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Regulating Striated Muscle Contraction: Through Thick and Thin

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Abstract

Force generation in striated muscle is primarily controlled by structural changes in the actin-containing thin filaments triggered by an increase in intracellular calcium concentration. However, recent studies have elucidated a new class of regulatory mechanisms, based on the myosin-containing thick filament, that control the strength and speed of contraction by modulating the availability of myosin motors for the interaction with actin. This review summarizes the mechanisms of thin and thick filament activation that regulate the contractility of skeletal and cardiac muscle. A novel dual-filament paradigm of muscle regulation is emerging, in which the dynamics of force generation depends on the coordinated activation of thin and thick filaments. We highlight the interfilament signaling pathways based on titin and myosin-binding protein-C that couple thin and thick filament regulatory mechanisms. This dual-filament regulation mediates the length-dependent activation of cardiac muscle that underlies the control of the cardiac output in each heartbeat.

1. INTRODUCTION

The contraction of striated muscle is generated by relative sliding of actin-containing thin filaments and myosin-containing thick filaments driven by transient interactions of the myosin motors with actin coupled to the hydrolysis of ATP. Regular arrays of thin and thick filaments are organized into ~2-μm-long contractile units called sarcomeres (**Figure 1**), which are arranged in series along the length of the muscle cell. The start signal for contraction is provided by the action potential that travels down transverse invaginations of the sarcolemma (T-tubules) into the interior of the muscle cell, activating the voltage-gated calcium channels, known as dihydropyridine receptors (DHPRs) (1, 2). In skeletal muscle the DHPRs are mechanically linked to the calcium channels situated in the membrane of the sarcoplasmic reticulum, called ryanodine receptors (RyRs). The voltage-induced activation of the DHPRs directly triggers the opening of the RyRs, causing a large increase in the cytoplasmic free calcium concentration within 2 ms from the action potential (3), followed by the recovery of the initial calcium concentration due to the Ca^{2+} reuptake in the sarcoplasmic reticulum driven by the calcium pumps. Instead, in cardiac muscle, the activation of the DHPRs that are uncoupled from the RyRs on the sarcoplasmic reticulum allows extracellular calcium ions to flow into the myoplasm. These calcium ions bind and open the RyRs, triggering the release of the calcium ions stored in the sarcoplasmic reticulum, a mechanism known as calcium-induced calcium release (1). Due to the differences in the mechanism of calcium release from intracellular stores, the increase in free calcium concentration after a single action potential in the cardiac muscle cell is ten times smaller and slower than that in skeletal muscle (4, 5) (**Figure 1*a***).

Until recently, the classical paradigm for the regulation of the interaction between myosin and actin in the muscle sarcomere was exclusively focused on the structural changes in the regulatory proteins of the thin filament, troponin and tropomyosin, triggered by the transient increase in intracellular calcium concentration following the electrical stimulation of muscle (6). In the classical view of thin filament regulation of muscle contractility, troponin and tropomyosin are regarded as so-called gatekeepers of the thin filament, switching off the thin filament at low calcium concentration in resting muscle and allowing myosin to bind actin when calcium ions are bound to troponin. Therefore, according to this view, the kinetics of force generation and relaxation in the force twitch triggered by the action potential is exclusively accounted for by the dynamics of the calcium transient and of the associated structural changes in the thin filament (**Figure 1*c***).

However, studies in the last decade shed light on a novel class of regulatory mechanisms, based on the thick filament, that regulate the contractility of skeletal and heart muscle in addition to the classical thin filament–based regulation (7–9). The evidence that the myosin motors at low calcium concentrations are switched off onto the surface of the thick filament backbone in an inhibited conformation, called the interacting-heads motif (IHM) (10, 11), challenged the classical paradigm of muscle regulation, suggesting that the myosin motors are not immediately available for the interaction with actin following electrical stimulation. Since then, it became clear that the contraction of striated muscle requires not only the activation of the thin filament by calcium, but also the removal of myosin inhibition on the thick filament, via independent mechanisms that do not necessarily require calcium (12, 13) (**Figure 1*c***). Similarly, mechanical relaxation of muscle triggered by the decrease in myoplasmic calcium concentration cannot be solely explained by the inactivation of the thin filament triggered by the removal of calcium from troponin. The mechanism of muscle relaxation is still unclear, but its rate depends primarily on the kinetics of detachment of myosin motors from actin, which is likely controlled by the coordinated inactivation of thin (14) and thick (15–17) filaments and by the intersarcomere dynamics (17, 18). Therefore, according to the myosin-based paradigm of muscle regulation that emerged from these studies, the thick

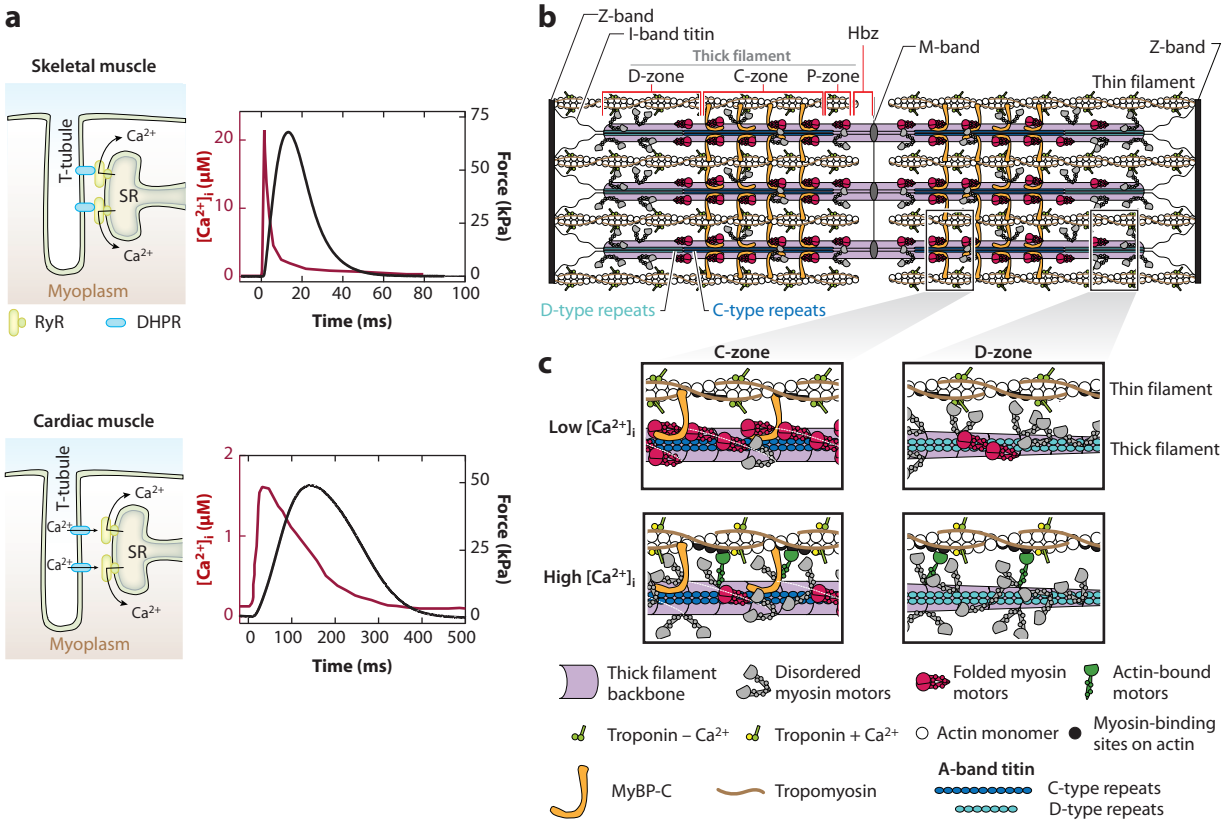

Figure 1

Excitation-contraction coupling in striated muscle and dual-filament regulation of muscle contraction. (*a*) Mechanism of calcium release from intracellular stores in skeletal (*top*) and cardiac (*bottom*) muscle. In skeletal muscle, the action potential in the T-tubule triggers activation of the voltage-gated calcium channels (DHPRs) coupled to the calcium channels (RyRs) of the SR, inducing a large but brief increase in the myoplasmic $[Ca^{2+}]_i$. In cardiac muscle, the voltage-gated calcium channels are uncoupled from the RyRs, so that the extracellular calcium entering the cell triggers the calcium release from the SR (calcium-induced calcium release). The time courses of $[Ca^{2+}]_i$ and force are shown in the right panels. (*Upper right*) Calcium transient (*red line*; data from 3) and force response (*black line*; data from 15) in EDL skeletal muscle from the mouse. (*Lower right*) Calcium transient (*red line*; data from 4) and force response (*black line*; data from 16) in rat ventricular cardiac trabeculae. (*b*) Schematic of the sarcomere, the structural unit of striated muscle. The thin filaments are anchored to the Z-bands and contain actin (*white circles*), tropomyosin (*brown lines*), and troponin (*light green*). Thick filaments (*purple*) are aligned in the center of the sarcomere, the M-band, and contain two antiparallel arrays of myosin motors. Each half-filament is divided into a proximal or P-zone, a C-zone containing MyBP-C, and a distal or D-zone. In the C-zone, MyBP-C molecules (*orange*) form links with actin filaments. Titin filaments span the half-sarcomere length and run along the thick filament surface (I-band titin is *black*, A-band titin C-type repeats are *blue*, and D-type repeats are *light blue*). (*c*) Snapshots of the structure of myofilaments at low (*top*) and high (*bottom*) $[Ca^{2+}]_i$ in the C-zone (*left*) and D-zone (*right*). At low $[Ca^{2+}]_i$, tropomyosin (*brown lines*) blocks the myosin-binding sites on the actin filament (*black*). In the C-zone of the thick filament (*top left*), most of the myosin motors are folded and helically ordered on the thick filament surface (*magenta*), whereas in the D-zone, the myosin motors are more disordered (*top right*; *gray*). (*Bottom right*) Calcium binding to troponin (*yellow circles*) triggers the partial activation of the thin filament. In the D-zone, the disordered myosin motors are immediately available to bind actin, whereas in the C-zone, they need to be released from the folded conformation before attaching to actin. Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; DHPR, dihydropyridine receptor; EDL, extensor digitorum longus; Hbz, half bare zone; MyBP-C, myosin-binding protein-C; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

filament with its regulatory proteins, myosin-binding protein-C (MyBP-C) and titin, modulates the number of myosin motors available for the interaction with actin during contraction. This review is focused on the regulatory mechanisms in thin and thick filaments that underpin the control of the dynamics of force generation in skeletal and cardiac muscle, and on the coordination of thin and thick filament–based regulatory pathways in a novel dual-filament mechanism of regulation of muscle contraction.

2. THIN FILAMENT–BASED REGULATION IN STRIATED MUSCLE

2.1. Structural Changes in the Thin Filament Induced by Calcium and Myosin Motors

In muscle cells at low calcium concentration, the tropomyosin strand on the thin filament is held by troponin in a position that blocks the myosin-binding sites on actin, inhibiting contraction (**Figure 1*c***). Following the electrical stimulation of muscle, calcium binding to troponin C (TnC) triggers structural changes in the troponin complex, which lead to the azimuthal motion of tropomyosin away from its inhibitory position on the thin filament, partially uncovering the myosin-binding sites on actin and enabling contraction. The control of the ON/OFF state of the thin filament by calcium ions is at the basis of the steric blocking mechanism of regulation of muscle contraction proposed nearly 50 years ago (19–22). Recent cryo-electron microscopy (cryo-EM) studies have provided high-resolution three-dimensional (3D) reconstructions of cardiac thin filaments in the absence of calcium and at saturating (23) and submaximal (24) calcium concentrations, greatly advancing our understanding of the structural basis of thin filament regulation by calcium (25, 26).

In vitro studies of the mechanism of thin filament regulation described a third structural state of tropomyosin on the thin filament, in which myosin motors strongly bound to actin in the ATP-free state (rigor state) further shift the tropomyosin strand away from the myosin-binding sites on actin (27, 28). These studies therefore introduced the concept of three regulatory states of the thin filament, corresponding to three azimuthal positions of tropomyosin, the blocked (Ca^{2+}-free), closed (Ca^{2+}-bound), and open (myosin-bound) states (**Figure 2**). The comparison of 3D reconstructions of isolated thin filaments in the presence and absence of calcium (22) and high-resolution structures of the thin filaments decorated with myosin motors in the rigor state (22, 29), together with the evidence of cooperative binding of myosin motors along the thin filament (30), provides support to that concept. More recently, cryo-electron tomograms of the sarcomere in skeletal myofibrils in the rigor state showed that, in the region of filament overlap (A-band), the myosin motors attached to actin hold the thin filaments in the open state, whereas in the absence of myosin at no overlap in the I-band, the thin filaments are in the closed state (31, 32). However, until recently the role of myosin binding to actin in the activation of the thin filament during contraction was unclear.

The mechanism of thin filament regulation has been studied in demembranated skeletal (33–35) and cardiac muscle (36–38) using fluorescence polarization from bifunctional rhodamine probes on TnC, a technique which allows measuring the orientation of specific protein domains in situ under physiological conditions (39). A recent study using those TnC probes has elucidated the contribution of Ca^{2+} and myosin to the regulatory structural changes in the thin filament in demembranated muscle fibers from rabbit skeletal muscle (40). The results of this study showed that at near-physiological temperature (26°C) and lattice spacing, conditions that are required to preserve the thick filament regulatory mechanisms in demembranated muscle fibers (41–43), the calcium sensitivity, and the cooperativity of the regulatory structural changes in the thin filament are increased by the attachment of force-generating myosin motors to actin. This is consistent

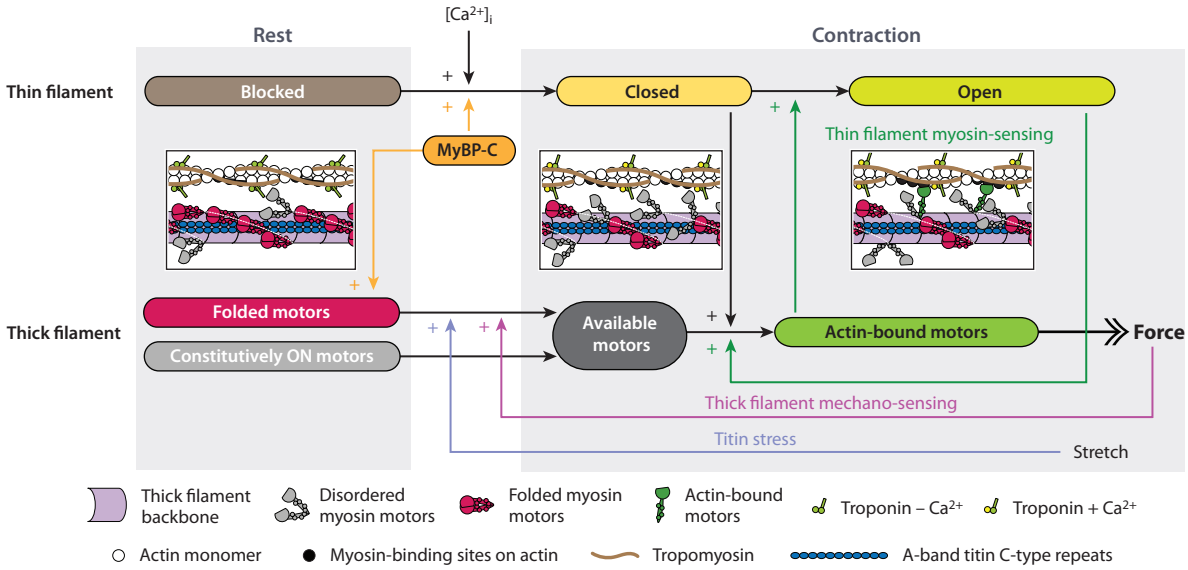

Figure 2

Schematic of dual-filament regulation in striated muscle. The contraction of striated muscle is controlled by two positive feedback loops modulating the activation of the thick (mechano-sensing) and thin (myosin-sensing) filaments, respectively. In resting muscle, the thin filament is blocked, as the myosin-binding sites on actin are covered by tropomyosin, and most of the myosin motors on the thick filament are unavailable to interact with actin, as they are folded and helically ordered on the thick filament surface. Few myosin motors are disordered. MyBP-C stabilizes the folded conformation of the myosin motors in skeletal muscle at rest or in cardiac muscle in diastole. MyBP-C sensitizes the thin filament to calcium in the C-zone. Calcium binding to troponin triggers the movement of tropomyosin on the actin filament partially uncovering the myosin-binding sites on actin (thin filament closed state). Myosin binding to actin induces a further movement of tropomyosin, triggering the transition of the thin filament to the open state. Filament stress triggers the release of folded motors from the filament surface (mechano-sensing), increasing the pool of available motors (*gray*) and consequently the number of actin-bound motors. In skeletal muscle, titin stress accelerates the release of folded motors during stretch of active muscle. Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; MyBP-C, myosin-binding protein-C.

with the effect of myosin on thin filament activation measured with fluorescent probes on the inhibitory subunit of troponin I in human slow-twitch fibers (44). These results suggest that during contraction of skeletal muscle the thin filaments are only partially activated by calcium and that full activation and cooperativity of the thin filaments require myosin binding to actin, extending the concept of the three regulatory states of the thin filament from in vitro studies to the myofilament lattice in physiological conditions. The myosin-dependent increase in calcium sensitivity and cooperativity of the orientation change of troponin are blunted by cooling the muscle fibers at 11°C (34), supporting the conclusion from mechanical studies that the cooperativity of thin filament activation depends not only on the attachment of myosin to actin, but also on the force generated by the myosin motors (45).

In demembranated cardiac muscle at 20°C, myosin attachment to actin increases the calcium sensitivity and cooperativity of the orientation change of both the C-lobe and N-lobe of TnC (38), although the myosin-dependent contribution to the activation of the thin filament is much smaller than that observed in skeletal muscle (40). This is consistent with the evidence from cryo-EM studies that the amplitude of the myosin-dependent motion of tropomyosin in the cardiac thin filament is smaller than that in the skeletal thin filament (46), suggesting that the mechanism of activation of the thin filament in cardiac muscle might be different from that in skeletal muscle. However, in relaxed cardiac muscle at temperatures below 27°C, the thick filament is partially

activated in the absence of calcium (47). Due to the coupling between thin and thick filament regulatory mechanisms (40), it is likely that changes in the regulatory state of the thick filament induced by the low temperature might also alter the signaling pathway of thin filament activation during contraction. Therefore, further work is required to determine the contribution of calcium and myosin to the regulatory structural changes in the cardiac thin filament and the coupling between thin and thick filament activation under more physiological conditions.

2.2. Structural Dynamics of the Thin Filament During Muscle Contraction

The kinetics of force generation during contraction depends on the speed of activation of thin and thick filaments following the electrical stimulation of muscle. How fast does the tropomyosin move on the thin filament during muscle activation? In vitro experiments using a pyrene label attached to tropomyosin have shown that kinetics of the transition of the thin filament from the closed to open state triggered by myosin motors in solution has a rate constant of ~1,000 s^{-1} (28, 48). The motion of tropomyosin in electrically stimulated skeletal muscle has been measured by X-ray diffraction, the only label-free technique that allows the structural dynamics of the myofilaments to be measured in situ on the millisecond timescale in the quasi-crystalline lattice of the muscle cell under physiological conditions (49). In their pioneering study, Kress et al. (14) measured the time course of the intensity of the second actin layer line reflection, associated with azimuthal shift in the position of tropomyosin on the thin filament, during the isometric contraction of frog skeletal muscle. Their results showed that at 22°C the kinetics of the structural changes in tropomyosin on the thin filament is much faster than the dynamics of force generation, whereas during relaxation after the end of the electrical stimulation, it is similar to the kinetics of the force decay (14). Similar dynamic changes in tropomyosin conformation were observed by Matsuo & Yagi (50) during twitch contractions of frog skeletal muscle at 16°C, elicited by a single electrical stimulus. These results confirmed that the motion of tropomyosin triggered by calcium binding to troponin is the first step in the signaling pathway of muscle activation. In overstretched muscle at no overlap the return of tropomyosin to its resting position during relaxation is faster than that at optimal filament overlap, suggesting that the myosin motors attached to actin might interfere with the recovery of the inhibitory position of tropomyosin (14). This is consistent with the evidence that forced detachment of myosin motors from the thin filament induced by a ramp shortening applied at the peak of the force twitch accelerates the recovery of the resting inhibitory position of tropomyosin (51).

The speed of thin filament activation following a rapid jump in calcium concentration produced by photolysis of caged calcium has been measured by X-ray diffraction with a time resolution of 4 ms in demembranated muscle fibers from rabbit psoas muscle at 4°C (52). In that study, the time course of the intensity of the tropomyosin reflection was fitted with a double-exponential function in which the faster component, representing ~60% of the motion of tropomyosin on the thin filament, was associated with the calcium-induced motion of tropomyosin and had a rate constant of ~160 s^{-1}, whereas the slower component (~40%) had a rate constant of ~18 s^{-1}, similar to the rate of force generation, and was associated with the attachment of myosin motors to actin. Moreover, the shortening of the muscle fiber at zero load applied at the plateau of contraction triggered the partial recovery of the inhibitory position of tropomyosin on the thin filament (52), suggesting a role of the myosin motors in the activation of the thin filament during contraction.

However, despite the remarkable combination of the X-ray diffraction technique with sophisticated physiological methods applied on demembranated muscle fibers, under the experimental conditions used in that study the physiological mechanisms of thin and thick filament activation in the muscle fiber are severely disrupted (41–43). A more recent X-ray diffraction study in

demembranated skeletal muscle fibers conducted at 30°C investigated the role of myosin in the activation of the thin filament in situ. This showed that during muscle shortening against a load, corresponding to ~30% of the tetanic force, the tropomyosin strand remains in the same conformation as that at the tetanus plateau, suggesting that even a small fraction of myosin motors attached to actin might hold the thin filament in the open state (53). Currently, there is to our knowledge no published X-ray diffraction study of the structural dynamics of the thin filament in electrically stimulated mammalian skeletal or cardiac muscle at near-physiological temperature. Measuring the motion of tropomyosin by X-ray diffraction in electrically paced cardiac muscle is even more challenging. Indeed, in cardiac muscle the changes in intensity of the tropomyosin reflection are expected to be smaller than those observed in skeletal muscle. This is because the thin filament is only partially activated at submaximal calcium concentration and also because the amplitude of the azimuthal motion of tropomyosin induced by calcium and myosin is smaller than that in skeletal muscle, according to cryo-EM reconstruction of thin filaments (46, 49). However, these limitations might be partially overcome in future studies exploiting the recent advancements in synchrotron technology and X-ray photon-counting detectors with higher sensitivity and higher temporal and spatial resolution (54).

Thanks to the high signal-to-noise ratio of fluorescence, the fluorescence polarization technique represents a powerful tool to measure with high specificity and high temporal resolution the structural dynamics of regulatory protein domains in the native environment of skeletal and cardiac muscle cells during contraction (55). Recently, a fluorescence polarization study using probes on TnC and the regulatory light chain (RLC) of myosin has characterized the speed of activation of troponin and the myosin motors with 0.12 ms time resolution in skeletal muscle fibers from rabbit psoas muscle activated by photolysis of caged calcium in near-physiological conditions (40). In those experiments calcium- and myosin-dependent structural changes in the thin filament could be kinetically separated, thanks to the difference of two orders of magnitude in their rate constants. Calcium binding to troponin triggers a fast reorientation of the troponin complex on the thin filament, with a rate constant of 4,000–7,000 s^{-1}, followed by a slower orientation change in troponin that tracks myosin binding to actin and the kinetics of force generation (~30 s^{-1}) (40). The attachment of force-generating myosin motors to actin triggers a positive feedback loop in the activation of the thin filament, called myosin-sensing (**Figure 2**), which dynamically couples the regulatory state of the thin filament to that of the thick filament during contraction at high loads and allows skeletal muscle to achieve rapid and cooperative activation following electrical stimulation.

In cardiac muscle the structural dynamics of the thin filament on the timescale of the heartbeat has not been elucidated. A time-resolved fluorescence polarization study using fluorescent probes on TnC in demembranated cardiac trabeculae activated by caged calcium showed that the calcium-induced structural changes in troponin are ten times faster than force generation (56), suggesting that also in cardiac muscle the calcium-dependent activation of the thin filament does not limit the rate of force generation. This result is consistent with the evidence from a recent X-ray diffraction study that the kinetics of force generation and relaxation in electrically paced intact cardiac muscle is largely controlled by the dynamics of structural changes in the thick filament (16), as discussed below.

3. THICK FILAMENT–BASED REGULATION IN STRIATED MUSCLE

3.1. Conformation of the Myosin Motors on the Thick Filament in Resting Muscle

The myosin filament is a centrosymmetric structure containing two arrays of myosin motors (**Figure 1*b***). Each array in the half-filament consists of 49 layers of motors with an axial

periodicity of ~14.5 nm. Each half-filament is associated with six titin molecules connecting the thick filament tips to the ends of the sarcomere in the Z-band. The thick filament–binding region of titin contains repeating immunoglobulin/fibronectin domains arranged in 11 × 11-domain (C-type) super-repeats or 6 × 7-domain (D-type) super-repeats, in the central and distal region of the thick filament, respectively (57, 58). MyBP-C is located in the middle third of each half thick filament with an axial periodicity of ~43 nm, and it is anchored to the myosin backbone via its C-terminal domain, whereas its N-terminal region is thought to be in a dynamic equilibrium between actin-bound and myosin-bound states, modulating the regulatory state of thin and thick filaments, respectively (59–62). The position of MyBP-C defines the C-zone of the thick filament, whereas the neighboring filament regions lacking MyBP-C are referred to as proximal (P) and distal (D) zones (**Figure 1*b***).

Recently it has become clear that in resting muscle the myosin motors are inhibited by folding onto the surface of the thick filament backbone and therefore they are not immediately available for the interaction with actin following electrical stimulation (**Figure 1*c***). The structural basis of myosin inhibition has been elucidated for the first time in cryo-EM studies of relaxed thick filaments from tarantula muscle (11). They show that the two motor domains of the myosin molecule are folded onto the myosin tail in an asymmetric conformation called the IHM, originally observed in two-dimensional crystals of smooth muscle myosin (10), which inhibits both the myosin ATPase and the interaction of myosin with actin. Early 3D reconstructions of the C-zone of human cardiac thick filaments also show the characteristic IHM structure (63–65), and subsequent studies demonstrated that folding of myosin motors into the IHM is a highly conserved mechanism for the inhibition of myosin II (66, 67). Recent cryo-EM studies have provided high-resolution structures of the purified human β-cardiac myosin IHM in the relaxed state (68) and the C-zone of cardiac thick filaments either isolated (69) or in the sarcomere of relaxed myofibrils (70) in the presence of the myosin inhibitor mavacamten. The in vitro and in situ reconstructions of the thick filaments have elucidated distinct conformations of the folded myosin motors in the C-zone and the structural organization of MyBP-C and the titin C-repeats on the surface of the thick filament (69, 70). Moreover, cryo-electron tomograms of the C-zone in relaxed myofibrils (70) and in chemically fixed cardiac muscle (71) revealed MyBP-C links between thin and thick filaments, although the regulatory role of such links in the interfilament signaling pathway of muscle regulation remains unclear.

Because the myosin ATPase is inhibited in the IHM, the folded state of myosin has been generally associated with the super-relaxed (SRX) or OFF state of myosin, a biochemical state of myosin characterized by a slow ATP turnover, identified in relaxed skeletal (72, 73) and cardiac muscle (73, 74). The equilibrium between the SRX state and disordered-relaxed (DRX) state of myosin can be pharmacologically modulated to alter the resting muscle metabolism (75, 76). However, a recent X-ray diffraction study using 2′-deoxy-ATP (dATP) to destabilize the folded state of myosin in relaxed porcine cardiac muscle showed that the structural transition of the myosin motors caused by dATP is not equivalent to the biochemical transition from the SRX state to DRX state (77). Therefore, the relationship between the SRX/DRX state of myosin and the regulatory structural states of the myosin motors remains to be elucidated.

The evidence that in resting muscle the myosin motors are sequestered in the folded state onto the filament surface led to the hypothesis that the contractility of skeletal muscle is largely controlled by regulatory structural changes in the thick filament (7), in addition to the classical calcium-dependent regulation of the thin filament. Moreover, it has been hypothesized that, in the heart, destabilization of the IHM by mutations in thick filament proteins that cause hypertrophic cardiomyopathy (HCM) might disrupt the thick filament–based regulation of contraction, potentially explaining the hypercontractile phenotype observed in HCM (60, 78–80).

The in situ conformation of the myosin motors on the thick filament in resting skeletal muscle (15, 81) and cardiac muscle in diastole (16, 82) derived from X-ray diffraction studies is largely consistent with the IHM, indicating that under physiological conditions most of the myosin motors are inhibited by folding onto the filament surface. However, a recent X-ray diffraction study on cardiac muscle described two populations of folded motors in diastole: the folded-helically ordered motors located in the C-zone and the folded nonhelically ordered motors located outside the C-zone (16) (**Figure 1*c***). The folded-helical conformation of the myosin motors might be stabilized by MyBP-C, in agreement with the 3D reconstructions of the C-zone of the thick filament (69, 70), and might account for the enrichment of the SRX state of myosin in the C-zone of the filament observed in isolated skeletal (83, 84) and cardiac (85) myofibrils. However, in cardiac myofibrils isolated from mice expressing a cardiac MyBP-C with an N-terminal truncation, the fraction of the SRX state remains higher in the central regions (P and C) of the thick filament (85), suggesting that other regulatory factors might contribute to the stabilization of the myosin SRX in these regions.

X-ray diffraction and fluorescence polarization studies showed that the physiological resting structure of the thick filament and the folded conformation of the myosin motors are largely preserved in demembranated skeletal (41, 42) and cardiac (86, 87) muscle under relaxing conditions, but only at temperatures higher than 26°C and after recovering the physiological lattice distance typical of the intact muscle using the osmotic agent dextran T-500. Therefore, under these conditions the demembranated muscle preparations represent a suitable model to investigate the regulatory mechanisms of the thick filament. Fluorescence polarization studies using bifunctional rhodamine probes on the RLC of myosin exchanged in demembranated skeletal muscle fibers (42) and cardiac trabeculae (87) concluded that in near-physiological conditions in the absence of calcium about two-thirds of the myosin motors on the thick filament are folded in the IHM, whereas one-third of the motors are more perpendicular to the filament axis. This is consistent with the fraction of folded and isotropic motors, respectively, in diastole determined in X-ray diffraction experiments (16). These isotropic motors might be a population of constitutively ON (12) or sentinel motors (66) (**Figures 1** and **2**), which are immediately available for the interaction with actin when the thin filament is partially activated by calcium. Although there are currently no high-resolution structures of the D-zone of the thick filament, it has been suggested that the isotropic motors are mainly located in the D-zone (16) (**Figure 1**), consistent with the enrichment of DRX myosin motors in this filament domain (83–85).

In conclusion, in resting muscle the myosin motors on the thick filament have multiple conformations that cannot be described in terms of only binary structural OFF/ON or biochemical SRX/DRX states. These myosin conformations might be confined to specific filament domains with different protein composition and, therefore, new structural approaches are necessary to characterize their spatial distribution and regulatory function in sarcomere subdomains under physiological conditions.

3.2. The Mechanism of Activation of the Thick Filament in Skeletal Muscle

Recent X-ray diffraction studies of amphibian skeletal muscle showed that activation of the folded myosin motors on the thick filament is directly controlled by filament stress, based on the evidence that the activation of the thick filament is prevented by imposing shortening at zero load after the start of the stimulation (12). The stress-dependent activation of the thick filament was further characterized in a fluorescence polarization study in demembranated muscle fibers, showing that the passive tension transmitted to the thick filament by titin partially activates the folded myosin motors, independently of calcium (13). According to the mechano-sensing mechanism the force

generated by the constitutively ON motors triggers a positive feedback loop in the activation of the thick filament (7) (**Figure 2**), which might account for the high cooperativity in the activation of the folded myosin motors in demembranated muscle fibers activated at constant calcium concentrations (13). The release of folded motors from the filament surface determines an increase in the number of actin-bound motors, which sensitizes the thin filament to calcium and increases the cooperativity of thin filament activation, triggering the myosin-sensing feedback loop in the activation of the thin filament (40) (**Figure 2**). Such a coupling between mechano-sensing in the thick filament and myosin-sensing in the thin filament might account for the rapid activation of skeletal muscle following electrical stimulation.

Recent studies showed that the mechano-sensing mechanism controls activation of the myosin motors also in the fast-twitch muscle of the mouse (88). However, there appear to be some differences in the structural dynamics of the thick filament in mammalian skeletal muscle, with respect to that in amphibian muscle (81, 89). First, a small component of thick filament activation, which consisted of a slight elongation of the filament backbone signaled by the increase in the spacing of the *M*6 reflection (S_{M6}), was detected during shortening at zero load after the start of the stimulation. Second, analysis of the *M*3 reflection, associated with the axial periodicity (14.5 nm) of the myosin motors along the thick filament, revealed a new component peak indexing at a slightly longer periodicity of ~14.8 nm, which appears in the early phase of force generation. This extra peak also appears during activation of demembranated skeletal muscle at submaximal calcium concentrations close to the threshold value for force generation (pCa 7–6.4) (90). This longer periodicity could be associated with a population of myosin motors interacting with the titin C-repeats on the thick filament (41). However, this hypothesis is not consistent with the recent high-resolution reconstructions of the C-zone of the thick filament that show a single axial periodicity matching that of the myosin helix (43 nm) (69, 70). An alternative hypothesis is that the myosin motors with longer periodicity might be associated with the D-type repeats of titin in the D-zone of the thick filament and might be involved in a transient activation state of the thick filament in the early phase of force generation (90). Finally, activation of the different zones of the thick filament during isometric contraction is sequential. The constitutively ON motors are preferentially located at the tips of the thick filament (**Figure 1**) and trigger the stress-dependent activation of the myosin motors in the P-zone, followed by the activation of motors in the C-zone (88). In conclusion, filament stress is the major determinant of thick filament activation in fast-twitch mammalian skeletal muscle, but an additional pathway of myosin activation independent of stress cannot be excluded.

The time course of structural changes in the thick filament triggered by a single action potential has been measured in extensor digitorum longus (EDL) muscle of the mouse by time-resolved X-ray diffraction (15). In response to a single electrical stimulus the length of the thick filament backbone, signaled by the X-ray parameter S_{M6}, slightly increases triggering the partial disruption of the helical order of the myosin motors on the thick filament (I_{ML1}) and the conformational change of the myosin motors from the folded to the perpendicular actin-attached state (L_{M3}), with time courses similar to those of force generation (**Figure 3**). The kinetics of thin filament activation triggered by the action potential has not been measured by X-ray diffraction in mammalian muscle, but according to the structural dynamics of troponin measured by fluorescence polarization in demembranated muscle fibers (40), the calcium-induced structural changes in the thin filament are expected to be transiently complete at the peak of the calcium transient, ~2 ms after the electrical stimulus. Therefore, according to these results, the speed of force generation in the twitch is largely rate limited by the dynamics of activation of the myosin motors on the thick filament. Mathematical models of muscle contraction that include the stress-dependent activation of the myosin motors in the thick filament simulate the time course of force generation during

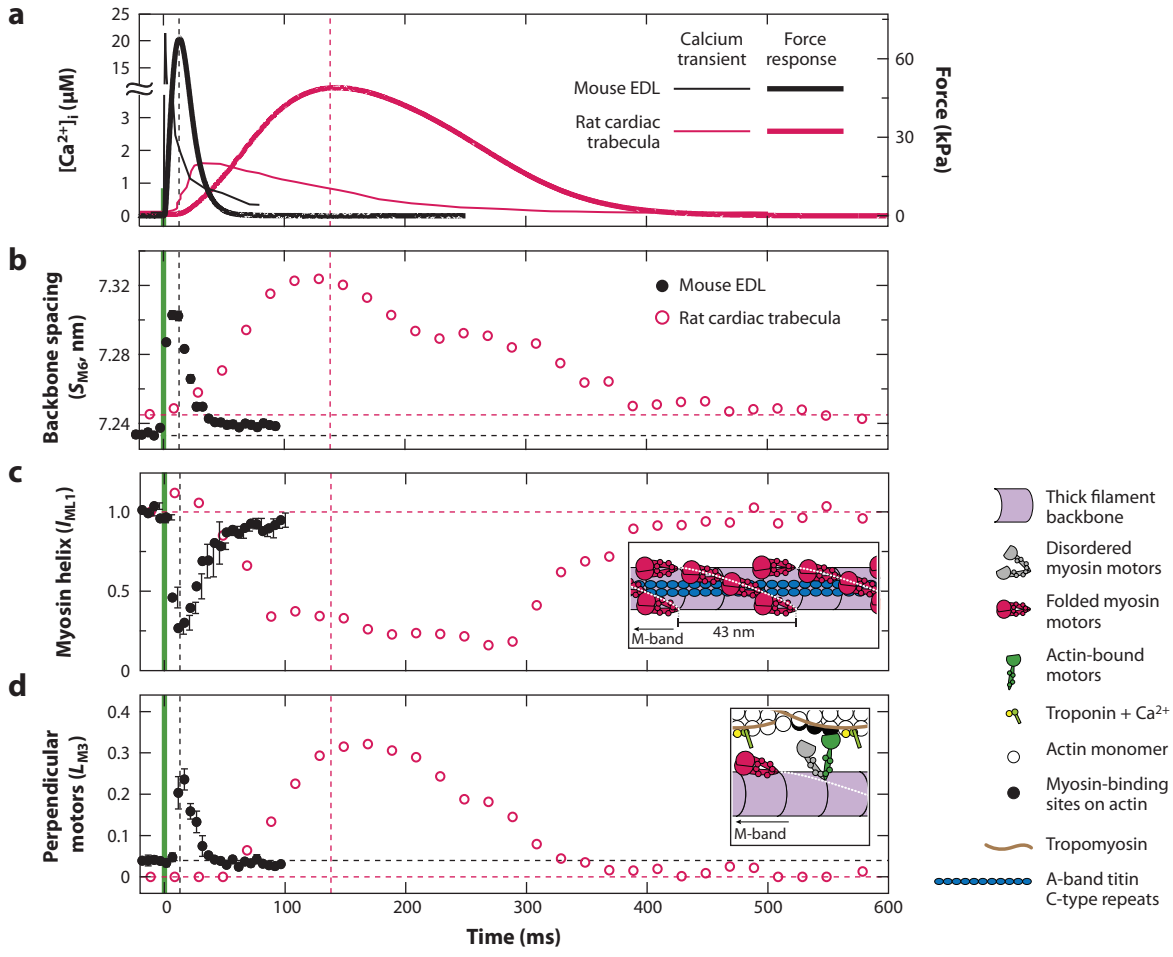

Figure 3

Structural dynamics of the thick filament triggered by a single action potential in skeletal and cardiac muscle. (*a*) Calcium transient (*thin black line*; data from 3) and force response (*thick black line*; data from 15) in EDL skeletal muscle from the mouse and calcium transient (*thin magenta line*; data from 4) and force response (*thick magenta line*; data from 16) in rat ventricular cardiac trabeculae. (*b*) Changes in the periodicity of the thick filament backbone reported by the spacing of the M6 reflection (S_{M6}). Spatial calibration for both skeletal and heart muscle is described in Reference 16. (*c*) Changes in intensity of the ML1 reflection (I_{ML1}) associated with the folded-helical conformation of the myosin motors on the filament surface (*inset*; *magenta*). (*d*) Changes in fractional intensity of the low-angle peak of the M3 reflection (L_{M3}) associated with the conformation of myosin motors, moving from a folded (*inset*; *magenta*) to more perpendicular (*inset*; *dark green*) actin-bound conformation. Black symbols indicate EDL skeletal muscle (data from 15), and magenta symbols indicate rat cardiac trabeculae electrically paced at 1 Hz (data from 16). Experiments performed at 26–28°C. Horizontal dashed lines indicate values at rest (*black*) and in diastole (*magenta*) in skeletal and cardiac muscle, respectively. Vertical dashed lines indicate peak force of the skeletal (*black*) and heart (*magenta*) muscle twitch. Vertical green lines mark the electrical stimulus. Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; EDL, extensor digitorum longus.

an isometric contraction (91), supporting the conclusion that the dynamics of force generation in skeletal muscle is largely determined by the structural dynamics of the thick filament.

The kinetics of the recovery of the resting spacing of the backbone and the folded-helical conformation of the myosin motors on the thick filament is similar to the kinetics of force relaxation (**Figure 3**), indicating that in the force twitch the inactivation of the thick filament is rate limiting

for the kinetics of mechanical relaxation. Muscle relaxation from the tetanus plateau occurs in two phases. In the first phase, called isometric relaxation, force declines at constant sarcomere length following myosin detachment from actin, but the myosin filament remains in the active state (15, 17). Instead, in the second phase, called chaotic relaxation due to sarcomere yielding, the thick filament partially recovers the resting structure with a kinetics slower than that of force (15). Although the mechanism of muscle relaxation is still not understood, these results suggest that thick filament inactivation alone cannot explain the kinetics of force relaxation from high loads and that the coordinated inactivation of thin and thick filaments might be required to inhibit the positive myosin- and mechano-sensing feedbacks, in thin and thick filaments, respectively.

3.3. The Mechanism of Activation of the Thick Filament in Cardiac Muscle

It has been recently proposed that activation of the thick filament in cardiac muscle is controlled by a mechano-sensing mechanism similar to that observed in skeletal muscle (8). This conclusion is based on the evidence from X-ray diffraction experiments on cardiac trabeculae from rat hearts showing that at the peak of the force twitch the activation level of the thick filament is proportional to the systolic force (82). A fluorescence polarization study using RLC probes exchanged in demembranated rat trabeculae showed that the activation of the myosin motors from the folded state at constant calcium concentrations and at different levels of RLC phosphorylation depends on filament stress, providing further support to the mechanism of stress-dependent activation of the thick filament (87). The mechano-sensing transition of the myosin motors has been included in mathematical models of cardiac muscle contraction to simulate the time course of force in the cardiac twitch and length-dependent activation of cardiac muscle (92–95).

However, more recently, an X-ray diffraction study on demembranated porcine cardiac muscle proposed that the thick filament in cardiac muscle can be directly activated by calcium (96). In that study activation of the thin filament by calcium was pharmacologically inhibited to determine the effect of constant calcium concentrations in the absence of force on the structure of the thick filament. The results showed that the thick filament is partially switched on at constant $[Ca^{2+}]$, suggesting a direct role of calcium in the activation of the myosin motors (96), although the calcium-binding site on the thick filament is unknown. Biochemical evidence showed that porcine cardiac myosin RLC can bind Ca^{2+}, but under physiological conditions the binding sites would be mainly occupied by Mg^{2+} in diastole, and the exchange of Mg^{2+} with Ca^{2+} during contraction would be too slow to trigger activation of the thick filament on the timescale of the heartbeat (97). An alternative hypothesis is that calcium signaling to the thick filament might be mediated by MyBP-C (98). Previous X-ray diffraction studies on intact papillary muscle isolated from the right ventricle of canine hearts reported an increase in the transfer of mass of myosin motors from the thick to thin filament in diastole at higher frequencies of stimulation (99, 100), suggesting that additional signaling pathways might control the regulatory state of the thick filament in cardiac muscle. In conclusion, further work is required to solve the controversy about the role of calcium and filament stress in the activation of the cardiac thick filament (101).

The structural dynamics of the thick filament during contraction of cardiac muscle have been recently elucidated in time-resolved X-ray diffraction studies on electrically paced cardiac trabeculae from rat hearts (16). Both the time course of the force twitch and kinetics of the regulatory structural changes in the thick filament, associated with the elongation of the thick filament backbone and the release of the myosin motors from the folded conformation, are ten times slower than those observed in skeletal muscle, although their amplitudes are similar (**Figure 3**). The difference in the speed of activation of the thick filament might be accounted for by the different myosin isoforms in these two muscle types and by the different time course of the intracellular calcium transient, which is ten times smaller and slower in the cardiac muscle cell (4) with respect

to that in skeletal muscle (3) (**Figure 3**). In cardiac muscle, activation of the myosin motors starts in the D-zone and propagates to the central regions of the thick filament. At the peak of the force twitch, the actin-bound myosin motors contributing to force generation are only confined to the C-zone of the thick filament (16), likely due to the sensitization of the thin filament to calcium in the C-zone induced by MyBP-C links (102, 103) (**Figure 2**).

During relaxation, deactivation of the thick filament occurs in the zonal sequence D-C-P, following the spatial gradient of force distribution on the filament backbone, in agreement with the mechano-sensing hypothesis, and it is accompanied by an increase in sarcomere inhomogeneity (16). However, in contrast with the fast reformation of the myosin helix observed in skeletal muscle, in cardiac muscle the recovery of the folded-helical conformation of the myosin motors occurs only at a later stage of mechanical relaxation (**Figure 3*c***) ($t > 300$ ms), suggesting that the helical order of the thick filament in the C-zone of the cardiac thick filament requires additional intermolecular interactions between myosin motors and MyBP-C. In conclusion, the zonal dynamics of thin and thick filament activation, and their coupling by an interfilament signaling pathway based on MyBP-C, control the strength and dynamics of contraction in cardiac muscle.

4. THE ROLE OF TITIN IN THE REGULATION OF MUSCLE CONTRACTILITY

Titin is traditionally regarded as a passive spring that resists lengthening of the sarcomere in resting muscle, the elasticity of which is associated with its I-band domain and is tuned to the operative sarcomere length range in cardiac and skeletal muscle (104, 105). The A-band domain of titin associated with the thick filament backbone acts as a molecular ruler and template for the assembly of the thick filament (58, 106). However, recent studies on skeletal muscle suggested that titin might have a direct role in the regulation of muscle contractility. In actively contracting skeletal muscle, titin exhibits a dynamic stiffness that is about 100 times higher than that in the muscle at rest (107), which is likely due to stiffening of the skeletal titin isoform induced by calcium (13, 108, 109). It has been proposed that, during contraction, titin switches from an extensible spring to a mechanical rectifier that allows free shortening and resists the elongation of the sarcomere (110). The increased stiffness of titin in the active sarcomere might buffer force imbalances due to sarcomere length inhomogeneity during high-load contractions, as suggested by the evidence that cleavage of titin in situ leads to sarcomere disorganization upon muscle activation (111). Fluorescence polarization experiments using probes on the myosin RLC in demembranated skeletal muscle fibers showed that the titin-based passive stress applied to the thick filament triggers the partial release of myosin motors from the folded state, suggesting a possible role of titin in the mechano-sensing transition of the thick filament (13) (**Figure 2**). Activation of the thick filament by the titin-based passive force was also recently shown in X-ray diffraction experiments on relaxed demembranated skeletal muscle fibers isolated from a mouse model in which titin could be specifically cleaved (112). More recently, X-ray diffraction experiments on electrically stimulated muscle fibers of the frog, in which force generation was suppressed using the myosin inhibitor para-nitro-blebbistatin, showed that, in the presence of calcium, the stress transmitted by titin to the thick filament backbone alters the resting conformation of the myosin motors (110).

Therefore, in skeletal muscle, titin stress triggers the mechano-sensing transition of the thick filament, but what is its physiological role in muscle contraction? Recent time-resolved fluorescence polarization experiments in demembranated skeletal muscle fibers activated by photolysis of caged calcium showed that the kinetics of activation of the myosin motors folded on the thick filament surface can be accelerated by stretching the muscle fiber during the early phase of contraction (40). The titin stress transmitted to the thick filament therefore triggers the rapid recruitment

of active myosin motors from the folded state (**Figure 2**) that can attach to actin and generate a high resistive force, accounting for the fast and efficient braking response of active skeletal muscle to the external stretch (113, 114).

In contrast to the effect observed in skeletal muscle, in cardiac muscle in diastole the passive tension generated by increasing the sarcomere length in the physiological range (1.9–2.3 μm) is small and does not trigger activation of the thick filament (16, 87, 115, 116). Moreover, the stiffness of the cardiac titin is already high in diastole and not altered in the presence of calcium (109), suggesting that the role of titin in buffering force imbalances between sarcomeres might be important in both systole and diastole. However, the regulatory role of titin in the control of the contractility of cardiac muscle is poorly understood, limiting our understanding of the mechanisms by which titin mutations can cause cardiac diseases (117–120).

5. MYOFILAMENT LENGTH–DEPENDENT ACTIVATION

The contractility of cardiac muscle is largely controlled by an auto-regulatory mechanism called the Frank–Starling law of the heart, according to which the ventricular stroke volume, i.e., the amount of blood pumped in each heartbeat, increases in response to an increase in the ventricular volume in diastole (121, 122). The cellular mechanism underlying this phenomenon consists of a stretch-induced sensitization of the myofilaments to calcium, called length-dependent activation (121, 123), which causes an increase in the strength of contraction in response to the stretch of the cardiac muscle cell in diastole (16). Length-dependent activation increases the efficiency of cardiac muscle contraction (124), and its disruption is associated with decreased contractile performance of the heart in human hypertrophic cardiomyopathies (125) and in patients with ischemic heart failure (126).

Despite decades of research, the length-sensing mechanism that modulates the calcium sensitivity of myofilaments in response to changes in sarcomere length is still unknown. It has been recently hypothesized that the stretch-induced potentiation of cardiac muscle contractility could be explained by an increase in diastole in the number of myosin motors available for the interaction with actin at longer sarcomere lengths (116). However, recent studies conducted by different laboratories suggest that this is not the case. X-ray diffraction experiments on electrically paced cardiac trabeculae isolated from rat hearts showed that increasing the sarcomere length in the range of 1.95 to 2.40 μm in diastole does not alter the relaxed structure of the thick filament (16, 115, 116). This result is in agreement with the conclusion from fluorescent polarization experiments on demembranated rat cardiac trabeculae showing that, in the absence of calcium, or at calcium concentrations close to the diastolic value (~0.1 μM), the folded myosin motors are insensitive to the passive force applied by titin to the thick filament (87). In contrast, a recent X-ray diffraction study on demembranated porcine myocardium concluded that the stretch in the absence of calcium triggers a partial release of myosin motors from the folded state (127).

In conclusion, most studies indicate that the length-dependent activation of cardiac muscle is not mediated by stretch-induced changes in the structure of the thick filament in diastole, although these results remain controversial. Stretch of the cardiac muscle cell at calcium concentrations in the systolic range (0.2–1 μM), which partially activate the thin filament, triggers the release of myosin motors from the folded state, contributing to the stretch-induced potentiation of cardiac contractility (87). Conversely, shortening of active cardiac muscle might induce deactivation of the myosin motors on the thick filament accelerating muscle relaxation (128). All together, these results suggest that length-dependent activation in cardiac muscle might be explained by a dual-filament mechanism that couples length-dependent structural changes in thin and thick filaments in the presence of calcium (87). The molecular mechanism of sarcomere length sensing

remains unclear, but it might involve interfilament communication pathways based on interactions between thin filament proteins and MyBP-C (87) or titin (121, 122).

6. CONCLUSIONS

The generation of force in striated muscle is controlled by a dual-filament regulatory mechanism that positively couples the calcium-dependent structural changes in the thin filament with the stress-dependent activation of the thick filament. In skeletal muscle, the activation of thin and thick filaments is coordinated by two positive feedback mechanisms, mechano-sensing in the thick filament and myosin-sensing in the thin filament, which account for the rapid and cooperative activation of muscle following electrical stimulation. An additional pathway based on titin stress might accelerate the kinetics of the mechano-sensing transition of the thick filament in response to a rapid increase in the external load, allowing skeletal muscle to act as an efficient brake. A dual-filament regulation mechanism also operates in the heart, although the coupling between thin and thick filament regulatory mechanisms has not been fully elucidated. The dynamics of contraction in cardiac muscle are controlled by the zonal dynamics of thin and thick filament domains that are coordinated by an interfilament signaling pathway based on MyBP-C. The coordinated activation of thin and thick filaments in this dual-filament model might underlie the length-dependent activation of cardiac muscle as the basis of the Frank–Starling law of the heart.

FUTURE ISSUES

1. What is the structural basis of mechano-sensing in the thick filament?
2. What is the mechanism of regulation of myosin motors in the D-zone of the thick filament?
3. Is the thick filament directly activated by Ca^{2+}?
4. How are thin and thick filaments switched off during muscle relaxation?
5. What is the role of titin and myosin-binding protein-C (MyBP-C) in the regulation of cardiac contractility?
6. What is the length sensor at the basis of length-dependent activation in cardiac muscle?

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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