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.

Funding: HSJ (KBSI-T29760); NIH: WL (HL36153), LST (HL38834, HL63774), RC (AR34711, RR08426).

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.

Circular dichroism and AUC analysis illustrated that the M-RIP peptide spanning residues 724-878 of M-RIP is a coiled coil and forms a dimer. The AUC analysis demonstrated that the C-terminal coiled-coil region of MYPT1 spanning residues 924-991 did not bind the M-RIP peptide, whereas the C-terminal random coiled-coil region of MYPT1 (synthetic LZ) spanning residues 991-1030 did bind, forming a heterotrimer. In addition, three individual glutamic acid residues (amino acids 998-1000) of MYPT1 were critical for binding. We replaced the glutamic acids either all three at a time or one at a time with glutamine residues. In addition, we replaced all three glutamic acids with aspartic acids. However none of these mutants bound to synthetic LZ demonstrating that these three glutamic acid residues are essential for binding.

1833-Pos

A Dynamic Approach Reveals Nonmuscle Myosin Influences the Overall Smooth Muscle Cross-Bridge Cycling Rate

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The mechanism of force maintenance in smooth muscle has yet to be elucidated, but recent evidence suggests that nonmuscle myosin IIB (NMIIB) contributes to the mechanical properties of smooth muscle. This study was designed to determine the affects of NMIIB on the overall cross-bridge cycling rate. Aortic smooth muscle strips from homozygous NMIIB KO ($B^{+/-}$) and WT littermates were stimulated to contract (80 mM KCl) and the force response to a sinusoidal change in length (~1% Lo) at frequencies between 0.25 and 125 Hz was recorded. The length perturbation and the corresponding force were expanded into Fourier series to calculate the stiffness and phase frequency responses and the data was illustrated in Bode diagrams. Steady state tension was significantly less for the $B^{+/-}$ than for the WT mice. Frequency analysis revealed two distinct regions in the Bode plots, and the individual regions were fit to find the asymptotes representing the low and high frequency regions. The intersection of the two asymptotes occurred at 12.86 ± 0.243 Hz for WT and 17.33 ± 0.261 Hz for $B^{+/-}$. Further, the slope of the relationship between tension/stiffness and frequency was significantly higher for the WT than $B^{+/-}$ mice. These data suggest in WT mice that the force per attached cross-bridge is higher and duty cycle longer. These data demonstrate a decrease in NMIIB produces a fall in the force per attached cross-bridge and an increase in the overall cross-bridge cycling rate. These data could suggest that a decrease in the relative expression of NMIIB would decrease steady state force more than stiffness to decrease both the force per attached cross-bridge and internal load to shortening and result in an increase in the overall cross-bridge cycling rate.

Key words: Nonmuscle Myosin, Stiffness, Frequency Response

1834-Pos

Structural Change of N-terminus in Smooth Muscle Myosin Regularly Light Chain using Accelerated Molecular Dynamics

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A novel accelerated molecular dynamics method (the Orthogonal Space Random Walk algorithm, OSRW) is applied to study the effect of the regulatory light chain (RLC) phosphorylation on the structure of the light chain binding domain of smooth muscle myosin. Smooth muscle myosin is activated by phosphorylation on the S19 (and T18 subsequently) at the N-terminus of the RLC that causes a conformational change from the closed inhibited asymmetric structure (Wendt et al. PNAS 2001) to the open structure by an unknown mechanism. The N-terminus also plays an important role in stabilizing the folded 10S conformation that is soluble at physiological ionic strength. However, X-ray structures of the RLC do not show the 24-residue N-terminus, which holds the phosphoration site. Thus, we are performing MD simulations on the 21 residues of the N-terminus as well as the RLC with part of the heavy chain. The phosphorylated N-terminus shows a bent α-helical conformation, where the S19 interacts with the R16. The unphosphorylated N-terminus has showed a straight α-helical conformation. Those simulations are carried out in explicit water under near-physiological conditions. The OSRW has demonstrated hundreds of times sampling capacity in compared with regular MD, which will promote our understanding on the phophorylation activation mechanism. This work is supported by NIAMSD.

1835-Pos

Additional Sites are Involved in the Regulation of Caldesmon by PAK Phosphorylation

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Caldesmon is an actin- and myosin-binding protein that is rich in smooth muscle. Caldesmon inhibits the actin activation of myosin catalyzed ATPase activity and may have additional functions in smooth muscle. The activity of caldesmon is controlled by phosphorylation and by binding to other factors such as Ca^{++}-calmodulin. Caldesmon is a substrate for p^{21}-activated kinase, PAK, which is reported to phosphorylate chicken gizzard caldesmon at two sites, Ser672 and Ser702. We investigated PAK phosphorylation of caldesmon using a 22kDa C-terminal caldesmon fragment. We also substituted Ser672 and Ser702 with either alanine or aspartic acid residues to mimic non-phosphorylated and constitutively phosphorylated states of caldesmon, respectively. We found that the aspartic acid mutation of caldesmon weakened calmodulin binding but had no effect on the inhibitory activity of caldesmon. Phosphorylation of the aspartic acid double mutant with recombinant PAK resulted in additional phosphorylation at Thr627, Ser631, Ser635 and Ser642. Phosphorylation at these sites by PAK was slow, but produced further weakening of calmodulin binding and reduced the inhibitory activity of caldesmon in the absence of calmodulin. Phosphorylation at the additional sites was without effect on Ca^{++}-Calmodulin binding if Ser672 and Ser702 were not phosphorylated, but was sufficient to release inhibition of actomyosin ATPase activity. This work raises the possibility that phosphorylation in the region of residues 627-642 significantly alters the activity of caldesmon.

1836-Pos

Purinoceptor Signaling in Arterial Smooth Muscle is Regulated by G-Protein-Coupled Recptor Kinase-2

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The regulation of arterial smooth muscle cell (SMC) contraction by adenine and uridine nucleotides, plays a key role in controlling systemic blood pressure. In SMCs, UTP activates P2Y receptors (subtypes 2/4/6), which couple via $G\alpha_{q/11}$-proteins to stimulate phospholipase C, increasing IP_3/Ca^{2+} concentrations and leading to SMC contraction. Continuous or repeated receptor stimulation reduces responsiveness to further stimulation, a process termed desensitization. Receptor desensitization is often regulated by G protein-coupled receptor kinases (GRKs), which phosphorylate receptors, enhancing their interaction with β-arrestins and uncoupling them from G-proteins.

We investigated the regulation of receptors responding to UTP, which mediates concentration-dependent contraction in rat mesenteric arteries. To characterize adaptations that occur on repeated UTP additions, changes in IP_3 and $[Ca^{2+}]_i$ were assessed using single-cell imaging. Receptor desensitization was assessed by challenging mesenteric SMCs with an EC_{50} concentration of UTP (10 μM) for 30 sec before (R1) and after (R2) the addition of a maximal UTP concentration (R_{max}, 100 μM, 30 sec) with 5 min washout periods. The change in R2 relative to R1 was used to characterize P2Y receptor desensitization. By extending the washout period after R_{max} a time-dependent recovery of IP_3/Ca^{2+} responses were observed. To evaluate the involvement of individual GRKs in this process, cells were transfected with catalytically-inactive, dominant-negative GRK mutants.

Using IP_3 generation to indicate receptor recovery (R2/R1%), over-expression of ^{D110A,K220R}GRK3 ($34 \pm 4\%$), ^{K215R}GRK5 ($38 \pm 7\%$), or ^{K215R}GRK6 ($29 \pm 8\%$) caused similar reductions in IP_3 levels to those in empty-vector-transfected cells ($23 \pm 5\%$). In contrast, expression of ^{D110A,K220R}GRK2 ($58 \pm 7\%$) markedly attenuated receptor desensitization (n=10-19 cells, >3 animals). Furthermore, siRNA-mediated knockdown (>75%) of GRK2 protein also attenuated agonist-induced receptor desensitization compared to control ($68 \pm 8\%$ versus $29 \pm 6\%$, respectively, n=8-12). In conclusion, this work implicates GRK2 as the pre-eminent GRK isoenzyme regulating UTP signaling in SMCs.

1837-Pos

TR-FRET Experiments and MD Simulations Resolve Structural States of Smooth Muscle Regulatory Light Chain

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