results by the substituted cysteine accessibility method upon addition of acetylcholine. Our MD simulation results are consistent with a mechanistic model in which the apo form, while predominantly sampling the "closed" conformation, can make excursions into the "open" conformation. The open conformation has high affinity for agonists, leading to channel activation, whereas the closed conformation upon further distortion has high affinity for antagonists, leading to inhibition.

Cardiac Muscle & Regulatory Proteins - I

1422-Pos Effects of Troponin Exchange on Length-Dependent Activation in Skinned Porcine Ventricular Muscle

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Board B398

The basis of the Frank-Starling mechanism of the heart resides in the intrinsic ability of myocardial fibers to produce higher active force in response to stretch (i.e., length-dependent activation). We have reported that titin-based passive force operates as one of the triggering factors in this phenomenon. In the present study, we investigated whether or not length-dependent activation is modulated at the thin filament level. We used skinned porcine left ventricular muscle that had been treated with 1% (w/v) Triton X-100. Quasi-complete reconstitution of thin filaments with skeletal troponin (sTn; prepared from rabbit psoas muscle) increased Ca^{2+} sensitivity of force and attenuated length-dependent activation. A control experiment showed that treatment of skinned porcine ventricular muscle with exogenous cardiac troponin (prepared from porcine ventricular muscle) did not alter length-dependent activation. We then investigated the effect of sTn reconstitution on cross-bridge kinetics by measuring the rate of force redevelopment (k_{tr}). k_{tr} increased upon sTn reconstitution at submaximal levels, suggesting the acceleration of cross-bridge formation and, accordingly, a reduction in the fraction of resting cross-bridges that can potentially produce active force. An increase in titin-based passive force, induced by manipulating the pre-history of stretch, enhanced length-dependent activation, in both control and sTn-reconstituted muscle. These results suggest that troponin plays an important role in length-dependent activation via on-off switching of the thin filament state, in concert with titin-based regulation.

1423-Pos Coupled Interactions of Troponin C Ca^{2+}-Binding Kinetics and Strong Crossbridge Formation in Cardiac Muscle Contraction

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Board B399

Striated muscle contractile regulation requires both Ca^{2+} binding to troponin C (TnC) and strong actomyosin crossbridge formation for thin filament activation and tension generation. We hypothesize that these processes are unique in skeletal and cardiac muscle and studied the coupled Ca^{2+} and crossbridge processes in demembranated rat cardiac trabeculae at 15°C. Ca^{2+} dissociation (k_{off}) from cardiac TnC (cTnC) was altered with whole cTn exchange using wild-type (WT) cTn (k_{off}=29.7±0.5s^{-1}, 15°C), L48Q cTn (7.3±0.1s^{-1}) or I61Q cTn (76.0±9.3s^{-1}). Strong crossbridge formation was reduced with 2,3-butandione monoxime (BDM) or inorganic phosphate (P_i) and increased with 2-deoxy-ATP (dATP). Maximal tension (F_{max}; pCa 4) after cTn exchange was ~85% for WT and L48Q cTn and ~20% for I61Q cTn. At saturating Ca^{2+} (pCa 4), 1–50mM BDM inhibited F_{max} with an inhibition constant (K_i) of 7.9±1.7mM for WT cTn, 9.3±1.5mM for L48Q cTn, and 5.0±1.4mM for I61Q cTn. F_{max} inhibition with 1–10mM P_i showed no difference in K_i between cTnC types (1.8±0.4mM for WT cTn), even though reconstituted F_{max} varied greatly. Increased crossbridge formation and cycling with dATP increased F_{max} by ~30% for WT and L48Q cTn and ~80% for I61Q cTn. In summary, when Ca^{2+} binding was increased (L48Q cTn), altering crossbridge formation or kinetics did not change the crossbridge component of thin filament activation. In contrast, reducing Ca^{2+} binding (I61Q cTn) resulted in greater capacity to increase activation via strong crossbridge formation but did not influence the ability to maintain activation as the crossbridge component was decreased. These data suggest that tight coupling exists between Ca^{2+} binding to cTn and strong crossbridge formation in cardiac muscle thin filament activation, unlike our previous results in skeletal muscle. Ongoing investigations include computational modeling approaches. HL61683, HL65497.

1424-Pos Measurements of Sarcomere Length in Intact Resting Rat Heart

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Board B400

Cardiac contractility is modulated by sarcomere length (SL). While SL is a well-characterized parameter in fixed myocardium and isolated cells or tissue, little is known about SL-values in intact heart.

Two-photon fluorescence microscopy was used to measure SL up to 300μm sub-epicardially in rat whole hearts (n=12), excised after cervical dislocation and Langendorff-perfused within 150s. Hearts were placed in a tailor-made silicone cradle and gently stabilized by nylon mesh. Tissue was stained by coronary perfusion with di-4-ANEPPS (Invitrogen) in normal Tyrode (5μM over 5min), and cardioplegically arrested for imaging (room temperature) using a Leica TCS-MP2 multi-photon microscope (excitation 840nm, collection 400–700nm). Fluorescence intensity profiles were collected perpendicularly to user-defined paths along the axis of individual cells, and SL was analyzed by Fourier transform. Path alignment is

software-optimized by 1°-rotation steps in the image's (x/y) plane to yield 'minimum apparent' SL. The theoretical maximum error in z-alignment (angle between cell axis and imaging plane) is identified, based on known path length and assumed maximum cell thickness (20μm). The 'theoretical minimum' SL is calculated using the cosine of this angle. The z-axis error angle is further constrained when measured near parallel-running small capillaries (diameter ~8–12 μm). Final SL is taken as the average of measured 'minimum apparent' (xy) and calculated 'theoretical minimum' (y) SL values (differences >15% were an exclusion criterion). Average diastolic SL is 2.07±0.17μm (mean ± SD, n=791, from 12 rats) and significantly different ($p<0.05$) from diastolic SL of acutely isolated cardiac myocytes 1.75±0.10μm (mean ± SD, n=111, 4 rats) analyzed using the same conditions and techniques. Thus, diastolic SL is considerably greater in whole heart preparations than in isolated cardiomyocytes. This has implications for interpolation of data between these two popular experimental models.

1425-Pos Differences in Apparent Troponin Dissociation Rates Between Cardiac and Skeletal Troponin in Skeletal Myofibrils

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Board B401

Troponin is the Ca2+-sensing protein of the thin filament. While cardiac troponin (cTn) and skeletal troponin (sTn) accomplish the same function, their subunit interactions within Tn and with actin-tropomyosin are different. To further characterize these differences, we utilized labeled Tn exchange in rigor myofibrils to estimate Tn dissociation rates from the 3 different regions of the thin filament (Swartz et al., J. Mol. Biol. *361*:420). ATPase activity showed that skeletal myofibrils containing > 95% cTn were more Ca2+ sensitive (pCa50 = 6.17) but less cooperative (nH = 1.64) than those containing sTn (pCa50 = 5.89, nH = 3.36). Analysis of Tn interaction by labeled Tn exchange using intensity ratio analysis showed that the time course at pCa 9 and 4 were quite different between cTn and sTn. Globally, apparent cTn dissociation rates were ~ 2 – 10 fold faster in all 3 regions of the thin filament while the effect of Ca2+ was ~ 5 fold less for cTn compared to sTn myofibrils. The apparent dissociation rates for cTn were 5 X 10-3 min-1, 150 X 10-3 min-1 and 260 X 10-3 min-1 while for sTn they were 0.6 X 10-3 min-1, 88 X 10-3 min-1, and 68 X 10-3 min-1 for B, C, and M-regions respectively. Normalization to their individual B-region rates give 30:50:1 for cTn compared to 150:110:1 for sTn (C:M:B). Thus, for the non-overlap region, Ca2+ enhances Tn dissociation 30 fold for cTn and 150 fold for sTn. The ATPase data suggest greater Ca2+ sensitivity for cTn compared to sTn, but this can be partially explained by cTn being less "off" or B-state in the nominal B-region at pCa 9 since cTn dissociation rate was 10 fold faster than sTn.

1426-Pos Ca^{2+}-independent Activation Of Striated Muscle With EMD 50733

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Board B402

EMD 50733 (EMD) is a positive inotropic agent that has been studied as a potential therapeutic intervention to improve cardiac pump function of patients in heart failure. While multiple studies have demonstrated a considerable improvement of Ca^{2+} mediated contraction in both cardiac and skeletal muscle, little is known about the effects of EMD at low Ca^{2+} levels present in resting muscle. Here we studied the effects of EMD at low [Ca^{2+}] on solution ATPase activity, thin filament *in vitro* motility and mechanics of myofibrils and skinned muscle cells. For both rat cardiac and rabbit skeletal myosin, 50 μM EMD had little effect in the absence of actin, but increased ATPase activity by ~50% in the presence of actin at 21°C. This indicates that EMD enhances actin-myosin interaction. In the *in vitro* motility assay, 10 μM EMD increased the fraction of moving, regulated skeletal thin filaments at pCa 9.0 (23°C) from 18% to 70% and speed of movement from 0.28 μm/s to 2.2 μm/s. This compared with 88% moving filaments at 4.61 μm/s at pCa 5.0 (no EMD). In mouse cardiac myofibrils (15°C), 50 μM EMD resulted in Ca^{2+} independent force (pCa 9.0) that was ~15% of maximal Ca^{2+} activated force, and this was abolished by addition of 50 mM BDM. In skinned rat trabeculae (15°C), 50 μM EMD increased chord stiffness at high (≥ 100 ML/s), but not low, rates of stretch. Together these mechanical data suggest that EMD enhances actin-myosin interaction independent of Ca^{2+}, resulting in an increased strong crossbridge population that can partially activate resting striated muscle. Continuing experiments investigate potential cardiac vs. skeletal muscle differences and molecular mechanisms of EMD action. Preliminary evidence suggests EMD may alter diastolic cardiac function.

Supported by HL65497, MUR-COFIN2006.

1427-Pos Simulating The Effect Of Lattice Spacing On The Frank-Starling Mechanism

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Board B403

The spatial relationship between thin and thick filaments is one mechanism regulating myocardial performance. It is thought that the interaction of sarcomere length and lattice spacing in constant

volume contraction is responsible for regulation of cross-bridge recruitment and alterations of force generation such as the Frank-Starling mechanism. Previous spatially explicit models operate in a one dimensional space that permits no alteration of filament spacing, and thus are unable to consider the role of lattice spacing in force development. Here, we develop a two-dimensional spatially explicit model of actin-myosin interaction.

We relax the assumption that cross-bridges behave as simple linear springs aligned with the axes of the myofilaments and instead employ a coupled linear/torsional spring mechanism for each myosin's mechanics. Thus we more accurately reflect the lever-arm model of force generation. The system's kinetics use two-dimensional, spatially explicit, stochastically-driven cross bridge cycling with a three state binding model. Cross-bridge recruitment is driven by constrained diffusion of myosin heads, and an iterative solution method is used at each time-step to balance all forces on each point along the myofilaments. Results address how the interaction of filament spacing and compliant realignment of binding sites can at least partially account for the Frank-Starling relationship, showing an increase in force generated at smaller filament spacings. This supports the idea that increased force development at longer sarcomere lengths is, at least partially, due to changes in lattice spacing.

[Supported by HL65497 and funds from the Komen Endowed Chair]

1428-Pos Calcium-Independent Alterations in Diastolic Sarcomere Length and Relaxation Kinetics in a Mouse Model of Lipotoxic Diabetic Cardiomyopathy

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Board B404

Previous studies demonstrated increased free fatty acid uptake and metabolism in MHC-FATP mice that overexpress fatty acid transport protein 1 (FATP1) in the heart under the control of the αMHC promoter. Echocardiographic studies revealed impaired left ventricular filling and atrial enlargement in MHC-FATP mice. Doppler tissue imaging and hemodynamic measurements confirmed diastolic dysfunction, in the absence of changes in systolic function. Experiments to test the hypothesis that diastolic dysfunction in MHC-FATP mice results directly from impaired ventricular myocyte contractile function revealed that mean diastolic sarcomere length is significantly shorter in isolated adult MHC-FATP cardiomyocytes than in wild type (WT) cells (1.79±0.01 vs.1.84±0.01 μm, $P<0.01$). In addition, the relaxation rate (-dL/dt) is significantly slower in MHC-FATP than WT myocytes (1.58±0.09 vs..1.92±0.13 μm/sec, $P<0.05$), whereas both fractional shortening and contraction rates are not different. Application of 40 mM BDM (a non-specific ATPase inhibitor that relaxes acto-myosin interactions) increases diastolic sarcomere length in both WT and MHC-FATP myocytes to the same length, suggesting that MHC-FATP myocytes are partially activated at rest. Direct measurements of intracellular Ca^{2+} revealed that the $t_{1/2}$ of calcium removal in MHC-FATP myocytes is faster indicating that Ca^{2+} handling is altered in these cells. This effect cannot, however, explain the observed reduction in relaxation rate. Moreover, diastolic sarcomere length was unaffected in either MHC-FATP or WT myocytes by removal of extracellular Ca^{2+} or chelation of intracellular Ca^{2+} with BAPTA-AM (100 μM), indicating that elevated intracellular Ca^{2+} does not underlie impaired diastolic function observed in MHC-FATP ventricular myocytes. Collectively, the data indicate that metabolic remodeling in MHC-FATP ventricles results in a Ca^{2+}-independent increase of diastolic tension, suggesting that alterations in cardiac lipid metabolism underlie the derangements in myocardial pump function evident in diabetic cardiomyopathy.

1429-Pos Short-range Force Responses To Stretch Are Increased In A Rat Model Of Aging-associated Diastolic Dysfunction

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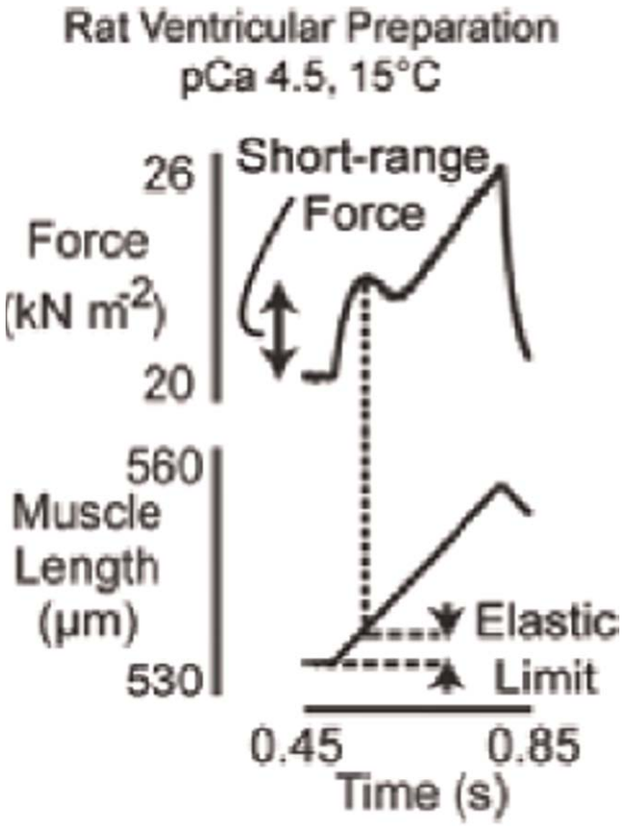
Board B405

Diastolic heart failure is more common in old patients and occurs when the cardiac ventricles are too stiff to distend fully at normal filling pressures. To determine which molecules might be responsible for the elevated stiffness, we examined the mechanical properties of permeabilized ventricular preparations isolated from 4, 18 and 24 month old Fischer 344 rats. These animals are an accepted model of aging-associated diastolic heart failure. Our results showed that the stiffness of preparations immersed in solutions with nanomolar free Ca^{2+} concentrations did not change with age. This implies that the aging-associated dysfunction observed in these animals is not due to changes in titin isoform expression or collagen content/cross-linking. In contrast, the mechanical properties of Ca^{2+}-activated preparations did vary with age. Specifically the short-range force responses and the elastic limits of the responses (Figure) were both greater ($p<0.02$) in preparations isolated from the 24 month old rats than in preparations from the 4 month old animals. Our working hypothesis is that the mechanical effects reflect increased expression of the slower beta-myosin heavy chain in the older animals.



1430-Pos Sex Specific Differences in Effects of CapZ on Cardiac Adrenergic Signaling and Sarcomeric Function

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Board B406

Our previous studies (Pyle *et al*. Circ Res. 2002) employing a transgenic mouse model (TG-CapZ) in which there is a ~7% reduction in expression of the actin capping protein, CapZ, demonstrated an increase in myofilament Ca^{2+} -sensitivity and altered sarcomere docking of PKC compared to wild-type controls (WT). Here we determined whether these *in vitro* effects translate to intact cardiac function in 3–6 month old mice as determined from pressure-volume loops obtained during beta-adrenergic blockage (Pro; s.c.) with or without the alpha-adrenergic agonist, phenylephrine (Phe; i.v.). As expected, indexes of cardiac performance in all groups were depressed following administration of Pro alone but reduction in dP/dt_{max} was greatest in males compared to females and E_{max}, a load-independent measurement of contractility, was further depressed in male TG-CapZ compared to all other groups. When Phe and Pro were administered concurrently all groups were able to mount an improved contractile response with the exception of male TG-CapZ. E_{max} in the male TG-CapZ group remained significantly depressed compared to both male WT and female TG-CapZ groups. Quantification of binding kinetics of alpha and beta adrenergic receptors demonstrated no differences between female and male controls and TG. There was no difference in pCa-force relations of skinned fiber bundles from control female and male hearts and TG male hearts at 1.9 and 2.2 microns SL. However, female TG fibers showed an increase in Ca^{2+}-sensitivity and a decrease in length dependence of activation when compared to male TG fibers. Our results indicate sex specific differences in sarcomeric signaling involving the Z-disk protein network.

1431-Pos Direct Measurements Of Ventricular Stiffness In Isolated Rat Hearts At 37°C

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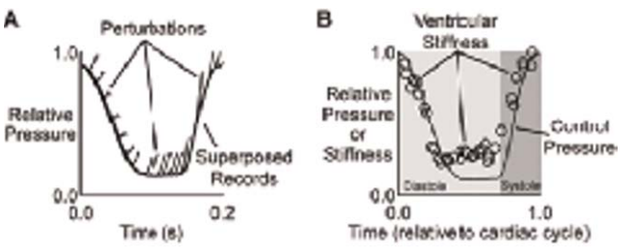
Board B407

Ventricular stiffness is an important determinant of cardiac performance because it influences the amount of blood that can enter the main pumping chambers during diastole. We have developed a new technique to measure the stiffness of the left ventricle of isolated rat hearts. Excised hearts are mounted on a Langendorff-perfusion setup and maintained at 37°C. The atria are removed and a small rubber balloon is inserted into the left ventricle. The balloon is partially filled with saline and connected to a custom-built fluid injector driven by a piezo-electric actuator. A Millar SP-869 sensor is then pushed into the balloon. This device measures the pressure and the volume inside the ventricle. Hearts are paced at 5 Hz and the injector is used to pump ~3 μl of additional saline in ~5 ms into the balloon at different time intervals after peak systole. The additional fluid is withdrawn during the subsequent cardiac cycle. Analysis of initial results shows that systolic myocardial stiffness is 3.8 ± 1.2 (SEM) times greater than diastolic stiffness. This suggests that attached cross-bridges are an important component of myocardial stiffness.



1432-Pos Evidence that Crossbridges both Influence and are Influenced by Nearest-Neighbor Regulatory-Units During Cardiac Myofilament Activation

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Board B408

We have formulated a theoretical model of myofilament activation that suggests a novel link between putative mechanisms of cooperative activation. Experiments in cardiac muscle showing steeply cooperative force-pCa relations and modulation of thin filament activation by crossbridges (XB) have been interpreted via two types of nearest-neighbor cooperative mechanisms: Regulatory-unit/regulatory-unit (RU/RU) cooperativity whereby RU tend to activate synchronously, and XB/RU cooperativity whereby XB enhance activation of neighboring RU. Whether these two mechanisms alone explain cooperative activation has not been established.

In order to analyze their respective roles, RU/RU and XB/RU cooperativity were incorporated into a Markov model whose states represent short chains of contiguous RUs along the thin filament. This construction enabled a computationally-tractable depiction of explicit nearest-neighbor interactions. Within each chain, RUs were assumed to be non-permissive, permissive, or permissive with attached XB. The network of Markov states was generated by considering all possible unique configurations of RUs within a chain of specified length. Markov states were related through transitions representing either activation or XB binding for an individual RU.

To satisfy microscopic reversibility around loops within the Markov network, it was necessary to include a third cooperative mechanism whereby the status of neighboring RUs influenced the rate of XB formation (RU/XB cooperativity). Simulations performed through numerical solution of model equations showed sharp convergence of steady-state and dynamic responses when 6 or more RUs were considered in each Markov state. The 6-RU model reproduces several key physiological responses, including steep cooperative activation, isometric twitch dynamics, and XB-mediated twitch prolongation. Thus, thermodynamic constraints inherent to the model suggest a link between all three forms of nearest-neighbor cooperativity in the sarcomere. This theoretical construction is supported by the model's ability to reproduce several experimentally observed phenomena.

1433-Pos Mechanical Role of LIM Protein FHL1 in Modulating Titin Based Passive Tension

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Board B409

LIM domain proteins have been shown to play a pivotal role in cardiomyopathy and heart failure in humans. Four and a half LIM domain protein-1 (FHL1) is a protein localized at the N2B region of titin. Titin is a major determinant of the diastolic mechanical properties of the heart. The N2B region has been proposed to modulate elasticity of titin through its phosphorylation by PKA or changes in titin isoform expression. To test the hypothesis that normal passive material properties of cardiac-muscle are dependent on the interactions between titin and FHL1, we generated knockout (KO) mice deleting FHL1 from the sarcomere. Stress-strain analysis on isolated papillary muscles from these mice reveals that FHL1KO muscles are significantly more compliant than controls. Treatment of FHL1KO muscles with PKA agonist, Isoproterenol, was found to have no significant effect on the compliance of KO specimens, unlike wildtype muscles. Furthermore, the magnitude of passive tension as a function of strain after Isoproterenol treatment of wildtype specimens is not statistically different from untreated KO specimens. Considering that titin is a major determinant of slack sarcomere length (SLo) and that an expression of the longer more compliant N2BA isoform may affect the SLo of cardiac myocytes, we measured the SLo of FHL1KO and wildtype myocytes and found no statistical difference (WT-SLo=1.86+/−0.03um; KO-SLo=1.87+/−0.03um). High resolution gel electrophoresis confirmed N2B titin as the major titin isoform expressed in both groups. Examination of the systolic function together with western-blot analysis of the PKA substrate phospholamban (PLB) (WT-PLB=0.117+/−0.045; KO-PLB=0.248+/−0.089) reveals baseline PKA activity to be unaffected by FHL1 deletion. We conclude that FHL1 functions to modulate titin based tension by acting as a negative regulator of titin phosphorylation, effecting baseline phosphorylation of titin and basal myocardial passive force levels.

1434-Pos Gene Expression Changes Caused By Mutation Altering Titin Isoform Splicing In Rat Cardiac Muscle And Skeletal Muscle

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Board B410

An autosomal dominant mutation that dramatically alters the alternative splicing pattern of titin has been described (Greaser et al., J. Mus. Res. & Cell Motil *26*:325–332, 2005). In spite of the dramatic change in titin size, the animals remain visibly healthy. Homozygous mutants can reproduce and several have lived to ages over one and a half years. Four rats have died spontaneously at approximately one year of age without any warning of distress (i.e. weight loss, rough coat, labored breathing, etc.). Hearts exhibit a atrial hypertrophy (mg atria wt/g body wt for Wt=0.15; for Hm=0.34). Electron micrographs of homozygous mutant ventricles showed occasional regions of myofibrillar disarray, but most areas appeared normal. The mutants also contained unusual clustering of mitochondria and often had very wide myofibrils. Changes in cardiac muscle transcriptional levels from mutant compared to normal rat ventricular were determined using Affymetrix Rat 230 2.0 arrays. Results showed 433 genes (from a total of 31099) were differentially expressed in mutant compared to normal rats. 239 genes were down-regulated and 194 genes were differentially up-regulated. Genes that exhibit the largest increase in expression are involved in the sarcomeric protein and regulation of muscle contraction, G-protein signaling and calcium regulation in cardiac cells. Those with the most significant decrease in expression are associated with cell adhesion, MAPK cascade and also muscle contraction and potassium/calcium channel. Eight of the interesting gene changes were confirmed using quantitative real time RT-PCR. In addition, four

gene changes (CARP, T-Cap, FHL2 and SERCA2) were also confirmed in skeletal muscle. In order to determine translational level change of those genes in cardiac muscle and skeletal muscle, and western blotting was also carried out using specific antibodies.

Supported by NIH HL77896.

1435-Pos Thyroid Hormone Regulates Developmental Titin Isoform Transitions via the PI3K/AKT Pathway

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Board B411

Titins, giant sarcomere proteins with major mechanical and signaling functions, are expressed in two main isoform-classes in mammalian heart: N2B (3,000kDa) and N2BA (>3,200kDa). A dramatic titin-isoform switch occurs during cardiac development, from fetal N2BA-titin (3,700kDa) expressed before birth to a mix of smaller N2BA/N2B isoforms found postnatally; adult rat hearts almost exclusively have N2B-titin. The isoform-switch, which can be reversed in chronic human-heart failure, greatly affects myocardial distensibility and mechano-chemical signaling. Here we determined factors regulating this switch, using as a model system primary cardiomyocyte (CM) cultures prepared from embryonic rats. In standard culture, the mean N2B-percentage initially was 14% and increased by ~60% within one week, resembling the in-vivo switching. The titin-isoform transition was independent of myocyte-hypertrophy and was not altered by pacing, increased contractility, contractile arrest, or cell-stretch; however, it was modestly impaired by decreasing substrate-rigidity and strongly dependent on serum components. The mean N2B-proportion was ~70% in 1-week-old cultures grown in standard medium, but only 45% in hormone-reduced medium, whereas addition of triiodo-L-thyronine (T3) again increased N2B to 62%. This T3-effect was not prevented by bisphenol-A, a specific inhibitor of the classical genomic pathway of T3-action, but could be completely reversed by the phosphatidylinositol-3-kinase (PI3K) inhibitor, LY294002. The presence of LY294002 suppressed a rapidly appearing T3-induced increase in Akt-phosphorylation and downregulated titin-N2B-mRNA transcripts within hours, as well. Thus, mechanical parameters such as matrix-stiffness may modulate titin-expression, but the main determinant of developmental titin-isoform transitions is T3-mediated signaling, acting via a rapid pathway through activation of PI3K/Akt.

1436-Pos Titin Phosphorylation By PKC

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Board B412

The presence in titin of various phosphorylation motifs has implicated it as a player in myocardial signaling. It has been found that titin phosphorylation by PKA reduces passive tension of cardiomyocytes. PKC has emerged as a major player in cardiac diseases including myocardial hypertrophy, ischemic heart disease, and congestive heart failure. Hence the aim of the present study was to determine whether PKC can also modulate titin properties. To establish titin phosphorylation by PKC mouse LV skinned fibers were incubated with 0.5 U/ml of PKC catalytic fragment in relaxing solution (RS), plus [γ-^{32}P]ATP, and incubated 2 h at RT. Samples were analyzed by gel electrophoresis and autoradiography. To determine the PKC target domain, recombinant titin fragments (N2B, PEVK and $Ig_{I1–I18}$) were incubated with 0.5 U/ml PKC in RS, plus [γ-^{32}P]ATP, for 2 h at RT. Samples were analyzed by 4–20% gradient gels and autoradiography. To ascertain the effect of PKC on myocytes mechanical properties passive tension and sarcomere length measurement were obtained from mouse skinned myocytes treated with 1 U/ml PKC in RS. Phosphorylation assays with mouse cardiac skinned fibers showed that PKC induced significantly increased of titin phosphorylation (195% higher than the control; $p<0.01$, N=8). We found that 50 μM chelerytrhine (PKC-specific inhibitor) completely abolished PKC induced phoshorylation of titin. In vitro phosphorylation assay with recombinant titin fragments suggested that PEVK, but not N2B or Ig, is a target of PKC. Mechanical experiments did not reveal a significant effect on passive tension generation of cardiomyocytes treated with PKC. These results suggest that PKC induces a specific and significant increase of titin phosphorylation with the PEVK element the most likely target domain. The functional significance remains to be established.

1437-Pos Proteolytic Fragments Of Cmybp-c Can Regulate Cardiac Contractility

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Board B413

cMyBP-C appears to have two structural regions, each associated with a different function. The N terminal region can bind to the head of myosin and/or actin and appears capable of modulating contractility. The C terminal region may form a collar around the thick filament and stabilize its structure, rendering it resistant to shear force and proteolysis. We have studied the effect of disruption of the putative collar on the ability of cMyBP-C to resist proteolysis by exposing skinned myocardial cells to increasing concentrations of the C5 fragment of cMyBP-C. During incubation in fresh contraction solution, addition of C5 reversibly lowers Ca sensitivity. If the Ca concentration of the first application is 10 ÂμM or more, a second application of the same solution produces a much stronger, but still reversible inhibition of force generation. With the higher concentration of C5 and a longer period between incubations, the second application causes an irreversible loss of resting and active force. Associated with the greater effects of a second application is the appearance of proteolytic fragments of exogenous C5 in the soak solution. The requirement for a relatively high concentration of Ca

suggest that the proteolysis is caused by calpain and its autolytic subfragments. The loss of resting tension suggests that the interaction between cMyBP-C and titin is involved and that this interaction is important in maintaining normal resting tension. Autolytic fragments of cMyBP-C may act as poison peptides.

1438-Pos Interaction of the Phosphorylation Domain of Cardiac Myosin Binding Protein-C with Myosin and Actin

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Board B414

Cardiac myosin binding protein-C (cMyBP-C) is a multi-domain regulatory protein whose role in the sarcomere is not clearly understood. The functional region of this protein is the linker region that is located between two immunoglobulin (IgI) motifs, C1 and C2 in the N-terminal region. This linker region consists of three phosphorylation sites and when phosphorylated, this increases systolic tension in the cardiac muscle. Additionally, mutations in genes encoding the linker cause familial hypertrophic cardiomyopathy (FHC). FHC is an autosomal dominant genetic disorder and it is one of the most common forms of congenital heart disease. Studies have found that cMyBP-C binds myosin S2 when dephosphoryated and this interaction is abolished upon phosphorylation and increases contactile force. Furthermore, previous studies have shown that the N-terminal region containing C1-linker-C2 (C1C2) binds to both myosin S2 and F-actin. Therefore we have cloned, expressed and purified it subfragments, C1-linker and linker-C2 to further localise the binding site for myosin and actin. Structural analysis using circular dichroism (CD) spectroscopy showed that, in the new constructs, both IgI domains, C1 and C2 folded to a beta sandwich and that alpha helices were present in the linker region of both constructs. Sedimentation binding studies found that C1-linker binds to both myosin and F-actin.

1439-Pos C1–C2 Domain Fragment Of Cardiac Myosin Binding Protein-C Inhibits Actomyosin Motility In A Phosphorylation-dependant Manner

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Board B415

Cardiac myosin-binding protein-C (cMyBPC) is a sarcomeric protein localized to the A-band. While the specific function of cMyBP-C remains enigmatic, mutations in cMyBP-C result in the development of cardiomyopathy. Structurally, cMyBP-C contains 11 domains, with the N-terminus exhibiting both actin and myosin- S2 binding capacity (i.e. domains C0–C2), whereas the C-terminus is capable of binding the myosin rod. Through these interactions, cMyBP-C may act as a tether limiting myosin's interaction with actin. To better define the functional significance of the C1–C2 domains, we compared the effect of whole, isolated cMyBP-C and a bacterially expressed C1–C2 fragment on skeletal myosin-generated actin filament velocity using an in vitro motility assay. In addition, as the C1–C2 linker contains three cardiac-specific phosphorylation sites, we sought to determine if cMyBP-C's interaction with actin and/or myosin can be regulated by phosphorylation. In the motility assay, whole cMyBP-C completely inhibits actin velocity at an estimated cMyBP-C to myosin molar ratio of 2:1 as does the C1–C2 fragment. Thus, much of whole cMyBP-C's inhibition can be attributed to the C1–C2 fragment. Interestingly, when C1–C2 is phosphorylated by protein kinase A, C1–C2 no longer inhibits actin filament velocity even at a 25:1 molar ratio. These results suggest that:

1. cMyBP-C may not function as a tether since the inhibitory effects of C1–C2 occur without binding to the myosin rod;
2. the C1–C2 inhibition can be physiologically regulated by phosphorylation.

We propose that cMyBP C's inhibitory effects are due to alterations in the kinetics of the actomyosin interaction. Future studies will determine whether the C1–C2 fragment's effects are mediated through its interaction with myosin and/or actin.

1440-Pos Protein Kinase A-Mediated Phosphorylation of cMyBP-C Increases Proximity of Myosin Heads to Actin in Resting Myocardium

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Board B416

Protein Kinase A-mediated (PKA) phosphorylation of cardiac myosin binding protein-C (cMyBP-C) accelerates the kinetics of cross-bridge cycling and may relieve the collar-like constraint of myosin heads imposed by cMyBP-C. We favor a mechanism in which cMyBP-C modulates cross-bridge cycling kinetics by regulating the proximity and interaction of myosin and actin. To test this idea, we used synchrotron low-angle X-ray diffraction to measure inter-thick filament lattice spacing and the equatorial intensity ratio, I_{11}/I_{10}, in skinned trabeculae isolated from wild-type (WT) and cMyBP-C null (cMyBP-$C^{-/-}$) mice. In WT myocardium, PKA treatment appeared to result in radial or azimuthal displacement of cross-bridges away from the thick filaments, as indicated by an increase ($\sim$33%) in I_{11}/I_{10} (0.22 $\pm$ 0.03 *versus* 0.33 $\pm$ 0.03). Conversely, PKA did not effect cross-bridge disposition in mice lacking cMyBP-C, as there was no difference in I_{11}/I_{10} between cMyBP-$C^{-/-}$ (0.40 $\pm$ 0.06) and PKA-treated cMyBP-$C^{-/-}$ myo-

cardium (0.42 ± 0.05). While lattice spacing did not change upon PKA treatment in WT (45.68 ± 0.84 nm *versus* 45.64 ± 0.64 nm), PKA treatment of cMyBP-C$^{-/-}$ myocardium increased lattice spacing (46.80 ± 0.92 nm *versus* 49.61 ± 0.59 nm). Konhilas *et al.* (2003) suggested that PKA phosphorylation of cTnI induces expansion of the myofilament lattice and that this effect may be greater in the absence of cMyBP-C. Our results support this interpretation and are consistent with the idea that there are direct interactions between cMyBP-C and actin, which if present would stabilize the lattice. These data support our hypothesis that tethering of cross-bridges by cMyBP-C is relieved by phosphorylation of PKA-sites in cMyBP-C, thereby increasing the proximity of cross-bridges to actin and increasing interaction probability.

Supported by an AHA predoctoral fellowship (BAC) and by NIH HL-R01-82900 (RLM).

1441-Pos Phosphorylation Of Myosin Binding Protein-C Altered In Hypertrophic Cardiomyopathy

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Board B417

Cardiac myosin binding protein C (cMyBPC) is a large regulatory protein within the sarcomere. cMyBPC phosphorylation increases systolic tension, and dissociates the N-terminal region of cMyBPC from the S2 neck region of myosin. cMyBPC mutations are associated with familial hypertrophic cardiomyopathy (FHC). We have cloned, expressed and purified the N-terminal region (immunoglobulin motifs C1 to C2) that encompasses the tri-phosphorylation sites (defined as sites A to C). Using in vitro mutagenesis we have also generated four FHC mutant forms of C1–C2. Four FHC-causing mutations are located in the phosphorylatable linker between Ig motifs C1 and C2; G278E, G279A, R326Q and L352P. The effect of these mutations on phosphorylation with PKA at 30°C was investigated. The G279A and R326Q mutations yielded the same rate of phosphorylation as the WT C1–C2; complete di-phosphorylation in less than 5 minutes and complete tri-phosphorylation by 2.5 hours. The G278E mutant was phosphorylated more slowly; complete di-phosphorylation by 30 minutes and complete tri-phosphorylation in $>$ 4 hours. Surprisingly, the L352P construct was phosphorylated more rapidly than WT; complete tri-phosphorylation in 2 hours. In all cases the phosphorylation order was the same as WT (first site B, then A and finally C). Structure prediction provides insights into the mechanisms underlying the changes in phosphorylation rate. Together these data suggest that an alteration in cMyBPC phosphorylation rate may underlie the pathogenesis of FHC caused by some mutations, although paradoxically the rate can be increased or decreased.

1442-Pos Effects of the MyBP-C Motif and C1 on Contractile Properties of Permeabilized Rat Trabeculae

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Board B418

Myosin Binding Protein-C (MyBP-C) is a regulatory protein involved in striated muscle contraction. The protein is comprised of 10 Immunoglobulin (Ig) and Fibronectin-III domains, referred to as C1–C10, with the unique MyBP-C motif sequence between C1 and C2. The cardiac isoform of MyBP-C has an additional Ig domain, C0, at its N-terminus. Recently, we showed that recombinant N-terminal domains affect actomyosin interactions. To better understand which N-terminal domains are important for these effects, we expressed recombinant proteins consisting of single Ig-containing fragments like C1m (the C1 domain and the motif) and larger, more inclusive fragments, such as C0C2 or C2C4. Effects of the recombinant proteins were then assessed in mechanical force experiments in permeabilized rat trabeculae to determine the Ca^{2+}-sensitivity and rate of force redevelopment (k_{tr}). For each trabecula a control tension-pCa relationship was run in the absence of added protein followed by a 15-minute incubation with protein (5–50uM) and a second tension-pCa curve. Force and k_{tr} in relaxing and maximally activating conditions were also determined. Results showed that proteins containing at least C1 and m showed an increase in the Ca^{2+}-sensitivity of force, a decrease in cooperativity of force and an increase in k_{tr} such that the Ca^{2+}-dependence of k_{tr} was eliminated in the presence of C1m. Measurable force was present in relaxing conditions with high protein concentrations indicating Ca^{2+}-independent activation. Proteins that lacked either the C1 domain or the motif did not affect force. These results suggest that C1 and the MyBP-C motif are required for the functional effects of MyBP-C.

This work supported by NIH HL080367 and NIH HL65497.

1443-Pos The Cardiac Myosin Binding Protein-C Motif and C1 Domain Activate Actomyosin Motility Independent of Ca2+

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Board B419

Cardiac myosin binding protein-C (cMyBP-C) is a regulatory protein associated with thick filaments in striated muscle. The MyBP-C motif, a linker region between N-terminal domains C1 and C2, may have a role in regulating actomyosin interactions through interactions with myosin S2. We have recently shown that the recombinant protein C1C2, which includes C1, C2, and the

motif, inhibits actomyosin motility in an *in vitro* motility assay using actin and heavy meromyosin (HMM) as the motor. In additional assays where Ca^{2+}-regulation was restored by inclusion of regulatory proteins (troponin and tropomyosin), C1C2 activated actomyosin motility in the absence of Ca^{2+}, while proteins lacking the motif did not. Comparable effects were observed in assays where S1 was used instead of HMM, suggesting that the motif-S2 interaction may not be essential for these effects. In order to further characterize the role of the N-terminus of cMyBP-C in the regulation of actomyosin interactions and to assess the importance of the motif, recombinant proteins containing cMyBP-C N-terminal domains with or without the motif were created and their effects assessed in *in vitro* motility assays using actin (or reconstituted thin filaments) and HMM. Results showed that proteins containing a combination of the C1, C2, and motif domains inhibited unregulated motility and Ca^{2+}-regulated motility in the presence of regulatory proteins and saturating Ca^{2+}. However, only proteins that contained both C1 and the motif activated Ca^{2+}-regulated motility in the absence of Ca^{2+}. These results suggest that both the C1 domain and the motif are required for activating effects in *in vitro* motility assays and that C1 and the motif may act as a functional unit to modulate actomyosin interactions.

This study was supported by NIH HL080367 and HL61683.

1444-Pos Ablation of Myosin Binding Protein C Accelerates the Kinetics of Cross-Bridge Cycling during the Myocardial Twitch

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Board B420

Myosin binding protein C (MyBP-C) is an accessory protein associated with the thick filaments of striated muscle. Previous measurements of k_{tr} and stretch activation in skinned myocardium from cardiac MyBP-C (cMyBP-C) knock out (KO) mice showed that cross-bridge interaction kinetics were accelerated by the deletion. Accelerated kinetics are at odds with the reduced ejection and slowed relaxation observed by echocardiography in the cMyBP-C KO hearts. Thus, we performed simultaneous intracellular Ca^{2+} and force measurements on intact papillary muscles to assess possible *in vivo* alterations of the force-pCa relationship. Normally, the Ca^{2+} peak occurs ahead of the force peak in a twitch. Any differences in the force-pCa relationship during the rise and fall of Ca^{2+} during twitches of KO and WT myocardium would contribute to alterations in the force *vs* Ca^{2+} hysteresis loop. At pacing rates of 1–3Hz, with or without dobutamine, KO papillary muscles exhibited significantly shorter time intervals between peak Ca^{2+} and peak force compared to WT, decreases in the area of the normalized force *vs* Ca^{2+} loop, and reductions in the increase in peak Ca^{2+} due to pacing. Neither relaxation time nor the duration of the Ca^{2+} pulse differed between KO and WT papillary muscles. The decreased time between the Ca^{2+} and force peaks indicates that cross-bridge kinetics were accelerated in KO myocardium, while the reduced area of the force-Ca^{2+} loop indicates that the activation response of the thin filament more closely followed the Ca^{2+} transient. Thus, cMyBP-C KO myocardium exhibited accelerated contraction kinetics and a lower peak Ca^{2+} during the twitch, both of which would contribute to depressed systolic function *in vivo*.

(Work supported by R37 HL82900).

1445-Pos Connectivity between Z-disc Proteins: Implications for Z-disc Stress-Sensor Function

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Board B421

The Z-disc is one of the nodal points of mechanosensing in cardiomyocytes. A complex consisting of T-cap, MLP, and titin (Z1–Z2) is thought to act as the Z-disc stretch sensor, possibly together with α-actinin, a major Z-disc protein that binds MLP and titin. Mutations in all four proteins are associated with DCM or HCM and it was postulated that a defect in the stretch sensor complex or associated proteins leads to an impairment of the transmission of the stretch-dependent mechanical signals. Here, several of the naturally found MLP mutations were created by site directed mutagenesis. Over-expression of these mutated MLP proteins in cultured embryonic rat cardiomyocytes did not reveal any obvious alterations in Z-disc organization. However, using GST-pull down assays we demonstrated that the MLP W4R mutation leads to a 50% reduction of the binding affinity to T-cap, compared to the wildtype protein. The MLP mutations, L44P and C58G, showed a 75% and 92% reduction, respectively, of the binding affinity to α-actinin-2. Stretch experiments with T-cap$^{(-/-)}$ myofibrils surprisingly showed unaltered positioning of the titin N-terminus in the Z-disc, detected by fluorescence staining with anti-Z1–Z2 antibodies. Only after removal of actin, using a gelsolin fragment, did the N-terminus detach from the Z-disc in T-cap$^{(-/-)}$, but not T-cap$^{(+/+)}$, myofibrils. We conclude that weakened interactions between the individual proteins of the Z-disc sensor do not cause gross disruption of the Z-disc structure, but likely affect the fine tuning of the Z-disc sensor.

1446-Pos Nitroxyl (HNO) Reversibly Modifies Critical Cysteine Residues to Induce Positive Inotropy in Isolated Murine Cardiomyocytes

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Board B422

Oxidants are increasingly appreciated as signals that may convey physiologically relevant messages, via reversible modification of thiol(-SH) moiety on critical cysteines. Nitroxyl (HNO) increases myocardial contractility, enhancing Ca^{2+} cycling, via increased Ca^{2+} release from ryanodine receptor (RyR2) and Ca^{2+} re-uptake into the SR via SERCA2a. In reconstituted RyR2, HNO-induced increase in open probability is reversed by the reducing agent dithiothreitol (DTT). Yet, direct evidence showing that HNO directly and reversibly targets -SH groups in intact myocytes is lacking. Cardiomyocytes were isolated from 2–6 mo old C57BL6 mice, and were resuspended in Tyrode's solution (1 mM Ca^{2+}) and field stimulated (0.5 Hz, 22–25°C) to assess sarcomere shortening (SS) (real-time imaging) and Ca^{2+} transients (Indo-1 fluorescence). First, we tested the effects produced by increasing concentrations (1, 2.5, 5 and 50μM) of the thiol-alkylator *N*-ethylmalemide (NEM). NEM enhanced SS in a dose-dependent manner: 13±10% (p=NS), 144±32%, 221±56% and 311±131%, respectively (all $p<.05$ vs base). This was paralleled by increased Ca^{2+} transients (20±13%, 63±19%, 74±42% at 2.5, 5 and 50μM, respectively, all $p<.05$), indicating that SH-alkylating agents *per se* are able to enhance contractility in intact cardiomyocytes. Opposite to HNO, NEM action was not blocked by DTT (increase of SS with NEM+DTT 147±22%, p=NS vs 2.5μM NEM). When the HNO donor Angeli's salt (AS, 0.5 mM) was infused during steady NEM response, no further inotropy was observed, proving available critical cysteine residues as the target of HNO. Similar results were obtained when cardiomyocytes were superfused with the fluorogenic maleimide CPM (20nM) that selectively blocks hyperreactive sulfhydryls, likely at RyR2 level. HNO requires available hyperactive thiols (-S.) to trigger/sustain inotropy in murine cardiomyocytes. However, differently from alkylating agents such as NEM, AS/HNO induces reversible, covalent modifications on its redox-sensitive targets.

1447-Pos Staurosporine inhibits Frequency Dependent Myofilament Desensitization in Intact Rabbit Trabeculae

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Board B423

Increases in heart rate change both contractility (force-frequency relationship or FFR) and relaxation rate (frequency dependent acceleration of relaxation or FDAR). These relationships are altered in heart failure and have been shown to contribute to the impaired contractility and/or relaxation. We have recently shown that myofilament calcium sensitivity decreases with frequency and this mechansim may contribute to FDAR. Phosphoprotein analysis revealed that both Troponin I and Myosin Light Chain-2 became more phosphorylated when frequency increased. We set out to test whether phosphorylation of these myofilaments are involved in the underlying molecular mechanism responsible for FDAR. Ultra-thin right ventricular trabeculae were isolated from New Zealand White rabbit hearts and iontophorically loaded with the calcium indicator bis-fura-2. Twitch force-calcium relationships and steady state force-$[Ca^{2+}]_i$ relationships were measured at frequencies 1 and 4 Hz at 37°C using potassium induced contractures. Staurosporine (100 nM), a non-specific Ser-Thr kinase inhibitor, or vehicle (DMSO) were included in the superfusion solution before and during the contractures. The EC_{50} of the force-calcium relationship shifted significantly 0.2 pCa units to the right upon an increase in frequency under control conditions (vehicle only, $P<0.01$). Staurosporine eliminated this shift such that there was virtually no change in sensitivity (0.04 pCa units to the left with an increase in frequency, P=n.s.). More specific kinase inhibitors such as KN93 (CaMKII) did not alter the normal shift in sensitivity observed with an increase in frequency. We conclude that a serine-threonine kinase is responsible for frequency dependent myofilament desensitization, and while we can eliminate CaMKII as the sole contributor, we cannot currently eliminate any of the other major possible candidates (PKC, PKG, PKA or a combination several).

1448-Pos Induction of Murine Cardiomyopathy by Alpha Tropomyosin Phosphorylation

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Board B424

Tropomyosin (TM) is a thin filament protein that regulates muscle contraction in a calcium dependent manner in cardiac muscle. Striated alpha TM is phosphorylated at serine residue 283. Embryonic mouse hearts have 60% of TM in the phosphorylated form whereas adult hearts contain less than 30% phosphorylated TM. To understand the role of TM phosphorylation in heart development and function, we developed a phosphomimetic TM transgenic mouse model, wherein serine 283 was replaced with aspartic acid to confer a constitutive negative charge to this amino acid, thus mimicking phosphorylation. We obtained different transgenic lines with high (100%), moderate (75%), and low (50%) expression of phosphomimetic TM specifically expressed in murine heart muscle. Detailed protein analysis shows that endogenous TM levels decrease with expression of the phosphomimetic TM. High expression mice die by 2 weeks of age due to severe dilation of both ventricles which culminates in heart failure. Moderate and low expression mice develop mild hypertrophic cardiomyopathy by 6 months and 20% of them die by 8 months of age. These results show that increased levels of phosphorylated TM in heart muscle leads to either dilated or hypertrophic cardiomyopathy. Physiological analysis of cardiac and myofilament function using the work performing heart model and skinned fiber bundles are currently in progress. In addition, thermal stability analysis of phosphomimetic TM by circular dichroism is being conducted. As such, this in vivo mouse model will enhance our understanding of the role of TM phosphorylation in cardiac development and sarcomeric function.

1449-Pos Binding Between TnI and Tropomyosin Regulates the Thin Filament

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Board B425

In the heart and in other striated muscles, the proteins of the troponin complex in conjunction with tropomyosin regulate force-producing interactions between actin thin filaments and myosin cross-bridges of the thick filament. Ca^{2+} dissociation from troponin inhibits muscle contraction, and involves repositioning of tropomyosin toward the myosin-binding part of actin. When Ca^{2+} dissociates from troponin C, a conformational change within troponin is believed to permit attachment of the TnI C-terminus (including a short, 'inhibitory peptide' region, and additional less defined elements) to actin and/or to tropomyosin. Crystallographic results suggest the TnI C-terminus does not fold as part of the isolated troponin core domain, particularly in the absence of calcium. To study this domain's function, we employed a human cardiac TnI fragment ($hcTnI_{131–211}$), that includes the residues of the inhibitory peptide (137–148) and extends to the C-terminus.

We found evidence of interaction between this C-terminal fragment of TnI and tropomyosin. In co-sedimentation assays, the peptide bound actin as expected, but also produced a co-sedimenting complex with tropomyosin (or equally with non-polymerizeable tropomyosin) in the absence of actin. The presence of the peptide appeared to enhance binding of tropomyosin to actin. Actin-myosin S1 ATPase assays showed that both full length TnI and $TnI_{131–211}$ were greatly inhibitory, but only the former inhibited fully at sub-stoichiometric concentrations. To better define the TnI-tropomyosin interaction site, tropomyosin internal deletion mutants, tropomyosin fragments, and smaller TnI peptides also are under study. Present data are supported by collaborative (Galinska-Rakoczy et al) EM reconstruction data showing the truncated cTnI draping over Tm and binding to subdomain 1 of actin. We predict this binding between cTnI and tropomyosin is a significant aspect of Ca^{2+}-sensitive regulation of actin and myosin in the sarcomere.

1450-Pos Impact of PKA Or PKC Contractile Protein Phosphorylation On Myosin Head Orientation As Measured By X-Ray Diffraction

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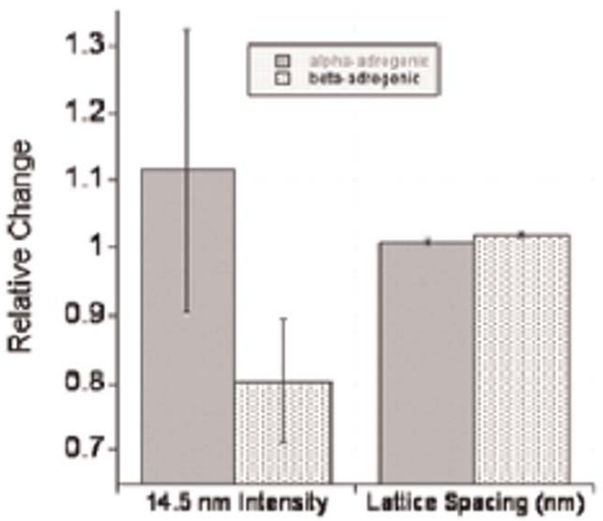
Board B426

Phosphorylation by PKA in skinned myocardium has been shown to induce either an increase or decrease in lattice spacing depending on which contractile proteins are phosphorylated Konhilas et al. (J. Physiol *547,3* 951–961 2003). Whether PKA or PKC mediated phosphorylation alters the structure of intact myofilament lattice proteins is still unknown. Here we measured the functional and structural parameters of isolated rat trabeculae (electrically stimulated at 1Hz, 1mM Ca^{2+}, 25^{OC}) with and without activation of either the PKC (Phenylephrine w/propanolol 10/1 $\hat{I}^1/_4$M) or PKA (Isoproterenol, 50 nM) pathways. Drug treatment induced a ~10% increase in twitch force. As shown in the included figure, stimulation of either kinase pathway induced significantly different changes in the orientation of the myosin heads, as assessed by the intensity of the 14.5 reflection (~10% increase PKA, ~20% decrease PKC) measured under diastolic conditions, with no significant change in the lattice spacing. We conclude that because selective activation of either PKA or PKC induces a similar force response with different (diastolic) orientations of the myosin heads, PKC enhances twitch force via structural changes in the thin filament.



1451-Pos Protein Kinase A Decreases Ca^{2+} Sensitivity Of Force In Skeletal Muscle After Reconstitution With Cardiac Troponin

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Board B427

It is well established that protein kinase A (PKA) decreases Ca^{2+} sensitivity of force in cardiac muscle via phosphorylation of troponin I (TnI). In the present study, we investigated whether or not PKA decreases Ca^{2+} sensitivity of force in skeletal muscle as well, after reconstitution with cardiac Tn, taking advantage of our quasi-complete Tn exchange technique (Biophysical Society, 2007). Troponins were extracted from porcine ventricular and rabbit psoas muscle (Ca^{2+} sensitivity: former < latter under control condition).

Without Tn exchange, PKA decreased Ca^{2+} sensitivity in cardiac (porcine ventricular) muscle, but not in skeletal (rabbit psoas) muscle. Reconstitution of cardiac thin filaments with skeletal Tn not only increased Ca^{2+} sensitivity, but abolished the PKA effect. This suggests that phosphorylation of TnI, but not that of myosin-binding protein C, is primarily responsible for the PKA-based reduction in Ca^{2+} sensitivity. Conversely, reconstitution of skeletal thin filaments with cardiac Tn decreased Ca^{2+} sensitivity, which was decreased further by PKA to a level similar to that observed in PKA-treated cardiac muscle. We confirmed that subsequent exchange for skeletal Tn restored Ca^{2+} sensitivity to the original level. These results suggest that PKA-based TnI phosphorylation results in a decrease in Ca^{2+} sensitivity regardless of the type of muscle, via alteration of the on-off switching mechanism of the thin filament state.

1452-Pos Impact of Troponin I phosphorylation on contractile function in human myocardium

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Board B428

Cardiac muscle contraction is regulated by intracellular calcium through the troponin complex (cTn). Previous animal studies have indicated that upon β-adrenergic stimulation, protein kinase A (PKA) is capable of phosphorylating the troponin subunit I (cTnI) at serines 23 and 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 other contractile proteins as well and species differences may be present as well. To address these points, a selective exchange procedure was used in which approximately 50% of the endogenous cTn in permeabilised human cardiomyocytes was exchanged with recombinant unphosphorylated human cTn (cTn-U) and with cTn, pre-treated with PKA to fully saturate serines 23/24 of cTnI (cTn-P). This permits the study of the specific effects of PKA-mediated phosphorylation of the cTn complex on contractile properties in human cardiac myocytes. Results obtained in cardiomyocytes isolated from healthy donor hearts, show a significant reduction in Ca^{2+} sensitivity upon exchange with Tn-P complex. The pCa_{50} values amounted to 5.55±0.02 for cTn-U and 5.45±0.03 for cTn-P. In both groups a further decrease in Ca^{2+} sensitivity to a similar level of 5.43±0.03 was observed after an additional treatment of the exchanged myocytes with PKA. In failing cardiomyocytes, in which phosphorylation levels of several contractile proteins are significantly decreased, the impact of an altered phosphorylation background has been studied. The data obtained in failing myocardium suggest that the decrease in myofilament Ca^{2+} sensitivity can be attributed solely to PKA-mediated cTnI phosphorylation.

1453-Pos Reduced Force Production And Ca2+ Sensitivity Of Force Activation During Low Blood Flow To The Heart Correlates With Altered Troponin I Phosphorylation

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A rat model of low myocardial blood flow was established to test the hypothesis that post-translational changes to proteins of the muscle filaments correlate with reduced contractility. Following three days of reduced blood flow by constriction of the left anterior descending artery, the rat hearts demonstrated a reduction in fractional shortening at rest, and a relative decline in fractional shortening when challenged with high dose versus low dose dobutamine, reflecting reduced energy reserves. Permeabilized fibres prepared from the low flow rat hearts demonstrated a reduction in maximal force and Ca2+ sensitivity as compared to their sham operated counterparts. An examination of contractile proteins by two-dimensional gel electrophoresis as well as phosphorylation site-specific antibodies demonstrated a significant reduction in Ser23/24 phosphorylation of troponin I (TnI) despite no significant change in total TnI phosphorylation. Furthermore, TnI from low flow myocardium had reduced relative affinity to Ca2+ bound troponin C (TnC) when compared to TnI from sham operated hearts. These results suggest a concomitant increase in TnI phosphorylation at sites unique from Ser23/24, most likely increased phosphorylation of protein kinase C target sites Ser43 and 45, reducing the affinity of TnI for TnC. Additional data will be presented examining the state of troponin T, regulatory and essential myosin light chain as well as myosin binding C-protein phosphorylation.

1454-Pos Kelch-Related Protein Krp1 Promotes Lateral Fusion of Myofibril Assembly Intermediates in Cultured Mouse Cardiomyocytes

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Board B430

Krp1 is a cardiac and skeletal muscle kelch-repeat protein hypothesized to promote myofibril assembly through interaction with N-RAP and actin. We have studied the distribution and function of Krp1 in cultured embryonic mouse cardiomyocytes. While immunofluorescence showed punctate Krp1 distribution throughout the cell, detergent extraction revealed a significant pool of Krp1 associated with cytoskeletal elements. To determine its role in myofibrillogenesis, Krp1 expression was reduced by siRNA. Cardiomyo-

cytes were fixed five days after transfection and stained for alpha-actinin and Krp1. Immunofluorescence revealed that cardiomyocytes with low levels of Krp1 often lacked mature myofibrils as defined by alpha-actinin localization in Z-lines. Instead, cells contained many dots of alpha-actinin, or Z-bodies. Despite the defect in myofibril accumulation, cells were otherwise healthy as indicated by lack of apoptosis, normal spreading over time in culture, and retention of intact organelles assessed by electron microscopy. To quantitate the change in phenotype, cardiomyocytes were categorized according to the dominant pattern of alpha-actinin organization. Almost all cells transfected with control siRNA were filled with well-aligned myofibrils, and only 5% accumulated dots of alpha-actinin. In contrast, 55% of cardiomyocytes transfected with Krp1 siRNA were filled with Z-bodies or narrow Z-lines that were often periodically spaced in series resembling newly forming myofibrils. Confocal microscopy of cells stained for actin, myosin, and myomesin demonstrated that these structures contained sarcomeric proteins with longitudinal periodicities similar to mature myofibrils, and electron microscopy showed normal thick and thin filaments. However, fibrils remained thin and separated. The data indicate that Krp1 is specifically required for lateral fusion of adjacent fibrils into mature myofibrils but is not necessary for periodic longitudinal organization of actin and myosin filaments.

1455-Pos Endothelin-1 and Phenylephrine Mediated Alterations in CapZ Actin Binding Affinity occur via PIP2 and PKC-Mediated Mechanisms

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Board B431

Cardiac myocytes undergo hypertrophy in response to agonist stimulation via cytoskeletal and sarcomeric remodeling. We hypothesized that reduced affinity of the actin capping protein (CapZ) for actin facilitates the hypertrophic response by enhancing sarcomeric addition and remodeling. Therefore, CapZ dynamics were analyzed by fluorescence recovery after photobleaching (FRAP) in neonatal rat ventricular myocytes (NRVM) treated with endothelin-1 (ET) (100nM, 4h treatment) or phenylephrine (PE) (10 μM, 24h treatment). We introduced GFP fusions of either wild-type or one of two actin binding deficient ß1 mutant (L262R or a C-terminal deletion) CapZ constructs via recombinant adenovirus into NRVM. Exogenous protein localized to the Z-disk, and cell surface area increased after ET treatment in cells infected with each of the 3 CapZ vectors, indicating a responsiveness to hypertrophic stimuli. FRAP analysis 30 min postbleach showed wtCapZ infected cells treated with ET recovered more completely than controls (77±9% vs. 50±6%, $p<0.001$, $n=8$). Similar results were found with PE (77±5%, $p<0.05$, $n=8$). Studies of mutant CapZ recovery demonstrated that L262R recovery was similar to untreated cells (55±7%, $n=12$), whereas the C-terminal deletion mutant was similar to that of agonist treated cells (73±12%, $n=5$). We hypothesized the increased CapZ exchange in ET and PE treated cells was PIP2 mediated. PIP2 sequestration with neomycin (500 μM, 4h pretreatment) blocked both ET (43±6%, $p<0.001$, $n=7$) and PE (36±4%, $p<0.001$, $n=11$) mediated recovery. The PKC inhibitor chelerythrine chloride (10 μM, 4h pretreatment) also blocked ET mediated recovery (53±10%, $p<0.001$, $n=6$). Our results suggest that ET and PE alter CapZ actin affinity through PIP2 dependent pathways; ET-1 alteration of affinity is also PKC dependent; and this alteration is mimicked by actin binding mutants of CapZ.

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1456-Pos RhoA/ROCK Signaling in Substrate Stiffness Control of Neonatal Rat Cardiomyocyte Maturation

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Board B432

Cardiac cells mature and adapt to a changing mechanical environment in the first postnatal week. In the absence of differences in adaptation or maturation, we would expect that cells on stiff substrates, which have nearly isometric contractions, would generate more force than cells on softer substrates due to greater actin-myosin overlap throughout the contraction and kinetic effects. However, we have found that the maturation of neonatal rat cardiomyocytes plated on collagen-coated polyacrylamide gels with elastic moduli from 1 to 50 kPa and cultured for 7 days depends on the stiffness of the gel. Cardiomyocytes generated greater mechanical force, had larger calcium transients and sarcoplasmic reticular (SR) stored calcium and had greater expression of some SR components on gels with an elastic modulus similar to the native myocardium, 10kPa, than on stiffer or softer substrates. We also observed stress fiber formation, with reduced sarcomere formation, in myocytes on very stiff surfaces. We hypothesized that the formation of stress fibers may compete with formation of well-defined, aligned sarcomeres on stiff surfaces and that inhibition of stress fiber formation, through inhibition of the RhoA/ROCK pathway might allow more complete cardiomyocyte maturation. To test this hypothesis, we inhibited several components of this pathway and measured functional parameters such as force development and calcium signaling. We found that inhibition of this pathway results in increasing force with increasing stiffness, as initially predicted for identical cells. We conclude that the activation of the ROCK pathway, leading to the formation of stress fibers, results in poor functional maturation, as measured by low force generation and SR calcium, of neonatal rat cardiomyocytes on very stiff substrates.

1457-Pos A Role For Rho-kinase In Ca^{2+}-independent Contraction Induced By Phorbol 12,13-dibutyrate

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