tetrad array. cDNA constructs carrying wt-β1a and C-term truncated clones were transiently expressed in mouse β1-null myotubes and tested for their ability to restore depolarization-induce Ca^{2+} release. Cells were then fixed and freeze-fractured to assess DHPR tetrad arrangement. Our data show that expression of wt-β1a fully restores EC-coupling, as well as normal DHPR tetrads arrays. Construct β14, lacking 14 aa of the C-terminal tail of β1a, still supports normal EC-coupling and also shows normal arrangement of tetrads. On the other hand, β14 clone containing several Leu/Ala mutations (β14L/A) that prevent EC-coupling failed to restore tetrad arrays. Immunofluorescence staining confirmed that all clones were expressed and targeted to the plasmalemma. These results suggest that 1) C-term tail of β1a plays an essential role in permitting and/or maintaining the precise positioning of four DHPRs relative to the four RyR subunits; 2) DHPR positioning in tetrads is essential to EC-coupling and 3) the molecular structure (aa sequence) of the C-term tail is relevant for the role of β1a in the DHPR-RyR structural and functional relationship.

Supported by NIH Grants: 5K01AR054818-02 (to CFP) and AR055104 to KG. Beam (CFA subcontract).

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Interactions between Dihydropyridine β1A Subunit and Ryanodine Receptor Isoforms

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Skeletal excitation contraction (EC) coupling requires a physical interaction between the L-type voltage gated dihydropyridine receptor (DHPR) in the transverse tubule membrane and the type 1 ryanodine receptor (RyR1) in the sarcoplasmic reticulum membrane. The C-terminus of the DHPR β_{1a} subunit influences EC coupling in skeletal myotubes (Beurg et al., Biophys J. 1999;77:2953-67, Sheridan et al., Biophys J. 2003;84:220-37, Sheridan et al., Biophys J. 2004;87:929-42). This may be through a direct interaction with RyR1, as we identified a hydrophobic interaction with L^{496}, L^{500} and W^{503} in the last 35 residues of β_{1a} that increases RyR1 channel activity in phospholipid bilayers (Rebbeck et al., Biophys J. 2011;100;922-30, Karunasekara et al., FASEB J. 2012). Additionally, the K^{3495}KKRR_ _R^{3502} motif in a RyR1 fragment (M^{3201}-W^{3661}) pulls down β_{1a} and facilitates EC coupling (Cheng et al., PNAS USA. 2005;102:19225-30). Our preliminary data indicate that substitution of these 6 basic residues with glutamines, abolishes the effect of β_{1a} on the full length RyR1. We also show that β_{1a} increases RyR2 activity in a similar manner to RyR1 except for significantly less activation of RyR2 by 10nM β_{1a} (10 and 100nM β_{1a} subunit increased RyR2 activity by 1.8- and 2.8-fold, in contrast to 2.6- and 2.8-fold with RyR1). Curiously, this reduced activation of RyR2 by 10nM β_{1a} is similar to lesser activation of the embryonic alternative spliced (ASI(-)) RyR1 isoform by 10nM β_{1a}, that lacks residues A^{3481}-Q^{3485}, compared with activation of adult (ASI(+)) RyR1 isoform by 10nM β_{1a}. Notably, as rabbit RyR2 lacks 4 of the 5 ASI residues. We conclude that β_{1a} may bind to a hydrophobic pocket conserved in the RyR1 and RyR2 and that this region is influenced by the presence of the alternatively spiced ASI residues and the polybasic K^{3495}-R^{3502} motif.

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3D Structural Illumination Microscopy of the Skeletal Muscle Excitation-Contraction Coupling Macromolecular Complex

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To study the structural organization of protein components associated with the membrane compartments involved in skeletal muscle excitation-contraction coupling, we stained enzymatically dissociated mouse FDB fibers with commercially available specific antibodies. Imaging was based on 3 dimensional structured illumination microscopy (3D-SIM, Zeiss Elyra system using a 63x/1.4 Plan-Apochromat lens and an EMCCD camera) or conventional confocal laser scanning microscopy for comparison (Zeiss LSM700). We investigated the localization pattern and distribution of RyR, Cav1.1 and of other protein components involved in excitation-contraction coupling. As expected, imaging of FBD fibers stained with anti -RyR Ab by conventional confocal microscopy revealed highly ordered band-line structures regularly distributed along the sarcomeres which strongly overlapped with Cav1.1. Analysis of the same FDB preparation at higher resolution (Dx-y 110-130 nm and Dz 280-340 nm) by 3D-SIM revealed a more distinct pattern of distribution. RyRs form 100 nm clusters, which are regularly separated and distributed along the longitudinal axis of the fiber. Anti-Cav 1.1 Ab stained clusters having a sarcomeric distribution co-localizing with RyRs. Cav 1.1 Ab also stained structures adjacent to but not overlapping with RyRs. These results suggest the power of the 3D-SIM approach to gain further insight into the structural organization of sarcotubular membranes in normal and diseased condition.

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Super-Resolution Localization and Distribution of Proteins within the Mammalian Couplon

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We have used 3D dSTORM to characterize the distribution and localization of the ryanodine receptor (RyR), the L-type calcium channel (Cav1.2) and the sodium-calcium exchanger (NCX) within the couplon of the rat cardiomyocyte. The images have a resolution of 20nm in X and Y and 50 nm in Z, and cover areas of up to 1200 μm^2 in XY with depths of up to 700nm in Z. In the case of RyR, hundreds of individual clusters could be identified and characterized. The clusters varied greatly in both size and structure; their internal structure showing little evidence of the checkerboard arrangement that has been thought to be predominant. RyR clusters were identified both in the transverse and axial tubules and in some cases could be seen to be separated by a gap typical of the width of a t-tubule. In general, the clusters of Cav1.2 were smaller and far denser than their RyR counterparts, with their centers appearing to be tightly packed. NCX was much more widely distributed than either RyR or Cav1.2 and formed a dense carpet along the cell surface with little clustering or identifiable detail. While small isolated clusters of NCX were present in the tubular system, there were other regions where the labeling was more widespread and the clusters were poorly defined.

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TRIC-B Channels Exhibit Labile Gating Properties; Evidence from TRIC-A Knockout Mice

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Trimeric intracellular cation channels (TRIC-A and TRIC-B) are located in the sarcoplasmic/endoplasmic reticulum (SR/ER) of most cells. Identifying the distinct biophysical properties of TRIC-A and TRIC-B is difficult because both channels are present in most tissues, yet this is crucial for delineating their individual physiological roles. Skeletal muscle SR vesicles (LSR) from TRIC-A knockout mice were incorporated into artificial membranes under voltage-clamp conditions as previously described [Pitt et al., 2010, *Biophys. J.* **99**, 417-426] and single-channel recordings of native TRIC-B were obtained in symmetrical solutions of 210 mM K-PIPES, pH 7.2. The maximum single-channel conductance of TRIC-B was 197 ± 2 pS (n=32; SEM). TRIC-B channels always exhibited sub-conductance gating states and while these were of a variable nature, the predominant sub-conductance levels were found at 156 ± 3 pS (n=17; SEM), 125 ± 2 pS (n=19; SEM), 96 ± 2 pS (n=19; SEM) and 62 ± 2 pS (n=27; SEM). TRIC-B channel gating was voltage-dependent and channels were inhibited at negative holding potentials. For example, the probability of dwelling in the full open channel level was 0.0478 ± 0.0194 at +30 mV but only 0.0010 ± 0.0008 at −30 mV (n=6; SEM; *p<0.05). Application of 300 mM KCl to the cytosolic channel side produced a parallel shift in the current-voltage relationship and a shift in the reversal potential to approximately −20 mV indicating that TRIC-B is not permeable to anions. Our study demonstrates that the single-channel properties of TRIC-B channels are exceptionally labile. This intrinsic variability may be important for enabling flexible physiological regulation of monovalent cation fluxes across the SR membrane.

Supported by the British Heart Foundation and the Japan Society for the Promotion of Science.

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Cardiac Ca^{2+} and Free Radical Disturbances in Mice with Arthritis

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Rheumatoid arthritis (RA) is a common inflammatory disease that afflicts ~1% of the population and is more common in women than in men. Cardiovascular disease is the leading cause of premature mortality in patients with RA. Still,

little is known about cellular mechanisms of cardiac dysfunction in inflammatory diseases. Hearts from patients with RA show signs of increased inflammatory activity in microvessels as well as in the cardiomyocytes themselves. Thus, the cardiovascular dysfunction in RA patients is likely to be due both to defective microcirculation and to direct effects on cardiomyocyte contractility. Here we show that cardiomyocytes from mice that displays many pathological characteristics of human RA (collagen induced arthritis; CIA) have altered cytosolic and mitochondrial Ca^{2+} handling together with decreased myofibrillar Ca^{2+} sensitivity, which suggest decreased contractile function. Neuronal nitric oxide synthase (nNOS) can produce nitric oxide and O_2^{-}*, which in combination can form peroxynitrite (ONOO-*) and result in oxidative stress. Total nNOS protein expression and nNOS associated with the major intracellular Ca^{2+} release channel, RyR2, were both increased in CIA compared with control hearts. This can cause increases in free radicals and oxidative stress and CIA hearts show mitochondrial O_2^{-}* accumulation with electrical stimulation and increased protein carbonylation, which are indicators of oxidative stress. The observed changes in Ca^{2+} and oxidative stress in hearts from mice with arthritis can, if sustained, cause arrhythmias and sudden cardiac death. Thus, our data implicate that stress-mediated signaling results in intricate interactions between mitochondrial free radical production, cellular Ca^{2+} handling and contractility and that these interactions are altered in cardiomyocytes of RA mouse models.

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Determinants of Abnormal Excitation-Contraction Coupling in Cardiomyocytes from Patients with Hypertrophic Cardiomyopathy

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Diastolic dysfunction and ventricular arrhythmias are the clinical hallmarks of hypertrophic cardiomyopathy (HCM). Studies on animal models of HCM highlighted the leading role of excitation-contraction coupling (ECC) abnormalities as cellular determinants of increased arrhythmogenicity and abnormal contraction. However, mechanisms of ECC anomalies have never been studied on human HCM samples, thereby limiting the development of novel therapies.

We aim to characterize the HCM-related ECC changes in cardiomyocytes isolated from the interventricular septum of HCM patients who underwent surgical myectomy for symptomatic obstruction, compared to non-failing non-hypertrophic surgical patients (controls).

Compared to controls, HCM cardiomyocytes displayed slower kinetics of Ca^{2+}-transients (duration at 90% decay= 719 ± 50ms vs. 419 ± 55ms at 1Hz) and higher diastolic Ca^{2+} (at 1Hz, 226 ± 16nmol vs. 139 ± 7nmol), especially at high stimulation rates. Moreover, the rate of spontaneous Ca^{2+} releases leading to delayed after depolarizations (DADs) was 5-fold higher.

Several mechanisms underlie such changes in HCM cardiomyocytes:

1)severe reduction of t-tubular density, leading to prolonged Ca^{2+}-transients raising phase;

2)reduced expression of SERCA, contributing to slower Ca^{2+}-transient decay;

3)increased Ca^{2+} current amplitude with prolonged inactivation, associated with 2-fold increased Calmodulin kinase II (CaMKII)-dependent phosphorylation of Ca^{2+} channel;

4)3-fold increase of CaMKII-dependent phosphorylation of ryanodine receptors (RyR2) and 15% higher SR Ca^{2+}-load, leading to increased RyR2 spontaneous activity and DADs;

5)enhanced late Na^{+} current (I_{NaL}) and the resulting increased intracellular Na^{+}, leading to altered function of Na^{+}/Ca^{2+} exchanger and reduced NCX-mediated Ca^{2+} extrusion;

The latter was tested by inhibiting I_{NaL} with ranolazine: via reduction of intracellular Na^{+}, ranolazine partially counteracted ECC abnormalities, leading to decreased diastolic Ca^{2+}, faster Ca^{2+}-transients, lower SR Ca^{2+}-load and rate of DADs.

Albeit multiple abnormalities contribute to ECC changes in human HCM, I_{NaL} inhibition with ranolazine represents a possible therapeutic option to reduce arrhythmias and diastolic dysfunction.

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Post-Myocardial Infarction Exercise Training Reverses Cardiac Excitation-Contraction Coupling Abnormalities by Activating Cytoplasmic CaMKII

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Post-myocardial infarction (MI) heart failure (HF) leads to abnormal excitation-contraction-coupling (ECC) causing cardiac dysfunctions attributable to hyper-activation of CaMKII. In contrast, exercise improves ECC in post-MI HF, but whether this is through CaMKII remains unknown. Our purpose was to test whether exercise reverses ECC dysfunction in post-MI HF through modulation of CaMKII. Permanent coronary artery ligation leading to HF was induced in Wistar rats. Four weeks after, regular treadmill running started (MI-TRN, n=14). Sham-operated (SHAM, n=12) and sedentary MI-HF (MI-SED, n=16) served as controls. MI-HF reduced exercise capacity 16% ($p<0.01$); exercise normalised this. Contractile function of twitch-stimulated cardiomyocytes was assessed using edge-detection and Fura-2 fluorescence microscopy with and without the CaMK inhibitor AIP (5uM). MI reduced contraction 40% ($p<0.01$) and increased contraction-relaxation times. This was explained by reduced Ca^{2+} transient amplitude and increased Ca^{2+} rise-decay times. Exercise corrected contraction and contraction-relaxation times, and improved Ca^{2+} handling. AIP abolished the exercise-induced improvement in contraction and Ca^{2+} transient amplitude (both $p<0.01$) and impaired relaxation and Ca^{2+} transient decay times ($p<0.01$) more in MI-TRN than MI-SED. Spontaneous Ca^{2+} waves and sparks were linescan confocal imaged in Fluo-3-loaded cardiomyocytes at normal (1.8mM) and high (5mM) extracellular Ca^{2+}. MI increased wave frequency 65% and amplitude 21% ($p<0.01$); this was more pronounced at high Ca^{2+} and reversed by exercise. MI and AIP had no effect on sparks but they were elevated by exercise ($p<0.01$). AIP had no effect on Ca^{2+} wave frequency in MI-SED but increased frequency and amplitude in MI-TRN ($p<0.05$). In conclusion, CaMKII modulated exercise-induced improvements in cardiomyocyte function following MI-HF. In particular, exercise improved sarcoplasmic reticulum (SR) Ca^{2+}-uptake and SR-loading by activating CaMKII, whereas Ca^{2+} release parameters were less affected. This suggests that exercise activated cytoplasmic, but not dyadic CaMKII.

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Calcium Handling Deficits Induced by Systemic Inflammatory Mediators in Rat Cardiomyocytes

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Background. Cardiac dysfunction in sepsis is thought to be caused by detrimental effects of systemic inflammatory mediators on cardiac muscle. Here we studied the effects of prolonged (18-24h) exposure of adult rat ventricular myocytes (ARVM) to bacterial lipopolysaccharide (LPS) and cytokines (tumor necrosis factor, TNF, interleukin 1, IL-1 and 6, IL-6).

Methods. We measured ARVM sarcomere shortening (SS) and calcium (Ca^{2+}) transients (ΔCa_i, with fura-2AM) elicited by external pacing at 37 °C.

Results. SS decreased after incubation with LPS (100 μg/ml), IL-1 (100 ng/ml) and IL-6 (30 ng/ml), but not with lesser doses of these mediators, or TNF (10 −100 ng/ml). A combination of LPS (100 μg/ml), TNF, IL-1 and IL-6 (each 100 ng/ml; ''Cytomix-100'') decreased SS to 58 ± 5% and ΔCa_i to 81 ± 4% of control ($p<0.05$; $n>38$ cells). Sarcoplasmic reticulum (SR) Ca^{2+} load (Ca_{SR}, measured with caffeine) was unchanged by Cytomix-100, however, SR fractional release ($\Delta Ca_i/Ca_{SR}$) was decreased to 78 ± 5% of control ($p<0.05$, $n>18$ cells). SR Ca^{2+} pump (SERCA) activity (as the time constant of Ca^{2+} decay) and expression (immunoblotting), and the expression of phospholamban were not changed by Cytomix, whereas Ca^{2+} entry into the cell (via L-type Ca^{2+} channels) was decreased (64 ± 4% of control, $p<0.05$, $n>24$ cells). Cytomix induced a 24 ± 8 fold increase in nitric oxide (NO) synthase 2 (NOS2) expression ($n=6$, $p<0.05$). The decrease in SS induced by Cytomix-100 was partially prevented by the NOS inhibitor L-NAME (SS was 81 ± 9% of control) and the radical oxygen species (ROS) scavenger Mn-TMPyP (SS was 66 ± 5 % of control, $p<0.05$, $n>18$ cells).

Conclusions. Exposure of ARVM to a mixture of LPS and inflammatory cytokines inhibits Ca^{2+} handling through activation of NO and ROS pathways.

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Anthracycline-Induced Dysfunction of Cardiac SR Ca^{2+} Handling - The Role of Thiol Oxidation

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Anthracyclines are powerful chemotherapy agents, whose use is limited due to the onset of potentially fatal cardiotoxicity. This cardiotoxicity manifests as acute and chronic effects including heart failure and arrhythmia with multifaceted etiology, involving ROS generation, altered Ca^{2+} handling and the