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Novel Regulatory Elements within Myofilaments of Vertebrate Striated Muscles—Who Knew

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Among the most influential advances in muscle biology during the 20th century was the discovery by Ebashi and Endo (1) of troponin, the primary Ca^{2+}-binding protein within the thin-filament regulatory strand of vertebrate striated muscles. This seminal report opened an important field of research that initially focused on determining the subunit composition of troponin and then on progressively higher-resolution studies of subunit structure, subunit interactions, and changes in either or both as a consequence of Ca^{2+} binding to troponin (2). Subsequent thought in the field about the possible role(s) of such binding in the regulation of muscle contraction has been influenced by a constellation of observations that are summarized only briefly here, without qualification or attribution. For example, myofibrillar ATPase activity varies with Ca^{2+} concentration in the presence of troponin but is maximal and unaffected by Ca^{2+} in its absence. Together with findings that contractile force and velocity vary with Ca^{2+} concentration, these results provided experimental support for the idea that Ca^{2+} binding by troponin serves as a graded switch regulating the activation state of the thin filament (2).

In its simplest application, the notion of switch-like activation of the thin filament due to Ca^{2+} binding to troponin has been viewed as a strictly responsiveness process in which cross-bridge number, force, and power in living muscle are controlled straightforwardly by varying the amount of Ca^{2+} released from the sarcoplasmic reticulum. Although this view of myofilament activation appears to have simplified the field of excitation-contraction coupling to just excitation and coupling, it does not take into account the pronounced nonlinearities in thin filament-mediated regulation or variations in regulation due to post-translational modifications of regulatory proteins in the thick or thin filaments. As examples, there is positive cooperativity in the binding of Ca^{2+} to troponin and in myosin cross-bridge binding to the thin filament, and each process dynamically reinforces the other. And the kinetics of force development are accelerated 10-fold when $[Ca^{2+}]$ is increased from threshold to maximum for force development, which is not explained by a simple Ca^{2+}-dependent on-off regulation of the thin filament. Because force (and power) and Ca^{2+} delivery are dynamically regulated in skeletal muscle and to a greater degree in cardiac muscle, it is reasonable to presume (but as yet difficult to test) that the kinetics and extent of each process are dynamically tuned to match the other. It seems likely that compensatory processes at the level of the cell or tissue in diseases such as hypertrophic cardiomyopathies are activated in response to a sustained imbalance in the tuning of contractile and Ca^{2+}-delivery systems.

In recent years, studies of populations of so-called "super-relaxed" (SRX) cross-bridges, first described by Cooke and colleagues ((3) and references therein), have explored the possibility that recruitment of SRX cross-bridges into force-generating states comprises an additional mechanism for regulating force in heart and skeletal muscles. SRX cross-bridges appear to assume an inactive conformation that is similar structurally (the so-called "interacting head motif") to the off state observed previously in relaxed smooth muscle, in which contraction is regulated via the thick and not thin filaments. But with force production and the resulting strain of the thick filament and an increased periodicity of cross-bridges along the filament, the number of cross-bridges in this conformation decreased, i.e., increased load activates cross-bridges for interactions with actin, thus matching the number of cross-bridges to the load on a muscle. This topic was reviewed in *Biophysical Journal* last year in a thoughtful, provocative article by Irving (4) summarizing the case for force-dependent recruitment of cross-bridges and the greater energetic efficiency that this confers to muscle. In a series of experiments in

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which fluorescence polarization probes were bound to the regulatory light chains of myosin, signals corresponding to the activated state of cross-bridges during Ca^{2+}-induced force generation in permeabilized muscle were also evident in the same muscles at rest by increasing muscle length to extend the elastic protein titin and thereby increase the mechanical stress and strain of the thick filament.

In this issue of *Biophysical Journal*, Ma et al. (5) tested the idea that stress applied to thick filaments in living skeletal muscle would recruit cross-bridges to an activated state, which they assessed using x-ray diffraction of muscle fibers at various lengths. Like the earlier experiments summarized in the review by Irving (4), thick-filament strain was induced by force development (although Ca^{2+} was delivered via tetanic stimulation of living fibers rather than infusion of buffered Ca^{2+} in skinned fibers) and the transition of cross-bridges to the activated state was determined by measuring intensity and spacing of reflections in x-ray diffraction patterns from these fibers. Results from these experiments were in some ways consistent with earlier findings in that increased thick-filament strain due to Ca^{2+} activated force was accompanied by increased numbers of activated cross-bridges. However, increased strain due to passive stretch of muscle in the presence of blebbistatin to inhibit cross-bridge force generation did not result in greater activation of cross-bridges. The results thus appear to dissociate cross-bridge activation in living muscles from greater thick-filament strain, unless that strain occurs in the presence of Ca^{2+} and/or cross-bridge-mediated force development. Alternatively, the activating effects on myosin due to mechanical stress might involve specific intermolecular interactions, e.g., myosin tail-tail, myosin-titin, or myosin-myosin binding protein-C interactions, that are specifically disrupted by the force generated within a myosin molecule due to cross-bridge binding to actin. As discussed by Irving (4), earlier studies by Haselgrove (6) reported changes in the x-ray pattern of electrically stimulated skeletal muscle that indicated an activation of myosin heads, i.e., loss of helical order, even when the muscle was stretched to lengths that greatly reduced both thick/thin-filament overlap and developed force. Irving suggested the intriguing possibility that thin-filament activation by Ca^{2+} is transmitted to the thick filament by a mechanism that is not yet known but might, for example, involve cMyBP-C as a link between the two filaments. The plot thickens!

Another potentially important finding by Ma et al (5). is that thick filaments become much stiffer when subjected to greater mechanical strain, a phenomenon that would be expected to affect measurements of force and the kinetics of force development in muscle. As pointed out by the authors, the stiffness of the thick-filament backbone has been assumed to be small and constant; however, if cross-bridges were to develop force against a more compliant thick-filament backbone at low forces, the rate constant of force development that is obtained would manifest the effects of the greater compliance to reduce the rate of force development. As mentioned in a preceding paragraph, the rate of force development increases by an order of magnitude as force is increased from threshold to maximum, as when $[Ca^{2+}]$ is raised. Unknown at this point is the extent to which the Ca^{2+} dependence of the kinetics of force development is due to a mechanical stress-induced stiffening of the thick filament at high $[Ca^{2+}]$ as opposed to processes such as the proposed Ca^{2+}- or force-dependent recruitment of SRX cross-bridges or slowing of force development at low Ca^{2+} due to the time taken for cooperative recruitment of cross-bridges to force-generating states (7).

Taken as a whole, the results of this and earlier studies strongly suggest that a single-dimensional view of force generation by myosin, i.e., that myosin binding to Ca^{2+}-activated thin filaments does nothing more than generate contractile force, is inadequate to explain either the regulation or the kinetics of force development and relaxation in vertebrate striated muscles. Future work will undoubtedly probe and identify the molecular elements of thick-filament-linked regulation, interactions within and between thick and thin filaments in this regard, and the roles played by each in determining muscle function.

In the expanding universe of muscle regulatory processes, Hill's (8) variation on a line by Shakespeare seems relevant: "There are more things in heaven and earth, Horatio, and even in muscles."

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