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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.

Supported by a grant from the British Heart Foundation

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

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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

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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*.

Supported by NIH HL080367 to SPH and a NSF Graduate Research Fellowship to JFS.

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

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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.

Supported by NIH HL080367.

2579-Pos Board B549

Altered Myofilament Targeting with Differential PKCδ Activation

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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

differential PKCdelta targeting to multiple myofibrillar proteins other than TnI. Further elucidation of alternative PKCdelta signaling to the myofilments is ongoing. Our results suggest Src-dependent phosphorylation of PKCdelta constitutes an alternative mechanism that allows for stimulus-specific PKCdelta ezymology and myofibrillar targeting within the myocardium.

2580-Pos Board B550

Protein Kinase A-based Modulation Of Ca^{2+} Sensitivity In Skinned Skeletal Muscle Fibers Reconstituted With Cardiac Troponin

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It is well known that protein kinase A (PKA) decreases Ca^{2+} sensitivity in cardiac muscle via phosphorylation of troponin I (TnI). In the present study, we directly tested whether PKA-based phosphorylation of cardiac TnI universally modulates Ca^{2+} sensitivity regardless of the type of muscle, by taking advantage of our Tn exchange technique (Terui et al., J Gen. Physiol. 131;275-283:2008). Troponins were extracted from porcine ventricular and rabbit fast skeletal muscles (Ca^{2+} sensitivity: former < latter). Without Tn exchange, PKA decreased Ca^{2+} sensitivity in cardiac (porcine ventricular) muscle, associated with enhanced phosphorylation of TnI. Reconstitution of cardiac muscle with the skeletal Tn complex (sTn) not only increased Ca^{2+} sensitivity but abolished the PKA effect, suggesting that phosphorylation of TnI, but not of myosin-binding protein C, is primarily responsible for the PKA-based reduction in Ca^{2+} sensitivity. Reconstitution of rabbit psoas muscle with the cardiac Tn complex (cTn) decreased Ca^{2+} sensitivity, as previously reported by us (Terui et al., J Gen. Physiol. 131;275-283:2008). PKA decreased Ca^{2+} sensitivity in cTn-reconstituted skeletal muscle, and subsequent exchange for sTn restored Ca^{2+} sensitivity to the original level. A similar result was obtained when skeletal muscle was reconstituted with the hybrid Tn complex (i.e., cTnI-cTnC-sTnT), suggesting that the troponin I-C complex, but not TnT, is essential for PKA-based modulation of Ca^{2+} sensitivity. These findings support the notion that PKA-based phosphorylation of TnI universally modulates Ca^{2+} sensitivity regardless of the type of muscle.

2581-Pos Board B551

Cardiac Troponin I Threonine 144 phosphorylation: impact on myofilament function

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Threonine 144 (T144) in the inhibitory region of cardiac troponin I (cTnI) is an important site for PKC mediated phosphorylation in the heart. In addition, presence of this residue is sufficient to impart length dependent activation (LDA) properties onto the cardiac sarcomere (Tachampa Circ. Res., 2008). Here we studied the functional impact of a charge mutation so as to mimic phosphorylation of this residue (T144E). Wild-type (WT) or cTnI-T144E containing recombinant troponin (cTn) complexes were exchanged for endogenous cTn in skinned rat cardiac trabeculae. Force and ATPase activity were measured as function of [Ca2+] at short (2.0 μm) or long (2.2 μm) sarcomere length. T144E induced decreased maximum force development (Fmax) and Calcium sensitivity (EC50), increased cross-bridge cycling rate (tension cost) but, in contrast, did not affect LDA (ΔEC50). We conclude that T144E affects cross-bridge cycling and recruitment independent of sarcomere length.

	WT cTn	T144E cTn
EC50 2.0 (μM)	4.0 ± 0.3	5.9 ± 0.4
EC50 2.2 (μM)	3.5 ± 0.2	5.2 ± 0.3
Fmax 2.0 (mN/mm2)	25.1 ± 4.7	13.0 ± 1.7
Fmax 2.2 (mN/mm2)	37.2 ± 4.7	24.5 ± 2.7
Tension cost 2.0	7.7 ± 0.5	12.0 ± 1.3
Tension cost 2.2	6.3 ± 0.5	9.4 ± 1.2
ΔEC50	0.6 ± 0.1	0.7 ± 0.2

2582-Pos Board B552

Effects of Cardiac Troponin C Mutants on TnC-TnI interaction and its modulation by PKA phosphorylation

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We are considering several cardiac TnC mutants as potential therapeutic strategies for cardiomyopathies. To judge their potential to improve *in situ* function it is important to understand how these mutants affect TnC-TnI interaction and its modulation by PKA-mediated phosphorylation of TnI Ser 23, Ser 24. In this study, we are characterizing two cardiac TnC mutations, Leu48Glu (L48Q) and Ile61Glu (I61Q), with increased and decreased (respectively) Ca^{2+} binding affinity. In previous studies we showed these mutations resulted in increased (L48Q) and decreased (I61Q) Ca^{2+} sensitivity of steady state force in skinned rat trabeculae. To determine if these changes in Ca^{2+} sensitivity were due to altered TnC-TnI interactions we generated a structural marker by attaching IANBD to Cys84 in the N-lobe of cTnC. Half-maximal IANBD fluorescence saturation of Ca^{2+} binding occurred at pCa7.42 for L48Q cTnC, 7.38 for wild-type (WT) cTnC and 7.30 for I61Q cTnC. In both the absence and presence of saturating Ca^{2+} (0.6 μM TnC) IANBD fluorescence increased with increasing TnI and saturated at different [TnI] in the order L48Q, WT, I61Q. Fluorescence half-maximal saturation occurred at 0.26μM(saturating Ca^{2+}) and 0.25 μM (no Ca^{2+})TnI for L48Q cTnC, 0.78 μM and 0.49μM for WT cTnC , and 1.45 μM and 0.69 μM I61Q cTnC according to the exponential function fit of the data. However, preliminary experiments suggest that when PKA phosphorylated [cTnI] was titrated to cTnC, in both the absence and presence of saturating Ca^{2+}, IANBD fluorescence enhancement with L48Q may be impaired. The data thus far suggest that single amino acid mutations that alter Ca^{2+} binding affinity of TnC can influence interaction with TnI and its modulation by PKA mediated phosphorylation of Ser 23, Ser 24. Supported by HL65497 to MR.

2583-Pos Board B553

Analysis of Cardiac Myofibrillar Troponin I Phosphorylation in Normal and Failing Human Hearts Using Phos-Tags

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Recently, we have used phosphate-affinity SDS-PAGE gels containing Phos-tag-acrylamide (a phosphate-chelating molecule), to determine the level of cardiac troponin I (cTnI) phosphorylation in human myofibrillar extracts. The Phos-tag moiety binds to, and retards, the mobility of phosphoproteins through the gel and results in the separation of the phosphoprotein bands according to their phosphorylation level.

Samples from time-courses of in-vitro PKA catalytic subunit-treated recombinant human cTnI and myofibrillar extracts from non-failing donor, hypertrophic obstructive cardiomyopathy (HOCM) and end-stage failing human heart tissue were analysed by phosphate-affinity SDS-PAGE. Separate gel bands corresponding to 1P, 2P, 3P, 4P and 5P cTnI were observed for the PKA-treated recombinant cTnI. Western blotting probed with the anti-cTnI antibody 14G5 and several different site specific phospho-cTnI antibodies demonstrated that all five of these phospho-species bound to a Ser24P-specific antibody, while a Thr144P-specific antibody only reacted with the 3P, 4P and 5P phospho-species of cTnI.

We observed 3 phospho-species of cTnI in the human heart tissue extracts, which correspond to 0P, 1P and 2P cTnI. Ratios of 0P cTnI were significantly higher in failing and HOCM (both 63 ± 4%) compared to donor (8 ± 2%) while ratios of 2P were significantly lower (failing = 6 ± 2%, HOCM = 8 ± 2%, donor 73 ± 6%). Western blots demonstrated that in human heart cTnI phosphorylation of Ser23/24 was mainly present in the 2P species (with a very small proportion in the 1P species) and that there was no phosphorylation at Thr144. Calculated levels of total cTnI phosphorylation in both HOCM (0.37 ± 0.03, n=50, $p<0.0001$) and failing heart (0.38 ± 0.03, n=24, $p<0.0001$) were significantly reduced from levels in donor heart (1.65 ± 0.04, n=38) and were comparable to previously determined measurements obtained from Pro-Q Diamond phosphoprotein gel staining.

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Effect of Troponin I Ser23/24 Bis-Phosphorylation on Ca^{2+}-Sensitivity is Dependent on PKA Phosphorylation of Other Contractile Proteins

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Upon β-adrenergic stimulation, protein kinase A (PKA) enhances cardiac Troponin I (cTnI) phosphorylation at ser23/24. PKA treatment leads to a decrease in myofilament Ca^{2+}-sensitivity. However, the specific effect of PKA-mediated phosphorylation of cTnI in human myocardium is unclear since PKA phosphorylates a broader set of contractile proteins, in particular myosin binding protein C (cMyBP-C).