Posters

Muscle Regulation I

1157-Pos Board B1

Purification and Proteomic Analysis of Human Membrane Lipid Rafts and Their Importance in Ca^{2+}-sensitization of Vascular Smooth Muscle (VSM) Contraction

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Hypercholesterolemia is a major risk factor for cardiovascular events. Among them, abnormal VSM contractions such as vasospasm are caused by Ca^{2+}-sensitization of VSM contraction. However, the relationship between cholesterol (CHOL) and the VSM Ca^{2+}-sensitization has not been clarified yet. Our recent studies showed that a sphingosylphosphorylcholine (SPC)/Src family tyrosine kinase (Src-TK) / Rho-kinase (ROK) pathway mediates the Ca^{2+}-sensitization, and SPC indeed induces severe vasospasm in vivo. We found that serum CHOL potentiates the SPC/Src-TK/ROK pathway leading to Ca^{2+}-sensitization in both human and rabbit: the extent of SPC-induced Ca^{2+}-sensitization correlated well with total CHOL and LDL-CHOL, but inversely correlated with HDL-CHOL. Moreover, the depletion of CHOL by β-cyclodextrin destroyed CHOL-enriched membrane lipid rafts and abolished the SPC-induced ROK translocation and Ca^{2+}-sensitization, suggesting the SPC-induced translocation of ROK to lipid rafts. Taken together, we suggested that not only CHOL, but also lipid rafts mediates the SPC-induced Ca^{2+}-sensitization. Understanding the mechanism(s) by which lipid rafts promotes Ca^{2+}-sensitization in human VSM requires the elucidation of lipid raft protein composition. As a first step, we succeeded for the first time in purifying lipid rafts from human VSM by sucrose density gradient ultracentrifugation, which were confirmed by western blot of raft marker proteins such as caveolin-1 and flotillin-1. Subsequently, using mass spectrometry (MALDI TOF-MS), proteomic analysis was performed to compare the protein compositions between lipid raft and non-lipid raft membrane fractions. The ongoing studies have identified so far previously unreported novel raft-localized proteins, in addition to the known proteins, including lipid- or GPI-anchored proteins and membrane proteins. We are attempting to accumulate functional data to suggest that some novel signaling molecules contribute to an SPC/Src-TK/ROK pathway leading to the VSM Ca^{2+}-sensitization.

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Phosphorylation-Induced Structural Changes in Smooth Muscle Regulatory Light Chain

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We are using site-directed spectroscopic labeling, fluorescence, and molecular dynamics simulations to define the phosphorylation-induced structural transition in smooth muscle myosin regulatory light chain (RLC). Smooth muscle is activated through phosphorylation of Ser 19 on RLC, but the N-terminal 24 amino acids of RLC do not appear in any crystal structure. EPR experiments (Nelson et al., 2005) have shown that phosphorylation induces a disorder-to-order transition within the N-terminal phosphorylation domain of the RLC, in which increased helical ordering relieves inhibitory head-head interactions. To define this structural change in atomic detail, we are combining molecular dynamics simulations with spectroscopic distance constraints. Simulations on the unphosphorylated 25-residue N-terminal fragment of the RLC reveal a disordered region in T9-K12, while the phosphorylated N-terminal domain maintains strong α-helicity over the same residues. This disorder-to-order transition is regulated by delicate balance between enthalpy and entropy involving R16. The same disorder-to-order transition has been observed by both simulations extended to include the entire RLC in complex with a portion of the myosin heavy chain. Furthermore, we have employed FRET distance measurements on di-Cys mutant RLC exchanged onto S1 and HMM, to provide geometric constraints for the simulations. This allows determination of both structure and dynamics of the regulatory domain in the absence and presence of phosphorylation. This work was supported by grants from NIH (AR32961, AR07612) and the Minnesota Supercomputing Institute. We thank Igor Negrashov for excellent technical assistance.

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The Involvement of Fyn Tyrosine Kinase in the Signal Transduction of Ca^{2+} sensitization of Vascular Smooth Muscle Contraction Mediated by Rho-kinase

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Rho-kinase (ROK)-mediated Ca^{2+} sensitization of vascular smooth muscle (VSM) contraction plays a critical role in abnormal VSM contraction such as vasospasm. Previously we found that sphingosylphosphorylcholine (SPC) induces the ROK-mediated Ca^{2+} sensitization through the activation of Src family tyrosine kinase (Src-TK) independently of a protein kinase C pathway. Since western blot analysis showed the presence of Fyn and c-Src among Src-TKs in VSM, we aimed to clarify which Src-TK is truly involved in the Ca^{2+} sensitization mediated by the SPC/ROK pathway. Immunofluorescent study showed that SPC induced the translocation of Fyn, but not c-Src, to plasma membrane in cultured VSM cells, which was blocked by eicosapentaenoic acid, a specific inhibitor of the SPC-induced Ca^{2+} sensitization. The siRNA-mediated knockdown of Fyn diminished the SPC-induced contraction remarkably in cultured VSM cells. In β-escin-permeabilized VSM strips, constitutively- active Fyn, which was produced by a baculovirus expression system, induced the Ca^{2+} sensitization, which was blocked by Y27632 (a ROK inhibitor). Dominant-negative Fyn blocked the Ca^{2+} sensitization induced by SPC, GTPγS (a direct activator of G-protein), and a GPCR agonist + GTP. Overexpression of constitutively-active and dominant-negative Fyn induced contraction and relaxation of cultured VSM cells, respectively. Furthermore, in order to identify the target(s) of Fyn, tyrosine-phosphorylated proteins were immunoprecipitated from the VSM cells which were treated with or without SPC. Subsequently, nanoflow liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) was used to identify the SPC-induced tyrosine-phosphorylated and tyrosine-dephosphorylated proteins and their phosphorylation sites. These findings clearly indicate that Fyn tyrosine kinase plays an essential role in the ROK-mediated Ca^{2+} sensitization of VSM contraction. Functional proteomic approach has been useful to reveal possible targets of Fyn, and their functional roles are currently under investigation.

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Simultaneous Measurement Of Force And Fluorescence In Single Guinea-pig Cardiac Myofibrils

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Kinetic studies of force activation and relaxation in the single myofibril preparation have enhanced our understanding of mechanisms that underlie regulation of muscle force. Likewise, stop-flow and steady-state measurement using fluorescently labeled TnC upon Ca2+ activation in reconstituted protein systems have provided data regarding the kinetics of thin filament regulation. However, to develop a detailed understanding and fully characterize muscle regulation, an integration of mechanical, biochemical, and structural information is necessary. We present the development of a new apparatus which allows simultaneous measurement of florescent probe signals from labeled contractile proteins (such as TnC), and force kinetics under active isometric force development to allow direct correlation between these parameters in a single myofibril. Single guinea-pig cardiac myofibrils were prepared in relaxing solution (PCa8.0) by mechanical homogenization; endogenous TnC was exchanged for recombinant mutant labeled human TnC (2.5 mg/ml; overnight 4°C; Alexa-350 label conjugated to cys-84). Myofibrils were attached to glass micro-tools with one serving as a cantilever of known stiffness to assess force (painted black for improved contrast) and the other attached to a high speed motor. Myofibrils were activated with a Ca2+ pulse delivered via a double barreled perfusion pipette attached to a stepper motor (~5 ms). Excitation UV-light (270-350 nm), chopped at 52 kHz using a photo-elastic modulator, was projected (400nm DC mirror) onto the attached myofibril. Force probe deflection was measured in bright-field using red light (670nm) while fluorescence emission was measured (420nm LP filter) using a photomultiplier. Data was sampled by a high-speed (2MHZ) A/D converter and demodulated using custom designed software. Preliminary results indicate that using this apparatus and techniques, ultra-low intensity TnC fluorescence signals can be obtained at high temporal resolution in parallel with bright-field optical measurement of force development.

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Activation and Relaxation Kinetics in Isolated Guinea-Pig Myofibrils: Impact of Sarcomere Length

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Myofilament length dependent activation is a universal property of striated muscle that is most pronounced in myocardium. The mechanisms that underlie length dependency are incompletely understood. Accordingly, here we tested the hypothesis that sarcomere length (SL) affects the rate of force activation and/or force relaxation in response to a saturating pulse of Ca2+. Isolated skinned guinea-pig myofibrils were attached to glass micro-tools positioned on the stage of an inverted microscope (15 C); one probe functioned as a force

probe cantilever, while the other probe was attached to a rapid displacement generator; the Ca2+ pulse was applied by rapid translation of a double-barreled perfusion pipette (de Tombe, AJP, 2007). Activations were performed at long (SL=2.06 ± 0.03 μm) and short (SL=1.85 ± 0.01; n=5) length. As expected, SL modulated maximum Ca2+ saturated force ~20%. Both the rate of force redevelopment following a rapid release-restretch maneuver (kTr; ~26%) and Ca2+ activated force development (kCa;~14%) were faster at the long SL. In contrast, SL did not modulate any parameters of force relaxation following rapid removal of activator Ca2+. Our data suggest that length dependent myofilament activation in the heart may be the result of differential modulation of activation dynamics in response to changes in sarcomere length.

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Tropomyosin: Long Range Perturbations In The Hydrophobic Interface

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Skeletal tropomyosin, (Tm), is an α-helical coiled-coil which binds to actin, and with troponin, regulates muscle contraction. We previously demonstrated that a conserved Asp 137 in the hydrophobic interface produces a dynamic region in the middle of Tm, and that this region is involved in the myosin dependent activation of the thin filament at high Ca^{2+}, (Sumida et. al. JBC 283 2008). The current work characterizes a long-range interaction between positions 137 and 190. The thermodynamic properties of wild type (WT) Tm and two single mutants, C190A and D137L, are compared with those of the double mutant, D137L/C190A, using differential scanning calorimetry, (DSC), and circular dichroism, (CD). CD measurements show that Ala 190 increases the fraction of helix unfolding in the 40°C pre-transition, before the main transition. DSC measurements support this finding, indicating a large enthalpic pre-transition, (ΔH=150kcal/mol), for the C190A mutant relative to D137L, D137L/C190A, or WT, (average ΔH=20kcal/mol). Additionally, Ala 190 increases the ΔCp, (heat capacity), of Tm ~5 fold, reflecting an increase in solvent exposure of hydrophobic residues in the pre-transition during unfolding.

Since the D137L/C190A and D137L mutants do not exhibit the large enthalpic pre-transition observed for C190A, the Leu mutation at 137 must stabilize the alanine effect observed for C190A mutation 77Å away. This demonstrates how a locally dynamic region near 137 is able to produce global effects along the thin filament, and in this manner provide the proper regulation of the myosin dependent activation of the thin filament. This observation may also contribute to our understanding about the manner in which single point mutations significantly affect function in cardio-myopathies such as FHC and DCM.

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Localization of The Tropomyosin-Binding Sites in Troponin T And Functional Suppression of An Error Splicing of Its C-Terminal Variable Region

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The interaction between troponin T (TnT) and tropomyosin (Tm) is pivotal in the Ca2+-regulation of muscle contraction. It has been known for three decades that TnT has two binding sites for Tm. The conserved middle and the C-terminal regions of TnT each contain a Tm-binding site and both sites are critical for the function of muscle thin filament. However, the precise locations of the Tm-binding sites have not been identified. By mAb competition assays, we located the middle region Tm-binding site of TnT in the beginning of the conserved sequence. Previous data showed that deletion of the C-terminal 14 amino acids in TnT did not reduce Tm-binding. Cardiac TnT with a longer deletion of the C-terminal 28 amino acids also retained the C-terminal Tm-binding site as shown by its dominant cardiomyopathy phenotype that indicates effective myofilament incorporation. In contrast, a truncation of slow TnT deleting the C-terminal 83 amino acids due to a nonsense mutation significantly lowered Tm-binding affinity and causes a recessive form of nemaline myopathy. Considering the known crystal structure of partial troponin, we further tested additional deletions to locate the C-terminal region Tm-binding site of TnT in the beginning of the T2 segment. Different from the dominant C-terminal truncation mutation of cardiac TnT, an error-splicing of the mutually exclusive exon 16 and exon 17 in fast skeletal muscle TnT significantly lowered Tm-binding affinity by deleting the C-terminal 28 amino acids and replacing it with a long non-sense peptide. The suppression of potentially harmful effects of the splicing error provides a mechanism to protect muscle function.

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Identification And Characterization Of Cardiac Troponin I From The Trout Heart

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Trout cardiac myofibrils are ~10-fold more sensitive to Ca^{+2} than those from mammalian hearts when measured at the same temperature. It has been demonstrated that trout cardiac troponin C (ScTnC) has 2.3 fold the Ca^{2+} affinity of human cTnC and is responsible for a 2-fold increase in cardiac myofibril Ca^{+2} sensitivity. The contributions of trout cardiac troponin I (ScTnI) to the Ca^{+2} sensitivity of the trout heart is currently unknown. The cDNA for ScTnI has been cloned using RACE-PCR. Sequencing results indicate that ScTnI is 59% and 56% identical to human cTnI and human skeletal troponin I, respectively, at the amino acid level. Interestingly, ScTnI lacks the ~30-residue N-terminal sequence present in mammalian cTnI that contains two protein kinase A (PKA) target residues at positions 23 and 24. ScTnI has been expressed and the influence of it on the Ca^{2+} activation of human cardiac troponin is currently being characterized using steady state Ca^{2+} binding assays and stopped flow kinetic analysis. This work is supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Canadian Foundation for Innovation (CFI) to TEG.

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PI3-Kinase Controls Smooth Muscle Contraction Via Regulation Of MLCP Activity

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We demonstrated for the first time that PI3-kinase plays a role in the regulation of smooth muscle contraction by controlling MLCP. The inhibition of PI3-kinase markedly inhibited Ca2+-induced contraction and GTPγS induced Ca2+ sensitization of α-toxin permeabilized vascular smooth muscle as well as K+-induced contraction of intact vascular smooth muscle. The contractile inhibition was accompanied by the decrease in MLC phosphorylation and MBS phosphorylation at Thr696 and Thr853, which are responsible for the inhibition of MLCP activity. On the other hand, the inhibition of PI3-kinase had no effect on MLCK activity. These results suggest that PI3-kinase is involved in the regulation of MLCP, thus regulating MLC phosphorylation. An Akt specific inhibitor, SH-6, had no effect on the contraction, suggesting that Akt, one of the major down-stream effecter of the PI3-kinase pathway is not involved in this mechanism. MBS phosphorylation at Thr853, a Rho kinase specific site, was decreased by the inhibition of PI3-kinase even at rest, when Rho kinase is not activated. These results suggest that PI3-kinase does not influence the MBS kinases, such as Rho kinase. In fact, we found that the PI3-kinase inhibition activated MBS phosphatase activity. Furthermore, we found that PI3-kinase inhibition increased MBS phsophorylation at the PKG site, suggesting the activation of PKG pathway. Since the activation of the cGMP/PKG pathway decreases MLC phosphorylation by activating MBS phosphatase (Nakamura et al., 2007), our results suggest that PI3-kinase regulates smooth muscle contraction by modulating the PKG pathway.

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Crossbridge-mediated Activation of Rabbit Skeletal Muscle Myofibrillar ATPase: a Role for the Calcium Binding Domains of Troponin C

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Activation of the thin filament in striated muscle is a cooperative process requiring both the binding of Ca^{2+} to troponin C (TnC) and the binding of myosin crossbridges to actin. The aim of this study was to assess the role of TnC domains in the crossbridge-mediated activation of rabbit skeletal muscle myofibrils in the absence of Ca^{2+}. Activation of myofibrillar ATPase was produced by addition of varying concentrations of myosin S1 modified by N-ethylmaleimide (NEM-S1), which facilitates crossbridge cycling by forcing tropomyosin into the open position on the filament. Comparisons were made of native myofibrils, myofibrils from which TnC was extracted, and myofibrils reconstituted with either a TnC mutant (TnC_{48-82}) in which Ca^{2+} activation was blocked by a disulfide bond in the N-terminal domain (Grabarek, et al, Nature, 345:132,1990) or a proteolytic fragment of TnC (TR2C) lacking the N-terminal Ca^{2+}- binding domain (Grabarek, et al, J. Biol. Chem. 265:13121, 1981). The ATPase activity of native myofibrils was increased ~170% by the addition of NEM-S1 (2-4μM). Following extraction of TnC the addition of the same concentrations of NEM-S1 produced ~60% activation. In both cases higher concentrations of NEM-S1 produced no further increase in activation. With the addition of either TnC_{48-82} or TR2C the degree of activation was higher (70-100%), but required higher NEM-S1 concentrations (4-8μM). These results suggest that both domains of TnC play a role in facilitating optimal crossbridge-mediated activation of the thin filament, presumably by providing alternative binding sites for troponin I.

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Structurally Unstable Regions in the Tropomyosin-Troponin Complex from Bovine Heart Muscle

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Regulation of contraction in striated muscles requires a semi-independent movement of various domains of the tropomyosin-troponin (TmTn) complex