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polarization from bifunctional sulphorhodamine (BSR) probes on the myosin regulatory light chain (RLC) in relaxed skinned fibers from rabbit psoas muscle. Mutants of chicken skeletal RLC, with native cysteines replaced by alanine and new cysteine pairs introduced at positions 95/103, 122/134, 131/138 or 151/158, were labeled by crosslinking cysteine pairs with BSR. BSR-RLCs were exchanged into skinned muscle fibers replacing ~20% of native RLC. The second- and fourth-rank order parameters of the orientation distribution of each BSR-RLC in the fiber, $<P_2>$ and $<P_4>$ respectively, were calculated from the measured polarized fluorescence intensities. At sarcomere length 2.4 μm the order parameters of each probe had a sigmoidal dependence on temperature in the range 3-33°C with half-maximal change at 18°C. Lattice compression by 5% dextran decreased the transition temperature to 13°C and increased the temperature-dependent change in the order parameters. These results show that the LCD becomes more parallel to the filament axis in relaxing solution at higher temperature and that osmotic compression of the myofilament lattice induces further tilting of the heads towards the filament axis, inducing the fully OFF state of the thick filament. At sarcomere lengths above 2.6 μm, slow ramp stretches (4% of fiber length in 0.25 s) applied in relaxing solution at 25°C in the presence of dextran produced large changes in RLC orientation towards the ON conformation seen during calcium activation, with partial reversal during force relaxation after the stretch. The correlation between RLC orientation and passive fiber tension provides evidence that myosin head orientation is sensitive to thick filament strain. Supported by Wellcome Trust, UK.

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Phospholemman-Dependent Regulation of Na/K-Atpase Modulates Constriction and Relaxation in Aortic Smooth Muscle

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The Na/K ATPase (NKA) plays a role in modulating vascular tone through both NO-dependent and independent pathways. Phospholemman (PLM) is a muscle-specific regulator of Na/K ATPase and, in cardiac muscle, links PKA, PKC, and NO-signaling pathways to NKA stimulation. PLM is expressed in vascular smooth muscle (VSM) and we hypothesized that its phosphorylation may modulate the vascular responses to agonists. We tested this using wild-type (WT), PLM knock-out (KO), and mutant PLM knock-in mice (3SA) in which the PKC and PKA phosphorylation sites (S63, S68, S69) are mutated to alanines. Agonist-induced constriction and relaxation were measured in aortic rings in an isometric wire myograph. Vascular NKA activity was assessed by K-induced relaxation (a surrogate measure of Na/K ATPase activation) and PLM phosphorylation by immunoblotting. In WT aortae, PLM phosphorylation (S63 and S68) was significantly increased in response to phenylephrine (PE) and K-induced relaxation was significantly higher in WT than KO mice ($85 \pm 7\%$ vs $58 \pm 4\%$ of PE-induced pre-constriction; $p<0.01$), suggesting PLM regulates NKA activity in VSM. The dose-response curve for PE-induced constriction was profoundly shifted up and to the left in 3SA aortae compared to WT suggesting PLM phosphorylation normally limits constriction. Ouabain (300μM) completely abolished this difference. Pretreatment with L-NAME (300μM) also potentiated constrictor responses to PE to a greater extent in WT than 3SA vessels. Relaxation induced by the NO donor spermine NONOate was blunted in vessels from 3SA mice: $67 \pm 6\%$ vs $84 \pm 2\%$ (U46619-induced constriction; $p<0.01$ cf WT). In summary, isolated aortic rings from mice expressing unphosphorylatable PLM showed markedly elevated constriction in response to PE and attenuated relaxation in response to an NO donor. Thus, PLM phosphorylation regulates the activity of vascular NKA and this contributes to modulation of aortic constriction and relaxation.

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Carbonic Anhydrase III Contributes to Fatigue Tolerance and Recovery of Skeletal Muscle

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Carbonic anhydrase III (CA3) is a metabolic enzyme with a potential role in regulating intracellular pH. CA3 is highly expressed in slow twitch skeletal muscles. Here we demonstrated that mouse tibialis anterior (TA), a fast twitch muscle, also expresses a high level of CA3 while its myofilament protein contents were similar to that of CA3-negative extensor digitorum longus (EDL) muscle. To investigate the function of CA3 in muscle contractility and tolerance to fatigue, we studied skeletal muscles of CA3 knockout (Car3-/-) mice. Sciatic nerve stimulation-generated in situ contractility of EDL and TA muscles were examined in comparison with wild type controls. The results of isometric twitch and tetanic contractions showed no significant difference between TA and EDL muscle of wild type mice or between Car3-/- and wild type TA muscles. Nonetheless, intermittent fatigue treatment revealed faster fatigue and slower recovery of wild type TA muscle than that of wild type EDL muscle. Car3-/- TA muscle exhibited slower and less fatigue but also slower recovery than that of wild type TA muscle, whereas the ultimate level of force recovery was unchanged. It is suggested that CA3 increases the sensitivity of muscle to fatigue, which might serve as an acute physiological protection. In the meantime, Western-blot detected a low molecular weight fast troponin T (TnT) variant specifically in TA muscle of adult Car3-/- mice, suggesting a chronically adaptive response through alternative RNA splicing. The role of CA3 in fatigue tolerance and recovery of skeletal muscle suggests a molecular therapeutic target for functional enhancement.

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Protein-Protein Interactions in Skeletal Muscle Calcium Transport Regulation

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We have detected regulatory interactions between the sarcoplasmic reticulum calcium-transporting ATPase (Ca-ATPase, aka SERCA) and its inhibitory subunit sarcolipin (SLN) from skeletal muscle. SLN inhibits SERCA by uncoupling calcium transport, reducing catalytic efficiency from two to one calcium ion transported per ATP molecule hydrolyzed. Phosphorylation of SLN is the switch that relieves SERCA inhibition; it is unknown whether SLN phosphorylation disrupts the inhibitory complex (dissociation model) or causes a structural rearrangement (subunit model). Crosslinking assays indicate that calcium binding to SERCA disrupts interaction with SLN, suggesting that SLN dissociates once per catalytic cycle during the key step of calcium uncoupling (Toyoshima, Nature 2013). We previously detected oligomeric interactions between the two proteins using fluorescence resonance energy transfer (FRET) microscopy, finding that that SLN binds to SERCA in a 1:1 binary complex in the presence of micromolar calcium and millimolar ATP (Autry, JBC 2011). Here we used FRET spectroscopy to detect regulatory interactions in the presence or absence of calcium and/or ATP, finding that the two proteins show similar binding in calcium-bound and calcium-free states of SERCA, regardless of nucleotide occupancy. We also assayed SLN-SERCA interaction by column chromatography on anion-exchange and nucleotide-mimetic resins; results indicate that SLN remains bound to SERCA following detergent solubilization in the presence of calcium. We conclude that calcium does not dissociate SLN from SERCA, a result consistent with SLN-mediated uncoupling of calcium transport. Addition of calcium/calmodulin-dependent kinase did not disrupt SLN-SERCA interaction; thus, we propose that the non-dissociative subunit model is the molecular mechanism that mediates SLN regulation of SERCA. Acknowledgments: FRET was performed in the Biophysical Spectroscopy Center at the University of Minnesota, with assistance from Fluorescence Innovations, Inc. (Greg Gillispie, President). This work was funded by NIH grants to DDT (R01-GM27906, P30-AR0507220, T32-AR007612).

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Fluorescence Comes of Age: Measuring Angstrom-Level Distance Changes Within Single Filaments of Regulated Actin

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Epifluorescence imaging reveals that samples of reconstituted regulated actin (rAc) filaments contain a heterogeneous mixture of dissociated troponin and tropomyosin, unregulated actin, single rAc filaments, an bundles of rAc filaments. Sample heterogeneity confounds the interpretation of spectroscopic measurements of rAc. We have addressed the issue of sample heterogeneity by combining the particle sorting capability of confocal imaging with time-resoled FRET spectroscopy. To perform the measurements, rAc is sparsely immobilized to a glass coverslip. Non-immobilized regions of rAc do not contact the glass and undergo Brownian motion that is apparent in fluorescence video-microscopy. We perform one (or more) single point TCSPC measurements on an isolated rAc filament that is identified in a confocal image prescan. Each point measurement involves ~50 rAc-bound Tn molecules within the confocal volume. We observe ~20 photons/Tn/second. Thus, a single 20 second point measurement produces ~20,000 photons, enough for reliable determination of fluorescence lifetimes. We have used this technique to determine Ca^{2+}-induced distance changes in troponin with rAc filaments. This method may be a robust and scalable platform for the screening of drugs that bind to rAc and modulate activation.