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Symposium 6: Membrane Associated Proteins that Regulate Muscle Contraction

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Chair: Angela Dulhunty

From DHPR to RyR and back again: What lies along the way?

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In skeletal muscle, excitation-contraction coupling depends on a bi-directional interaction between the dihydropyridine receptor (DHPR), a voltage-gated calcium channel in the plasma membrane, and the type 1 ryanodine receptor (RyR1), a homo-tetrameric calcium release channel in the sarcoplasmic reticulum (SR). As a consequence of this bi-directional interaction: (i) the DHPR, in response to depolarization of the plasma membrane, elicits Ca^{2+} release *via* RyR1 without an intervening second messenger, (ii) RyR1 increases the amplitude of Ca^{2+} currents *via* the DHPR, and (iii) DHPRs within the plasma membrane are organized into groups of four (tetrads) such that each DHPR is apposed to a subunit of RyR1. A number of approaches have been used to probe the protein-protein interactions that link the DHPR and RyR1, including expression of cDNAs in muscle cells null for DHPR subunits or for RyR1, biochemical analyses of binding, and application of peptides to isolated RyR1. However, these have not yet produced a consistent picture. We have been examining several alternative approaches for establishing the spatial interrelationships between DHPRs and RyR1. To determine the orientation of DHPRs within tetrads, the fluorescent proteins ECFP or EYFP were fused to sites of α_{1S} or β_{1a}. Between N- and C-terminals, fluorescence resonance energy transfer (FRET) occurred between α_{1S} subunits adjacent within tetrads, but not between adjacent β_{1a} subunits, consistent with the idea that the N- and C-terminals are oriented towards, and away from, the center of tetrads for α_{1S} and β_{1a}, respectively. As a second approach, we have been determining which sites of the DHPR may be in close proximity to RyR1. This is accomplished by attachment of an ECFP-EYFP tandem (“CY”, 23 residue linker) or a biotin acceptor domain (BAD: 70 or 97 residues) to DHPR sites. For CY-β_{1a} and α_{1S}-CY, FRET efficiency increased after expression in dyspedic myotubes (no RyR1) compared to dysgenic myotubes, suggesting that RyR1 may closely appose the β_{1a} N-terminal and α_{1S} C-terminal. In the case of the BAD fusions, expressing myotubes were fixed and permeabilized and exposed to fluorescently labeled NeutrAvidin (~60 kDa). NeutrAvidin had access to BAD at the N- and C-terminals of β_{1a} and to the α_{1S} N-terminal and II-III loop (“peptide A” region). NeutrAvidin did *not* have access to α_{1S}-BAD in dysgenic myotubes, but did have access to α_{1S}-BAD in dyspedic myotubes. Thus, two independent approaches suggest that the C-terminal of α_{1S} may be closely apposed to RyR1.

Regulation of ryanodine receptors from skeletal and cardiac muscle by components of the cytoplasm and lumen

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Contraction in skeletal and cardiac muscle occurs when Ca^{2+} is released from the sarcoplasmic reticulum (SR) through ryanodine receptor (RyR) Ca^{2+} release channels. Ca^{2+}, Mg^{2+} and ATP are key regulators of RyRs. Skeletal (RyR-1) and cardiac (RyR-2) RyRs are modulated differently by these ligands and these differences may underlie the different characteristics of excitation-contraction (EC) coupling in skeletal and cardiac muscle. RyRs are regulated by two Ca^{2+}/Mg^{2+}-dependent mechanisms. They are activated at ~1 µmol/l [Ca^{2+}] and inhibited at mmol/l [Ca^{2+}] in the cytoplasm. Mg^{2+} can inhibit RyRs by binding at the Ca^{2+} activation and inhibition sites. ATP strongly activates RyR-1 in the virtual absence of cytoplasmic Ca^{2+} while in RyR-2, ATP primarily enhances Ca^{2+} activation.

The Ca^{2+} load of the SR is an important stimulator of Ca^{2+} release in skeletal and cardiac muscle. It is known that luminal Ca^{2+} stimulates RyRs but the mechanisms for this are not understood. In cardiac muscle, the release of Ca^{2+} from the SR strongly reinforces RyR activation, a process called Ca^{2+}-induced Ca^{2+} release (CICR). Although CICR should provide an explosive, positive feedback in Ca^{2+} release, the quantity of Ca^{2+} released from the SR has a graded, stable dependence on the magnitude of the Ca^{2+} inflow through the DHPRs.

In order to understand the mechanisms controlling Ca^{2+} release in skeletal and cardiac muscle, single RyRs and RyR arrays were incorporated into artificial lipid bilayers. SR vesicles were prepared from the back and leg muscles of New Zealand rabbits and from sheep hearts. Animals were killed by barbiturate overdose prior to muscle removal. SR vesicles containing RyRs were incorporated into artificial planar lipid bilayers which separated baths corresponding to the cytoplasm and SR lumen. The baths contained 30-230 mmol/l $CsCH_3O_3S$, 20 mmol/l CsCl, 10 mmol/l TES (pH 7.4) plus various amounts of Ca^{2+}, Mg^{2+} and ATP. Channel activity was recorded using Cs^+ as the current carrier.

Several proteins influence the way RyRs are regulated by luminal Ca^{2+}. The luminal proteins, calsequestrin (CSQ), triadin and junctin are associated with RyRs. By dissociating CSQ from RyR-1 it was shown that CSQ inhibits RyRs and can enhance the activating effect of luminal Ca^{2+}. In addition, CSQ dissociates from RyRs when luminal Ca^{2+} exceeds 4 mmol/l. These observations reveal several possible mechanisms by which CSQ can act as a sensor for luminal [Ca^{2+}].

The action of luminal Ca^{2+} on RyR-1 and RyR-2 was strongest in the absence of cytosolic Ca^{2+} and the potency of the luminal Ca^{2+} was enhanced by membrane potentials favouring Ca^{2+} flow from lumen to cytoplasm. At these voltages, RyR-1 activity rose ~5-fold by raising luminal [Ca^{2+}] from zero to ~100 µmol/l while a further increase to mmol/l levels caused ~30% fall from peak activity. RyR-2 had a more exaggerated Ca^{2+} dependence than RyR-1. RyR-2 activity increased ~100 fold between zero and 100 Ca^{2+} and decreased by 90% from peak activity at 1 mmol/l Ca^{2+}. Luminal Mg^{2+} inhibited RyRs by competing with luminal Ca^{2+} for both activating and inhibiting luminal sites. Thus Ca^{2+} and Mg^{2+} regulated RyR activity in a very similar way from both the luminal and cytoplasmic sides.

RyRs showed coupled gating when conditions favoured Ca^{2+} flow from the luminal to cytoplasmic baths. The rate constant for channel opening was increased by the opening of other RyRs in the bilayer. This indicates that the close packed RyR arrays seen in muscle are retained during isolation and bilayer incorporation. In these arrays, luminal Ca^{2+} can permeate an open channel to activate neighbouring RyRs. Coupled openings were followed by a rapid and complete closure of all the channels that occurred within 10 ms. This may be the first inactivation phenomena demonstrated *in vitro* that could possibly explain the rapid termination of Ca^{2+} sparks and the graded control of Ca^{2+} release in cardiac EC coupling.

Structural and functional characterisation of the interaction of the dihydropyridine receptor II-III loop with the ryanodine receptor

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In skeletal muscle, the dihydropyridine and ryanodine receptors (DHPR & RyR) are two membrane proteins that play a central role in excitation-contraction coupling. It is now widely accepted that an interaction between these two proteins is involved in triggering the release of calcium *via* the RyR into the SR. Recent attention has focused on the exact site of interaction and the loop between the second and third repeats of the skeletal DHPR $\alpha 1$ subunit (II-III loop) has been shown to be a critical region interaction site. In an attempt to correlate the structure of this loop with its function our group has previously determined the structure of several functionally active peptides derived from the II-III loop, however structural data for the whole II-III loop at a molecular level has remained elusive.

In this study we focus on the structure/function relationship of the full DHPR II-III loop. This protein has been fully expressed, purified and fully assigned by multidimensional NMR techniques. The conformation of the protein exists as a series of helical elements and turns arranged in an open type structure. The location of the binding site on the RyR has been identified and this fragment has been expressed, purified and refolded. We show by fluorescence experiments that these proteins interact with micromolar affinity. We highlight the regions of the II-III loop that are important for interaction with the RyR.

ClC-1 chloride channel - matching its properties to a role in skeletal muscle

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ClC-1 is a member of a large family of Cl^- channels. It is primarily expressed in skeletal muscle, and is essential for maintaining normal electrical excitability of the muscle. Mutations in the gene encoding ClC-1 have been shown to cause myotonia, an impairment of skeletal muscle relaxation after voluntary contraction. Myotonia results from an increase in muscle excitability that can be detected in electromyograms in the form of myotonic runs. In humans, there are two forms of this disease: autosomal recessive Becker-type myotonia congenita, and autosomal-dominant myotonia or Thomsen disease.

ClC-1, as the other members of this family, is a dimeric, double pored channel, with each monomer forming an individual conduction pathway. ClC-1, which has been studied extensively using electrophysiological techniques, shows a complex gating behaviour. It displays two types of gating — a faster gating process that opens and closes each protopore independently (the ‘fast’ or ‘single pore’ gates), and a slower gating process that closes both protopores simultaneously (the ‘slow’ or ‘common’ gate). Both types of gating depend on permeating anions, and intracellular and extracellular pH. Recent results show that gating of ClC-1 is also regulated by intracellular nucleotides.

Dependence of ClC-1 on pH and ATP makes it a likely contributor to a complex mechanism that regulates muscle contractility in exercise and fatigue. The exact role of ClC-1 in muscle physiology, however, is yet to be established.