direction. This data suggests that Vin contributes to the passive mechanical properties of the myocardium, and disrupting the mechanical linkage between the cytoskeleton and the cell membrane reduces the overall stiffness of the myocardium.

2571-Pos Board B541

Quantitative Assay of Skeletal Muscle alpha-actin Expression In Normal and Pathological Human and Mouse Hearts

O'Neal Copeland[1], Gianna Ravenscroft[2], Kristen Nowak[2], Nigel Laing[2], **Steven Marston[1]**.
[1]Imperial College London, London, United Kingdom, [2]University of Western Australia, Perth, Australia.

We have developed a polyclonal antibody specific to skeletal muscle actin (ACTA1) in the presence of cardiac actin (ACTC) and have used it to quantify the skeletal actin content of human and mouse cardiac muscle. Heart muscle myofibrils were separated by SDS-PAGE and Western blotted. The membrane was stained first with MemCode total protein stain and then probed in with the anti-skeletal muscle actin antibody, visualised by ECL. The ECL band signal was normalised to MemCode-stained actin band. Skeletal muscle myofibrils were used as a 100% skeletal actin standard. For the negative control we used myofibrils from skeletal muscle of a skeletal actin knockout mouse crossed with a transgenic mouse over-expressing cardiac actin in skeletal muscle. There was no detectable signal from the skeletal actin antibody in the pure cardiac actin control.

Human non-failing donor heart muscle contained $21 \pm 2\%$ skeletal actin (n=9). This is comparable to previous estimates using N-terminal sequencing or Mass spectroscopy. In both end-stage failing heart muscle and in myectomy samples from HCM muscle the skeletal actin content was much higher ($58 \pm 5\%$ in both cases, n=12, 11). The increase in skeletal actin content of myopathic muscle was highly significant ($p < 0.001$). Mouse heart muscle (C57BL/6 strain) contains $26 \pm 3\%$ skeletal actin (n=7). This is similar to human heart. ACTC DCM mutation E361G expressed at 50% in mouse heart has $16 \pm 3\%$ (n=8) skeletal actin but ACTC HCM mutation E99K expressed at 50% is not significantly different from NTG $24 \pm 2\%$ (n=5). We conclude that in human heart, acquired heart failure or failure secondary to HCM is associated with an increased content of skeletal muscle actin. In contrast, in mouse genetic models of HCM and DCM skeletal muscle actin content may be lower than normal.

2572-Pos Board B542

Structural and Functional Characterization of Cardiac Troponin T Mutations in the TNT1 Domain That Cause Familial Hypertrophic Cardiomyopathy

Pia J. Guinto, Edward P. Manning, Rachel K. Moore, Steven D. Schwartz, Jil C. Tardiff.
Albert Einstein College of Medicine, Bronx, NY, USA.

FHC is a primary cardiac muscle disorder that is one of the most common causes of sudden death in young people. FHC ''hotspot'' mutations at residue 92 in cardiac troponin T (cTnT) flank the proposed α-helical TNT1 tail domain whose flexibility has been suggested to be important in normal protein-protein interactions within the thin filament. Through Molecular Dynamics (MD) simulations, we showed that FHC mutations Arg92Leu, Arg92Trp, and Arg92Gln cause local α-helical structural changes and increased flexibility at a critical hinge region 18 Angstroms distant from the mutation. We have extended this MD analysis via the use of a self-defined coordinate to measure localized bends in the helix and found that forces acting on this bending coordinate are lower in mutants than wildtype. This quantitatively suggests a less restrictive bending motion in mutants explaining the increased flexibility of the hinge region. To determine how primary biophysical changes induced by these mutations cause complex cardiomyopathies we hypothesize that flexibility alterations and changes in force within compaction-expansion regions in mutational segments lead to electrostatic perturbations, possibly interfering with cTnT-TM complex formation and thin filament function. *In vitro* motility assays with wildtype cTnT and hotspot FHC-cTnT mutants are in progress to directly correlate predicted alterations in electrostatic properties with resultant functional changes. Moreover, contractile and Ca^{2+} transient measurements on isolated myocytes address downstream myocellular responses to the mutation's primary perturbation on structure and function. Data showed normal percent shortening in Arg92Leu myocytes while Arg92Trp percent shortening was significantly impaired compared to Non-Tg ($4.740 + 1.165$ vs. $6.971 + 2.098$, $p<0.001$). Completion of these studies will directly address the links between thin filament structure/function, downstream myocellular responses, and resultant distinct cardiovascular phenotypes.

2573-Pos Board B543

Structural and Functional Characterization of cTnT in Familial Hypertrophic Cardiomyopathy

Rachel K. Moore, John Wilson, Pia Guinto, Michael Riegelhaupt, Gary Gerfen, Jil Tardiff.
Albert Einstein College of Medicine, Bronx, NY, USA.

Familial Hypertrophic Cardiomyopathy (FHC) is a primary disease of the cardiac sarcomere. Many disease-causing mutations in the thin filament protein cTnT are found within the TNT1 region. Residues 160-163 represent a mutational hotspot within a highly charged region (158-RREEEENRR-166). In this region, this highly alpha helical domain may unwind to create a flexible hinge that is necessary for function, the structure and dynamics of which may be affected by FHC mutations. We are investigating the structure and function of this region using in vitro motility (IVM) assays and SDSL-EPR. The purpose of our IVM experiments is two-fold: to functionally analyze our spin labeled proteins and to gain insight into the function of TNT1 in the presence of cysteine substitutions and FHC mutations. Preliminary IVM data shows a progressive increase in the severity of the functional effects of cysteine substitution and spin labeling across the putative hinge region ($153<168<172$), suggesting that this region is dynamically important and may be making critical interactions with other components of the sarcomere. Preliminary CW-EPR spectra show an increase in isotropic rotational rate at residue 153 (upstream of the putative hinge region) between cTnT alone and in the troponin ternary complex, suggesting that there is a decrease in alpha helical character at this residue in the ternary complex. Introduction of Δ160E further increases the isotropic rotational rate, suggesting an increase in flexibility due to backbone changes induced by the mutation. To further investigate structural and functional changes caused by FHC mutations within the putative hinge region, we will continue to expand our IVM functional analyses with additional cysteine substitutions, as well as FHC mutations at residues 160 and 163. Double label SDSL-EPR are currently underway that will provide secondary and tertiary structural information.

2574-Pos Board B544

FHC-linked Mutations in the Myosin Regulatory Light Chain Interfere with RLC Phosphorylation in Transgenic Mice

Katarzyna Kazmierczak, Alexander Raytman, Michelle Jones, **Danuta Szczesna-Cordary**.
University of Miami Miller School of Medicine, Miami, FL, USA.

Previous studies have shown that MLCK-phosphorylation of the ventricular regulatory light chain (RLC) increases myofilament Ca^{2+} sensitivity, maximal level of force and rate of tension development thereby enhancing the systolic function of the heart. We have investigated the effect of FHC (familial hypertrophic cardiomyopathy) mutations in the myosin RLC on its phosphorylation measured in rapidly frozen ventricular samples from transgenic (Tg) mice. We observe that the Aspartate to Valine substitution in Tg-D166V mice and Arginine to Glutamine in Tg-R58Q mice result in a decreased phosphorylation of RLC detected in left ventricular samples from Tg-mutant compared to Tg-WT mice expressing the human ventricular RLC. The level of RLC phosphorylation was determined by Western blotting utilizing human specific phospho-RLC antibodies (gift from Dr. N. Epstein, NIH). Our data from skinned Tg-D166V and Tg-R58Q papillary muscle fibers show that both RLC mutations lead to a decreased maximal level of force and to slower kinetics of force generating myosin cross-bridges compared to WT fibers. Studies in intact papillary muscle fibers show prolonged force transients for both D166V and R58Q mutants. In addition, the hearts of aging mutant mice demonstrate histopathological changes and frequent occurrences of fibrotic lesions. Clinical studies revealed that both D166V and R58Q mutations are associated with severe FHC phenotypes with multiple cases of sudden cardiac death. Our results suggest that phosphorylation of RLC plays an important role in regulating cardiac function and its deficit may contribute to malignant FHC phenotypes. Reduced RLC phosphorylation observed in the D166V and R58Q hearts correlates with our cellular findings and could be responsible for delayed force transients, slower cross-bridge kinetics and decreased force observed in Tg-D166V and Tg-R58Q papillary muscle fibers. Supported by NIH- HL071778 (D.S-C.).

2575-Pos Board B545

Mouse HCM Model Expressing E99K ACTC Mutation Reproduces Phenotypes As Found In Human Patients

Weihua Song[1], Daniel J. Stuckey[2], Emma Dyer[1], Dominic Wells[3], Sian E. Harding[1], Carolyn A. Carr[2], Kieran Clarke[2], Steven B. Marston[1].

[1]NHLI, Imperial College London, London, United Kingdom, [2]University of Oxford, Oxford, United Kingdom, [3]Imperial College London, London, United Kingdom.

The mutation Gly99lys (E99K) in the cardiac actin (ACTC) gene was reported to cause Hypertrophic Cardiomyopathy in extensive clinical studies. Transgenic (TG) mice expressing 50% E99K mutant cardiac actin in their hearts were generated and studied. The mice show high mortality between 28 and 45 days old (70% females, 34% males).

Thin filaments reconstituted with purified mouse f-actin from the survivors and human heart tropomyosin and troponin were studied by *in vitro* motility assay. The E99K thin filaments were 2.5 ± 0.6 times more Ca^{2+} sensitive than NTG thin filaments ($p = 0.05$). E99K actin thin filaments also exhibited a reduced response to troponin dephosphorylation (EC_{50} E99K/E99KdP = 1.1 ± 0.1 compared with 3.0 ± 0.3 for NTG /NTGdP).

7 month-old E99K TG mice (n=9) and their NTG littermates (n=7) were studied using *in vivo* cine MRI. Abnormal cardiac morphology and significantly lower ejection fractions (56.5 vs. 65.2%) and reduced stroke volumes (26.0 vs. 42.3 μl) were observed in TG mice. Peak LV ejection rates were also reduced (188 ± 41 vs. 252 ± 49 μl/min). LV mass was similar between groups, but septal wall thickness was increased (1.5 vs. 1.0 mm).

Left ventricular function of 9 month-old female E99K NTG (n=4) and TG (n=5) mice were studied with an *in vivo* conductance catheter. In TG mice ejection fraction was 20.2% less, end-diastolic pressure was 39.6% higher and relaxation rate was 50.0% slower.

We conclude that the basic effect of E99K mutation is increased Ca^{2+}-sensitivity and blunted response to troponin dephosphorylation and this leads to the high rate of sudden death at early ages, alterations to cardiac function and hypertrophy as observed in patients with hypertrophic cardiomyopathy.

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2576-Pos Board B546

Protein Kinase A Catalyzed Phosphorylation of Cardiac Myosin Binding Protein C Decreases Calcium Sensitivity of Force and Increases Cross-Bridge Cycling Kinetics in Murine Myocardium

Peter P. Chen, Jitandrakumar R. Patel, Inna N. Rybakova,
Jeffery W. Walker, Richard L. Moss.
University of Wisconsin, Madison, WI, USA.

At the myofilament level, increases in cardiac output in response to increased sympathetic tone is achieved via protein kinase A (PKA)-mediated phosphorylation of cardiac myosin binding protein C (cMyBP-C) and cardiac troponin I (cTnI). However, despite the physiological importance of β-adrenergic stimulation in maintaining cardiac performance, the respective roles of cMyBP-C and cTnI phosphorylations in the myofibrillar force response of working myocardium are not completely understood. Using transgenic mouse lines either (1) expressing mutant non-phosphorylatable cTnI ($cTnI_{ala5}$) or (2) expressing $cTnI_{ala5}$ on a cMyBP-C null background ($cMyBP\text{-}C^{-/-}/cTnI_{ala5}$), we assessed the calcium sensitivity of force (pCa_{50}) and the rate of force redevelopment (k_{tr}) in skinned myocardial preparations following treatment with PKA and/or reconstitution with purified recombinant cMyBP-C. Before mechanical measurements, all preparations were treated with 2,3-butanedione monoxime (BDM) to reduce regulatory light chain (RLC) phosphorylation to near zero. In $cTnI_{ala5}$ myocardium, PKA phosphorylation of cMyBP-C resulted in a decrease in pCa_{50} and an increase in k_{tr}. However, no changes in either variable were observed in $cMyBP\text{-}C^{-/-}/cTnI_{ala5}$ myocardium in response to PKA treatment. Following reconstitution of $cMyBP\text{-}C^{-/-}/cTnI_{ala5}$ myocardium with cMyBP-C, k_{tr} decreased to the values observed in $cTnI_{ala5}$ myocardium, demonstrating that incorporation of cMyBP-C slowed the rates of cross-bridge attachment and transitions to strongly bound, force generating states. Subsequent treatment of reconstituted $cMyBP\text{-}C^{-/-}/cTnI_{ala5}$ myocardium with PKA produced a rightward shift in pCa_{50} and an increase in k_{tr}. Together, these results suggest that in the absence of cTnI phosphorylation (and RLC phosphorylation), PKA phosphorylation of cMyBP-C decreases calcium sensitivity of force and speeds cross-bridge cycling kinetics in murine myocardium.

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PKA Phosphorylates Serine 307 of Murine Cardiac Myosin Binding Protein-C In Vitro

Justin F. Shaffer[1,2], Weitao Jia[2], Julie A. Leary[2], Samantha P. Harris[2].
[1]University of Washington, Seattle, WA, USA, [2]University of California, Davis, Davis, CA, USA.

Cardiac myosin binding protein-C is a regulatory protein associated with sarcomere A-bands that modulates actomyosin interactions in a phosphorylation dependent manner. The MyBP-C motif, a highly conserved sequence in the N-terminus of cMyBP-C, contains three to five protein kinase A (PKA) phosphorylation sites, depending on species. In the human isoform, three PKA sites have been identified (S275, S284, and S304). Three homologous sites exist in the murine isoform (S273, S282, and S302) along with a potential fourth site, S307, which is not present in human cMyBP-C. In this study, we investigated the effects of PKA phosphorylation of murine cMyBP-C by treating a recombinant protein, C1C2 (which contains the C1, motif, and C2 domains), with PKA and assessing phosphorylation levels using IEF gels, ProQ Diamond staining, and mass spectrometry. The wild-type C1C2 has a pI of ~8 and PKA treatment (C1C2P) shifted the pI to ~5-6 as determined by 1-D IEF gels. A mutant C1C2 (3S/D), containing aspartic acid for serine substitutions at S273D, S282D, and S302D, was still phosphorylated upon treatment with PKA as indicated by increased ProQ Diamond staining. However, a mutant 4S/D C1C2 (containing the additional mutation S307D) showed a pI near that of C1C2P and was not further phosphorylated by PKA. Mass spectrometry and MASCOT analysis of C1C2P confirmed that S307 was phosphorylated by PKA. These results suggest that murine S307 can be phosphorylated *in vitro*. Further studies are needed to investigate the phosphorylation state of murine cMyBP-C *in vivo*.

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Functional Effects of cMyBP-C Phospho-Mimics in Permeabilized Trabeculae

Kristina L. Bezold, Justin F. Shaffer, Samantha P. Harris.
University of California, Davis, CA, USA.

Myosin Binding Protein C (MyBP-C) is a sarcomeric protein that has both structural and regulatory roles in striated muscle contraction. Cardiac (c) isoforms of MyBP-C can be phosphorylated by protein kinase A (PKA) at three to five sites within a unique regulatory region referred to as the MyBP-C motif. We have previously shown, using permeabilized rat trabeculae, that the recombinant protein C1C2, which contains the motif, significantly increased Ca2+ sensitivity of force and increased rates of tension redevelopment (ktr) at submaximal [Ca2+]. To investigate whether these effects are modulated by phosphorylation of the motif, we used the catalytic subunit of PKA to phosphorylate C1C2. In addition, we used site directed mutagenesis to mutate three key serine residues (Ser273, 282, 302) to aspartic acids to mimic phosphorylation at these sites. Results demonstrated that either 10uM phosphorylated C1C2 (C1C2P) or 10uM phospho-mimic C1C2 (C1C23S/D) increased Ca2+ sensitivity of force and increased rates of tension redevelopment (ktr) at submaximal [Ca2+]. However, the phospho-mimic C1C23S/D was more effective than C1C2P in producing these effects. Together these results indicate that the 3 Ser to Asp phospho-mimic does not fully mimic effects of PKA phosphorylation of C1C2 and that the functional effects of C1C2 in permeabilized cardiac trabeculae are mediated at least in part through phosphorylation-independent mechanisms.

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2579-Pos Board B549

Altered Myofilament Targeting with Differential PKCδ Activation

Tanganyika Wilder, Aaron C. Hinken, R. John Solaro.
University of Illinois at Chicago, Chicago, IL, USA.

Post-translational modification of troponin (Tn) and other myofilament proteins by protein kinase C (PKC) isozymes plays a prominent role in regulating myocardial contraction. Conventionally, phosphorylation of conserved serine and threonine residues in the c-terminus activates PKCdelta, a novel calcium independent isozyme. Several recent investigations including our work (Sumandea et al. J Biol Chem. 2008;283(33):22680-9) have led to the probability of alternative activation of PKCdelta by tyrosine residue phosphorylation through a redox-sensitive mechanism. Previous work determined PKCdelta phosphorylation of adult, cardiac rat myocytes in vitro reduces skinned myocyte tension generation at sub-maximum with no change at maximum calcium concentrations. Conversely, tyrosine phosphorylated PKCdelta reduces the maximal Ca-activated tension with no decrease in submaximal tension production. Biochemical data indicated a shift in TnI residue targeting with tyrosine phosphorylated PKCdelta from exclusive phosphorylation of S23/24, to include T144. To test the hypothesis that kinase targeting to TnI-T144 was sufficient to blunt effects of conventional PKCdelta targeting to TnI-S23/24, myofibrillar function was assessed following exchange of TnI with pseudo-phosphorylated residues. Pseudo-phosphorylation at residues S23/24 decreased Ca-sensitivity of force production and increased tension cost. Pseudo-phosphorylation at TnI-144 had minimal affect on mechanical parameters. However, the combination of pseudo-phosphorylations at residues S23/24 and T144 did not successfully blunt desensitization. Further biochemical assessments have determined