Angeli's salt (AS, 0.5 mM), Ca^{2+} transient amplitude (-15 ± 5 *vs* 17 ± 7% in WT, p<.001), Ca^{2+} transient decline, and caffeine-induced SR Ca^{2+} release were unchanged in PLN-/- myocytes. However, PLN-/- myocytes still displayed, albeit blunted, a significantly increased SS response (48 ± 10 *vs* 80 ± 17% in WT, p<.05) likely due to HNO-evoked myofilament Ca^{2+}-sensitizing effects. When WT SR vesicles were incubated with 0.25 mM AS, the Ca^{2+} uptake rate was increased (0.32 *vs* 0.67 s^{-1}; p<.001; n=8). No stimulation was observed in vesicles from PLN-/- mice. AS/HNO increased dephosphorylation in SERCA2 co-expressed with WT PLN (0.47 *vs* 4.64 s^{-1}), but failed to activate dephosphorylation in microsomes expressing SERCA2a and Cys$^{->}$Ala PLN (0.21 *vs* 0.18 s^{-1}). We conclude that PLN is essential for the HNO-mediated increase in Ca^{2+} uptake by SERCA2a, and that modification of PLN thiols is central to this modulation. Enhancing Ca^{2+} uptake by HNO may benefit heart failure patients that often display depressed SR function.

2650-Pos Board B620

Junctate Interacts with SERCA2a in Mouse Cardiomyocytes

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Junctate is a newly identified sarco(endo)plasmic reticulum (SR/ER) associated Ca^{2+} binding protein, which is an alternative splicing form of the same gene generating aspartyl β-hydroxylase and junctin. Recently, we showed evidence that junctate over-expressing transgenic mice led to altered SR functions and development of severe hypertrophy (*J. Mol. Cell. Cardiol.* 44:672-682, 2008). The present study was undertaken to investigate the direct interaction of junctate with SERCA by various molecular methods. The studies of co-immunoprecipitation and immunolocalization using anti-SERCA2a and anti-junctate antibodies showed that junctate and SERCA2a were co-localized in the SR of mouse cardiomyocytes. GST- pull down assay also showed the direct interaction between junctate and SERCA2a. By deletion mutation experiments, we have found that the C-terminal region of junctate (79-278 aa) is the site for the interaction with SERCA2a and the interaction is inhibited by millimolar Ca^{2+} concentration. Furthermore, transiently over-expressed junctate in cardiomyocytes by Adenovirus system for 24hrs resulted in decreased decay time of Ca^{2+} transients (Ad-LacZ: 0.329 + 0.009 vs. Ad-Junctate: 0.271 + 0.012 sec, n=4, p < 0.05) under the condition where there was no significant alterations of protein expression or phosphorylation of major SR Ca^{2+} cycling proteins. Our data suggest that junctate plays an important role in the regulation of SR Ca^{2+} cycling through the interaction with SERCA2a in the mammalian heart.

2651-Pos Board B621

Stimulation of P2X Purinergic Receptors Increases Calcium Spark Frequency, but Does Not Normalize Calcium Transient Synchronization, in Mouse Cardiomyocytes from the Calsequestrin Model of Cardiomyopathy (CSQ)

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Sarcoplasmic reticulum (SR) Ca^{2+} release is impaired in cardiomyocytes from failing hearts. In studies of cardiomyocytes from CSQ hearts Ca^{2+} spark frequency and synchronization of Ca^{2+} release were reduced. Interestingly, binary overexpression of CSQ and the human P2X4 purinergic receptor prolongs CSQ survival. Our objective was to determine if amelioration of the CSQ phenotype through Binary (CSQ+P2X4) overexpression was due to purinergic effects on Ca^{2+} release function. Cardiomyocytes isolated from the hearts of wild-type (WT), P2X4, CSQ or Binary mice were loaded with Fluo-4AM, superfused with a modified Tyrode's solution (22°C) and paced at 0.5 Hz in the presence/absence of the P2-receptor agonist 2-MeSATP (3 μM). Line-scans were recorded with a Zeiss LSM510. Under basal conditions, 2-MeSATP responsive cardiomyocyte Ca^{2+} spark frequencies (sparks/μm/sec) did not differ (WT 1.04 ± 0.23; P2X4 1.78 ± 0.28; CSQ 1.60 ± 0.87; Binary 0.79 ± 0.27; p=0.73). When 2-MeSATP was applied, Ca^{2+} spark frequency increased significantly compared to basal for each genotype (WT 2.89 ± 0.32; P2X4 5.79 ± 1.02; CSQ 5.13 ± 1.53; Binary 3.45 ± 0.89; p<0.01). These data suggest that a P2X4R-mediated mechanism can influence SR Ca^{2+} load and/or release. Effects of purinergic stimulation on coordination of SR Ca^{2+} release were investigated by determining the dyssynchrony index (DI) in paced cardiomyocytes. Under basal conditions the CSQ and Binary DIs were dramatically increased compared to WT (13.59 ± 1.39 and 14.28 ± 1.89 vs 4.34 ± 0.93; p<0.01). Application of 2-MeSATP did not decrease the DI in myocytes from failing CSQ and Binary hearts (15.82 ± 3.03 and 13.85 ± 1.01). Conclusion: Cardiac P2X purinergic receptor stimulation increases Ca^{2+} spark frequency, suggesting a beneficial effect on SR Ca^{2+} loading or release. However, P2X receptor activation does not normalize DI, determined from confocal line-scans, in CSQ or Binary cardiomyocytes.

2652-Pos Board B622

Effect Of Extracellular Ca^{2+} On Intracellular Ca^{2+} Dynamics In Intact Hearts Of Wildtype And Calsequestrin 2 Ko Mice

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Free [Ca^{2+}] in the lumen of the sarcoplasmic reticulum (SR) is a critical factor controlling Ca^{2+}-induced Ca^{2+}-release (CICR). Ca^{2+}-binding protein calsequestrin 2 (Casq2) located in SR lumen is important component in the regulation of CICR. One of the possible roles of Casq2 could be to prevent the depletion of the luminal Ca^{2+} stores during Ca^{2+} release. In order to modify the SR Ca^{2+} content we changed extracellular Ca^{2+} concentration in hearts from wildtype and Casq2 KO mice. The dynamics of intra-SR Ca^{2+} depletion, myoplasmic free Ca^{2+} and time course of the action potentials (APs) were measured from the epicardial layer of murine hearts using Pulsed Local Field Fluorescence Microscopy. Lowering extracellular Ca^{2+} resulted in smaller amplitude of Ca^{2+} transients, acceleration of the restitution of CICR and diminishing Ca^{2+} alternans. The ablation of Casq2 led to noticeable changes in the dynamics of CICR especially at low extracellular Ca^{2+}. The prolongation of the release can be explained by the modification of the properties of the ryanodine receptors (RyR2) in the absence of Casq2. The restitution of CICR, which was already accelerated by low extracellular Ca^{2+} was even faster in hearts of KO mice. In addition, the decline in cytosolic Ca^{2+} level observed in response to low extracellular Ca^{2+} was more pronounced in KOs. APs (conducted in the presence of blebbistatin) display a prolongation of the phase 2 of cardiac APs at 37°C as extracellular Ca^{2+} was decreased. Interestingly, an opposite effect was observed at room temperature (21°C). In conclusion, the results obtained on transgenic mice lacking Casq2 suggest that this protein can be engaged in controlling amplitude of CICR not only as a Ca^{2+} buffer but also a modulator of RyR2.

2653-Pos Board B623

Newly Synthesized Calsequestrin and Triadin-1 Traffic In Two Sarcoplasmic Reticulum Compartments In Heart Cells

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Cardiac calsequestrin (CSQ) is a major protein of junctional sarcoplasmic reticulum (jSR) in the heart, where it may serve dual roles of Ca buffering and ryanodine receptor regulation. CSQ is probably a polymer in jSR, but it remains unclear whether CSQ serves its functions as a monomer or polymer, and whether its localization in heart cells is affected by its polymerization. To investigate CSQ trafficking and concentration in jSR in heart cells, we compared acute overexpression by adenoviral constructs where CSQ was fused to tetrameric DsRed (CSQ-tetRed), monomeric DsRed (CSQ-monoRed), or epitope tag (CSQ-HA). CSQ-tetRed exhibited a prominent and unique distribution pattern in ER cisternae surrounding the nucleus. Retention near its apparent site of biosynthesis likely reflected tetramerization of DsRed with loss of further trafficking. Interestingly, analyzed using either anti-CSQ antibodies or anti-DsRed antibodies, the bright perinuclear CSQ-tetRed fluorescence was virtually invisible, likely due loss of epitope accessibility when polymerized. In contrast to CSQ-tetRed localization as seen by DsRed fluorescence, the immunofluorescence pattern of CSQ-tetRed showed CSQ traversing the cardiac secretory pathway towards the cell periphery. Moreover,immunostaining patterns for newly-synthesized CSQ, compared with native rat CSQ, suggested that endogenous jSR sites were less likely to incorporate newly-synthesized CSQ. Newly synthesized cardiac triadin-1 (TRD) was distinguished from native rat triadin-1 using species-specific anti-TRD antibodies. Newly synthesized TRD associated with CSQ-tetRed in perinuclear cisternae, but also trafficked to junctional SR. Mutant TRD, lacking the CSQ-binding site, did not reside in early biosynthetic compartments but co-localized with native CSQ in junctional SR. These data indicate that SR proteins CSQ and TRD are synthesized in a perinuclear compartment, can bind to one another even in this proximal compartment, and traffic to SR junctions within the cellular periphery.

2654-Pos Board B624

Polymerization of Calsequestrin Inside the Secretory Pathway is Isoform-Specific and Occurs on Either Side of ER Exit Sites

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In heart and fast twitch skeletal muscle, cardCSQ and skelCSQ concentrate in an ER/SR compartment known as junctional SR. Junctional SR is morphologically distinct in the two cell types, and mechanisms of CSQ trafficking and concentration within junctional SR remain undetermined. A model for CSQ polymerization has recently been developed that could explain traditional observations of a matrix of CSQ inside junctional SR lumens. CardCSQ, for example, is very efficiently retained in proximal ER tubules, as long as its native

structure is not critically modified, or the ER milieu significantly altered. In the present study, we found that skelCSQ trafficked differently from cardCSQ in nonmuscle cells and neonatal heart cells. In fact, the distribution of skelCSQ was unique among known ER markers. SkelCSQ concentrated in a membrane compartment that was juxtaposed and distal to ER containing the cardiac isoform. SkelCSQ was contained in novel streaming tubules and vesicles aligned on tufts of microtubules. Consistent with immunofluorescence microscopy were the structures of the two Asn316-linked glycans on CSQ isoforms, with skelCSQ glycans trimmed beyond the Man9,8 that are indicative of proximal ER. Despite the complete non-overlap of skelCSQ and cardCSQ compartments, the two proteins co-localized in early ER when co-overexpressed, suggesting heteropolymer formation. The present study indicates that skelCSQ, in contrast to cardCSQ, evades ER polymerization, and polymerizes in the next distal secretory compartment, an early subcompartment of ERGIC. We conclude that different subcellular localizations for skelCSQ and cardCSQ result from a difference in the lumenal requirements for polymerization of each of the two CSQ isoforms, leading to ER retention (cardCSQ) or retention in a contiguous intermediate compartment (skelCSQ).

2655-Pos Board B625

Calumenin Knock-down (kd) Enhances Ca^{2+} Cycling Ability In Hl-1 Cells

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Calumenin is a multiple EF-hand Ca^{2+}-binding protein localized in the sarcoplasmic reticulum (SR) lumen. Evidence of the interaction between calumenin and SERCA2 in rat cardiac SR was shown recently (*Mol. Cells*, 26:265-269, 2008). To elucidate the possible role of calumenin in cardiac excitation-contraction (E-C) coupling, calumenin was knocked down by transfection of mouse cardiac cell line (HL-1 cells) with calumenin specific siRNA oligonucleotides. After 72 hrs of transfection, calumenin protein level was reduced by 75% without any obvious changes in the expression levels of other E-C coupling proteins such as RyR2, SERCA2, NCX, CSQ and PLB. A field stimulation (1Hz) of KD cells ($n = 58$) led to significantly increased Ca^{2+} transient peak height (1.02 ± 0.02 *vs.* 0.82 ± 0.03 fura-2 ratio at 340 and 380 nm, $p < 0.05$), decreased time to peak (0.093 ± 0.001 *vs.* 0.107 ± 0.003 s, $p < 0.05$) and time to 50% baseline (0.172 ± 0.005 *vs.* 0.235 ± 0.006 s, $p < 0.05$) as compared to control cells ($n = 44$). On the other hand, the SR Ca^{2+} load remained unchanged in KD cells. Pull-down experiments with GST fusion proteins showed that calumenin interacts with both RyR2 and SERCA2 in a Ca^{2+} dependent manner. Taken together, the present results suggest that calumenin is related to SR Ca^{2+} homeostasis. Currently, the molecular interactions between calumenin and SERCA2 or RyR2 are being examined by using various deletion mutants.

2656-Pos Board B626

Interaction between Cardiac Ryanodine Receptor and FK506-Binding Protein Revealed by Cryo-EM and FRET

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Type 2 ryanodine receptor (RyR2) is the major calcium release channel in cardiac muscle. Abnormal calcium release through a dysfunctional RyR2 has been implicated in certain types of sudden cardiac death and heart failure. A 12.6kDa FK506 binding protein (FKBP12.6) tightly associates with RyR2, and stabilizes the close state of RyR2 calcium channel. One proposed mechanism that underlies RyR2 channel dysfunction is the destabilization of the RyR2-FKBP12.6 interaction. In the present study, we mapped the location of green fluorescent protein inserted after residue Tyr-846, near the amino-terminal diseases-causing mutation hotspot, in the three-dimensional (3D) structure of RyR2 by cryo-electron microscopy (cryo-EM). The location of the inserted GFP was found to be close to the previously mapped FKBP12.6 binding site. Based on the structural information that we have learned from 3D cryo-EM, we designed a fluorescence resonance energy transfer (FRET) pair by inserting a yellow fluorescent protein in RyR2 after residue Tyr-846, and attaching a cyan fluorescent protein to FKBP12.6. By monitoring the FRET signals between the donor and acceptor, we are investigating the interaction dynamics between RyR2 and FKBP12.6.
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2657-Pos Board B627

Effect of Stem Cell Transplantation on the Calcium Signaling in Adult Ventricular Myocytes

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Bone marrow derived stem cells (MSCs) are often discussed as a potential source for cardiac replacement tissue. Transplantation of undifferentiated cells into cardiac infarct regions has been shown to decrease infarct size and preserve cardiac function but the impact the cells have through paracrine effects or intercellular coupling remains to be determined. To determine how MSCs influence the excitability of cardiac myocytes we established a co-culture between freshly isolated mouse ventricular myocytes and dissociated MSCs. After 3 hrs of co-culture the cells were loaded with the Ca^{2+} indicator Fluo-4/AM and the Ca^{2+} handling properties of ventricular myocytes were analyzed at a stimulation frequency of 0.5 Hz. In comparison to control myocytes (ctrl) cardiomyocytes that co-localized with MSCs (co-MSC) exhibited a significantly increased Ca^{2+}-transient amplitude (F/F_o: ctrl: 2.3 ± 0.5, $n = 8$; co-MSC: 3.5 ± 1.2, $n = 4$). In addition, the transient duration at 50% (APD_{50}: ctrl: 457 ± 61 ms to co-MSC: 360 ± 33 ms); and 90% inactivation (APD_{90}: ctrl: 1.31 ± 0.15 s; co-MSC: 1.08 ± 0.16 s) was significantly shortened. We have previously demonstrated that stem cell derived cardiomyocytes and adult myocytes can establish intercellular coupling within 1 hour of co-culture. However, in heterocellular pairs of ventricular myocytes and MSCs no change MSC $[Ca^{2+}]_i$ could be determined upon stimulation of the myocyte. The data indicate that MSCs modulate substantially the Ca^{2+} signaling properties of adult ventricular myocytes and therefore could have as substantial anti-arrhythmic effect upon transplantation. It remains to be determined if intercellular coupling is necessary to establish this effect.

2658-Pos Board B628

Skeletal Myotubes from Adult Mice in a Cardiac Environment

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The present study was designed to evaluate the functional impact of cardiac environment on the differentiation of skeletal myoblasts and Ca2+ signaling in electrically stimulated myotubes. Adult mice FDB muscle myoblasts were cultured alone or in co-culture with cardiomyocytes contractile monolayers. After 5 days of differentiation in a cardiac environment, the number of skeletal myogenic cells and myotubes were fourfold higher than control. Cardiac environment changed parameters of myotube calcium transients (Fluo-4AM) associated with Ca2+ removal mechanisms without affecting parameters related to Ca2+ release. The values (mean ± sem) of half width (HW) and single exponential decay time constant (τ), obtained from mono-cultured myotubes (MMy) were 209.42 ± 34.72 ms and 415.16 ± 55.12 ms (n=9), while for co-cultured myotubes (CMy) they were 554.94 ± 67.38 ms and 1340.17 ± 330.73 ms (n=9), respectively. HW and τ from neighboring cardiomyocytes (CM) were 274.78 ± 50.17 ms and 387.72 ± 47.98 ms (n=5). The transient rise time (RT) values for MMy and CMy were 33.46 ± 8.79 (n=9) and 29.66 ± 8.97 ms (n=9) while the amplitude values (ΔF/F) for the two cases were 0.94 ± 0.09 (n=9) and 1.00 ± 0.07 (n=9), respectively. In the absence of external Ca2+ (0.5mM EGTA) the parameters associated with Ca2+ removal were not affected, while those related to release mechanisms were as follows: RT = 105.38 ± 24.93 ms (n=5) and 80.87 ± 18.28 ms (n=5) for MMy and CMy respectively, and $\Delta F/F = 0.56 \pm 0.11$ (n=5) and 0.71 ± 0.12 (n=5) for MMy and CMy, respectively. Thus, transient sensitivity to extracellular Ca2+ was not affected by coculture since both CMy and MMy were similarly modified by exposure to 0Ca2+. We conclude that at an early coculture stage, a cardiac environment facilitates skeletal muscle differentiation without affecting functional attributes characteristic of skeletal muscle, with the exception of a selective effect on Ca2+ removal parameters.

2659-Pos Board B629

Relationship of Ryanodine Receptors to the Sarcolemma in Rabbit Ventricular Myocytes

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To investigate the spatial relationship between the sarcolemma and ryanodine receptors (RyRs) we dual-labeled cells using Alexa fluor dyes and simultaneously imaged them with confocal microscopy. We deconvolved the images and subjected them to digital processing. We obtained three-dimensional reconstructions from cells in two configurations: lying flat and standing on end. In the flat configuration, RyR clusters appeared to be arranged in sheets near Z-disks. The distance between sheets was ~2 μm. Although some clusters are closely associated with detectable sarcolemma, the majority of them are not (>70%). With cells standing on end in agar we obtained XY scans orthogonal