) L-type Ca^{2+} channel isoform to activate the cardiac RyR2 channel (Cheng and Wang, 2002). Although the lack of requirement for extracellular Ca^{2+} for skeletal-type EC coupling was first described over 40 years ago by Armstrong et al. (1972), expression of non-permeable or non-gating mutant $Ca_V1.1$ channels in myotubes harvested from *dysgenic* (α_{1S} null) mice has since demonstrated the lack of requirement for Ca^{2+} flux on the molecular level. Dirksen and Beam (1999) expressed an α_{1S} subunit mutant carrying a glutamate to lysine swap in the pore domain of Repeat III (residue 1014) as a means to test this idea. The E1014K mutation was designed to eliminate inward divalent permeation under standard patch-clamp recording conditions while sparing EC coupling (see also Bannister and Beam, 2009, 2011). Similarly, Eltit et al. (2012) examined another mutant α_{1S} subunit carrying a mutation associated with malignant hyperthermia in humans. This mutation, an arginine to tryptophan exchange at position 174 occurred at a site intuitively involved in channel gating – the innermost positively charged residue in the voltage-sensing S4 α-helix of Repeat I (Carpenter et al., 2009). Like E1014K, the R174W mutation had little effect on SR Ca^{2+} release but eliminated L-type Ca^{2+} current during 200 ms step depolarizations. Interestingly, the R174W mutation wiped out L-type Ca^{2+} current by a distinctly different mechanism from the E1014K swap. As the R174W channel could be opened by a combination of pharmacological (Bay K 8644) and electrophysiological (long/strong depolarization) manipulations, it was posited that the mutation caused a profound depolarizing shift in channel activation that stabilized the closed state of the channel (Bannister and Beam, 2013b). A speculative explanation for the inability of R174W to achieve transitions required for the open confirmation without Bay K 8644 and long/strong depolarization was that the passage of the voltage sensor through the field of the plasma membrane is impeded by the bulky tryptophan residue.

Recent evidence has confirmed that Ca^{2+} flux via $Ca_V1.1$ is dispensable for EC coupling in both fish and mammals *in vivo*. Schredelseker et al. (2005) demonstrated that zebrafish and other higher teleosts naturally lack L-type Ca^{2+} current even though they have two distinct α_{1S} subunit isoforms (α_{1S}-a and α_{1S}-b; Schredelseker et al., 2010). Interestingly, each of these channels has evolved different mechanisms to curtail Ca^{2+} flux via their respective pores. For α_{1S}-a, permeation has been eliminated by a tryptophan–glycine doublet taking the place of a methionine–glutamate pair in the S5–S6 linker of Repeat I while an aspartate for asparagine swap in Repeat II prevents Ca^{2+} flux through the selectivity filter of α_{1S}-b. In mammals, Lee and colleagues (2015) generated an E1014K knock-in mouse model that displayed no obvious locomotor dysfunction. In addition to providing proof that EC coupling in mammalian skeletal muscle does not require Ca^{2+}

Department of Medicine-Cardiology Division, University of Colorado Denver-Anschutz Medical Campus, 12700 East 19th Avenue, Room 8006, B-139, Aurora, CO 80045, USA.

*Author for correspondence (roger.bannister@ucdenver.edu)

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List of abbreviations

EC	excitation–contraction
ECCE	excitation-coupled Ca^{2+} entry
EM	electron microscopy
FDB	flexor digitorum brevis
RGK	Rad-Rem-Rem2-Gem/Kir
RyR1	type 1 ryanodine-sensitive intracellular Ca^{2+} release channel
SR	sarcoplasmic reticulum
Stac3	SH3 and cysteine-rich domain-containing protein 3
YFP	yellow fluorescent protein

flux through $Ca_V1.1$ *in vivo*, this work also identified the L-type Ca^{2+} channel as a regulator of excitation–transcription coupling and provided nearly conclusive evidence that the source of excitation-coupled Ca^{2+} entry (ECCE) into skeletal muscle is L-type Ca^{2+} flux through the conventional divalent-conducting pore of $Ca_V1.1$.

$Ca_V1.1$ is the voltage sensor for skeletal-type EC coupling, but how it communicates the signal to RyR1 remains unclear

In 1973, muscle contraction was directly linked to intramembrane gating charge movement (Schneider and Chandler, 1973) and this relationship of charge movement to SR Ca^{2+} release was found to be similarly affected by 1,4-dihydopyridine antagonists of $Ca_V1.1$ (Ríos and Brum, 1987). These physiological studies conducted without the benefit of molecular cloning provided fairly convincing evidence that a 1,4-dihydropyridine-sensitive, voltage-sensing molecule was responsible for transmitting the EC coupling signal to the SR. Independently, the known perinatal lethality of the *dysgenic* mouse strain was found to be a consequence of the tissue-specific absence of an L-type Ca^{2+} channel in skeletal muscle (Beam et al., 1986). Subsequently, reintroduction of the newly cloned $Ca_V1.1$ α_{1S} subunit rescued EC coupling, L-type Ca^{2+} current and charge movement in myotubes cultured from *dysgenic* mice (Tanabe et al., 1988; Adams et al., 1990). The success of this latter experiment transformed the field by enabling the study of modified α_{1S} subunits in a relatively native environment, though this statement must be qualified in that most studies that have employed this approach have expressed a variant of the adult rabbit channel in developing mouse cells (e.g. Tanabe et al., 1990; Nakai et al., 1998; Wilkens et al., 2001; Kugler et al., 2004; Papadopoulos et al., 2004; Bannister et al., 2009). For this reason, there is a possibility of context-dependent results that may not be entirely reflective of events occurring in adult muscle fibers. Even so, this approach has been quite useful in the definition of regions of the α_{1S} subunit important for both voltage sensing and Ca^{2+} conduction (see Table 1; Tanabe et al., 1990; Nakai et al., 1998; Wilkens et al., 2001; Kugler et al., 2004).

As the functional role(s) of each of the intracellular segments of the α_{1S} subunit have already been summarized in earlier reviews (Bannister, 2007; Bannister and Beam, 2013a), I will only discuss them briefly here. The relatively short α_{1S} amino terminus is dispensable for both EC coupling (Bannister and Beam, 2005) and channel function, while the α_{1S} III–IV loop influences EC coupling indirectly through its ability to regulate gating (Bannister et al., 2008). Regions of the carboxyl terminus proximal to Repeat IV are responsible for proper targeting of the channel to plasma membrane–SR junctions and possibly for interactions with RyR1 (Slavik et al., 1997; Proenza et al., 2000b; Flucher et al., 2000; Lorenzon et al., 2004; Papadopoulos et al., 2004; Lorenzon and Beam, 2007). In contrast, the distal portion of the carboxyl terminus is cleaved post-translationally and diffuses away from the junction into the I-bands on its way to being degraded; electrophysiological analysis of carboxy-terminal α_{1S} deletion mutants in *dysgenic* myotubes and native L-type Ca^{2+} channels in adult muscle flexor digitorum brevis (FDB) fibers showed that cleavage of the distal portion of the carboxyl terminus has very little, if any, functional consequence (Beam et al., 1992; Ohrtman et al., 2015). The α_{1S} I–II loop is the site for the interaction with the highly conserved guanylate-kinase-like domain of the β_{1a} subunit and therefore is essential for channel trafficking, ultrastructural arrangement and possibly EC coupling (Table 1; discussed below).

Table 1. Known essential components of the skeletal muscle EC coupling complex

Essential component	Established function(s) in the EC coupling process	References
$Ca_V1.1$ α_{1S} (principal)	Voltage sensor for EC coupling, L-type Ca^{2+} channel, tetrad formation	Schneider and Chandler (1973); Beam et al. (1986); Ríos and Brum (1987); Tanabe et al. (1988); Takekura et al. (1994)
$Ca_V1.1$ β_{1a} (auxiliary)	α_{1S} trafficking, modulation of $Ca_V1.1$ current kinetics, tetrad formation	Gregg et al. (1996); Strube et al., (1996); Schredelseker et al. (2005, 2009)
Stac3/$Ca_V1.1$ ε (auxiliary)	α_{1S} trafficking, modulation of L-type Ca^{2+} current kinetics	Horstick et al. (2013); Nelson et al. (2013); Polster et al. (2015)
RyR1	SR Ca^{2+} release channel, tetrad formation, modulator of $Ca_V1.1$ channel gating properties	Nakai et al. (1996); Protasi et al. (1998)

The four (known) essential components of the excitation–contraction (EC) coupling macromolecular are listed in the left column. Mice null for any of these components die perinatally from respiratory paralysis. The known functions of these proteins, relevant to the EC coupling process, are listed in the middle column. A short list of references is listed in the right column.

While most parties agree that the α_{1S} II–III loop is an essential structure for EC coupling (Tanabe et al., 1990; Lu et al., 1994), views have differed on which portions of the loop are key in supporting EC coupling. The divergent views have arisen from information obtained in different experimental systems. Data obtained by application of synthetic or recombinant peptides to RyR1s in reconstituted lipid bilayers have indicated that a short amphipathic helix in the amino-terminal portion of the α_{1S} II–III loop could open RyR1 (encompassing residues 671–690; El-Hayek et al., 1995; El-Hayek and Ikemoto, 1998). In the past few years, the idea that this region gates RyR1 *in vivo* has lost some steam because this region can be scrambled, deleted or disrupted through the introduction of large amounts of foreign sequence without a great effect on the ability of $Ca_V1.1$ to engage EC coupling (Proenza et al., 2000a; Ahern et al., 2001a,b; Flucher et al., 2002; Lorenzon et al., 2004; Papadopoulos et al., 2004; Bannister et al., 2009). By contrast, α_{1S} residues 720–764/5 in the central portion of the loop were found to be 'critical' for EC coupling, RyR1-dependent enhancement of $Ca_V1.1$ open probability and the tetradic arrangement of $Ca_V1.1$ within triad junctions (see below) because the corresponding sequences of $Ca_V1.2$ α_{1C} subunit could not substitute for this portion of the loop (Nakai et al., 1998; Grabner et al., 1999; Wilkens et al., 2001). While these results regarding the critical domain's importance for EC coupling have been mostly consistent, a couple of studies conducted in myotubes have raised exceptions. First, Ahern and colleagues (2001b) observed that a

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component of EC coupling persists in the absence of both the critical domain and the amino-terminal region of the loop that activates RyR1 in bilayers. In the context of the critical domain, this result is difficult to comprehend because simple deletion of the critical domain killed EC coupling in the same study. Second, a modified α_{1S} subunit with the *Musca domestica* α_{1M} II–III loop still forms tetrads despite being incapable of skeletal-type EC coupling (Takekura et al., 2004).

The carboxy-terminal portion of the loop which connects the critical domain with Repeat III is also involved in the events that are requisite for EC coupling. The significance of this segment was more difficult to tease out owing to its considerable conservation with other L-type Ca^{2+} channels, but this obstacle was overcome by the introduction of yellow fluorescent proteins (YFPs) at sensitive loci within the loop (Bannister et al., 2009). This observation could potentially explain why the α_{1S}–α_{1M} chimera described above could form tetrads. As the carboxy-terminal portion of the α_{1M} II–III loop shares sequence similarity with this region of α_{1S}, these regions could be important for anchoring $Ca_V1.1$ within triad junctions.

Taken together, the results of most studies that have assayed EC coupling in *dysgenic* myotubes with modified α_{1S} subunits have indicated that the integrity of the central and carboxy-terminal portions of the α_{1S} II–III loop is critical for conformational coupling with RyR1, though precisely how this contiguous portion of the α_{1S} II–III loop supports RyR1 gating remains frustratingly unclear. In another past review article, Kurt Beam and I presented a model in which the critical domain interacts with an undefined junctional moiety, causing Ca^{2+} flux, while the carboxy-terminal portion supports junctional anchoring (again with a yet to be defined partner) and provides a conduit for transmission of the EC coupling signal from the membrane-bound voltage-sensing regions of the channel (Beam and Bannister, 2010). The model has provided a viable hypothesis regarding the mechanics of the α_{1S} II–III loop during EC coupling, but it has yet to be rigorously tested, in part, because of the lack of structural information available on the $Ca_V1.1$ complex. This obstacle is now beginning to fade.

Until 2012, the published cryo-electron microscopy (EM) structures of the $Ca_V1.1$ heteromultimer were largely amorphous owing to the limitations of the technique at the time. The resolution has now been reduced from 23 Å (Wolf et al., 2003; Fig. 1, top left) in early structures to 15 Å in a very recent paper from Hu and colleagues (2015; Fig. 1, top right). Ahead of the latter structure, Szpyt et al. (2012) presented a lower resolution structure (~19 Å) that identified many components of the $Ca_V1.1$ channel complex. The crystal structure of the Na_VAb bacterial Na^+ channel (Payandeh et al., 2011) was used to estimate the position and orientation of the α_{1S} subunit with the cryo-EM structure. An engineered YFP tag helped locate the β_{1a} subunit and then its orientation was set based on the published crystal structure of the β_{2a} subunit (Opatowsky et al., 2004). Labeling of the channel with a monoclonal antibody directed specifically to the critical domain suggested that this region is situated in relatively close proximity to RyR1. The higher resolution structure presented by Hu and colleagues (2015) expanded on this work, showing that β_{1a} is situated lower relative to the plasma membrane than previously thought and giving a subtle hint of definition to the α_{1S} II–III loop, which extends into the myoplasm beyond β_{1a}. In both cases, the myoplasmic orientation of these portions of $Ca_V1.1$ supports the idea that either one (or both) may interact with RyR1 *in vivo*.

Together, these recent cryo-EM structures represent a substantial step forward in the definition of $Ca_V1.1$ structure, as

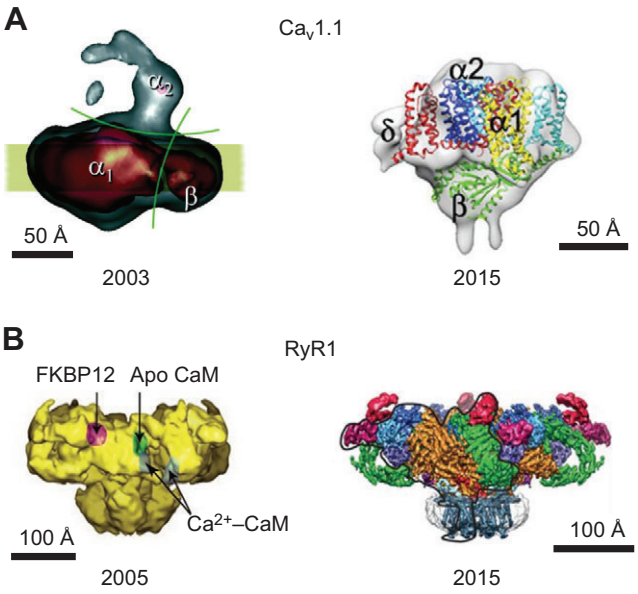

Fig. 1. Comparison of $Ca_v1.1$ and RyR1 cryo-electron microscopy (EM) structures. Top: cryo-EM structures of the L-type Ca^{2+} channel $Ca_V1.1$, originally published by Wolf et al. (2003) and Hu et al. (2015). On the right, note the low position of the β_{1a} subunit and the 'legs' posited to be the II–III loop. Bottom: cryo-EM structures of the type 1 ryanodine-sensitive intracellular Ca^{2+} release channel RyR1, originally published by Samsó et al. (2005) and Efremov et al. (2015). FKBP12 and apo/Ca^{2+}–calmodulin (CaM) binding sites are indicated on the RyR1 structure in the bottom left panel. The black line in the bottom right panel defines the position of a single RyR1 monomer within the structure. Panel A, left, was reprinted from Wolf et al. (2003) with permission from Elsevier; panel A, right, was reprinted from Hu et al. (2015) with permission from Nature Publishing Group; panel B, left, was reprinted from Samsó et al. (2005) with permission from Nature Publishing Group; and panel B, right, was reprinted from Efremov et al. (2015) with permission from Nature Publishing Group.

the jump from 23 Å to 15 Å takes us from a schmoo to the near resolution of secondary structures postulated to be important for $Ca_V1.1$–RyR1 communication. The inherent weakness of this approach is that it only provides a snapshot of a dynamic physiological process. It may be difficult to discriminate between resting closed states and closed states that are involved in EC coupling. Moreover, the absence of other elements of the complex is problematic.

New high-resolution cryo-EM stuctures of RyR1 are a big step towards understanding gating of the SR Ca^{2+} release channel

It is well established that RyR1 is the SR Ca^{2+} release channel (Table 1; Lanner et al., 2010). As far as ion channels go, RyR1 is a colossus. A single RyR1 subunit weighs in around 565 kDa and a functional channel requires four of these subunits, giving a final 2.2+ MDa. The myoplasmic amino-terminal region accounts for nearly 90% of this mass. The amino termini of RyR1s are clearly visible as electron-dense moieties bridging the myoplasm between the plasma membrane and the SR in transmission electron micrographs of triad junctions. Because these structures have an uncanny resemblance to the feet of a caterpillar, the amino terminus of RyR1 is widely referred to as the 'foot' (Franzini-Armstrong, 1970; Protasi et al., 1998). In freeze-fracture replicas of junctions, the tetrameric foot aligns four $Ca_V1.1$ channels in register (Block et al., 1988; Protasi et al., 1998, 2002; Sheridan et al., 2006). This tetradic arrangement of $Ca_V1.1$ particles can be modified by altering the conformation of RyR1 with a high concentration of ryanodine

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(Paolini et al., 2004). Based on these ultrastructural observations, it is thought that some region of the $Ca_V1.1$ multimer interacts with the top part of the foot to catalyze conformational changes that open the RyR1 pore some 10 nm away.

Despite much experimental effort, the $Ca_V1.1$ complex interaction locus on RyR1 also remains a mystery. The results of chimeric studies have identified large tracts of RyR1 which may be involved in intermolecular interactions that support EC coupling (Protasi et al., 2002; Perez et al., 2003; Sheridan et al., 2006), but these results have been difficult to interpret because a strong correlation amongst the abilities of the chimeras to support EC coupling, the higher L-type channel open probability and tetrad arrangement could not be easily drawn. The second conserved SPRY domain of RyR1 (residues 1085–1112) has been implicated as being an important element for communication with $Ca_V1.1$ based largely on its ability to bind the amino-terminal portion of the α_{1S} II–III loop *in vitro* (Cui et al., 2009), but structural analysis suggests it forms an interior link between the amino-terminal gating ring of the channel and the 'clamp' region (Lau and Van Petegem, 2014). The functional relevance of the SPRY2 domain has yet to be determined (Tae et al., 2011).

The assignment of function to portions of RyR1 may have become a lot easier because of the simultaneous publication of three high-resolution cryo-EM structures of the entire RyR1 tetramer in *Nature* earlier this year (Efremov et al., 2015; Yan et al., 2015; Zalk et al., 2015). Ten years ago, the resolution of the ryanodine receptor structure was around 10 Å (Samsó et al., 2005; see also Serysheva et al., 2005; Fig. 1, bottom left). Now, the resolution is 4–6 Å (Fig. 1, bottom right). Unlike the structures of $Ca_V1.1$ described above, specific domains can be discriminated in each of these new RyR1 structures. Information provided by these elegant structures should be highly useful in determining the point(s) of interaction with the $Ca_V1.1$ multimer and identifying the conformational rearrangements in the foot that open the RyR1 pore. However, these structures are still below the level of resolution needed to fully understand intramolecular transitions within the foot domain that are critical for channel pore gating. In this regard, vivid atomic structures of RyR1 subdomains will continue to be revealing (see Lobo and Van Petegem, 2009; Tung et al., 2010; Yuchi et al., 2012; Lau and Van Petegem, 2014).

What about β_{1a}?

The idea that the $Ca_V\beta_{1a}$ subunit may be an intermediary link in communication between $Ca_V1.1$ and RyR1 has been around since the generation of the β_1 null mouse nearly 20 years ago (Gregg et al., 1996). This mouse died perinatally and displayed the same kypothic, underdeveloped morphology as both *dysgenic* and *dyspedic* (RyR1 null) mice. Moreover, myotubes cultured from β_1 null pups lacked voltage-induced Ca^{2+} transients and had substantially reduced L-type Ca^{2+} current amplitude and charge movement, all of which were rescued by exogenous expression of β_{1a} (Gregg et al., 1996; Strube et al., 1996). Although these data clearly demonstrated that β_{1a} is an essential component of the EC coupling complex, a direct role for β_{1a} in conformational coupling between $Ca_V1.1$ and RyR1 has been very difficult to test because β_{1a} serves two other functions that are prerequisite for such coupling. Specifically, β_{1a} promotes efficient membrane expression of $Ca_V1.1$ and facilitates the tetradic ultrastructural arrangement of $Ca_V1.1$ channels within plasma membrane–SR junctions. Consistent with this requirement, β_{1a} has been found to interact with a short stretch of positively charged residues in RyR1 *in vitro* (K3495–R3502; Cheng et al., 2005).

Though the complete role of β_{1a} remains ambiguous, several studies have contributed valuable pieces of information towards the elucidation of its precise function(s). Following closely on the heels of the generation of the β_1 null mouse, a series of studies employing expression of β_{1a} deletion mutants and β_{1a}–β_{2a} chimeras in null mouse myotubes demonstrated that the distal carboxyl terminus of β_{1a} was a critical component for the maintenance of skeletal-type EC coupling (Beurg et al., 1999; Sheridan et al., 2003a,b, 2004). In broad agreement with these data, a carboxy-terminal fragment of β_{1a} corresponding to its final 35 residues (V490–M524) was shown to stick to RyR1 *in vitro* and to promote channel opening in planar lipid bilayers (Rebbeck et al., 2011). A similar, but shorter peptide (V490–M508) activated RyR1 in a later study (Hernández-Ochoa et al., 2014). This peptide also proved to have an effect in a cellular context, as it potentiated both EC coupling and L-type Ca^{2+} current by ∼50% when introduced into FDB fibers via a patch pipette. The results of the work performed with modified β_1 constructs in null myotubes and via application of peptides to RyR1-containing bilayers can be interpreted in a variety of ways; however, each in itself poses a good argument for the importance of the β_{1a} carboxyl terminus in the events that are necessary for EC coupling.

The importance of the β_{1a} carboxyl terminus has driven subsequent experiments to identify the minimal structural determinants of this region that are particularly relevant. In an early study, simultaneous mutation of three hydrophobic residues (L478A, V485A, V492A) within a heptad repeat was reported to ablate the ability of β_{1a} to restore EC coupling and L-type Ca^{2+} channel activity in β_1 null myotubes (Sheridan et al., 2004). The results of this study have since been challenged as these combined mutations failed to affect $Ca_V1.1$ targeting, tetrad formation, charge movement or EC coupling in β_1 null (*relaxed*) zebrafish myotubes (Dayal et al., 2010). In addition, short peptides mimicking the β_{1a} carboxyl terminus with alanine substitutions at positions corresponding to L478, V485, V492 failed to increase RyR1 activity in bilayers (Karunasekara et al., 2012). These challenges have cast grave doubt on the importance of the heptad repeat, but a conclusive test is still lacking.

As noted above, β_{1a} residues V490–M508 have been identified as being critical for biochemical interactions with RyR1, the activation of RyR1 in lipid bilayers and the potentiation of EC coupling and L-type Ca^{2+} current in FDB fibers (Karunasekara et al., 2012; Hernández-Ochoa et al., 2014). A hydrophobic pocket formed by residues L496/L500/W503 was shown to support these actions of the V490–M508 peptide, but the critical importance of these residues was challenged in an even more recent study in which the authors stably expressed a β_{1a} L496A/L500A/W503A mutant in a β_1 null cell line (Eltit et al., 2014). Myotubes expressing this construct formed tetrads and produced impaired, but still substantial, EC coupling.

So yet again, we run into ambiguity regarding β_{1a}; there appears to be general agreement on the idea that the β_{1a} carboxyl terminus is important for EC coupling, but the particular portion is currently a matter of contention. Needless to say, structural information would be quite useful for delineating the function of the carboxyl terminus and for the identification of the contributing elements. Unfortunately, the disordered carboxyl termini of other β subunit isoforms were beyond resolution in earlier atomic structures (Chen et al., 2004; Opatowsky et al., 2004; Van Petegem et al., 2004). As this experimental obstacle would be expected to preclude the definition of the β_{1a} carboxyl terminus as well, more inventive approaches are needed to answer the β_{1a} question.

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In this regard, the Grabner laboratory has been progressive in its use of the zebrafish model system to investigate the β_{1a} subunit. Their finding that β_{1a} is a critical element for the alignment of $Ca_V1.1$ with RyR1 is arguably the most significant advance in our knowledge of the function of β_{1a} in EC coupling since the generation of the β_1 null mouse (Schredelseker et al., 2005, 2009). Continuing on with this approach, they determined that $Ca_V1.1$ voltage sensing is facilitated by a cooperative intramolecular relationship between the carboxyl terminus and a conserved SH3-like domain of β_{1a} (Dayal et al., 2013).

My laboratory has recently published what we believe to be much needed new insight into the β_{1a} question, as we have uncoupled the voltage sensor from the SR Ca^{2+} channel by targeting the β_{1a} subunit (Beqollari et al., 2015). We have done so by overexpressing a known β_{1a}-interacting protein – the Rad-Rem-Rem2-Gem/Kir (RGK) protein Rem. Specifically, electroporated Rem markedly reduced voltage-induced myoplasmic Ca^{2+} transients in FDB fibers without greatly affecting $Ca_V1.1$ targeting, intramembrane gating charge movement or releasable SR Ca^{2+} store content (see Fig. 2). By contrast, a β_{1a}-binding-deficient Rem triple mutant (R200A/L227A/H229A) had little effect on myoplasmic Ca^{2+} release in response to membrane depolarization. Our work may prove to be significant because Rem disrupts $Ca_V1.1$–RyR1 communication in intact, differentiated muscle fibers without deleting or altering the peptide sequences of the endogenous components of the Cav1.1–RyR1 complex. The mechanism underlying this observation is not yet defined, but, at the very least, our data present Rem as a probe for use in future tests of the importance of the β_{1a} subunit in $Ca_V1.1$–RyR1 communication.

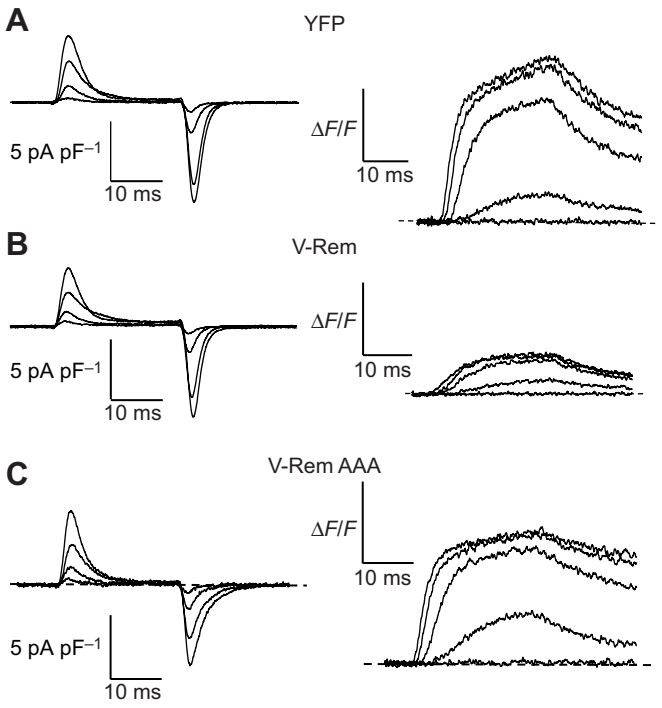

Fig. 2. Rem uncouples EC coupling in differentiated muscle fibers. Representative recordings of intramembrane gating charge movements are shown for transfected flexor digitorum brevis (FDB) fibers expressing control yellow fluorescent protein (YFP; A, left), V-Rem (B, left) or β_{1a} subunit binding-deficient V-Rem AAA (R200A/L227A/H299A; C, left). Representative recordings of myoplasmic Ca^{2+} transients are shown for FDB fibers overexpressing YFP (A, right), V-Rem (B, right) or V-Rem AAA (C, right). Figure modified from Beqollari et al. (2015) with permission from the publisher.

Stac3 – the new kid in EC coupling town

Seventeen years passed between the identification of the β_{1a} subunit as being essential for EC coupling and the identification of SH3 and cysteine-rich domain-containing protein 3 (Stac3) as the fourth essential molecular component of the complex. Stac3 is a 334 residue modular scaffolding protein that contains a polyglutamic acid region, a domain resembling the C1 domain of protein kinase C, and two SH3 domains (Bower et al., 2012; Horstick et al., 2013). It was originally characterized as a regulator of muscle cell growth and differentiation (Reinholt et al., 2013; Ge et al., 2014).

Using a forward genetic screen of zebrafish mutants, an immotile mutant line (mi34) of fish was identified (Horstick et al., 2013). Although mi34 larvae were immotile, they were still able to generate muscle action potentials. An EC coupling impairment was evident because muscle obtained from mi34 embryos could not produce myoplasmic Ca^{2+} transients in response to electrical stimulation but had somewhat normal caffeine-induced SR Ca^{2+} release. The defect was attributed to a premature stop codon resulting from altered alternative splicing in the Stac3 transcript. The critical role of Stac3 in skeletal-type EC coupling was confirmed by the rescue of depolarization-induced Ca^{2+} transients by exogenous expression of wild-type Stac3 in mi34 embryo muscle.

The work in zebrafish was soon supported by a study that examined the role of Stac3 in mouse muscle (Nelson et al., 2013). Like *dysgenic*, *dyspedic* and β_1 null pups, mice null for Stac3 died perinatally from asphyxiation. The perinatal lethality of Stac3 null pups occurred for the same reason as the three other known EC coupling-impaired mouse strains – their diaphragms were incapable of contracting despite the presence of sarcolemmal action potentials. Application of the RyR agonist 4-chloro-*m*-cresol triggered sufficient SR Ca^{2+} release to cause contraction, demonstrating that SR Ca^{2+} stores and RyR1-mediated Ca^{2+} release were relatively normal despite the targeted ablation of Stac3.

Disappointingly, neither of the studies that described the requirement for Stac3 gave much insight into its mechanism of action. Neither group assayed voltage-sensor activity by recording L-type Ca^{2+} currents or gating charge movement. Thus, it remains unclear, at this fleeting moment in time, whether Stac3 is an essential link for communication between $Ca_V1.1$ and RyR1 or whether it is merely required for proper delivery of $Ca_V1.1$ to triad junctions (Table 1). In this regard, Stac3 has recently been shown to facilitate the delivery of $Ca_V1.1$ to the plasma membrane of non-muscle cells (Polster et al., 2015) and presumably serves the same function in muscle cells. Moreover, the ability of Stac3 to retard inactivation of $Ca_V1.2$-mediated current indicates that Stac3 also stays associated with L-type Ca^{2+} channel complexes following membrane insertion (Polster et al., 2015). As promotion of membrane expression and modulation of kinetics are the definitive functions of ion channel subunits, Stac3 really ought to be considered as a new $Ca_V\varepsilon$ subunit of the $Ca_V1.1$ channel complex. Given its critical role, the investigation of Stac3, or rather $Ca_V\varepsilon$, as a direct facilitator of EC coupling may face some of the same obstacles that have complicated the study of the other essential $Ca_V1.1$ auxiliary subunit, β_{1a}. Still, we are now able to investigate the function of $Ca_V1.1$ in a reduced system with very little background from other channels. This finding not only enables the structure–function analysis of Stac3 but also provides a system to investigate the biophysical properties of modified α_{1S} and β_{1a} subunits.

Synthesis

What goes on in the myoplasmic gap between $Ca_V1.1$ and RyR1 is still mysterious a quarter of a century following the identification of

these two channels as voltage sensor and SR Ca^{2+} release channel, respectively, but real progress is being made in elucidating this basic mechanism that is essential for life and motion. The zebrafish model system has produced significant gains in our knowledge and has the potential to be even more useful down the road. In particular, the identification of Stac3 as an essential component for communication between $Ca_V1.1$ and RyR1 is a big step as it could well be the elusive intermediary protein. More information regarding the function of Stac3 should be forthcoming shortly after publication of this article as multiple groups are now pursing this topic at pace. Just as there is now great curiosity about Stac3, there is also renewed interest in β_{1a}. Even though β_{1a} has been known to be indispensable for EC coupling for almost 20 years, the jury is still out on its precise role; it may be a conduit for communication between α_{1S} and RyR1, but such a role is very difficult to test because of its established role in supporting $Ca_V1.1$ membrane expression.

As I mentioned 8 years ago, the really big gains in our understanding of the dynamics of EC coupling will come with good structure (Bannister, 2007). Now, vivid high resolution cryo-EM and atomic structures of the components of the complex are finally beginning to materialize. It is truly an exciting time for those of us who find the intricacies of EC coupling fascinating. I much look forward to reviewing the advances of the next 8 years in 'Bridging the myoplasmic gap III'.

Note added in proof

We did not have to wait long for a substantial improvement of $Ca_V1.1$ structure. During the proofing stage of this article, Wu et al. (2015) published a vivid cryo-EM structure of the $Ca_V1.1$ channel complex at 4.2 Å resolution.

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Competing interests

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