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with methyl-β-cyclodextrin (*MCD*) and then measured SOCE in these myotubes. Reduced SOCE was observed following MCD extraction, similar to the limited SOCE we previously observed in young *mg29-/-* and wild-type aged muscle fibers. Further studies will determine if mutation of conserved residues in the MARVEL domain of MG29 modulate SOCE function in, and the lipid composition of, skeletal muscle cells.

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Differential Role of Calsequestrin Isoforms on Calcium Entry in Skeletal Muscle FDB Fibres

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JP45 is a sarcoplasmic reticulum membrane protein interacting with Cav1.1 and calsequestrin (CASQ). The JP45/CASQ complex enhances Cav1.1 channel calcium activity to promote calcium entry to maintain an adequate level of releasable SR calcium during tetanic contraction. In this study, we investigated FDB fibres from JP45/CASQ1 double KO (DKO), JP45/CASQ2 double KO (DKO) and JP45/CASQ1/CASQ2 triple KO (TKO) mice to assess the role, if any, of JP45 and CASQ2 on calcium entry. Mag-Fluo-4 was used to monitor rapid Ca^{2+} transients evoked by a 0.5 ms pulse at supramaximal field stimulation. In the presence of 1.8 mM Ca^{2+} in the extracellular solution, the peak calcium signal in FDB fibers from WT and JP45/CASQ2 DKO was similar, in JP45/CASQ1 DKO and JP45/CASQ1/CASQ2 TKO FDB fibres, peak calcium was lower compared to WT. The half time of the decay of the Ca^{2+} transient in FDB fibres from JP45/CASQ1 DKO, JP45/CASQ2 DKO and JP45/CASQ1/CASQ2 TKO was significantly slower compared to WT. Such a difference disappeared when Ca^{2+} was substituted by 100 μMLa3+ in the extracellular medium. The effect of La3+ was more evident upon stimulation of FDB fibres with repetitive 0.5 ms pulses at 100 Hz. The calcium signal evoked by a train of pulses at 100 Hz for 300 ms was strongly inhibited by La3+ in fibers from JP45/CASQ1 DKO and JP45/CASQ1/CASQ2 TKO, but negligibly affected in WT and in JP45/CASQ2 DKO. Mn2+ quenching experiments in fura-2 loaded FDB fibers from JP45/CASQ1 DKO and JP45/CASQ1/CASQ2 TKO show that calcium influx during tetanic stimulation is blocked by 50 μM nifedipine an inhibitor of Cav1.1 calcium currents. These results show that ablation of CASQ2 in a JP45 KO background does not affect calcium entry via Cav1.1.

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Elevation of NO Increases Ca^{2+} Entry and Resting Ca^{2+} and Na^{+} Concentrations in Skeletal Muscle Cells

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Protein S-nitrosylation is a reversible post-translational modification and can have profound effects on protein function in skeletal muscle. S-nitrosylation has been proposed to exert regulatory effects on ion channels such as RyR1 and TRPCs, by modifying the redox state of critical thiols. Increased nitric oxide (NO) levels may play a crucial role in the alteration of intracellular resting $[Ca^{2+}]r$ and [Na+]r homeostasis in skeletal muscle. The present work aimed to characterize the contribution of NO using the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP) on intracellular Ca^{2+} and Na+ homeostasis in Wt myotubes. In quiescent Wt myotubes 100 μM SNAP increased Ca^{2+} entry, $[Ca^{2+}]r$ and [Na+]r. These effects could be blocked with either Gd3+ or BTP-2 and partially reversed by DTT, a known inhibitor of S nitrosylation, suggesting that the effect of SNAP was through a reversible increase in the TRPC channel activity. Furthermore, we found that RyRs are not the principal target for S-nitrosylation because SNAP incubation increases $[Ca^{2+}]r$ and [Na+]r in RyR1/RYR3 null myotubes. It is known that NO production is elevated in mdx muscle. Here we found that DTT or BTP-2 significantly reduced $[Ca^{2+}]r$ in mdx myotubes and DTT was able to restore a normal Ca^{2+} entry. We then looked at the effects of SNAP on K^{+} induced Ca^{2+} release and SR Ca^{2+} content in WT and mdx myotubes. SNAP did not significantly decrease sarcoplasmic reticulum Ca^{2+} content in either WT or mdx myotubes but reduced the magnitude of the Ca^{2+} transient induced by K^{+} in Wt but not in mdx myotubes. These results suggest that nitrosative modifications play a key role regulating TRPC activity in muscular dystrophy and their role in the Ca^{2+} and Na+ overload seen in dystrophic muscles.

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A Compartment Model to Investigate the Roles of SR Membrane Channels during E-C Coupling

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During excitation-contraction (E-C) coupling, efficient Ca^{2+} release by ryanodine receptor (RyR) channels from the sarcoplasmic reticulum (SR) requires that counterions move into the SR. These counterion fluxes are thought to effectively clamp the SR membrane potential (Vm) near 0 mV, sustaining the trans-SR Ca^{2+} driving force. Likewise, Ca^{2+} uptake by sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps is facilitated by the existence of counterion fluxes. The SR also contains TRIC (SR K^{+} channels) and Cl^{-} channels in addition to RyRs and SERCA pumps. Thus, SR Vm during and between RyR-mediated Ca^{2+} releases is determined by multiple channels whose function spatially and temporally varies. The contribution of each channel to SR Vm control is unclear and debated. We used a compartment model to examine counterion (K^{+}, Cl^{-} and Mg^{2+}) fluxes across the SR membrane during E-C coupling. The model allowed us to study the roles of the different ion channels, as well as the time evolution of SR Vm and local changes in intra-SR/cytosolic ion concentrations. Our results show that RyR Ca^{2+} efflux is indeed electrically balanced by K^{+}, Cl^{-} and Mg^{2+} countercurrents. When RyRs are open, Mg^{2+} initially moves into the SR and then surprisingly moves back out. Redundant pathways exist to carry K^{+} countercurrent (RyR and TRIC) during SR Ca^{2+} release. When RyRs close, the K^{+} must exit through TRIC channels to maintain resting SR Vm at 0 mV. Our analysis indicates TRIC channels are essential for resting SR K^{+} re-equilibration that is needed to re-establish the normal trans-SR Ca^{2+} driving force.

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Model of a Propagating Action Potential (AP) in a Twitch Skeletal Muscle Fiber Mounted in a Double-Vaseline-Gap Chamber - Currents Involved in Shaping the AP

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Following the peak of the action potential (AP) in a twitch fiber from frog, a very rapid repolarization occurs to an approximately constant plateau level (the ''after potential'') ~30 mV more positive than the resting potential (RP) lasting several msecs before returning to the RP (Adrian & Peachey 1973; J. Physiol. 235:103-131). In order to help understand how currents shape the AP with such fibers mounted in a double-Vaseline gap chamber, we developed a finite-difference model of a propagating AP for this preparation. The model includes time- and voltage-dependent gating of the Na and K currents based on the Hodgkin-Huxley (HH) models. The assumed properties of the Na, K, and leak conductances for the surface membrane are from Table 1B of the above reference. The specific conductances for these currents in the T-system were assumed to be the same as those for the surface. An access resistance for the T-system was adjusted to give an ~1-ms delay between the surface AP and that in the T-system. The model results indicate that the ''after potential'' can be explained by Na current resulting from recovery from inactivation - a new explanation for the ''after potential'' to our knowledge. Modelled APs with a Ca-activated K current, $I_{K(Ca)}$, replacing the HH K current in the model are consistent with measured APs at different Ca contents (Lamboley et al., 2013; Biophys. J. 104(2):290a). The model supports a relatively straightforward way for analyzing these results leading to our conclusion that $I_{K(Ca)}$ is the main determinant of the rapid repolarization phase of the AP. Consequently, $I_{K(Ca)}$ comprises a very important negative feedback mechanism for controlling Ca^{2+} release from the sarcoplasmic reticulum.

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CaMKII-Dependent Phosphorylation of RyR2 Causes Domain Unzipping and Reduced Calmodulin Binding, But Dantrolene Reverses These Effects

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Increased diastolic SR Ca^{2+} (Ca) leak via cardiac ryanodine receptors (RyR2) can cause cardiac arrhythmias and dysfunction in heart failure (HF). CaMKII-dependent RyR2 phosphorylation (at S2814) is thought to be a critical

promoter of leaky RyR2 in HF. A conformational change in RyR2 (''domain unzipping'') observed in HF can also increase SR Ca leak. Calmodulin (CaM) quiets RyR2 gating, and the CaM affinity for RyR2 is reduced in HF. Further, we have shown reciprocal negative coupling between CaM binding to RyR2 and accessibility of the unzipping RyR peptide DPc10 (Oda et al. 2013). That is, unzipping with DPc10 reduced CaM binding and increased Ca leak, whereas CaM quieted leak and inhibited DPc10 access. Here, we tested the hypothesis that RyR2 phosphorylation by CaMKII increases Ca leak by favoring the same unzipped-conformation that exhibits reduced CaM affinity. We either activated endogenous myocyte CaMKII or used knock-in mice expressing non-phosphorylatable RyR2-S2814A or phosphomimetic RyR2-S2814D. We used FRET to directly detect binding of fluorescently labeled DPc10, FKBP12.6, and CaM to RyR2 in permeabilized cardiomyocytes. In phosphomimetic S2814D vs. S2814A myocytes, CaM-RyR2 affinity was reduced 3-fold (Kd= 72 $\pm$ 9 nM vs. 20 $\pm$ 2 nM), but FRETmax was unaltered. Access of DPc10 was also 2-fold faster in S2814D versus S2814A myocytes. Dantrolene (1 μM), which stabilizes the zipped conformation, restored CaM-RyR2 binding affinity in S2814D, slowed DPc10 wash-in, and lowered Bmax. Forced RyR2-S2814D saturation with 500 nM CaM also slowed DPc10 wash-in and reduced Bmax. We conclude that RyR2 phosphorylation by CaMKII causes pathological conformation changes (unzipping) that reduce RyR2-CaM affinity and ability to quiet SR Ca leak. Dantrolene effectively reverses these effects.

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Determination of the Junctional Space [Ca^{2+}] Set by Ryanodine Receptor Leak in Slow- and Fast-Twitch Muscle Fibres

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The tubular (t-) system of skeletal muscle forms a junction with the sarcoplasmic reticulum (SR), with some 12nm between the membranes. In the resting muscle, [Ca^{2+}] within the small volume bound by the junctional membranes will be determined by the leak of Ca^{2+} through the SR ryanodine receptors (RyRs), the Ca^{2+} handling ability of the t-system and diffusion of Ca^{2+} from the junctional space (js). The $[Ca^{2+}]_{js}$ is expected to be higher than $[Ca^{2+}]_{bulk}$ with a standing gradient set between the RyRs and SR Ca^{2+}-pumps. The value of $[Ca^{2+}]_{js}$ is unknown but has implications for signalling cascades initiating in this nanodomain. To determine $[Ca^{2+}]_{js}$ we exploited the fact that t-system Ca^{2+} uptake activity will be set by $[Ca^{2+}]_{js}$. T-system Ca^{2+}-uptake activity was tracked with rhod-5N trapped in the t-system of mechanically skinned fibres of rat slow- and fast-twitch muscles on a confocal microscope. Chronic depletion of $[Ca^{2+}]_{SR}$ with caffeine reduced $[Ca^{2+}]_{t\text{-}sys}$ to 0.1 mM via chronic activation of store-operated Ca^{2+} entry. We then exposed Ca^{2+}-depleted preparations to 50-800nM $[Ca^{2+}]_{cyto}$ in 50mM EGTA to allow observation of t-system Ca^{2+} uptake rates at known $[Ca^{2+}]_{bulk}$. Experiments were repeated in the presence of 1mM tetracaine to block RyR Ca^{2+} leak and allow $[Ca^{2+}]_{js}$ to equilibrate with $[Ca^{2+}]_{bulk}$. Rhod-5N signals and $[Ca^{2+}]_{t\text{-}sys}$ were calibrated and t-system Ca^{2+} fluxes were derived. $[Ca^{2+}]_{bulk}$ and peak t-system Ca^{2+} fluxes were fitted by Hill curves. Vmax was significantly depressed in slow- compared to fast-twitch fibres. The k_D for both fibre types was right-shifted by tetracaine. It followed that at 100nM $[Ca^{2+}]_{bulk}$, $[Ca^{2+}]_{js}$ was 165 and 220nM in slow and fast-twitch fibres, respectively. These results show that t-system Ca^{2+} fluxes can be used as a nanodomain sensor of RyR leak.

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Effect of Calcium in the Cardiac Ryanodine Receptor Inter-Molecular Contacts

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The cardiac ryanodine receptor (RyR2) is the major calcium (Ca^{2+}) release channel on the sarcoplasmic reticulum (SR) in cardiomyocytes. The RYR2 gene encodes a large ~565 kDa protein that forms homotetramers with molecular weight ~2.26 MDa. They are organized in a large squared cytoplasmic domain of 290 x 290 x 100 Å connected to a smaller, square tapering prism shaped transmembrane domain of 120 x 120 x 60 Å. The corners of the cytoplasmic domain are named the clamp domains and the flat, slab-shaped sides define the handle domains.

The dyads are structural elements formed by the close apposition of the plasmalemma and the junctional sarcoplasmic reticulum (jSR), while a couplon is the functional element within the dyad formed by juxtaposition of cardiac L-type voltage-gated Ca^{2+} channels (Cav 1.2) in the plasmalemma with RyR2 in the adjacent jSR. The clustering of RyR2 into functional Ca^{2+} release units is central to current models for cardiac excitation-contraction (E-C) coupling.

Classical studies done in dyads suggested RyR2 inter-molecular contacts through their clamp domains, but more recent studies propose multiple and complex RyR2 arrangements, which can be modulated by diverse local factors like Mg2+ concentration, phosphorylation and redox state.

In the present study the effect of the Ca^{2+} in RyR2-RyR2 contacts was investigated. RyR2s purified from pig cardiac muscle were incubated in 100μM Ca^{2+} or in 2 mM EGTA, negatively stained, imaged on the electron microscope and image-processed, yielding well-resolved images of RyR2 dimers. In addition to the clamp-clamp domain interactions, we find clamp-handle domain contacts in multiple configurations, and that their relative proportions depend on the presence or absence of Ca^{2+}. These different RyR2-RyR2 interactions could have a vital role in various physiological and pathological conditions.

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Malignant Hyperthermia Susceptibility Mutation Ca_v1.1 R174W Dramatically Alters RyR1 Single Channel Function

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Nearly 200 variants in the gene encoding the skeletal muscle RyR1 Ca^{2+} channel are associated with MHS but only 30 were demonstrated to perturb channel function. We previously showed that mouse MHS mutations RyR1_R163C or RyR1_T4826I have abnormally high open probabilities and elevated [^{3}H]ryanodine binding when compared to wild type. Recently, a small number of MHS families were shown to express mutations, not in the RyR1, but rather in the gene encoding the skeletal muscle L-type Ca^{2+} channel (Ca_V1.1). We therefore generated a knock-in mouse line expressing Ca_V1.1_R174W that confers MHS in human kindred. Homozygous (HOM) mice survive, but trigger with fulminant MH when exposed to halogenated volatile anaesthetics, have chronically elevated resting myoplasmic Ca^{2+}, and lack L-type Ca^{2+} current in adult *Flexor digitorum brevis* fibers (Bannister et al, this meeting). It is unknown however if MHS mutations residing within Ca_V1.1 are capable of modifying RyR1 structure/function thereby contributing to disrupted intracellular Ca^{2+} homeostasis. To address this question, SR membranes were prepared from the skeletal muscles of age-matched WT and HOM Ca_v1.1_R174W for biochemical and electrophysiological analysis. Unexpectedly, we found significantly abnormal properties of HOM as compared to WT in: *(1)* enhanced [^{3}H]Ry binding; *(2)* elevated levels of RyR1/FKBP12 expression, and *(3)* exceptionally higher P_o with channels in BLM at all $[Ca^{2+}]_{cis}$. Even at $[Ca^{2+}]_{cis}$=120nM, an inhibitory condition to RyR1 in normal WT muscle fibers, RyR1 channels from Ca_V1.1_R174W muscle maintained a conformation accessible for ryanodine binding. These results demonstrate that a MHS mutation residing outside of RyR1 is capable of persistently modifying RyR1 channel function. These findings have significant implications about the molecular mechanisms that lead to MHS and fulminant MH. (Grant supports: AR052534 to PDA, KGB, PMH, CFA, INP; AR055104 and MDA277475 to KGB).

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Effects of MH and CCD Mutations in the Central Region on RyR1 Channels

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Type 1 ryanodine receptor (RyR1) is a Ca^{2+} release channel in the sarcoplasmic reticulum and the major target for muscle diseases, e.g., malignant hyperthermia (MH) and central core disease (CCD). It is widely believed that MH and CCD mutations cause hyperactivation of the Ca^{2+}-induced Ca^{2+} release (CICR), resulting in abnormal Ca^{2+} homeostasis in skeletal muscle. However, it remains unclear how the disease-associated mutations affect CICR. We have recently characterized several disease-associated mutations in the amino-terminal region by live-cell Ca^{2+} imaging and [^{3}H]ryanodine binding and found that these mutations divergently affect the gain (i.e., peak activity) and the sensitivity to activating Ca^{2+} of CICR. In this study, we extended this approach to 15 MH and MH/CCD mutations in the central region (1592-2508). The disease-associated mutations increased the gain and the sensitivity