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COMMENTARY

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Calcium activation through thick and thin?

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A historical perspective of the super-relaxed (SRX) state, interacting heads motif (IHM), and impact of calcium on muscle contractility.

An exciting new manuscript by Drs. Weikang Ma, Suman Nag, and colleagues, titled "Cardiac myosin filaments are directly regulated by calcium," has the potential to shift the paradigm for our understanding of the mechanisms governing striated muscle contraction (Ma et al., 2022). Nearly all modern biology, biochemistry, and physiology textbooks contain a chapter dedicated to muscle structure and function. These chapters illustrate striated muscle contraction and relaxation involves the cycling of calcium ions into and out of muscle sarcomeres. These sarcomeres are tiny contractile units that contain an array of myosin-based thick and actin-based thin filaments, which slide past one another to generate mechanical force and motion during a contraction. It has long been postulated that the regulatory proteins that decorate the thin filaments block myosin binding sites on actin, in the absence of calcium. Once calcium enters the sarcomere, it is bound by a thin filament regulatory protein, which unblocks initial binding sites, allowing myosin molecules to bind actin. In 2010, Dr. Cooke and colleagues published a seminal manuscript that proposed a mechanistic twist on these mechanical processes (Stewart et al., 2010). They proposed that the availability of individual myosin heads protruding from the thick filament backbone was equally as important as thin filament activation for regulating the contraction and relaxation of the sarcomere.

In 2011, I was a relative newcomer to muscle field, which is rich in historical discovery. Yet, I was electrified by the excitement and debate Dr. Cooke's presentation triggered at the Muscle and Molecular Motors Gordon Conference at Colby-Sawyer College. By this point in time, Dr. Cooke had performed elegant biochemical assays to demonstrate the presence of two distinct lifetimes by which nucleotides are released from myosin molecules in the absence of actin binding sites, within relaxed skeletal and cardiac muscle preparations. Dr. Cooke hypothesized that the very slow rate of nucleotide release observed in these biochemical assays, termed the super-relaxed (SRX) state, resulted from the structural docking of myosin heads along the thick filament backbone, termed the interacting heads motif or IHM, observed in electron micrographs of relaxed tarantula thick filaments (Alamo et al., 2008). Since this time, the hypothesis that the biochemical SRX state results from the formation of the IHM on the surface of the thick filament has caught on fire. Due to observation that disruptions in the SRX state and IHM may result in cardiac diseases, there is precedence for the development of novel therapeutics (Nag and Trivedi, 2021; Schmid and Toepfer, 2021; Craig and Padrón, 2022). Thus, the SRX state and IHM are currently among the hottest areas of research in the muscle field.

Dr. Cooke's biochemical data regarding the SRX state and hypothesis about its connection to the IHM represented a pivotal discovery in the field. Recent experimental data and modeling suggest that the initial binding of myosin molecules to actin generates strain that propagates through the backbone of the thick filament. This strain is proposed to destabilize myosin heads that adopt the IHM, and promote their rapid release from the surface of the thick filament (Fusi et al., 2016; Irving, 2017). Thus, this rapid structural change brought about by mechanosensation would increase the availability of the myosin heads to bind the calcium-activated thin filament.

In this new manuscript, Drs. Ma and Nag and colleagues propose an alternative explanation of the mechanism that regulates the availability of the myosin heads between cardiac muscle contractions, which is supported by a combination of data from x-ray diffraction and in vitro biochemical assays. They interpret their data to suggest that at low calcium concentrations most myosin molecules adopt the IHM, which holds them in an inactive state. They propose that the calcium ions released into the sarcomere non-specifically interact with the backbone of the thick filament, to release the myosin heads from the IHM and

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increase their availability to initiate contraction. This new hypothesis implies that calcium ions play dual roles in the sarcomere, regulating both the initial availability of binding sites on actin, and the availability of the myosin molecules needed to bind these sites and generate mechanical forces.

Drs. Ma and Nag and colleagues performed their x-ray diffraction experiments on relaxed, demembranated cardiac muscle preparations, incubated in buffers containing a range of calcium concentrations and a small molecule inhibitor that prevented thin filament activation. Their data demonstrated that incubation of the muscle preparations in low concentrations of calcium (10 nM) resulted in the myosin heads laying along the thick filament backbone, presumably in the IHM, whereas incubation in activating concentrations of calcium shifted the position of the myosin heads away from the thick filament backbone, even when thin filament activation was blocked with the inhibitor. These observations that calcium can impact the structure of the thick filament are consistent with previous structural data from vertebrate myosin thick filaments. Dr. Craig and colleagues demonstrated that myosin molecules in relaxed native cardiac thick filament preparations adopt the IHM in the absence of calcium (Zoghbi et al., 2008), while Dr. Podlubnaya et al. (2000) demonstrated that addition of 0.1 mM calcium to synthetic thick filaments preparations resulted in the protrusion of the myosin heads from the thick filament backbone.

Drs. Ma and Nag and colleagues complemented their x-ray diffraction data with biochemical assays, similar to those utilized by Dr. Cooke. Their biochemical data demonstrated incubation of synthetic cardiac thick filaments (i.e., those polymerized from cardiac myosin molecules in vitro) in activating concentrations of calcium reduced the fraction of molecules found in the SRX state. This observation was unique to their thick filament preparations, as calcium had no effect on the SRX state of non-filament forming fragments of myosin, which lacked the coiled-coil tails required for thick filament assembly. They collectively interpreted their data to suggest that the release of calcium into the sarcomere increased the availability of the myosin heads to bind actin.

The data from these biochemical assays and interpretations are exciting, but they are partly confounded by an anomaly. These data demonstrate that both synthetic thick filaments composed of full-length cardiac myosin molecules, and S1 cardiac myosin molecules lacking the S2 and tail portions of the heavy chain, were characterized by the same degree of SRX activity (15 vs. 13% SRX; 0.03 vs. 0.03 s^{-1} ATPase) at non-activating calcium levels (10 nM). Based on the original hypothesis that the biochemical SRX state results from the IHM, one would predict that the fraction of myosin molecules that demonstrate SRX ATPase activity would be high in synthetic thick filaments. This is because the heads of full-length myosin molecules should readily adopt the IHM on the backbone of the thick filament. In contrast, one would predict that S1 myosin would demonstrate little to no SRX ATPase activity, because S1 myosin cannot form the IHM. However, their biochemical data did not support these predictions. This disconnect between the SRX state and IHM has been reported before (Chu et al., 2021), suggesting the need for more critical evaluation of the overall connection between the biochemistry and structure of myosin. The relationship between the SRX state and IHM may be probed by determining whether SRX ATPase activity is found in native thick filament preparations from *Drosophila* flight muscle, because myosin molecules within these thick filament do not appear to adopt the IHM (Daneshparvar et al., 2020).

Despite this disconnect, is there additional evidence that myosin adopts the IHM between contractions and calcium plays a role in destabilizing this structure to increase the availability of myosin molecules during a cardiac muscle contraction? In the current manuscript, the muscle and myosin filament preparations were incubated in calcium prior to making the experimental measurements. These steady-state conditions are very different than those observed in vivo, where calcium is quickly released into the sarcomere and rapidly removed, as discussed (Podlubnaya et al., 2000). However, studies performed by Dr. Matsubara and colleagues during the 1970s using intact muscle preparations may provide insight into the positioning of the myosin heads within the sarcomere during a contraction, and the impact of calcium on these structural interactions in vivo.

Dr. Matsubara initially demonstrated that myosin heads are rapidly transferred between the thick and thin filaments during each contraction, in intact cardiac muscle preparations, using x-ray diffraction (Matsubara et al., 1977). While Dr. Matsubara observed that myosin heads enter a "quiescent" state close to the thick filament backbone, which may be the IHM, this phenomenon only occurred when intact muscle was stimulated to contract with a frequency of <4 contractions per minute (Matsubara et al., 1977). This is 15–25 times less frequent than observed in a beating human heart. Dr. Matsubara then demonstrated that a fraction of the myosin heads normally extend from the thick filament and linger near the thin filament, but the extent of this lingering was is dependent of the contractile frequency (Matsubara et al., 1978). Dr. Matsubara ultimately concluded these frequency-dependent changes in the distribution of the myosin molecules near the thin filament between contractions may result in physiologically significant alterations of cardiac contractility (Matsubara et al., 1979; Matsubara, 1980). This is because the fraction of the myosin heads that linger near the thin filament between cardiac muscle contractions may be more likely to engage the activated thin filament in a subsequent contraction.

Based on a combination Dr. Matsubara's transient measurements and these new steady-state data, it is conceivable that increasing the frequency that the thick filament is bathed in calcium may have a residual impact on thick filament structure, and fine-tune the availability of the myosin heads before the thin filament is activated. Yet, other elegant studies have suggested the shift in the position of the myosin heads is independent of calcium (Fusi et al., 2016). To resolve the question as to whether these new results presented by Drs. Ma and Nag are physiologically relevant, it is conceivable that Dr. Matsubara's original studies could be repeated with the addition of this new thin filament inhibitor. These data would provide a better sense of whether the frequency-dependent lingering of the myosin heads near the thin filament was due the direct effect of calcium,

or a residual effect of thin filament activation. These new data and controversies highlight that this is an exciting time for muscle research. Additional experimental evidence in other model systems should be utilized to test these hypotheses so we can update our textbooks and improve our current understanding of these critical processes.

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