1823-Pos
Effects of Human Cardiac Troponin T Mutations Associated with Cardiomyopathy
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Mutations in troponin, an important muscle protein complex, can result in cardiomyopathy by interfering with the normal muscle activity of the heart. Troponin T (TnT) is the largest subunit of troponin and is involved in binding the troponin complex to the thin filament. Investigation of two mutations associated with cardiomyopathy in TnT, I90M and R173Q, showed different physiological characteristics. The TnT I90M mutation was identified as the causative agent of familial hypertrophic cardiomyopathy (FHC) in a large multi-generational Chinese family and at least two family members with this mutation died of sudden cardiac death. Another mutation in TnT, R173Q, was identified as the underlying cause of dilated cardiomyopathy (DCM). Patients with the TnT R173Q mutation experienced prenatal onset DCM and supraventricular tachycardia at a young age. Functional troponin complexes containing wild-type or mutant TnT's demonstrated similar maximal actomyosin ATPase activity. The inhibitory ability of the troponin complexes containing the I90M mutation was significantly reduced relative to wild-type TnT. Most RCM mutations investigated to date showed a reduced ability to inhibit actomyosin ATPase activity but the RCM mutation, R173Q, did not affect the inhibitory ability of troponin. The mutations showed increased (I90M) and decreased (R173Q) calcium sensitivity of actomyosin ATPase activity consistent with what has been observed for most FHC and DCM mutations. The mutations reduced the rate of degradation of these proteins by calpain relative to wild-type TnT. Overall, these results suggest that although calcium sensitivity may be an indicator of the type of cardiomyopathy no clear trends in maximal or minimal ATPase activity exist that can be used to characterize DCM and FHC mutations.

1824-Pos
Functional Consequences of a Novel Cardiac Troponin T Mutation Linked to Infantile Restrictive Cardiomyopathy
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A novel double deletion in cardiac troponin T (cTnT) of two highly conserved amino acids (N100 and E101) was identified in the cardiac cDNA of a pediatric transplant recipient. The patient previously presented with restrictive and hypertrophic cardiomyopathy. Family work-up was negative, and she was found to harbor a *de novo* mutation. Electron microscopy revealed the presence of myofibrillar disarray and fibrosis. To further define this cTnT mutation as a cause of the disease, functional studies were performed. Functional effects of the single and double cTnT mutants (ΔN100, ΔE101 and ΔN100/ΔE101) were analyzed in porcine skinned papillary muscle. Fibers were displaced with exogenous cTnT mutants or WT, Ca^{2+} unregulated force was measured and then reconstituted with binary cTnI.cTnC complex. The ΔN100 and ΔE101 mutations showed opposing changes in the Ca^{2+} sensitivity of force development compared to WT. The ΔN100 mutation increased this by 0.29 pCa units and the ΔE101 mutation, in contrast, decreased it by 0.28 pCa units. Interestingly, the ΔN100/ΔE101 mutation shifted the Ca^{2+} sensitivity to the left (+ 0.19 pCa units). This finding is compatible with the preserved systolic function in this patient. ΔE101 was the only mutation that decreased the maximal force recovery compared to WT. In contrast, ΔN100 and ΔN100/ΔE101 did not show significant changes in this parameter. Both ΔN100 and ΔN100/ΔE101 exhibited decreased cooperativity of force development, suggesting altered intra-filament protein-protein interactions. These data show that residue N100 has a predominant effect over E101 and its absence is much more deleterious for cTnT function. In addition, the strength of the functional data validates this novel cTnT deletion mutant as the cause of this cardiomyopathy. Supported by NIH HL-42325(JDP), AHA 0825368E (JRP), AHA 09POST2300030 (MSP) and CIHR GMHD 79045 (GA).

1825-Pos
Biophysical and Biochemical Studies of Human Slow Skeletal Troponin T Isoforms in Slow Skeletal Muscle
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A paucity of information exists concerning the functional roles of the human slow skeletal troponin T isoforms (HSSTnT isoforms) in different muscle types. Three HSSTnT isoforms have been found in slow skeletal muscle: HSSTnT1 (+ Exons 5 and 12), HSSTnT2 (+5, −12), HSSTnT3 (−5, −12) and HSSTnT4 (−5, +12, only found at the mRNA level). Soleus rabbit skinned fibers were displaced with HSSTnT1, 2, 3 or 4 and reconstituted with human SSTnI-C/STnC complex. The extent of Tn displacement was analyzed by measuring the Ca^{2+} unregulated force (UF) at pCa 8.0 after SSTnT treatment. The UF ranged from 63 to 73%. The Ca^{2+} sensitivity increased between SSTnT isoforms: isoform 1 ($pCa_{50} = 5.73$) < isoform 2 ($pCa_{50} = 5.80$) < isoform 3 ($pCa_{50} = 5.84$). HSSTnT4 yielded a $pCa_{50} = 5.78$. Using a reconstituted fast skeletal muscle system, the actomyosin ATPase activity containing different HSSTnT isoforms was determined. The HSSTnT isoforms did not alter ATPase activation or inhibition in the presence or absence of Ca^{2+}. Potential interactions between human cardiac troponin C (HcTnC), rabbit skeletal tropomyosin (RsTm) and human cardiac troponin I (HcTnI) with SSTnT were mapped. Dot blot analysis using HRP conjugated proteins revealed new interactions between SSTnT peptides and HcTnC, RsTm and HcTnI. These results may help identify the functional differences that occur between SSTnT isoforms due to their alternative splicing. Supported by NIH HL-042325(JDP) and AR-050199 (JDP) and AHA 0825368E (JRP).

1826-Pos
Does the DCM Functional Phenotype Predominate over that of HCM and RCM?
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In vitro investigations into Hypertrophic Cardiomyopathy (HCM) and Restrictive Cardiomyopathy (RCM) show that mutations in cardiac Troponin T (cTnT) produce a pathogenic state via increase in myofilament Ca^{2+} sensitivity, whereas mutations in cTnT that cause Dilated Cardiomyopathy (DCM) decrease Ca^{2+} sensitivity and maximal force. Our aim was to determine whether combinatory mutations of an HCM, RCM and a DCM in cTnT yield an intermediate or dominant functional phenotype that could be correlated with the clinical condition seen in patients. Standard laboratory methods were used for cloning, expression and purification of the WT and mutants: ΔK210 (DCM), I79N (HCM), ΔE96 (RCM), ΔK210/I79N, ΔK210/ΔE96 and ΔE96/I79N. Porcine papillary skinned fibers were displaced with cTnT WT or mutant and reconstituted with HCTnI-TnC. The Ca^{2+} sensitivity of force development, maximal force and basal force were evaluated. The extent of TnT displacement was analyzed by measuring the unregulated tension at pCa 8.0 after cTnT treatment and none of the mutants showed an inability to displace the native cTn complex. Both double mutants (ΔK210/I79N and ΔK210/ΔE96) containing the DCM mutant showed a rightward shift in the Ca^{2+} sensitivity with a decrease in maximal force. In addition, the ΔK210 mutation rescued the impaired relaxation produced by the RCM mutation (ΔE96). From the skinned fiber data, ΔK210 has a dominant effect over I79N and DE96 mutations in cTnT. Circular dichroism measurements demonstrated that all three double mutants had lower alpha helical content than WT. In contrast, single mutants I79N (significantly) and DK210 (showed a tendency) to increase alpha helical content. These results suggest that cTnT can exist in multiple conformations that may be responsible for these distinct functional phenotypes. NIH HL-67415 and HL-42325 (JDP), AHA 0825368E (JRP).

1827-Pos
Cardiomyopathy-Causing Deletion K210 in Cardiac Troponin T Alters Phosphorylation Propensity of Sarcomeric Proteins
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Ca^{2+} desensitization of myofilaments is indicated as a primary mechanism for the pathogenesis of familial dilated cardiomyopathy (DCM) associated with the deletion of lysine 210 (ΔK210) in cardiac troponin T (cTnT). ΔK210 knock-in mice closely recapitulate the clinical phenotypes documented in patients with this mutation. Considerable evidence supports the proposition that phosphorylation of cardiac sarcomeric proteins is a key modulator of function and may exacerbate the effect of the deletion. In this study we investigate the impact of K^{210} deletion on phosphorylation propensity of sarcomeric proteins. Quantitative analysis of cardiac myofibrils isolated from ΔK210 hearts identified a decrease in basal phosphorylation of cTnI (46%), cTnT (29%) and MyBP-C (31%) compared with wild type controls. Interestingly, immunoblot analyses with phospho-specific antibodies show augmented phosphorylation of cTnT-Thr^{203} (28%) and decreased phosphorylation of cTnI-$Ser^{23/24}$ (41%) in mutant myocardium. *In vitro* kinase assays indicate that ΔK210 increases phosphorylation propensity of cTnT-Thr^{203} three fold without changing cTnI-$Ser^{23/24}$

phosphorylation. Molecular modeling of cTnT-ΔK210 structure reveals changes in the electrostatic environment of cTnT helix (residues 203-224) that lead to a more basic environment around Thr^{203}, which enhances PKC-dependent phosphorylation. In addition, yeast two-hybrid assays indicate that cTnT-ΔK210 has enhanced binding to cTnI compared with cTnT-wt, and may impair Ca^{2+} sensing/transmission leading to myofilament desensitization. Collectively, our observations suggest that cardiomyopathy-causing ΔK210 has far-reaching effects influencing posttranslational modifications of key sarcomeric proteins, and potentially cTnI-cTnT interaction.

1828-Pos

Em and Single Particle Analysis of Troponin at Low and High Ca^{2+}

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Crystal structures of the troponin ''core-domain'' formed in the presence and absence of Ca^{2+} display a bilobed TnC subunit mounted on a semi-rigid scaffold formed from major stretches of TnI and TnT. A central coiled-coil of TnI and TnT is flanked by single TnI and TnT helices to form the W-shaped supporting structure, which appears to be little changed by the binding of Ca^{2+} (Takeda et al., 2003; Vinogradova et al., 2005). In contrast, at low Ca^{2+}, the central helix joining C- and N-lobes of TnC melts, and the ''regulatory'' C-terminal domain of TnI dissociates from the N-lobe of TnC (Vinogradova et al., 2005). Consistent with biochemical studies, the C-terminal TnI sequences in the thin filament are thought to latch onto actin and constrain tropomyosin in the blocked state at low-Ca^{2+}. Their dissociation from actin at high-Ca^{2+} and association with the N-terminal lobe of Ca^{2+}-saturated TnC may relieve the constraint (Galinksa-Rakoczy et al., 2008). These conclusions remain uncertain, however, because troponin is only semi-rigid (so crystal packing forces may have influenced the structure) and the troponin complex used for crystallization contained truncated subunits. Here we have studied isolated, intact troponin molecules using negative stain electron microscopy and single-particle image processing. Averaged projection views and 3D reconstructions of the isolated molecules show many of the same features seen in the crystal structures. Comparison of reconstructions of low and high Ca^{2+} data suggests that the TnC N-lobe of cardiac troponin may be further from the core domain in the EM than in the crystal structure.

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1829-Pos

Nanobiology of the Cardiac Myofilament

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The cardiac myofilament is a protein assembly that provides Ca-regulated force development enabling the heart to undergo alternating periods of contraction and relaxation. Troponin (Tn), a three-member protein assembly within the myofilament, acts as a Ca-sensitive switch. Here, using single pair FRET in freely diffusing assemblies of Tn, we show that Tn incompletely activates after binding regulatory Ca. The reserved population of inactive Tn appears to function as a nanoscopic form of cardiac reserve that can be can be manipulated by cell signaling mechanisms to fine-tune cardiac contractility. The results are discussed in terms of an energetic model of the cardiac myofilament.

1830-Pos

Investigating the Effect of Cardiomyopathy-Causing Mutations in Cardiac Troponin-T on Calcium Buffering *In Situ*

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In vitro investigation of the effects of cardiomyopathy-causing mutations in thin filament regulatory proteins has demonstrated that hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) are caused by distinct primary alterations of cardiac contractility and myofilament calcium affinity. We hypothesise that chronically altered calcium-buffering by mutant thin filaments leads to altered calcium handling and, via calcium-dependent signalling pathways, contributes to disease pathogenesis. We aim to study the *in situ* effect on calcium flux of a HCM and a DCM causing mutation in human cardiac troponin T (cTnT) (R92Q, R131W respectively), by adenoviral mediated expression of mutant protein in adult guinea pig cardiomyocytes. The adenoviral vectors co-express GFP and western blot analysis of FACS-sorted, GFP-expressing cells showed that recombinant cTnT comprised 45-50% of the total cTnT in these cardiomyocytes, 48 hours after infection. Analysis of unloaded sarcomere shortening showed that at an excitation frequency of 2 Hertz, cardiomyocytes infected with R131W cTnT elongated the time to 50% relaxation and reduced the magnitude of contraction, whilst R92Q cTnT reduced the time to 50% relaxation and increased the contractile magnitude compared to wild type. Analysis of calcium transients of the same cells using fura-2 loading, indicates that the R92Q mutation reduces calcium transient amplitude, whilst the R131W mutation increases the time to complete calcium reuptake, with no change to the transient amplitude, despite the observed decrease in contraction. We are currently assessing the caffeine transients of these cells to investigate alterations to overall SR load and measuring alterations to components of calcium-dependent signalling cascades which may link the acute effects of cTnT mutations to macroscopic remodelling observed in the pathological disease states of HCM and DCM.

1831-Pos

The Small Molecule Smooth Muscle Myosin Inhibitor, CK-2018571, Selectively Inhibits ATP Hydrolysis and Relaxes Smooth Muscle *In Vitro*

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Smooth muscle contraction is driven by cyclical, nucleotide-dependent changes in myosin conformation that alter its affinity for actin, produce force, and generate movement. We used a high throughput screen to identify compounds that inhibit the ATPase activity of smooth muscle myosin; optimization of the initial hit compounds has resulted in compounds with nanomolar affinity. A potent representative of this chemical series, CK-2018571, inhibits the steady-state ATPase activity of human smooth muscle myosin at low nanomolar concentrations, approximately 10-fold lower than are required to inhibit non-muscle myosin, the most closely related myosin II. Selectivity between smooth and striated myosin IIs are >100-fold. Transient kinetic studies demonstrate that CK-2018571 inhibits the myosin-catalyzed hydrolysis of the γ-phosphate group of ATP, with no effect on nucleotide binding or release from the enzyme. Actin co-sedimentation assays indicate that CK-2018571 stabilizes a weak actin-binding conformation of myosin in the presence of ATP. Consistent with this mechanism, CK-2018571 relaxes skinned rat tail artery muscle tissue at low micromolar concentrations. Importantly, this relaxation occurs regardless of whether the skinned muscle has been activated by calcium or by thiophosphorylation of the myosin regulatory light chain, supporting evidence that CK-2018571 relaxes smooth muscle tissue by direct inhibition of activated smooth muscle myosin. The ability of CK-2018571 to relax intact tracheal smooth muscle and aortic ring preparations at micromolar concentrations suggests this mechanism may prove useful in diseases of smooth muscle hypercontractility, such as hypertension and asthma.

1832-Pos

Direct Interaction between the C-terminus of the Myosin Light Chain Phosphatase Targeting Subunit and Myosin Phosphatase-Rho Interacting Protein

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Both the Ca^{2+} signal and the alteration of the Ca^{2+} sensitivity of the contractile apparatus regulate smooth muscle contraction. Myosin light chain kinase (MLCK) phosphorylated the 20 kDa regulatory myosin light chain (MLC20) resulting in contraction. Myosin light chain phosphatase (MLCP) dephosphorylates MLC20 causing relaxation. Thus, the balance between the activities of MLCK and MLCP determines the level of MLC20 phosphorylation.

MLCP consists of a 38 kDa catalytic subunit (PP1cδ), a 110 kDa targeting subunit (MYPT1), and a 21 kDa small subunit (M21). MYPT1 provides the substrate specificity and the regulation of phosphatase activity. It was reported that myosin phosphatase-Rho interacting protein (M-RIP) bound MYPT1 and thus targeted MLCP to the actomyosin contractile filament based on yeast-two hybrid and cell biological assays.

To determine if MYPT1 binds to M-RIP directly, we performed analytical ultracentrifugation (AUC) study using purified peptides of MYPT1 and M-RIP.