Calcium, Cross-Bridges, and the Frank-Starling Relationship
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The steep relationship between active force and length in cardiac muscle is based on a length dependence of myofilament Ca\(^{2+}\) sensitivity. However, it is not muscle length but the lateral spacing between actin and myosin filaments that sets the level of Ca\(^{2+}\) sensitivity, mainly through modulation of myosin-mediated activation of the thin filament.

At the turn of the 20th century, Otto Frank and Ernest Starling carried out their classic studies on the relationship between end diastolic volume and systolic function in the isolated heart. Their findings, still valid, have since been absorbed by many generations of students of medicine and physiology, and they invariably enter into the discussion of such subjects as cardiac output regulation, exercise, and congestive heart failure. Yet at the beginning of the 21st century, investigators are still trying to identify the intracellular signaling pathways responsible for the seemingly simple relationship between myocardial fiber length and the work output of the heart. Recent years have seen a proliferation of studies of length-dependent behavior of intact cardiac muscle, isolated cardiac myocytes, and various types of skinned fiber preparations in which the membranes have been removed, usually by detergent treatment. These studies together have provided a reasonably convincing picture of how relatively small changes in sarcomere length can be transduced into large changes in developed force. However, the detailed molecular interactions that adjust molecular motor function to myocyte length remain to be clarified. Further progress can be expected with the current availability of powerful molecular-genetic techniques, which can probe the relationship between specific molecular alterations and functional changes at the cell and organ levels.

The response of cardiac muscle to stretch

If a strip of cardiac muscle is mounted in a muscle chamber under isometric conditions and stimulated at a fixed frequency, an increase in sarcomere length along the ascending limb of the force-length curve (sarcomere length 1.7–2.4 \(\mu\)m) produces a biphasic increase in twitch force. Immediately following the initial stretch, there is a large increase in force that is unaccompanied by a significant change in the amount of Ca\(^{2+}\) released into the cytoplasm (1). After a delay of several minutes, there is a further slow increase in force that occurs in conjunction with a slow increase in the height of the Ca\(^{2+}\) transient. The published data show considerable variability, but in most studies the slow phase accounts for ~20–30% of the total force increase in response to the stretch. The initial force increase is primarily due to an increase in myofilament Ca\(^{2+}\) sensitivity, with possibly a small...
with no significant change in the height of the Ca\textsuperscript{2+} transient, a response that they interpreted as indicating an increase in myofilament Ca\textsuperscript{2+} sensitivity. With stimulation extended over a period of several minutes, there were parallel increases in both force and the height of the Ca\textsuperscript{2+} transient. Concurrently, Hibberd and Jewell (9) used detergent-skinned rat trabeculae immersed in Ca\textsuperscript{2+}-buffered solutions to study the effect of sarcomere length on the relationship between force and pCa. They showed that with an increase in sarcomere length from 1.9 to 2.4 \textmu m there was a clear leftward shift of the force-pCa curve, denoting an increase in Ca\textsuperscript{2+} sensitivity. The latter is generally quantified in terms of the pCa associated with half-maximal force generation (pCa\textsubscript{0.5}). These fundamental observations have been repeatedly confirmed, and it is now accepted that a length-dependent shift in Ca\textsuperscript{2+} sensitivity is the key component of the Frank-Starling relation. Associated with the altered Ca\textsuperscript{2+} sensitivity is a parallel change in the affinity between Ca\textsuperscript{2+} and the regulatory binding site on cardiac troponin C. These findings raise a fundamental question: how does the regulatory protein complex that mediates Ca\textsuperscript{2+} activation “sense” muscle length? As will be discussed below, this question is closely connected to the more basic problem of how the contractile system is activated by the sudden rise in cytosolic Ca\textsuperscript{2+} triggered by the action potential.

**Thin filament activation**

Regulation of the cross-bridge cycle in striated muscle is mediated by the regulatory protein complex (troponin-tropomyosin) bound to the thin filament (see Ref. 7 for a comprehensive review). The backbone of the thin filament, F-actin, is formed by the polymerization of G-actin subunits to form a two-stranded helix, one end of which is anchored to the Z disc. Tropomyosin (Tm) is an elongated dimer (~42 nm long) composed of two highly \(\alpha\)-helical polypeptides that form a coiled-coil structure. The Tm lies in the grooves of the actin double helix, where each Tm overlaps seven actin monomers. Attached to each Tm is a troponin (Tn) complex. Each Tn is comprised of three subunits, namely troponin C (TnC), the Ca\textsuperscript{2+}-binding subunit, troponin I (TnI), the inhibitory subunit, and troponin T (TnT), which binds the complex to Tm. The TnC isoform expressed in cardiac muscle (as well as in slow skeletal muscle), designated cTnC, has three Ca\textsuperscript{2+}-binding sites, in contrast to the four found in the fast skeletal muscle isoform (sTnC). Two of these sites, located in the COOH terminus, are high-affinity sites (K \(\sim 10^{-7}\) M) that also bind Mg\textsuperscript{2+} (Ca\textsuperscript{2+}/Mg\textsuperscript{2+} sites). These seem to play a role in keeping the cTnC bound to the thin filament (7). The single binding site located in the NH\textsubscript{2} terminus has a lower affinity (K \(\sim 10^{-6} - 10^{-5}\) M) for Ca\textsuperscript{2+} than the COOH terminal sites, but it is highly specific for Ca\textsuperscript{2+} and functions as the regulatory site for the initiation of cross-bridge cycling. The TnI isoform that is expressed in cardiac muscle (cTnI) is unique in that it contains a 32-amino acid NH\textsubscript{2} terminal extension that has sites for phosphorylation and that can play a role in the modulation of myofilament Ca\textsuperscript{2+} sensitivity (14).

With the introduction of the “steric blocking” model in the 1970s, a key function in Ca\textsuperscript{2+} activation was assigned to Tm.
(7). Since each Tm overlaps seven actin subunits and has attached to it one Tn, the complex A,TmTn was considered to be the fundamental thin filament regulatory unit. This unit could exist in an “on” or “off” state depending on the position of the Tm in the groove of the actin filament. Early X-ray diffraction studies indicated that the binding of Ca²⁺ to TnC caused the Tm to shift from the periphery toward the center of the groove, thereby allowing myosin heads easier access to binding sites on actin. In this simple two-state model, the Ca²⁺-ion is the sole activator of the thin filament. However, it has been known for some time that there is a second thin filament activator, and that is myosin itself. Myosin activation is most simply demonstrated by placing a skinned muscle fiber in a Ca²⁺-free relaxing solution and gradually lowering the MgATP concentration. Actin and myosin bind tightly at very low MgATP concentrations. When MgATP concentration is sufficiently low (~10⁻⁵ M), the attachment of a critical number of strong-binding cross-bridges (rigor bridges) to actin causes a cooperative upregulation of the thin filament, seen as an increase in myosin ATPase activity and the development of tension. In addition, the attachment of rigor bridges to actin causes an increase in Ca²⁺-TnC binding affinity. There is now ample evidence that the force-generating cross-bridges formed under more physiological conditions (3.5 mM MgATP, 10⁻⁷ to 10⁻⁵ M Ca²⁺) cause the same cooperative activation of the thin filament. A complete review of this evidence is beyond the scope of this article, but we may cite biochemical experiments with reconstituted thin filaments that showed that if the ratio of myosin heads to actin subunits was sufficiently low the myosin ATPase activity remained low even in the presence of Ca²⁺ concentrations that would fully saturate the binding sites on TnC (7, 11). At the structural level, electron microscopic and X-ray diffraction studies have shown that the amount of Tm movement produced by Ca²⁺ binding to TnC in isolated thin filaments (in the absence of myosin) was significantly less than that seen when myosin heads were strongly bound to actin (15). Thus full activation of the thin filament results from a concerted effect of Ca²⁺ binding to TnC and myosin binding to actin.

Biochemical studies carried out during the past decade by Geeves, Lehrer, and co-workers (11) have provided strong support for a three-state model of thin filament activation. According to this model, in the presence of physiological concentrations of MgATP, but in the absence of Ca²⁺, Tm is in its optimal blocking position and the cross-bridges are largely detached from actin (“blocked” state). With the binding of Ca²⁺ to TnC, there is a partial movement of Tm, which allows the myosin heads to bind weakly to actin (“closed” state). With Ca²⁺ still bound to the regulatory site on TnC, the weak actin-myosin complex (A state) can then isomerize to a strong-binding complex (R state), which is responsible for the generation of force and which shifts the thin filament into the “open” state. The A → R transition forces Tm further into the groove of the actin filament, thereby allowing for the recruitment of more cross-bridges. This recruitment makes a major contribution to the highly cooperative nature of the force-pCa relationship. As shown by Lehrer and Geeves (11), this system has the main features of an allosteric regulatory system in which myosin (or myosin-ADP complex) is the true activating ligand, actin is the catalytic subunit, Tm is the regulatory subunit, and the Tn±Ca²⁺ is the allosteric effector (positive when Ca²⁺ is bound to TnC, negative when Ca²⁺ is dissociated from TnC).

Strong-binding cross-bridges and Ca²⁺ sensitivity

Returning to the question first posed, evidence has been accumulating that sarcomere length modulates the level of Ca²⁺ sensitivity through variation in the number of strong-binding actin-myosin interactions. The basic idea is that with increase in sarcomere length along the ascending limb of the force-length curve there is a more favorable disposition of the myosin heads relative to actin, thereby increasing the probability of strong-binding actin-myosin interactions taking place. That is, an increase in sarcomere length favors myosin-mediated activation of the thin filament. In support of this view, measurements of radioactive Ca²⁺ binding to cTnC in skinned bovine cardiac fibers showed that the length dependence of the Ca²⁺-cTnC interaction was based on a length dependence of strong-binding actin-myosin interactions rather than length itself (10). The length dependence of Ca²⁺ binding was eliminated by cross-bridge interaction inhibitors that prevented either rigor or force-generating bridges from attaching to actin. More recently, Fitzsimons and Moss (5) showed that labeling of the actin of skinned cardiac myocytes with strong-binding myosin heads (chemically modified) reversibly eliminated the length dependence of Ca²⁺ sensitivity. Thus changes in the geometry of the myofilament lattice modify Ca²⁺ sensitivity via effects on myosin-mediated activation of the thin filament.

Length vs. width

The hypothesis that strong-binding cross-bridge interactions are responsible for the length modulation of Ca²⁺ sensitivity encountered an early difficulty with the observation, first made in the 1970s, that in both skeletal and cardiac muscles the Ca²⁺ sensitivity is increased when skinned fibers are stretched along the descending limb of the force-length curve (1). In this region, the potential for cross-bridge interactions should decrease as the overlap of actin and myosin filaments is diminished. An alternative hypothesis that takes this objection into account is that the Ca²⁺ sensitivity is determined not by sarcomere length but by a derivative of sarcomere length, namely interfilament spacing. The filament lattice of striated muscle is an isovolumic system. Hence, as the sarcomere length is increased, the lateral spacing between actin and myosin filaments is reduced. If this spacing influences the probability of cross-bridge attachment, the Ca²⁺ sensitivity would not be a simple function of actin-myosin overlap but would also depend on interfilament spacing.
Interfilament spacing at a fixed sarcomere length can be changed quite easily by immersing skinned fiber preparations in buffer solutions containing a high-molecular-weight polymer that cannot diffuse into the interfilament space. The resulting osmotic withdrawal of fluid from the interfilament space causes a reversible reduction in fiber diameter, the magnitude of which is a function of the polymer concentration. One of the more commonly used polymers is Dextran T-500. Early studies with skinned skeletal muscle fibers showed that moderate lattice compression at a constant sarcomere length caused an increase in Ca\(^{2+}\) sensitivity (1). More quantitative studies with skinned cardiac myocytes have shown that if a given reduction in fiber diameter is produced by either stretch or osmotic compression, the observed changes in Ca\(^{2+}\) sensitivity are in quite close agreement (12). In a study in our laboratory (6), skinned muscle bundles at different sarcomere lengths were subjected to varying degrees of osmotic compression with Dextran T-500. It was then possible to analyze both Ca\(^{2+}\) sensitivity and Ca\(^{2+}\)-cTnC affinity as a function of sarcomere length under conditions in which interfilament spacing could change with sarcomere length or remain relatively constant. It was clear that both Ca\(^{2+}\) sensitivity and Ca\(^{2+}\) binding were correlated with interfilament spacing and not at all with sarcomere length (Fig. 2). Thus the shift in Ca\(^{2+}\) sensitivity that underlies the Frank-Starling relation depends not on a change in sarcomere length per se but on the change in lateral separation between actin and myosin filaments.

### Length-dependent activation and the three-state model

In terms of the above discussion, one might postulate that the spacing between actin and myosin filaments determines the “effective” concentration of myosin heads in the vicinity of a given actin filament. An increase in this effective concentration, whether the result of stretch or osmotic compression, should facilitate myosin-mediated activation of the thin filament. As pointed out above, myosin activation entails the participation of two of the three thin filament states. The myosin must first form a weak bond (A-state) with thin filaments in the closed state, the latter state being favored by the binding of Ca\(^{2+}\) to cTnC. With Ca\(^{2+}\) bound to the regulatory site on cTnC, the cross-bridge isomerizes to the strongly bound R-state, in which force can be developed. This state is coupled to a shift of the thin filament to the open state, in which Tm is pushed further into the groove of the actin filament, thereby promoting recruitment of additional force-generating cross-bridges. According to this scheme, the fraction of thin filament regulatory units in the open state is correlated with the level of Ca\(^{2+}\) sensitivity. Is it the blocked ↔ closed or the closed ↔ open transition that is length sensitive? A means of answering this question was provided by the discovery that in the absence of Ca\(^{2+}\) the lowering of ionic strength to approximately <50 mM caused the blocked ↔ closed equilibrium to shift far to the right without any major effect on the closed ↔ open equilibrium (8). Thus variation in ionic strength can be used as a probe of the contribution of weak-binding cross-bridges to length-dependent activation since these bind to thin filament regulatory units in the closed state. Studies in this laboratory (12) have shown that as the KCl concentration of the buffer is reduced to <50 mM normal Ca\(^{2+}\) regulation is retained but the length dependence of Ca\(^{2+}\) sensitivity (as well as Ca\(^{2+}\)-cTnC affinity) is eliminated (Fig. 3). These data provide strong evidence that the number of weakly bound cross-bridges is a function of filament lattice geometry as well as pCa. The effect of lattice spacing on the blocked ↔ closed transition is bypassed when the ionic strength is lowered. Thus the three-state model can be expanded to include a geometric factor that can, along with Ca\(^{2+}\) and ionic strength, determine the size of the population of weakly bound cross-bridges (Fig. 4).

### Problems for future investigation

The aspect of length modulation about which we have the least information is the nature of the signaling pathways...
within the thin filament that adjust Ca\(^{2+}\) sensitivity to the number of actin-myosin bonds. It is evident that when myosin heads bind strongly to actin, protein-protein interactions are initiated that ultimately increase the affinity of Ca\(^{2+}\) for the regulatory binding sites on cTnC. It might be pointed out that in skeletal muscle measurable effects of force-generating complexes on the binding of Ca\(^{2+}\) to TnC have not been detected, although there are length-dependent changes in Ca\(^{2+}\) sensitivity (7). Interestingly, this statement holds true for slow skeletal muscles that express cTnC rather than sTnC. Thus the Ca\(^{2+}\)-binding properties of cTnC are not intrinsic to the molecule but are subject to modulation by protein-protein interactions that still remain to be defined. Arteaga et al. (3) have recently studied length-dependent cardiac activation in transgenic mouse hearts in which the cTnI has been replaced by the slow muscle isoform (ssTnI). Skinned myocytes obtained from the transgenic hearts showed a much lower change in pCa\(_{50}\) for a given length change compared with the wild type. Evidence is presented that ssTnI substitution, like reduced ionic strength, leads to an increase in the number of cross-bridges in the weak-binding state. Thus cTnI appears to be an integral component of the length-sensing mechanism in that it can modulate weak actin-myosin interactions as a function of sarcomere length. It will be recalled that ssTnI lacks the NH\(_2\) terminal extension characteristic of cTnI. Further experiments will be needed to determine whether this segment of cTnI plays the key role in length modulation and to delineate the specific roles of TnT, Tm, and actin. It will also be of great interest to determine what effect genetically engineered changes in the length dependence of Ca\(^{2+}\) sensitivity have on cardiovascular function in the intact organism.

Although it appears that the length-dependent shift in Ca\(^{2+}\) sensitivity can be almost fully accounted for by changes in actin-myosin separation, contributions by other factors cannot be excluded. Recently, a role for the giant elastic protein titin has been suggested (cardiac titin ~ 2500 kDa). Titin, which extends from the Z line to the M line, accounts for most of the elasticity of striated muscle fibers. The elasticity of titin is primarily a function of immunoglobulin-like domains lying in the I band segment of the molecule. Cazorla et al. (4) reported that in skinned cardiac myocytes the Ca\(^{2+}\) sensitivity is correlated with passive tension rather than sarcomere length. Moreover, degradation of titin by mild trypsin treatment caused an ~50% reduction in the change in pCa\(_{50}\) produced by a given length change. A role for titin is plausible inasmuch as it is known to interact with thin filament proteins. However, in experiments in which correction was made for interfilament spacing (6), an independent effect of titin on Ca\(^{2+}\) sensitivity was not seen (Fig. 2). Confirmation of this finding in other types of skinned myocyte preparation is needed. One would also like to be able to relate Ca\(^{2+}\) sensitivity to the actual actin-myosin separation, as measured by X-ray diffraction, rather than the more crude measurement of fiber diameter. Additional studies along these lines may be very informative.

**Summary**

The classic Frank-Starling relationship of the intact heart requires that the myocyte contain a length-sensing mechanism that adjusts the activity of the cross-bridges to the volume of blood in the relaxed ventricle. There is now a consensus that a length dependence of myofilament Ca\(^{2+}\) sensitivity is a key component of this mechanism. The Ca\(^{2+}\) sensitivity is a function of the level of myosin-mediated activation of the thin filament. The latter can be modulated by length-dependent variations in the lateral spacing between the actin and myosin filaments. Further studies are needed to identify the signaling pathways that link cross-bridge attachment and regulatory protein function.

Because of editorial restrictions, many important contributions could not be cited. See Refs. 7 and 14 for a more comprehensive list of recent papers in this field.

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**References**

Measurements of Vesicle Recycling in Central Neurons

Neurotransmitter-containing vesicles in presynaptic nerve terminals are essential for synaptic transmission. The vesicles undergo a cycle that leads to transmitter release by exocytosis followed by endocytosis of the vesicles. Here we discuss new optical methods that have been developed for direct measurement of vesicle recycling (see Fig. 1). In this brief review, we will focus on experimental evidence obtained with optical methods.

The number of fused presynaptic vesicles is crucial for understanding mechanisms of neurotransmitter release from synaptic boutons of central neurons. These boutons contain thousands of vesicles with a diameter of about 50 nm that are released into the synaptic cleft by exocytosis of the vesicular membrane. The released neurotransmitter diffuses across the synaptic cleft and binds to specific receptors in dendritic spines causes either excitation or inhibition of electric activity in postsynaptic dendrites. The number of fused presynaptic vesicles is crucial for understanding the quantal nature of transmitter release.

Measurements of vesicle recycling are important because it is not known whether this process is limited by the rate of vesicle fusion or the rate of vesicle retrieval. This is crucial information for understanding mechanisms of neurotransmitter release from synaptic boutons. Therefore, it is not surprising that in the literature considerable uncertainty exists about the quantal nature of transmitter release in CNS synapses. In recent years, a number of new methods have been developed for direct measurement of vesicle recycling and transmission of the evoked electrical signals and their structural correlates. As a matter of fact, postsynaptic electrophysiological experiments give only indirect information about presynaptic structural changes, such as synaptic clefts and synaptic vesicles. Therefore, it is not surprising that in the literature considerable uncertainty exists about the quantal nature of transmitter release in CNS synapses.

Modulations of synaptic transmission are thought to be important for learning and memory. They can be potentiated or depressed by coincident activation of pre- and postsynaptic structures in axons and dendrites. The number of fused presynaptic vesicles is crucial for understanding mechanisms of neurotransmitter release from synaptic boutons of central neurons. Therefore, it is not surprising that in the literature considerable uncertainty exists about the quantal nature of transmitter release in CNS synapses.