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COMMENTARY

Excitation–Contraction Coupling

From α_1s splicing to γ_1 function: A new twist in subunit modulation of the skeletal muscle L-type Ca^{2+} channel

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The L-type Ca^{2+} channel of skeletal muscle ($Ca_V1.1$) is part of a multi-protein complex involved in excitation–contraction (EC) coupling. Some of the proteins in this structure are essential for the plasma membrane control of internal Ca^{2+} release, others play a modulatory role. The auxiliary subunit γ_1 is highly specific for this channel even though it is not required for voltage-activated Ca^{2+} release. A recent study by El Ghaleb et al. (2022) in the *Journal of General Physiology* presents new evidence for a functional interaction of γ_1 with the channel molecule that is influenced by alternative splicing.

EC coupling in skeletal muscle

In skeletal muscle fibers, a single action potential triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) that raises the free myoplasmic Ca^{2+} concentration from <0.1 to >10 µM within ~2 ms (Hollingworth and Baylor, 2013). Ca^{2+} binding to troponin C initiates contraction by unblocking the actin binding sites for myosin cross bridges. The rapid mobilization of an exceptionally large amount of stored Ca^{2+} is made possible by (1) a steep chemical gradient for Ca^{2+} across the SR membrane, established by active ATP-driven Ca^{2+} pumping and efficient SR-luminal buffering, (2) a large increase in SR Ca^{2+} permeability mediated by ryanodine-sensitive channels (ryanodine receptor RYR1) and (3) a sophisticated protein machinery coupling the RYR1 gating to a voltage sensor in the membrane of the transverse tubules (TTs), i.e., narrow cannels which conduct the electrical excitation from the surface of a muscle cell towards its center. $Ca_V1.1$, serves as the voltage sensor in this process (Bannister and Beam, 2013; Hernández-Ochoa and Schneider, 2018). Its original role, i.e., delivering Ca^{2+} from the external space to the cytoplasm, got suppressed during vertebrate evolution in exchange for functional adjustments to serve as a voltage-dependent controller of the efflux of Ca^{2+} from the SR (Mackrill and Shiels, 2020). In some vertebrate muscles (all higher teleost fishes), this protein has even become completely non-conductive for Ca^{2+}, caused by point mutations in the selectivity filter region (Schredelseker et al., 2010). Therefore, a trigger Ca^{2+} influx eliciting SR Ca^{2+} release, as found in vertebrate heart muscle (Ríos, 2018), is not required in the skeletal muscle of these species. That this is also true for vertebrates possessing Ca^{2+}-conductive $Ca_V1.1$ was demonstrated by eliminating extracellular Ca^{2+} (Armstrong et al., 1972; Spiecker et al., 1979) and most recently by studying homozygous mutant mice presenting one of the Ca^{2+} permeation-blocking "fish mutations" (Dayal et al., 2017).

The exact mechanism of functionally coupling the TT membrane to the SR membrane across the ~12 nm junctional gap is still elusive. Very likely, it is a chain of conformational changes involving $Ca_V1.1$–RYR1 physical interaction and the $Ca_V1.1$ II–III loop (connecting homologous domains II and III) as a major determinant. Other proteins contribute to the molecular machinery for Ca^{2+} release control (Avila et al., 2019; Shishmarev, 2020). The essential components have recently been identified by reconstituting functional voltage-controlled Ca^{2+} release from the endoplasmic reticulum in a non-muscle cell line (Perni et al., 2017). The characteristic sigmoidal voltage-dependence of Ca^{2+} release could be established in tsA201 cells, although the signals remained far from the robust Ca^{2+} transients found in skeletal muscle cells. The set of co-expressed proteins that did the job consisted of RYR1, STAC3 (SH3 and cysteine-rich domain-containing protein 3), JP2 (junctophilin 2), and the L-type Ca^{2+} channel subunits $Ca_V1.1$ (α_{1s}) and β_{1a} (Fig. 1 A).

The enigmatic γ subunit

In skeletal muscle cells, $Ca_V1.1$ is associated with two further auxiliary subunits, $\alpha_2\delta$ and γ. The γ subunit, a polypeptide exhibiting four transmembrane α helices is highly specific for skeletal muscle (Biel et al., 1991; Jay et al., 1990). Single-particle cryo-EM revealed associations between transmembrane

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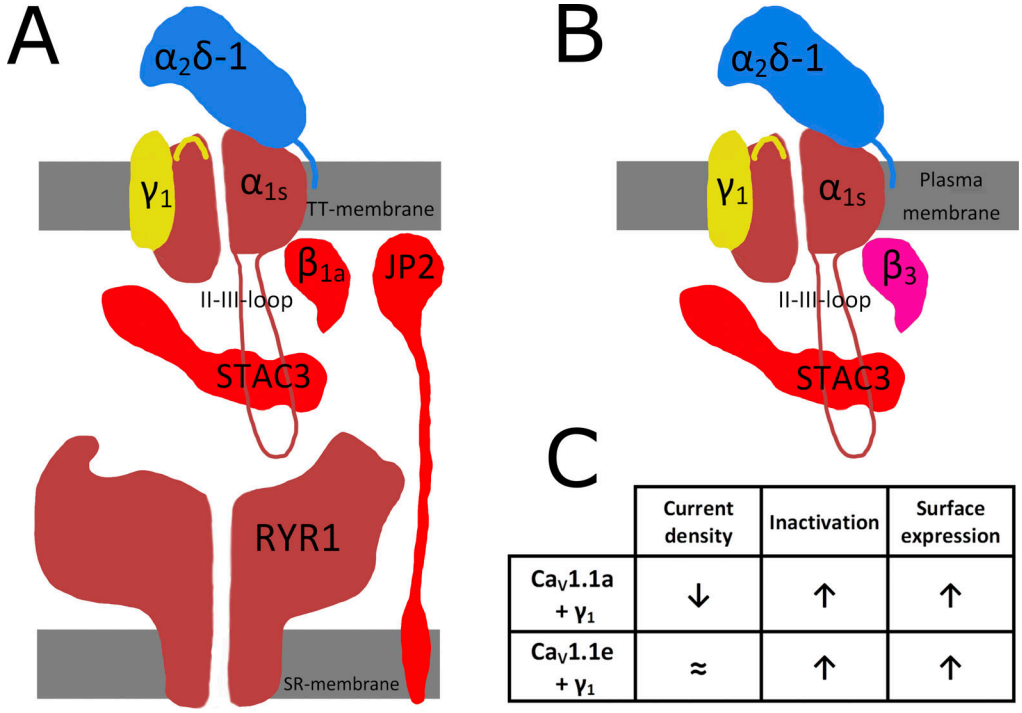

Figure 1. **The EC coupling multi-protein complex of skeletal muscle. (A)** Proteins involved in TT-SR junction formation and TT membrane voltage control of SR Ca^{2+} release. The TT of mammalian skeletal muscle fibers express the $Ca_V1.1$ complex responsible for the L-type Ca^{2+} inward current, which consists of the channel forming α_{1s} protein and auxiliary subunits $\alpha_2\delta$-1, β_{1a}, and γ_1. Up to four $Ca_V1.1$ channels can be associated with one homo-tetrameric Ca^{2+} release channel RYR1. Conformational communication with RYR1 requires further proteins, STAC3, and junctophilins (JP1 and JP2). Highlighted in red is the minimal set of molecular components that allowed functional reconstitution of voltage-dependent Ca^{2+} release after heterologous expression in non-muscle cells (Perni et al., 2017). **(B)** Proteins expressed in the study by El Ghaleb et al. (2022) in HEK293 cells to investigate the impact of the γ_1 subunit and a 19 amino acid stretch in the domain IV S3–S4 linker of α_{1s} that is absent in the embryonic splice variant $Ca_V1.1e$ and present in adult $Ca_V1.1a$ (both structures indicated in yellow). **(C)** Alterations of functional characteristics of $Ca_V1.1a$ and $Ca_V1.1e$ caused by co-expressing γ_1. Inactivation (VDI) and surface expression are comparably enhanced, but L-type Ca^{2+} current density is only reduced from a relatively high level in combination with the adult splice variant $Ca_V1.1a$ (El Ghaleb et al., 2022).

segment 2 (TM2) of this protein and domain IV of α_{1s} (Wu et al. 2015, 2016). Known as γ_1, this subunit was the first discovered representative of a protein family whose most other members modulate glutamate receptor function in neurons by serving as transmembrane AMPA receptor regulatory proteins (TARPs; Jackson and Nicoll, 2011). They are structurally related to the claudin family of tight junction proteins.

γ_1 knockout mice showed neither movement abnormalities nor changes in electrically evoked contraction in fast and slow twitch muscle (Ursu et al., 2001; Ahern et al., 2001). Voltage-dependent Ca^{2+} current and Ca^{2+}-release activation measured in single adult muscle fibers of the γ_1-null mice were indistinguishable from wild type (Ursu et al., 2004). However, voltage-dependent inactivation (VDI) of both Ca^{2+} current and Ca^{2+} release was found to be altered such that the voltage of half-maximal availability was displaced by 16 and 14 mV, respectively, to more depolarized potentials, i.e., a stronger prolonged depolarization is needed to obtain the same degree of inactivation in γ_1-null muscle. Probably resulting from this reluctance to inactivate, muscle fiber bundles of the γ_1-null mouse showed significantly larger contractures during application of high-K^+ solutions, causing long-lasting depolarization to about −17 mV (Ursu et al., 2001; Melzer et al., 2006).

The very slow VDI (taking seconds for completion) and the even slower recovery (requiring minutes for full restauration) are characteristics of $Ca_V1.1$-mediated Ca^{2+} current next to its remarkably slow activation kinetics. Ca^{2+} release, even though activated much more rapidly by depolarization than the L-type Ca^{2+} current, shares the slow kinetics of VDI. Structural studies on bacterial Na_V channels, the likely evolutionary precursors of Ca_V channels, indicate that VDI results from a collapse of the pore caused by movements of the S6 segments of the four homologous domains (Catterall et al., 2017). This mechanism may also apply to $Ca_V1.1$. Certain Ca^{2+}-antagonistic drugs affect Ca^{2+} release in skeletal muscle by enhancing VDI (Ríos and Pizarro, 1991; Melzer et al., 1995; Zhao et al., 2019). We could show that such antagonists (a phenylalkylamine and a benzothiazepine drug) and γ_1 influenced each other with regard to their effects on VDI and dihydropyridine binding, respectively, qualifying γ_1 as a muscle-intrinsic Ca^{2+} antagonist (Andronache et al., 2007). Consistent with this notion, binding sites for those groups of antagonists have been identified on S6 segments, notably in domains III and IV of cardiac $Ca_V1.2$ and skeletal muscle $Ca_V1.1$ (Catterall and Swanson, 2015; Catterall et al., 2020; Zhao et al., 2019) and for γ_1 in nearby regions, i.e., the III–IV linker and S4 of domain IV of $Ca_V1.1$ (Wu et al., 2016).

The change in VDI was a consistent effect of the γ_1 subunit, even when it was experimentally co-expressed with the α_{1c} subunit of the cardiac L-type channel $Ca_V1.2$ (Sipos et al., 2000) and when studying mature (fibers) and immature skeletal

muscle cells (myotubes; Ursu et al., 2004; Ahern et al., 2001; Freise et al., 2000). In myotubes derived from mice younger than 4 wk, a second effect, a lower Ca^{2+} current amplitude as compared to wild type, has been reported (Freise et al., 2000; Ahern et al., 2001; Held et al., 2002). Both changes could be reversed by transient expression of γ_1 in the knockout myotubes. The difference in amplitude but not in the shifted voltage dependence of inactivation got lost when myotubes were cultured from older animals indicating independence of these two functional modifications (Held et al., 2002). The paper by El Ghaleb et al. (2022) likewise describes a dissociation of γ_1 effects on Ca^{2+} current amplitude and fractional VDI and relates the impact on current size to a structural change in the α_{1s} subunit caused by alternative splicing.

$Ca_V1.1$ splicing changes the functional impact of γ_1

In previous work from the same laboratory, a remarkable change in Ca^{2+} current properties had been discovered when studying (in a $Ca_V1.1$-null myotube-expression system) a splice variant of $Ca_V1.1$ that lacks exon 29 encoding 19 amino acids in the loop linking segments S3 and S4 of homologous domain IV (Tuluc et al., 2009; Benedetti et al., 2015). The characteristics of this variant ($Ca_V1.1e$), which predominates in embryonic muscle cells, are (1) a lower-voltage threshold of activation, (2) a larger maximal conductance, and (3) a more rapid turn-on during step depolarization compared to the adult splice variant $Ca_V1.1a$. Thus, the presence of the 19 amino acid stretch in the IV S3–S4 linker helps to suppress Ca^{2+} influx in adult muscle. One advantage of reducing $Ca_V1.1$ conductance would be to prevent the corresponding electrical current from interfering with the Na^+-based action potentials. Continued expression of the $Ca_V1.1e$ variant in adult muscle is of clinical relevance, as it is correlated with weakness in myotonic dystrophy (Tang et al., 2012).

In the present study (El Ghaleb et al., 2022), a non-muscle system was employed to investigate both variants further. HEK293 cells already constitutively expressing muscle $\alpha_2\delta$-1 and a β subunit (non-muscle β_3) were used to generate two cell lines hosting STAC3 in addition. STAC3 is known to significantly enhance the expression of $Ca_V1.1$ and to bind to the II–III loop of α_{1s} (Polster et al., 2018). These cells were then transfected with plasmids encoding $Ca_V1.1a$ and $Ca_V1.1e$, respectively. Surprisingly, in this setting, the adult splice variant $Ca_V1.1a$ did not show the expected much-lower current density that was observed when $Ca_V1.1$-null myotubes were used for expression (Tuluc et al., 2009), whereas it did exhibit the higher-voltage threshold of activation compared to $Ca_V1.1e$. Some additional determinant for suppressing the current was apparently missing. Because of its structural position adjacent to domain IV of α_{1s} (Wu et al., 2016), γ_1 was considered as a candidate for the missing factor. Indeed, co-expressing γ_1 (Fig. 1 B) reduced the current maximum in the $Ca_V1.1a$ containing cells but not in those expressing $Ca_V1.1e$, therefore re-establishing a similar situation as found in the myotube expression system (Fig. 1 C). Using an elegant fluorescence-labeling approach, the increase in surface expression caused by γ_1 was found to be comparable for both $Ca_V1.1$ variants. Consequently, a difference in channel density incorporated in the plasma membrane was ruled out by the authors as a possible cause for the difference in current density.

The team went on to look for possible determinants enabling direct ionic interactions between γ_1 and α_{1s}. Based on structure modelling, they applied side-directed alanine mutations to remove charged residues on both the S3–S4 linker and the γ_1 subunit. Because these changes lacked the expected result, it is concluded that γ_1 affects the channel conformation by a different allosteric mechanism involving the S3–S4 linker of domain IV that leads to reduced conductance. Obviously, the effect of γ_1 on VDI is independent of this mechanism.

Conclusion

In summary, this study adds further pieces to the EC coupling puzzle. It is in line with previous results obtained using myocytes from young γ_1 knock-out mice (Ahern et al., 2001; Freise et al., 2000; Held et al., 2002) showing that the γ subunit can exert two independent inhibitory effects on the L-type channel, (1) enhancing voltage-dependent inactivation and (2) reducing maximal Ca^{2+} conductance; and it highlights the importance of alternative splicing of α_{1s}. The present results indicate that the change in conductance caused by γ_1 is possible only in combination with the adult splice variant $Ca_V1.1a$. Yet, in mature muscle fibers and in myotubes of adult γ_1-null mice Ca^{2+} current was not significantly affected whereas the absence of γ_1 led to an increase at an earlier developmental stage (e.g., myotubes cultured from neonatal γ_1-null mice; Ursu et al. 2001, 2004; Freise et al., 2000; Held et al., 2002). The reason for this apparent discrepancy requires further investigation. The presence of the ryanodine receptor may be an important factor because of its reciprocal interactions with $Ca_V1.1$ (Huang et al., 2011; Benedetti et al., 2015).

The approach of assembling proteins of the EC coupling machinery in a non-muscle cellular environment is a powerful supplement to targeting these components in muscle cells. Obviously, it would be of interest to see if the present results are invariant to adding further elements of the EC coupling system, primarily RYR1 (and the muscle-specific β_{1a} in replacement of β_3). One also wonders whether there are any consequences of these findings for the Ca^{2+} release control by voltage. Further efforts are required to identify the molecular interactions leading to the differential γ_1 effects on conductance and inactivation. Generating chimeras between γ_1 and one of its non-muscle relatives, as has been done by Arikkath et al. (2003) may be promising. Interesting in this context is also the observation by Held et al. (2002) of a comparable differential response to cAMP analogs pointing to different levels of protein kinase-A–dependent phosphorylation as a cause of the conductance differences seen in their experiments (see above). Finally, the surface expression of $Ca_V1.1$ in the HEK293 cell expression system may permit to determine, by patch clamping, which alterations in single channel properties underlie the observed changes in current density. In any case, using this general experimental approach will hopefully continue to uncover important structure–function relations on the way to a full understanding of the link between muscle electricity and force development.

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