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COMMENTARY

Getting into the thick (and thin) of it

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The heart is a highly regulated system in which a combination of mechanisms work together to match cardiac output to the needs of the body. Loss of this regulation characterizes many cardiomyopathies, where muscle is either hypo- or hypercontractile. For many years, x-ray diffraction has been used to study the structural basis of myofilament length-dependent activation (LDA; de Tombe et al., 2010; Ait-Mou et al., 2016; Reconditi et al., 2017; Piazzesi et al., 2018), which results in increased calcium sensitivity and maximal force at longer sarcomere lengths (the Frank-Starling law of the heart; Ter Keurs et al., 1980; Allen and Kentish, 1985; de Tombe et al., 2010). X-ray diffraction has also been used to study the effects of phosphorylation of sarcomeric proteins on myofilament structure and how such structural changes might relate to increased force during systole (a positive inotropic effect). In a recent issue of the *Journal of General Physiology*, Lombardi et al. provided new insights into this story using small-angle x-ray diffraction (Caremani et al., 2019). They built upon their previous work on a strain-dependent model of thick filament activation (Linari et al., 2015), in which thick filament activation during systole depends on positive feedback between the stress on the filament and the graded process of switching "ON" individual myosin motors (Reconditi et al., 2017; Piazzesi et al., 2018) from their low energy-consuming "OFF" state. A key concept in this model, which is dependent on systolic sarcomere length, is that the number of motors available for force production is related to the stress on the thick filament and thus to the loading conditions of the contraction.

Caremani et al. (2019) performed x-ray diffraction experiments on membrane-intact twitching trabeculae isolated from rat hearts using the small-angle x-ray diffraction instrument on Beamline ID-02 at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. This allowed a novel approach in which the x-ray pattern from the sarcomere repeat and the conventional two-dimensional x-ray diffraction pattern from the myofilaments were measured in the same protocol, allowing sarcomere length to be well-controlled. The authors were also able to obtain high-quality patterns from the short time window during the diastolic phase of the contractions. In addition to the strong equatorial reflections, the authors resolved the fine detail in the reflections from the thick and thin filaments on the meridian of the pattern as well as the intensity of the first myosin layer line. This is an impressive technical achievement given the small size of the preparations. The information in these diffraction features confirmed key components of the Linari et al. (2015) thick filament activation model. But the authors posed a different question: how much of the increased force at longer sarcomere length or in the presence of 10^{-7} M isoprenaline (a β-adrenergic stimulator of protein phosphorylation) can be attributed to processes related to thick filament activation? They found that, in diastole, none of the signals attributed to the OFF state of the thick filament were significantly affected by either increases in sarcomere length or phosphorylation of myofilament proteins. They therefore concluded that the control of thick filament activation is downstream from Ca^{2+}-dependent thin filament activation, and that myosin motors return to their OFF state during diastole independently of inotropic intervention.

An unexpected finding of their study is that intact muscle shows structural changes that are different from those observed in skinned muscle by x-ray diffraction (Colson et al., 2007, 2012; Palmer et al., 2011) and in isolated filaments by EM (Kensler et al., 2017). Both of these studies suggested that cardiac myosin-binding protein C (cMyBP-C) phosphorylation causes myosin heads to be released from the thick filament backbone in relaxed muscle so that they can more readily interact with actin, thus explaining the increased force in response to adrenergic stimulation. In contrast, the results of Caremani et al. (2019) suggest there is no change from the OFF state with isoprenaline in membrane-intact rat trabecular tissue in diastole. How can this discrepancy be explained? It appears likely that the release of myosin heads observed in the earlier studies reveals an underlying weakening of their binding to the thick filament surface. The conditions needed for EM, or the change in osmotic environment of the myofilaments caused by skinning, might

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then lead to their dissociation. Under the crowded, physiological milieu of the intact muscle, with its smaller interfilament lattice spacing, the heads might retain their ordered positions, despite weakening of their interactions, because the forces causing them to move are smaller or absent. An immediate conclusion is that data from skinned muscle and isolated filaments must be interpreted with caution. On the other hand, data from intact muscle also do not provide a full picture. While showing the structural impact of cMyBP-C phosphorylation in intact muscle, the results of Caremani et al. (2019) do not reveal any loosening of head interactions, which appears to be a key part of the mechanism. One possibility is that there is indeed head movement, but in the crowded intact lattice, it is too small to detect with x-ray diffraction. Thus, only by combining data from all three approaches (isolated filaments, skinned muscle, and intact muscle) are the fullest insights provided.

According to the results of Caremani et al. (2019), the mobilization of heads characteristic of the OFF–ON transition in the strain-dependent thick filament activation model occurs only during calcium activation, when active force is generated. They suggest that the increase in force observed with both LDA and phosphorylation of myofilament proteins increases the gain of the positive feedback between the stress on the thick filament and motor recruitment. What might be the origin of this gain? An interesting observation made by the authors was a reduction in intensity of reflections relating to cMyBP-C (the M1 cluster) and the first order troponin reflection (T1) in the presence of isoprenaline. Since phosphorylation of both proteins is stimulated by isoprenaline, the results suggest that this may lead to structural changes that underlie their inotropic effect. As mentioned above, phosphorylation of MyBP-C at low [Ca^{2+}] is believed to cause a weakening of the interactions between myosin heads, leading to increased head mobility and thus force, i.e., an increase in "gain."

Together, these experiments present a satisfying story, but there are some missing pieces. For example, because the authors did not observe changes relating to thick filament activation mechanisms with increases in sarcomere length, one might conclude that there are no structural events related to LDA in diastole, either with or without the effects of phosphorylation. The authors suggest that some x-ray intensity changes they did see in diastole are due simply to improved ordering of filaments, in contrast to the results of other investigators (discussed below).

An important contribution of the paper is the increased understanding of the molecular basis of LDA—the observation that the myocardium generates more force at longer sarcomere lengths for the same amount of activating calcium (increased calcium sensitivity). This is the mechanism underlying the Frank-Starling law, which posits that the heart generates higher systolic pressure with higher end-diastolic volume. For many years, it was believed that increased calcium sensitivity associated with LDA was due to the reduction in lattice spacing when muscle is stretched. Further work (summarized in de Tombe et al., 2010) showed, however, that not all changes in calcium sensitivity could be explained in this way, so this cannot be the fundamental mechanism. Other work showed that the degree of titin-based passive tension correlated with increased calcium sensitivity (Cazorla et al., 2001; Lee et al., 2010; Methawasin et al., 2014). Because titin is the principal contributor to the length-dependent passive tension of cardiac muscle (Granzier and Irving, 1995; Linke and Fernandez, 2002; Granzier and Labeit, 2004), the correlation suggests that titin is the strain-sensor that links changes in sarcomere length to increased calcium sensitivity and force.

A recent study (Ait-Mou et al., 2016) involving experiments on intact twitching rat papillary muscle, similar to those of Caremani et al. (2019) on trabeculae, shed light on this question. WT myocardium was compared with that from a rat model expressing a titin isoform that produces greatly reduced passive force upon stretch. As expected, LDA was much lower in the model due to the reduction in passive tension. In WT myocardium, the increase in titin-based passive tension with sarcomere stretch produced structural changes in both the thick filaments (indicated by changes in the thick filament meridional reflections), and thin filaments (indicated by changes in the third order troponin meridional reflection). These changes were absent in the mutant myocardium with its greatly reduced titin-based passive tension and, hence, thick filament strain. These observations motivated a model in which titin-based strain in the thick filaments is communicated to the regulatory apparatus on the thin filament, leading to enhanced calcium sensitivity. While a number of candidate structures for communicating between the thick and thin filaments were discussed, their identity remained unresolved.

Another study (Zhang et al., 2017), which used blebbistatin to inhibit force production and fluorescent probes on troponin C (TnC) and the regulatory light chain (RLC), monitored structural changes in thin and thick filament components in skinned ventricular trabeculae during stretch. Changes in TnC were observed that were distinct from those during calcium activation, and independent of the presence of force generating myosin heads. These results were in accord with similar fluorescence experiments by Ait-Mou et al. (2016) on skinned cardiomyocytes using fluorescently labeled cardiac TnC, from which it was concluded that stretch induced a conformational rearrangement of troponin that was distinct from changes induced by Ca^{2+}. It appears, then, that increasing sarcomere length in cardiac muscle changes the structure of troponin in the thin filament in such a way that its sensitivity to calcium is increased and is correlated with titin-based strain. Further, this occurs in the absence of active, force-generating myosin heads. The fluorescence studies were done on skinned myocardium, and given the discussion above, one should be cautious about extrapolating the behavior to intact muscle. Nonetheless, the results suggest that LDA involves contributions from structural alterations in the troponin complex that are independent of myosin head binding, consistent with the x-ray results of Ait-Mou et al. (2016).

To transmit the titin-based strain in the thick filaments (the primary length sensor), there must be a communication pathway to the regulatory apparatus on the thin filament. What is the communicator? As discussed by Ait-Mou et al. (2016) and Zhang et al. (2017), cMyBP-C is a likely candidate, as it is anchored to

the thick filament via its C-terminal region but can bridge to the thin filaments and bind to actin via its N-terminal domains (Shaffer et al., 2009; Luther et al., 2011; Kampourakis et al., 2014; Mun et al., 2014; Risi et al., 2018). Moreover, such a cMyBP-C bridge would be dynamic and regulated by phosphorylation (Colson et al., 2012; Rosas et al., 2015; Mamidi et al., 2016; Previs et al., 2016). One scenario is that strain in the thick filament is transmitted via cMyBP-C directly to the troponin–tropomyosin complex, altering its structure. Alternatively, thick filament strain could simply alter the number and nature of the cMyBP-C interactions with actin. Regardless of the mechanism, evidence for a critical role for cMyBP-C in this process is apparent from the observation that its ablation blunts the length-dependent increase in Ca^{2+} sensitivity (Mamidi et al., 2014).

Caremani et al. (2019) do not address the structural changes responsible for increased calcium sensitivity at increased length, particularly those in troponin. They suggest that the intensification of the first order (T1) troponin reflection that they observe is due to an increased myofilament ordering caused by a reduction in the degree of double overlap of thin filaments as the sarcomere lengthens. This mechanism may account for the observed reduction in the I_{11}/I_{10} intensity ratio at longer sarcomere lengths, which is otherwise difficult to explain. Ait-Mou et al. (2016), however, observed changes in the second and third order troponin meridional reflections (T2 and T3 respectively), but in opposite directions. T2 intensity was slightly reduced and T3 approximately doubled when going from 2.0 to 2.4 µm sarcomere length. These opposing effects are not easily explained by an increase in filament order alone, and when combined with the fluorescence studies, they suggest that structural changes occur within the troponin complex itself.

Ait-Mou et al. (2016) proposed that the thin filament changes were coupled to changes in thick filament structure resulting from increased titin-based passive tension, as both were observed in a 10-ms time window just before a twitch. The most important finding of Caremani et al. (2019) is that, if there are any changes in thick filament structure with increase in sarcomere length in diastole, they are not of the type associated with the transition of the thick filament from the OFF to the ON state. But this does not exclude the possibility of any changes in thick or thin filament structure in diastole. Both Ait-Mou et al. (2016) and Caremani et al. (2019) show that the thick filaments are slightly longer at the longest sarcomere lengths they studied (2.4 and 2.2 µm, respectively) in conditions where there is significant titin-based passive tension to strain the thick filament. Both studies show that, although the myosin-based meridional x-ray reflections generally increase in intensity with increasing sarcomere length, the individual reflections do so by differing amounts. This implies subtle changes in filament structure, most likely due to the increased thick filament strain, as these changes vanish with the more compliant mutant titin. In any event, the differential response of individual reflections suggests that they arise, at least in part, from structural changes in the filaments themselves, not just increases in filament ordering.

Thick filament structural changes due to increased titin-based passive tension under resting conditions are also indicated by fluorescence studies on skinned rabbit psoas muscle (Fusi et al., 2016). The results showed that the orientation of the RLC C-lobe changed in a graded way with force and that this was independent of whether that force was actively or passively generated, i.e., it did not depend on $[Ca^{2+}]$. Given the differences between skinned versus membrane-intact muscle and skeletal versus cardiac muscle, one should be cautious about extrapolating the results. Nevertheless, one can expect modest, but possibly important, changes to thick filament structure, particularly with respect to myosin head orientation, in response to passive stress.

Caremani et al. (2019) have produced a stimulating model for increased force with LDA or phosphorylation. However, the above discussion suggests that it is possible to separate events leading to increased calcium sensitivity during diastole from those leading to increased force in systole. It is the increased force in systole, due to either LDA or phosphorylation of myofilament proteins, that can be explained by a common thick filament activation mechanism that may be modulated by cMyBP-C. The suggestion by Caremani et al. (2019) that LDA and phosphorylation of myofilament proteins effectively modulates the "gain" in a stress-sensitive thick filament activation mechanism is very attractive. In addition, it is possible that increased calcium sensitivity with length change may come first, during diastole, followed by the increased force in systole as active bridges develop and are modulated by the thick filament activation mechanism. This two-step change has been suggested by the fluorescence studies of Zhang et al. (2017). We agree with the authors that the stress-sensing mechanism that switches ON myosin motors is modulated by the inotropic mechanisms that they studied. Their conclusion that this "opens a new scenario in which hypertrophic cardiomyopathy-causing mutations would operate by lowering the force threshold of the switch that controls the thick filament activation" is a compelling hypothesis that warrants vigorous investigation.

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