that the neonatal cardiac isoform of TnI, ssTnI, confers pH-insensitivity in this regard compared to the adult cTnI isoform. However ssTnI confers deleterious effects of impaired relaxation in the adult myocyte. Alignment and functional studies have demonstrated that this pH-insensitivity is derived from ssTnI residue H132. Introduction of a histidine at the cognate position in cTnI (A164H) mitigates the pH-sensitivity of the calcium-force relationship in cardiac myocytes while retaining relaxation enhancement via the N-term domain relative to ssTnI. We are establishing a time-resolved fluorescence methodology for detecting alterations in the calcium sensitivity of the thin filament during ischemia. We have engineered a single cysteine mutation for labeling with environmentally sensitive fluorophores designed to detect Ca^{2+} and pH-sensitive structural changes in cTnI and cTnC. We will discuss progress using this approach to interrogate troponin function in ischemia mimetic conditions.

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Molecular Mechanism of Cardiomyopathy-Causing Mutations in Alpha-Tropomyosin

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Primary cardiomyopathy is one of the most common cardiac disorders, affecting more than 1 in 500 individuals. Primary cardiomyopathies are most frequently caused by inherited single amino acid substitutions, in a single allele encoding one of the cardiac sarcomeric proteins.

Alpha-tropomyosin is a key cardiac sarcomeric protein, which interacts structurally and functionally with all other components of the sarcomeric contractile apparatus and thereby regulates cardiac muscle contraction in response to Ca^{2+}. There are more than fifteen substitutions identified throughout the length of alpha-tropomyosin which can result in cardiomyopathy. However, the fundamental biochemical and biophysical mechanism(s) by which these single amino acid substitutions affect sarcomeric function and cause cardiomyopathy is (are) unclear. Also, there is no clear relation between the location of these substitutions in alpha-tropomyosin and the nature of the resulting cardiomyopathy. Working with a collection of less-characterised, cardiomyopathy-associated mutations in human cardiac alpha-tropomyosin, we find that even if two mutations are associated with the same cardiomyopathy, the molecular dysfunction caused by the two mutations could be different. Previously it has been characterized that most HCM mutations in alpha-tropomyosin show weaker binding to actin. However, we find that the HCM-associated alpha-tropomyosin L185R mutant binds to F-actin with a greater affinity and co-operativity. In a co-sedimentation assay to measure the binding of alpha-tropomyosin and F-actin, the Kd decreases from 200 ± 20 nM (wild-type) to 100 ± 30 nM (L185R), and the Hill's coefficient increases. This mutation, and some others, have been characterized in further detail, within the frame-work of the three-state model of the regulated thin filament, to provide novel insights into the mechanisms underlying cardiomyopathies.

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Skeletal Muscle Myopathy Mutations in Tropomyosin Gene TPM3 affect Thin Filament Transitions between the Inactive and Active States

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It has recently been discovered that nemaline myopathy and cap myopathy can be caused by mutations in β-tropomyosin (*TPM2* gene) and that a third skeletal muscle myopathy, congenital fibre-type disproportion, is predominantly due to mutations in slow α-tropomyosin (*TPM3* gene). We have expressed tropomyosin TPM3 mutations of residues R90, R167 and R244 in a baculovirus-Sf9 insect cells system and studied reconstituted actin-tropomyiosin mutant filaments biochemical properties. We analysed the effects of these TPM3 mutations on the biochemical properties of tropomyosin namely secondary structure, actin binding and the equilibrium constants of transitions between the 'open', 'closed' and 'blocked' states of thin filament and the cooperativity of these transitions. We also assessed their functional effects on the actomyosin ATPase. We found that TPM3 mutations did not affect secondary structure of tropomyosin and its interaction with actin. In contrast these mutations affected troponin-tropomyosin inhibition and activation of actomyosin ATPase. TPM3 mutations also affected the Blocked-closed transitions in the cooperative allosteric mechanism of regulation of the actomyosin complex. These findings are in agreement with recent structural studies of tropomyosin bound to actin in the closed and blocked conformations. We propose that destabilisation of the actin-tropomyosin interface by charge change in the blocked state may represent the underlying biochemical defect for the onset of skeletal muscle myopathies linked to these mutations.

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Direct Visualization of Cooperative Binding of Troponin-Tropomyosin to F-Actin

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In cardiac muscle, troponin (Tn) and tropomyosin (Tm) on f-actin (Ac) provide Ca^{2+}-dependent regulation of force development by myosin motors. Ca^{2+} activation is a highly cooperative process that is thought to be due to cooperative structural interactions among Tn, Tn, and Ac. Previous measurements have quantified cooperative binding associations through indirect methods coupled with modeling. We sought to characterize cooperative interactions within regulated actin by directly observing individual actin filaments. Tn, Tm, and Ac were labeled, respectively, with AF 546 (green), ATTO 655 (red) and phalloidin AF488 (blue dyes). Regulated actin was reconstituted at 1:1:1 stoichiometry of Tn:Tm:Ac7. The samples were diluted, immediately deposited on aminosilanized glass coverslips, and imaged in an epifluorescence microscope. From the co-localization of the three dye colors, we observe that (1) Tn-Tm binding to actin is a highly cooperative nearly all-or-nothing process, where actin had TnTm bound along its entire length or had no TnTm bound at all; (2) Tn-Tm not bound to actin are dissociated from each other. Filaments had different lengths, and Tn-Tm bound preferentially longer filaments. Regulated filaments were on average 4.75 times longer than unregulated filaments in presence and absence of Ca^{2+}. In the Mg^{2+}- and Ca^{2+}-saturated state, the average binding constant of TnTm to actin is 0.55 ± 0.08 uM-1 and 0.7 ± 0.2 uM-1 respectively. Dissociation rate of TnTm bound to f-actin at 20 was very slow (< 1 hr-1). Our results suggest that surface immobilized regulated actin, combined with single particle analysis and particle sorting, is a promising method for examining the structure of Tn as a member of regulated actin.

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The Accuracy of Cardiac Myofilament Simulations is Enhanced by Permitting Calcium-Independent Tropomyosin Transitions

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Conceptual and computational models have generally assumed that each regulatory unit (RU) of the thin filament remains in the blocked state until Ca^{2+} binds to troponin C. This includes our previous model (Campbell et al., *Biophys J* 98:2254, 2010), which simultaneously recapitulated key attributes of myofilament activation. However, the model failed to fully reproduce exchange experiments in which some fraction of myofilament troponin C is replaced with a non-Ca^{2+} binding mutant (xTnC). In simulations, xTnC caused much greater reductions in tension than were shown experimentally (Gillis et al., *J Physiol* 580:561, 2007). This effect was caused by the assumption of strong cooperative inhibition among nearest-neighbor RUs, which was required to produce basic myofilament activation behavior. We hypothesized that permitting some Ca^{2+}-independent RU activation while maintaining cooperative inhibition would reconcile this discrepancy. In a new model, blocked-to-closed RU transitions were allowed without bound Ca^{2+} but at greater energetic cost (ΔG). Monte Carlo simulations showed that reducing ΔG to a finite value of 4.7 kJ/mol maintained cooperative myofilament activation while reproducing xTnC experiments (Figure). These results suggest that thin filament function does not require perfect Ca^{2+} switch fidelity.