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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We have combined time-resolved fluorescence resonance energy transfer (TR-FRET) and molecular dynamics (MD) simulations to elucidate structural changes in the phosphorylation domain (PD) of chicken gizzard smooth muscle regulatory light chain (RLC) bound to smooth muscle myosin. The PD is absent in crystal structures, leaving uncertainty about the mechanism of regulation. Donor-acceptor pairs of probes were attached to three site-directed di-Cys mutants, each having one Cys at position 129 in the C-terminal lobe and the other at position 2, 3, or 7 in the N-terminal PD. Labeled RLCs, with and without phosphorylation at S19, were reconstituted into myosin S1. Time-resolved FRET identified two structural states of the RLC, closed and open, which are present in both unphosphorylated and phosphorylated biochemical states. Phosphorylation shifts the equilibrium toward the open state by 20-30%. Molecular dynamics simulations, guided by these and previous spectroscopic studies, confirm the existence of the open and closed structural states and produce atomic resolution models of these states. In the closed state, the PD interacts with the surface of the C-terminal lobe. In the open state, the PD is more helical and straight, resides farther from the C-terminal lobe, and is more mobile. Phosphorylation stabilizes the open state by forming a specific salt bridge between R16 and phosphorylated S19. This conformational shift is consistent with a mechanism of regulation that catalyzes large structural changes within myosin at a low energetic cost. This work was supported by grants from NIH (AR32961, AR07612) and the Minnesota Supercomputing Institute. We thank Igor Negrashov for excellent technical assistance.

1838-Pos

Stimulated Actin Polymerization Induces Force Potentiation in Swine Carotid Artery

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The phenomenon of post-tetanic potentiation, in which a single submaximal contraction or series of submaximal contractions strengthens a subsequent contraction, has been observed in both skeletal and cardiac muscle.

In this study, we describe a similar phenomenon in swine carotid arterial smooth muscle. We find that a submaximal K^+ depolarization increases the force generation of a subsequent maximal K^+ depolarization - we term this ''force potentiation.''

Force potentiation was not associated with a significant increase in crossbridge phosphorylation or shortening velocity during the maximal K^+ depolarization, suggesting that the potentiated force was not caused by higher crossbridge cycling. We found that measures of stimulated actin polymerization (higher prior Y118 paxillin phosphorylation, higher prior F actin, and transition to a more solid rheology evidenced by lower noise temperature and phase angle) present prior to the maximal K^+ depolarization predicted the degree of force potentiation.

Increased prior contraction alone did not induce force potentiation since readdition of Ca^{2+} to Ca^{2+}-depleted tissues induced a partial contraction that was not associated with changes in noise temperature or with subsequent force potentiation.

These data suggest that stimulated actin polymerization may produce a substrate for increased crossbridge mediated force, a process we observe as force potentiation.

1839-Pos

Is Slower Myosin Cross-Bridge Kinetics in Tg-D166V Preparations Due to Decreased Myosin RLC Phosphorylation?

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In this report we have investigated a particularly malignant phenotype of Familial Hypertrophic Cardiomyopathy (FHC) associated with the 166 Aspartic Acid to Valine (D166V) mutation in the ventricular myosin regulatory light chain (RLC). We show that the rates of myosin cross-bridge attachment and dissociation are significantly different in isometrically contracting cardiac myofibrils from transgenic (Tg)-D166V compared to Tg-WT mice. A single molecule approach was taken where the fluorescence anisotropy of rhodamine phalloidin labeled actin protomers was measured in cardiac myofibrils undergoing isometric contraction. Orientation of an actin molecule oscillated between two states, corresponding to the actin-bound and actin-free states of the myosin cross-bridge. The rates of cross-bridge attachment as well as cross-bridge dissociation were significantly decreased in isometrically contracting Tg-D166V myofibrils (binding, 1.4 s^{-1}; detachment, 1.2 s^{-1}) compared to Tg-WT myofibrils (binding, 3 s^{-1}; detachment, 1.3 s^{-1}). The duty ratio of the cross-bridge cycle, equal to the fraction of the total cycle time that cross-bridge remains attached to actin, was 47% inTg-D166V myofibrils and 30% in Tg-WT. Immunoblotting of cardiac myofibrils used for kinetics studies demonstrated a large reduction in RLC phosphorylation in Tg-D166V vs. Tg-WT myofibrils. These data are in accord with our previous findings in skinned and intact papillary muscles showing slower fiber kinetics and prolonged force transients in Tg-D166V fibers compared to Tg-WT preparations (Kerrick et al., FASEB J. 23: 855, 2009). Similarly, the level of RLC phosphorylation in muscle extracts from Tg-D166V ventricles was decreased. Our cellular and single molecule data suggest that a mutation-dependent decrease in RLC phosphorylationcould initiate the slower kinetics of the D166V cross-bridgesand ultimately lead to abnormal cardiac muscle contraction. Supported by NIH-HL071778 (DSC), NIH-AR048622 (JB) and NIH-HL090786 (to JB and DSC).

1840-Pos

Biochemical Phenotypes Associated with the Myosin Essential Light Chain Mutations

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To investigate the effects of familial hypertrophic cardiomyopathy (FHC) mutations in the ventricular myosin essential light chain (ELC) we have exchanged the human ventricular wild type (WT) and two FHC ELC mutants (A57G, E143K) for the endogenous porcine ELC in isolated and purified pig cardiac myosin.To elucidate the importance of the long N-terminus of cardiac myosin ELC for the actin-myosin interaction, we also exchanged the N-terminal truncation mutant ELC-Δ43 into native porcine myosin. The phenotype associated with the A57G mutation consists of a classic asymmetric hypertrophy with varying pathology and disease progression including sudden cardiac death (SCD) (Lee, *et al.* (2001) *Am Heart J***141**, 184-9). The E143K mutation is associated with a restrictive cardiomyopathy and SCD phenotype (Olson *et al.* (2002) *Circulation***105**, 2337-40). We hypothesized that FHC ELC mutants may bind to the myosin heavy chain with a lower affinity than ELC-WT, thus affecting the structural integrity of the thick filaments in muscle. SDS-PAGE demonstrated that indeed the A57G mutation yielded lower percent exchange in cardiac porcine myosin compared to WT. We further hypothesized that as a consequence the interaction of ELC-mutant myosin with actin will be affected. All ELC-mutant myosins were tested for their ability to bind actin using fluorescence spectroscopy and pyrene-labeled F-actin. We observed a significantly decreased binding affinity for both FHC mutants while the binding of the ELC-Δ43 mutant myosin to F-actin was stronger than WT. The latter supports the hypothesis that the N-terminus of ELC acts as a molecular constrain inhibiting the actin-myosin interaction. Lower binding affinity of myosin containing the A57G and E143K mutations might be responsible for the development of the pathologic cardiac phenotype observed in patients carrying these FHC mutations. Supported by NIH HL071778 and NIH HL090786.