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 ± 9 nM vs. 20 ± 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