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Calcium-Induced Conformational Changes in the Cardiac Isoform of the Troponin Complex Monitored By Hydrogen/deuterium Exchange and Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

George M. Bou-Assaf[1,2], Jean E. Chamoun[1], Piotr G. Fajer[1,2], Mark R. Emmett[2], Alan G. Marshall[1,2].
[1]Florida State University, Tallahassee, FL, USA, [2]National High Magnetic Field Laboratory, Tallahassee, FL, USA.

Calcium-induced conformational changes in the cardiac troponin complex have been determined from solvent accessibility data measured by solution-phase hydrogen/deuterium exchange (HDX). The troponin complex was digested with protease XIII and the peptides identified by FT-ICR mass spectrometry. To uniquely assign proteolytic fragments of the same mass, experiments were performed with troponin subunits isotopically depleted in ^{13}C and ^{15}N. For instance, the peptide with monoisotopic m/z = 592.98^{3+}could be either TnT fragment 76-91 or to TnI fragment 155-170, also known as the switch peptide. Isotopic depletion uniquely identified the TnT fragment.

Comparison of the HDX rates in the isolated subunits and those observed in the reconstituted complex identified inter-subunit interfaces, in general agreement with the x-ray structure of the core complex (Takeda *et al.* Nature, 2003 **424** (6944)). HDX experiments for the complex in the presence and in the absence of calcium revealed a multitude of conformational changes, some of which were not apparent from comparison of Ca-saturated and Ca-free x-ray structures of skeletal troponin (Vinogradova *et al.* PNAS, 2005 **102** (14)). For example, the C-lobe of TnC, which was not expected to change upon Ca binding, showed different HDX rates in the presence and in the absence of Ca for peptides 105-119 and 150-153. Similar effects were observed for residues 24-27 of TnC, but smaller differences were found for the TnI switch peptide (158-162), the N-terminus of TnI, and the IT coiled-coil. These observed changes will be discussed in the context of the cardiac and skeletal crystal structures. This work was supported by NIH (R01 GM78359), NSF Division of Materials Research through DMR-06-54118, and the State of Florida.

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Structure of Troponin Complex:FRET and Molecular Dynamic Simulations

Jayant J. Jayasundar[1], Jun Xing[1], John M. Robinson[2], Herbert C. Cheung[3], Wen-ji Dong[1].
[1]Washington State Univ, Pullman, WA, USA, [2]University of Alabama at Birmingham, Birmingham, AL, USA, [3]University of Alabama at Birmingham, Birmingham, AB, USA.

Cardiac troponin regulates the functioning of the heart. Ca^{2+}binding to cTnC and PKA-phosphorylation of the unique N-extension (Nxt) of cTnI play critical roles in regulation. However details of the Ca^{2+} and PKA phosphorylation signal transduction is still elusive. To acquire detailed structural information of troponin during activation/deactivation, we performed molecular dynamic simulations of the cardiac troponin complex combined with FRET distance measurements as restraints. Over one hundred inter-site distances were obtained from time-resolved FRET measurements at Ca^{2+}-free and Ca^{2+}-saturating conditions with/without PKA phosphorylation. These distance distributions were used as distance constraints during the simulations to resolve the detailed structure, particular the previously unresolved key functional regions of troponin. Results show that under Ca^{2+}-free condition the Nxt of cTnI interacted with the Ca^{2+}-binding sites on the N-domain of cTnC. The inhibitory region of cTnI and the D/E linker of cTnC were disordered. Upon Ca^{2+} binding in the absence of PKA phosphorylation, the Nxt of cTnI interacted with cTnC helix A and the cTnI mobile domain. The inhibitory region of cTnI and the D/E linker of cTnC become ordered. The results suggest that Ca^{2+}-induced folding and unfolding of the D/E linker of troponin C, coupled with a change in orientation of the regulatory region of troponin I within the N-domain hydrophobic pocket of troponin C, regulates the dynamics of the inhibitory region of troponin I. When Ser23/24 was phosphorylated, the Nxt of cTnI moved away from the surface of the cTnC, perturbing the interaction between the Nxt and cTnC helix A, leading to significant unfolding of helices A and D of cTnC. The D/E linker of cTnC and the inhibitory region of cTnI became mobile. These finding shed new light onto the modulation role of PKA phosphorylation of cTnI on thin filament regulation.

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Functional and Structural Changes Induced By cTNT-Related FHC Mutations in TNT1 Alter Actomyosin Binding Interactions

Rachel K. Moore, Pia J. Guinto, Candice Dowell-Martino, Jil C. Tardiff.
Albert Einstein College of Medicine, Bronx, NY, USA.

Familial Hypertrophic Cardiomyopathy (FHC) is a primary disease of the cardiac sarcomere. Many FHC mutations in hcTnT are found within the TNT1 domain, with a mutational hotpot at residues 160-163. These residues fall within a highly charged region (158-RREEEENRR-166), which may create a flexible hinge necessary for function, the structure and function of which is affected by FHC mutations. We are investigating the effects of these hotspot mutations using in vitro motility (IVM) assays, SDSL-EPR, and transgenic mouse models. IVM data indicate that mutations Δ160E and E163R disrupt actin binding to heavy meromyosin under standard assay conditions. By reducing the ionic strength of the motility solutions, thin filament binding and sliding are restored suggesting that mutations in this region cause disease by disrupting the weak electrostatic interactions between the thin filament and myosin necessary for crossbridge formation. CW-EPR spectra show an increase in spin label isotropic rotational rate at hcTnT residue 153 (upstream of the putative hinge region) between Troponin ternary complexes containing Δ160E verses WT hcTnT, suggesting an increase in flexibility due to backbone changes induced by the mutation. We are expanding our SDSL-EPR experiments with additional cysteine substitutions superimposed onto 160-163 mutant proteins to provide further data regarding secondary structural changes imposed by these mutations. These results correspond with our Δ160E mouse model showing dose dependant myofilament disarray. Preliminary observations of an E163R model suggest that this mutant is less severely affected, tolerating a higher transgene dose. The structural and functional changes observed in vitro may contribute to the structural impairment seen in vivo. By correlating our IVM and SDSL-EPR findings with in vivo data generated from the Δ160E and E163R models, a mechanism of disease for these hotspot mutations can be determined.

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PKA Phosphorylation Has No Effect on the Force-PCA Relationship or Length Dependent Activation Following L48Q cTNC-Tn Exchange in Rat Demembranated Trabeculae

Frederick S. Korte, Maria V. Razumova, Erik R. Feest, Dan Wang, Michael Regnier.
University of Washington, Seattle, WA, USA.

The molecular mechanism behind the Frank-Starling law of the heart is thought to be due in part to changes in myofilament lattice spacing and/or increased myosin proximity to actin binding sites at longer sarcomere lengths (SL). We previously showed that cardiac trabeculae have a left-shifted force-pCa relationship and eliminated SL-dependence of this relationship following passive exchange with cTn containing a mutant (L48Q) cTnC (with enhanced TnC-TnI interaction strength). This result suggests L48Q cTnC reduces the cross-bridge dependent component of thin filament activation. Experiments here were designed to test the hypothesis that cTnI phosphorylation can restore SL dependence in L48Q cTnC exchanged trabeculae by decreasing Tn Ca^{2+} affinity and/or TnC-TnI interaction strength. Interestingly, PKA treatment did not reduce Ca^{2+} sensitivity of force or restore SL dependence in L48Q cTnC-cTn exchanged trabeculae. PKA treatment of native and WT cTn exchanged trabeculae right-shifted and also reduced SL dependence of the force-pCa relationship. Other laboratories investigating the effect of PKA on SL dependence have produced discordant results, possibly due to PKA-induced phosphorylation of proteins other than TnI (i.e., MyBP-C and titin) Therefore, planned future experiments will investigate exchange of PKA pre-treated cTn complexes, S23/24D (phosphomimic) cTnI, and S23/24A (non-phosphorylatable) cTnI with WT and L48Q cTnC. Experiments will be coupled with solution measurements cTnI-cTnC affinity using spectrofluorimetry to provide a more detailed understanding of how PKA phosphorylation and cTnI-cTnC interaction strength influence SL-dependence in cardiac muscle. Support provided by NIH HL65497 (MR) and T32 HL07828 (FSK).

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The Effect of Hypertrophic Cardiomyopathy (HCM) Mutations of Tropomyosin on Force Generation and Cross-Bridge Kinetics in Thin-Filament Reconstituted Bovine Cardiac Muscle Fibers

Fan Bai[1], Adam Weis[1], Aya Takeda[2], Bryant Chase[2], Masataka Kawai[1].
[1]Department of Anatomy and Cell Biology, The University of Iowa, Iowa City, IA, USA, [2]Department of Biological Science, Florida State University, Tallahassee, FL, USA.

Three HCM mutations (V95A, D175N and E180G) of Tropomyosin (Tm) were examined using the thin-filament extraction and reconstitution technique. Effects of Ca^{2+}, ATP, phosphate and ADP concentrations on force and its transients were studied at 25°C and compared to WT. E180G showed larger isomeric tension (1.89 ± 0.11) than WT (1.59 ± 0.08). Tension of two other mutants (V95A 1.53 ± 0.09, D175N 1.47 ± 0.07) was not different from WT. pCa_{50} (Ca^{2+} sensitivity) of V95A (6.20 ± 0.06) and E180G (6.51 ± 0.02) was larger than WT (5.85 ± 0.03), while that of D175N (5.88 ± 0.05) remained the same. The cooperativity was reduced in all three mutants (V95A 1.70 ± 0.11, D175N 1.87 ± 0.09, E180G 1.91 ± 0.14) compared to WT (2.79 ± 0.25). Four equilibrium constants were deduced using sinusoidal analysis. The largest effect was on K_5 (Pi association constant) which was reduced to ~0.5X in all

mutants, while K_4 (force generation step) was unchanged. V95A showed significantly lower K_2 (cross-bridge detachment step: 0.93 ± 0.06) than WT (1.37 ± 0.13). D175N and V95A showed significantly lower K_1 (ATP association constant, 0.91 ± 0.13 and 0.86 ± 0.16, respectively) than E180G (1.84 ± 0.33) and WT (1.60 ± 0.35). However, the cross-bridge distribution was not significantly different among 4 Tms, indicating that force/cross-bridge in E180G is larger than WT, but it is unchanged in V95A and D175N. In conclusion, all three mutants showed significant deviations in force/cross-bridge, pCa_{50}, cooperativity or cross-bridge kinetics; in particular, E180G had the largest effect. Because E180G and D175N are located in the Tm-Troponin (Tn) interaction region and result in the net charge increase, and E180G causes the largest hydropathy change, we infer that both electrostatic and hydrophobic interactions between Tm and Tn play vital roles in maintaining normal muscle functions.

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UNC-45 Knock-Down in Drosophila Heart Targets Myosin Accumulation and Yields Severe Myofibrillar Disarray and Cardiac Dysfunction

Girish C. Melkani[1,2], Karen Ocorr[2], Rolf Bodmer[2], Sanford I. Bernstein[1].
[1]Department of Biology, Molecular Biology and SDSU Heart Institute, San Diego State University, San Diego, CA, USA, [2]Development and Aging Program, Burnham Institute for Medical Research, La Jolla, CA, USA.

UNC-45 belongs to the UCS (UNC-45, CRO1, and She4p) domain protein family, whose members interact with various myosins and are required for myosin function. The in vivo RNAi approach using the Gal4/UAS system permits us to knock down Drosophila unc-45 (dunc-45) transcripts in a tissue-specific manner. DUNC-45 knock-down with muscle-specific driver 24bGal4 leads to embryonic and larval lethality. However, DUNC-45 knock-down in the heart allows survival to adulthood, but lifespan is dramatically reduced, with ~90% dead within three weeks. Optical heartbeat analysis of semi-intact hearts from various age adults was carried out using high-speed video and movement analysis algorithms. Analysis of several cardiac parameters (e.g., diastolic and systolic diameters, heart period and arrythmicity index) showed severely compromised cardiac function. For example, ~80% of 1, 2 and 3 week old knock-down hearts do not show contraction or relaxation of normally highly-contractile regions (primarily in abdominal segment three). The remainder of the heart shows a greatly reduced and irregular beating pattern. Another striking response to DUNC-45 knock-down was dilation of the third segment of the heart. For example, the average diastolic diameter for one week old control fly heart is 76 μm. However, in the one-week-old knock-down heart the average diastolic diameter is 128 μm. Cardiac arrhythmias were observed in one-week-old flies, whereas arrhythmias were only observed after three weeks of age in wild type flies. Immunofluorescent images of relaxed hearts using myosin antibody showed irregular heart muscle patterning and missing myofibrils in DUNC-45 knock-down adults. Heart defects of the DUNC-45 knock-down heart were rescued to some extent by transgenic over-expression of DUNC-45. As UNC-45 is essential for accumulation of myosin, its knock-down in the heart results in myofibrillar disarray and reduced contraction.

Platform C: Voltage-gated Na Channels

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The Nachbac Pore: Creation and Characterisation of a KcsA-Like Sodium Channel

Andrew M. Powl[1], Maurits R.R. de Planque[2], Hywel Morgan[2], B.A. Wallace[1].
[1]Birkbeck College, University of London, London, United Kingdom, [2]Southampton University, Southampton, United Kingdom.

Voltage-gated sodium channels (VGSC) are integral membrane proteins responsible for the transient flux of sodium ions across cell membranes in response to changes in membrane potential. In humans as well as lower eukaryotes they are essential for homeostasis and normal functioning, and mutations in them are associated with a range of disease states. Although potassium channels, which are members of the same large family of voltage-gated channels have been well characterized, much less known about the structural features of sodium channels. For potassium ion channels, an important advance in understanding resulted from the determination of the three dimensional structure of the bacterial potassium channel KcsA, a simplified channel composed only of two transmembrane segments per subunit present in the tetrameric structure. In 2001, Ren *et al* found that bacteria also possess simplified versions of sodium channels, although in this case the individual subunits of all the homologues that have been identified thus far possess six transmembrane segments, which include both a pore-forming subdomain (S5-S6) and a voltage-sensing subdomain (S1-S4). Here we report on the creation of a smaller KcsA-like pore-only version of a sodium channel from the *B. halodurans* VGSC (pNaChBac), engineered to contain S5-S6 plus the C-terminal region of the NaChBac channel. The NaChBac pore has been expressed and purified from *E. coli*membranes, solubilised in detergent micelles, reconstituted into lipid vesicles and characterized for its secondary structure and thermal stability, as well as its electrophysiological properties from single-channel recordings, providing new insight into features of sodium channel structure and function.

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A Central Role For Mitochondria in the Regulation of Cardiac Sodium Current

Man Liu[1], Georgia Gaconnet[1], Barry London[2], Samuel C. Dudley[3].
[1]Cardiology, University of Illinois at Chicago, Chicago, IL, USA, [2]The Cardiovascular Institute, University of Pittsburgh, Pittsburgh, PA, USA, [3]Cardiology, University of Illinois at Chicago, the Jesse Brown VAMC Chicago, Chicago, IL, USA.

Background: A mutant glycerol-3-phosphate dehydrogenase 1-like, A280V (A280V GPD1-L) reduces cardiac Na^+ current (I_{Na}) and causes Brugada Syndrome. Recent data suggest that this effect is dependent on alterations in NADH, reactive oxygen species (ROS), and PKC activation. Since NADH and PKC can activate ROS production from mitochondria, we investigated the role of this organelle in mediating the effects of mutant GPD1-L and NADH on I_{Na}.
Methods: HEK cells stably expressing the cardiac Na^+ channel were used, and effects on I_{Na} were assessed by whole-cell patch clamp recording.
Results: A280V GPD1-L caused a 2.48 ± 0.17-fold increase of intracellular NADH level (n=3; $P<0.001$). Cytosolic NADH application (100 μM) or co-transfection with A280V GPD1-L resulted in significant decrease of I_{Na} ($52 \pm 9\%$ or $81 \pm 4\%$, respectively; $P<0.01$), which was reversed by 5-50 μM chelerythrine, 5 μM superoxide dismutase (SOD), 5-10 μM mitoTEMPO (a specific inhibitor to block mitochondrial superoxide generation), 1-5 μM rotenone (a complex I inhibitor), and 40-80 μM 4′-chlorodiazepam (an inhibitor of mitochondrial benzodiazepine receptor). The decreased I_{Na} induced by 30 nM PMA ($60 \pm 7\%$, $P<0.01$) was prevented by SOD. Antimycin A (a complex III inhibitor known to produce ROS) at 20 μM decreased I_{Na} ($51 \pm 4\%$, $P<0.01$). L-NAME (an inhibitor for uncoupled NOS), cyclosporin A (an inhibitor for mitochondrial permeability transition pore), and KN-93 (an inhibitor of CAMKII) had no effect on NADH reducing Na^+ current.
Conclusions: A280V GPD1-L appears to regulate $Na_v1.5$ by altering the oxidized to reduced NAD(H) balance, which then activates mitochondrial ROS production through a PKC-dependent signaling mechanism. This ROS production leads to reduced I_{Na}. This signaling cascade may help explain the link between altered metabolism, conduction block, and arrhythmic risk.

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NaV-Mediated Sodium Currents Are Necessary For Vertebrate Appendage Regeneration

Kelly Ai-Sun Tseng, Michael Levin.
Tufts University, Medford, MA, USA.

Mammals have a limited ability to regenerate tissues. In contrast, amphibians such as frogs can restore lost developmental structures, including the lens and tail. A detailed understanding of natural regeneration is important for developing therapies for organ repair. Recently, ion transport has been implicated as a regulator of regeneration. While voltage-gated sodium channels play a well-known and important role in propagating action potentials in excitable cells, we have identified a novel role in regeneration for the ion transport function mediated by the voltage-gated sodium channel, NaV1.2. After Xenopus tadpole tail amputation, a regeneration bud (containing progenitors required for regenerative growth) is formed within 1 day at the injury site, and a new tail is re-grown by 7 days. NaV1.2 is expressed early in the bud, and its function is required for regeneration. Inhibition of its activity causes regenerative failure by greatly reducing expression of downstream genes that drive tail outgrowth and patterning, leading to decreased proliferation and altered axonal patterning in the regeneration bud. Significantly, NaV1.2 is not expressed under non-regenerative conditions, suggesting that its activity is a determinant of regenerative ability. Most importantly, pharmacological induction of a brief, transient sodium current into the regeneration bud after tail amputation is sufficient to restore full regeneration of the tail during the refractory period (an endogenous developmental period when regeneration is blocked). Our study demonstrates that sodium transport is a critical mechanism for controlling regeneration, and suggests that short-term modulations of ion transport could represent an exciting new approach to tissue repair in mammals.