and is detrimental to myocardial function. We previously demonstrated that high levels of peroxynitrite decrease myocardial contraction by reducing phospholamban (PLB) phosphorylation through a protein phosphatase-dependent mechanism. However, we did not examine the direct effect of peroxynitrite on protein phosphatase activity in the myocardium or the specific protein phosphatase which is activated. Here we test: 1.) the effect of SIN-1 (peroxynitrite donor) on protein phosphatase activity in whole heart homogenates using a colorimetric assay, and 2.) the effect of SIN-1 on the interaction of PLB with protein phosphatase 1 (PP1) and protein phosphatase 2a (PP2a) using co-immunoprecipitation. SIN-1 induced a 63% increase in total protein phosphatase activity (1.6 ± 0.2 vs. 2.6 ± 0.3 nmol/min/mg, $p<0.05$ vs. Control), which was abolished with specific PP1/PP2a inhibition using okadaic acid (1.4 ± 0.2 nmol/min/mg, $p<0.05$ vs. SIN-1). Since okadaic acid prevented the effects of SIN-1, we next examined the effect of SIN-1 on the interaction of PLB with PP1 and PP2a. SIN-1 increased the interaction of PLB with PP2a by 350% ($0.6 \pm$ 0.3 vs. 2.7 ± 0.7 A.U., $p<0.05$ vs. Control), but had no effect on the interaction with PP1. The peroxynitrite scavenger, urate, prevented both the SIN-1-induced increase in protein phosphatase activity and the interaction of PLB with PP2a, thus implicating peroxynitrite as the causal species. The results of this study provide further insight into the mechanism through which high levels of peroxynitrite serve to decrease PLB phosphorylation and myocardial contraction. Therefore, increased peroxynitrite production may play a key role in heart failure where protein phosphatase activity is increased and PLB phosphorylation is decreased, ultimately leading to contractile dysfunction.

2646-Pos Board B616

Epac Effect on the Cardiac RyR: Involvement of PLC, PKC and IP3R

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Epac is a protein directly activated by cAMP whose actions are independent of PKA. We recently show that Epac induces activation of CaMKII and phosphorylation of the Ca^{2+} release channel, the RyR, in rat cardiac myocytes. The effects included an increase in the Ca^{2+} sparks frequency and a slight decrease in the $[Ca^{2+}]_i$ transient amplitude. Here we investigated the signaling cascade from Epac activation to its effects on Ca^{2+} release. Ventricular myocytes were enzymatically isolated from rat heart ventricles. Cells were loaded with the fluorescence Ca^{2+} indicator Fluo-3 AM and viewed by confocal miscroscopy. $[Ca^{2+}]_i$ transients were evoked by field stimulation at 1 Hz. Ca^{2+} sparks were recorded in quiescent cells and SR Ca^{2+} load was estimated by rapid caffeine exposure. Epac activation was analyzed in presence of 8-CPT and of various antagonists. The possible involvement of Rap was checked on cells infected with adenoviruses coding for Rap-GAP and GFP. The results show that Rap is not involved in Epac effect on cardiomyocyte Ca^{2+} release. Inhibition of PLC by U73122 completely prevented Epac actions on Ca^{2+} sparks and $[Ca^{2+}]_i$ transients, indicating that PLC is involved in Epac actions. Blocking PKC by chelerytrine completely prevented Epac effect on $[Ca^{2+}]_i$ transient but not on Ca^{2+} sparks, suggesting that there are two separates pathways. Because PLC activation produces IP3, we checked whether activation of IP3 receptors (IP3R) is involved in Epac actions. Blockade of IP3R by 2-APB attenuated the effects of Epac on Ca^{2+} release events. Thus we conclude that activation of Epac by cAMP leads to Ca^{2+} release events modulation via a cascade involving PLC, PKC and IP3R. The resulted increase in the local Ca^{2+} release might be involved in the prohypertrophic actions of Epac on cardiac myocytes.

2647-Pos Board B617

A Quantitative Assessment Of Selective Pharmacological Inhibition Of Serca In Isolated Rabbit Working Hearts

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Decreased SERCA2a activity has been associated with contractile dysfunction in animal models of heart failure. An isolated working rabbit heart preparation and direct SERCA activity measurements were used to assess the level of SERCA inhibition necessary to terminate cardiac output under a standardised set of haemodynamic conditions. Hearts were perfused with a physiological extracellular solution whilst preload and afterload were set at $10cmH_2O$ and $85cmH_2O$ repectively. Ventricular function was assessed through the use of a pressure-volume catheter. Following initial stabilisation of cardiac function, 2.8μM thapsigargin (TG) was added to the circulating solution. Functional parameters were assessed continuously before and during application of TG. Cardiac function steadily declined in the presence of TG until the working heart configuration could not be sustained. The time to termination of aortic flow ranged from 15 to 60min. In the last minute prior to failure haemodynamic characteristics were markedly impaired (steady state *vs*. TG, n=4, $p<0.05$). Peak systolic pressure (107.78 ± 3.66 *vs*. 82.40 ± 2.15mmHg) and the maximum rate of rise of pressure (dp/dt_{max}) (1951 ± 177 *vs*. 995 ± 22mmHg.s^{-1}) were significantly reduced whilst dp/dt_{min} (-2422 ± 178 *vs*. -1470 ± 122mmHg.s^{-1}), relaxation time constant (0.028 ± 0.004 *vs*. 0.066 ± 0.009ms) and end diastolic pressure (8.33 ± 1.85 *vs*. 11.65 ± 0.62mmHg) were significantly increased. In all hearts coronary flow was maintained (80.5 ± 1.26 *vs*. 79.50 ± 1.7ml.min^{-1}). On cessation of aortic flow the left ventricle was snap frozen and homogenised in a protease-phosphatase buffer solution before biochemical analysis. Oxalate-dependent SERCA-mediated Ca^{2+}-uptake was used to assess SR Ca^{2+} uptake at a range of homogenate protein concentrations. Initial measurements indicate that termination of aortic flow occurs when SERCA activity (V_{max}) is reduced to <15% of control (DMSO vehicle). These data indicate the minimum level of SERCA activity required to sustain cardiac output in the rabbit working heart preparation.

2648-Pos Board B618

Contribution of Cycle Length History to Myocardial Contractility in Isolated Rabbit Myocardium under Physiological Conditions

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Modulation of contractile force via changes in heart rate can occur through processes that are either immediate (intrinsic) and/or through processes that involve prolonged exposure to a given situation and act via post-translational modification. Because the contractile strength of the steady state force-frequency relationship (FFR) and post-rest potentiation (PRP) involve both instant intrinsic responses to cycle length as well as slower acting components such as post-translational modification based mechanisms, it remains unclear how cycle length intrinsically affects cardiac contraction and relaxation. To dissect the intrinsic impact of cycle length changes from slower acting signaling components of the FFR, twitch contractions of right ventricular rabbit trabeculae at 5 different cycle lengths were randomized around a physiological stimulation baseline of 2.85 Hz. Patterns of previous cycle lengths that resulted in changes in force and/or relaxation times were identified. We found that the duration of the cycle length prior to the analyzed twitch contraction (primary) positively correlated with force. In sharp contrast, the cycle length one more removed from the analyzed twitch (''secondary'') was found to have a negative correlation with force. The ''tertiary'' cycle length impacted force similar to the secondary cycle length, albeit with a lesser magnitude. Using this novel stimulation protocol we can quantify the intrinsic effect of cycle length on contractile strength, as well as avoiding run-down and lengthiness that are often complications of FFR and PRP assessments. The data show that the history of at least 3 cycle lengths prior to a contraction influences myocardial contractility under near physiological conditions, and the secondary/tertiary cycle lengths affect cardiac twitch dynamics in the opposite direction than primary cycle length with decreasing importance as the cycle length is further removed from the current beat.

2649-Pos Board B619

Nitroxyl (HNO) Modifies Cysteine Residues in Phospholamban to Increase Myocyte Ca^{2+}-Cycling and Contractility

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HNO donors enhance cardiac inotropy by increasing SR Ca^{2+} re-uptake/release. Given its thiophylic nature, HNO likely modifyies critical cysteine residues in E-C coupling proteins. Phospholamban (PLN) is a potential target for HNO, and its genetic removal or mutation of PLN cysteines should abolish/blunt HNO cardiac effects. Cardiomyocytes were isolated from PLN knockout (PLN-/-) and wildtype (WT) mice, field-stimulated and assessed for Ca^{2+} transients and sarcomere shortening (SS). HNO effects on the SR-Ca^{2+}ATPase (SERCA2a) were evaluated by isolating SR vesicles from PLN-/- and WT mice and measuring Ca^{2+} uptake by stopped-flow mixing. Dephosphorylation of SERCA2a (a measure of E_2P hydrolysis) was investigated in ER microsomes from Sf21 insect cells expressing SERCA2a ± PLN (WT or Cys 36-41-46->Ala mutant). PLN-/- myocytes showed enhanced myocyte contraction and a blunted response to isoproterenol. When challenged with the HNO donor

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