The myosin interacting-heads motif present in live tarantula muscle explains tetanic and posttetanic phosphorylation mechanisms

Raúl Padrón1,2, Weikang Ma3,1, Sebastian Duno-Miranda4, Natalia Koubassova4, Kyoung Hwan Lee3,4, Antonio Pinto1, Lorenzo Alamo1, Pura Bolaños1, Andrey Tsaturyan4, Thomas Irving5, and Roger Craig6

1Division of Cell Biology and Imaging, Department of Radiology, University of Massachusetts Medical School, Worcester, MA 01655; 2Biophysics Collaborative Access Team, Department of Biological Sciences, Illinois Institute of Technology, Chicago, IL 60616; 3Centro de Biología Estructural, Instituto Venezolano de Investigaciones Científicas, Caracas 1020A, Venezuela; 4Institute of Mechanics, Moscow State University, 119992 Moscow, Russia; and 5Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas, Caracas 1020A, Venezuela

This contribution is part of the special series of inaugural Articles by members of the National Academy of Sciences elected in 2018.

Contributed by Raúl Padrón, April 7, 2020 (sent for review December 6, 2019; reviewed by H. Lee Sweeney and Rene Vandenboom)

Significance

Muscle contracts when myosin heads on the thick filaments bind to actin in the thin filaments, producing force, thin filament sliding, and sarcomere shortening. Two important questions on how muscle works are: 1) How myosin heads of a thick filament are “turned on” to enable their binding to actin; and 2) how, after a tetanus, a muscle produces a stronger twitch force than the twitch force produced before the tetanus (posttetanic potentiation). Muscles from tarantula provide insights into both questions: 1) Tarantula thick filaments are not primarily activated by mechanical stress, as in vertebrates, but by phosphorylation; 2) posttetanic potentiation in tarantula muscles occurs by phosphorylated heads that remain released and disordered.

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uscle contraction results from the force exerted by the myosin heads on Ca2+-activated thin filaments, following release of the heads from the thick-filament backbone. We want to understand how the helically ordered heads on the relaxed thick filament sense the Ca2+-activation of the thin filaments so that their release is coordinated with thin filament activation. Initial evidence for such a “thick-filament activation” mechanism was experimentally found in 2015 when mechanosensing was proposed for vertebrate muscle (1). Our first goal here is to inquire if a vertebrate-like mechanosensing mechanism is also present in muscles from invertebrates.

The structure of thin filaments from vertebrate and invertebrate muscle has been studied extensively by electron microscopy (EM) negative staining (2, 3) and low-angle X-ray diffraction (XRD) (4–6). Invertebrate filaments (Limulus, scallop, tarantula) were found to be particularly amenable to structural studies, as their heads were well ordered in true helices, in contrast to the poorer ordering in pseudohelices found in vertebrates (7). Three-dimensional (3D) reconstructions from frozen-hydrated tarantula thick filaments showed that the helices of heads on the filaments were formed by a two-headed assembly that we called the myosin interacting-heads motif (IHM) (8, 9), formed by the interaction of a blocked head (BH) and a free head (FH) (10) with the subfragment-2 (8) (Fig. L4). The head-head interaction was essentially identical to that found in single molecules of myosin II in the relaxed state (10, 11). The switch-2 “closed” state of the myosin head was found to be required for establishing the helical arrangement of IHMs around the thick filament backbone, as shown by XRD (12) and EM (13). The IHM has been found to be present in all animals studied so far by EM of isolated myosin molecules (14, 15) and isolated thick filaments of skeletal, cardiac, and invertebrate smooth muscle (16). In all thick filaments the IHMs are located approximately parallel to the filament axis, the only exception

skeletal muscle | thick filament activation | phosphorylation | posttetanic potentiation | myosin interacting-heads motif

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www.pnas.org/cgi/doi/10.1073/pnas.1921312117

PNAS | June 2, 2020 | vol. 117 | no. 22 | 11865–11874

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1921312117/DCSupplemental.


Reviewers: H.S., University of Florida; and R.V., Brock University.

The authors declare no competing interest.

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Data deposition: Data for this paper are available in the Open Science Framework repository at https://osf.io/anuary/view_only_v1=5491c6ba8bca4add9d979306147346e0.

R.P. and W.M. contributed equally to this work.

To whom correspondence may be addressed. Email: raul.padron@umassmed.edu.

1Department of Molecular Physiology and Biophysics, Cardiovascular Research Institute, University of Vermont, Burlington, VT 05405.

2Massachusetts Facility for High-Resolution Electron Cryo-Microscopy, University of Massachusetts Medical School, Worcester, MA 01655.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1921312117/DCSupplemental.
being insect flight muscle (*Lethocerus*), with a perpendicular IHM (17). The IHM has been key to the concept of super-relaxation (18) and understanding the structural pathogenesis of hypertrophic and dilated cardiomyopathy (19). We show that an IHM-based structure (Fig. 1C) [PDB ID code 3JBH (20)] explains the XRD pattern from relaxed skinned tarantula muscle (21). Our second goal here is to assess if the IHM is present in living relaxed tarantula muscle as well as skinned muscle by recording XRD patterns from resting leg muscle in the whole animal (in vivo) or from excised legs (ex vivo).

The modulation of skeletal muscle contraction by myosin light-chain phosphorylation has been extensively studied (22, 23). Studies on purified myosin from *Limulus* skeletal muscle (24) [an arthropod, like tarantula, exhibiting a similar thick-filament structure (25)] showed it was regulated by a Ca^{2+}-calmodulin myosin light-chain kinase (MLCK)-dependent phosphorylation of its myosin regulatory light chain (RLC), and being dephosphorylated by a myosin light-chain phosphatase (MLCP). While myosin from vertebrate skeletal and cardiac muscle can also be phosphorylated, however, there was no direct effect on the in vitro actin-activated Mg-ATPase activity (26). In purified myosin from tarantula muscle, unlike *Limulus*, phosphorylation does not directly regulate myosin activity but is associated with enhanced actin-activated myosin Mg-ATPase activity (27). EM of negatively stained thick filaments showed that RLC phosphorylation induced the release and disordering of the helically ordered heads (27), consistent with changes in the equatorial X-ray intensities from skinned tarantula muscle (28) and with similar studies of *Limulus* (29) and rabbit skeletal muscle (30, 31). Thus, in vitro, release of heads upon phosphorylation is associated with either switching on of thick filament activity (*Limulus*) or enhancement of thick filament activity (tarantula and vertebrate). Further structural studies showed that tarantula thin filaments are regulated by actin/tropomyosin, as in vertebrate skeletal muscle (32).

Tarantula muscle has a phosphorylatable RLC (27) that can be mono- or biphosphorylated (33) on serines Ser35 and Ser45, as detected by mass spectrometry (MS) (34). On this basis, an IHM-based phosphorylation mechanism was proposed in which phosphorylation of the FH makes possible phosphorylation of the BH from the next myosin molecule along the helix, the so-called cooperative phosphorylation activation (CPA) mechanism (34, 35), which can account for the thick-filament structural changes we observed in tarantula filament homogenates upon Ca^{2+}-activation (27). In this mechanism (Fig. 2B), the selective action of MLCK on the RLC of the BH and FH (Fig. 2A) allow their sequential cooperative release from the thick-filament backbone by Ser35/Ser45 mono- or biphosphorylation (Fig. 2B).

The CPA mechanism (Fig. 2B) proposes that the FHs of relaxed muscle are constitutively monophosphorylated (mono-P) by PKC on Ser35 (34) (Fig. 2B, a), supported by our 18O MS studies that show that on Ca^{2+}-activation, de novo 18O-ATP incorporation occurs only on Ser45 but not on Ser35 (36). In vitro motility assays show that unregulated rabbit F-actin filaments slide along relaxed isolated thick filaments of tarantula muscle in the absence of Ca^{2+} (34). We therefore proposed that the Ser35 mono-P allows the FHs to move away from and toward the filament backbone by Brownian motion, becoming so-called swaying heads (Fig. 2B, a, double curved arrows) (34). Constitutively ON heads (1) [or sentinel heads (37)], functionally similar to the swaying heads of tarantula, have also been hypothesized for vertebrate thick filaments, supported by in vitro motility evidence (38). In the CPA model, the BHs are non-phosphorylated because of steric blocking of their Ser35 by the FHs (Fig. 2B, a) (34, 35). In the presence of Ca^{2+} (Fig. 2B, b), thin filaments become activated, and the swaying (Ser35 mono-P) FHs produce initial force. Simultaneously, Ca^{2+} activates MLCK and the FHs slowly become phosphorylated on Ser45 (adding to their Ser35 phosphorylation, so that they are fully released from the filament backbone) (Fig. 2B, c). These biphosphorylated heads cannot dock back to the filament as their RLC N-terminal extensions (NTEs) containing the phosphorylatable Ser45s are elongated (39) and rigid (40) (Fig. 2B, c), which can only be reversed by dephosphorylation by MLCP. This semipermanent release of the FHs removes the steric hindrance of the BH Ser45, so that some BHs become mono-P by MLCK on Ser45 and are released and sway away (due to the mono-P) from the filament backbone (Fig. 2B, d) and are now able to produce additional force (Fig. 2B, e). Frieto et al. (34) suggested that this mechanism may be limited to arthropods, which—as with tarantula—also exhibit RLCs with long NTEs having consensus sequences for two constitutive/potentiating (PKC/MLCK) phosphorylatable serine pairs, in contrast with vertebrate muscles, with short NTEs and a single MLCK-phosphorylatable serine. As the CPA mechanism was devised in the context of filament homogenates or isolated filaments, our third goal here was to test it in live muscle by assessing its structural predictions, using time-resolved synchrotron XRD patterns during a tetanus to evaluate the location and disorder of the heads, and measuring the non-, mono-, and bi-P RLC phosphorylation using urea-glycerol (U-G) gels.

It has been known for over 150 y (41) that force produced by skeletal muscle can be potentiated by its previous activity. There have been several explanations proposed for this so-called posttetanic potentiation (PTP), with the early work reviewed in ref. 42. The potentiation of force in vertebrate skeletal muscle is stimulated either at lower frequency (treppe or staircase) or higher frequencies resulting in fused tetani (reviewed in refs. 22, 23, and 43), as well as postactivation potentiation and post-activation performance enhancement in human muscle (reviewed in ref. 44), remain an important target of physiological and biophysical research. Our last goal here was to explore the structural basis of PTP in tarantula muscles using XRD.

Here we report: 1) That in thick filaments of live tarantula muscle, the backbone elongation induced by muscle force is
much smaller than in frog, implying that mechanosensing is either absent or only of minor importance in tarantula thick filament activation; 2) the IHM structure is present in live, relaxed tarantula muscle and fully explains the observed XRD pattern; 3) there is structural evidence in live tarantula muscle for an IHM-based phosphorylation mechanism of thick filament activation; and 4) this mechanism also provides a structural basis for PTP.

Results and Discussion

The IHM Structure Is Present in Relaxed Live Tarantula Muscle. We recorded XRD patterns from muscles of the femur either in the intact, living animal (in vivo), in whole legs removed from the animal (ex vivo), or with the muscle removed from the femur and skinned (Fig. 3 A–C). The in vivo preparation (Fig. 3E) had the advantage that the femur muscles are intact and, in contrast to the ex vivo preparation (Fig. 3D), irritated by hemolymph carrying O₂ and metabolites. The in vivo and ex vivo patterns are essentially identical, displaying (see labels in Fig. 3A): nine 43.35-nm-based myosin layer lines (MLL1 to MLL9); three meridional reflections due to the 14.45-nm-based axial separation of myosin heads (M3, M6, M9); four troponin meridional reflections (Tn1 to Tn4) at 37.21, 19.12, 12.76, and 9.56 nm; two actin ~35.4-nm-based layer lines at 5.9 and 5.1 nm (ALL6 to ALL7); and four equatorial reflections 10, 11, 20, and 30. The myosin layer line intensities (IMLL1–IMLL6) of the ex vivo and skinned XRD patterns (Fig. 4A) after background subtraction (45) show peaks at the same reciprocal radii, indicating that skining has no effect on the average radial position of the myosin heads above the backbone. The axial spacings of the M3 and M6 meridional reflections (S_{M3} and S_{M6}) are 0.11- and 0.05-nm longer, respectively, than in vertebrate (frog) muscle (1), consistent with other invertebrate muscles (46). The M3 and M6 splitting observed in frog, due to the interference between myosin head arrays on opposite ends of the thick filaments (1), was not detected, possibly because the thick filaments in tarantula muscle are much longer, about 4 to 5 μm (47), so that the interference fringes become too close to be resolved (48, 49). Tarantula skeletal muscle lacks the accessory protein MyBP-C (50) so there was no meridional reflection at 44.2 nm.

Analysis of the XRD patterns in relaxed and contracting tarantula muscle is simplified by the almost total absence of lattice sampling due to random rotational orientations of the thick filaments (5), unlike vertebrate skeletal muscle where the filament organization gives rise to significant lattice sampling. We used a thick-filament model (Fig. 1C), based on cryo-EM 3D reconstruction that clearly resolves the FHs and BHs of the IHM (Fig. 1 A and B, a), to assess the separate contribution of BHs and FHs to the XRD pattern. To evaluate the correctness of this IHM-based model as a description of the thick-filament structure in the live muscle lattice, we compared the calculated IMLL1–IMLL6 (with all FHs docked) of this IHM-based model with those of the ex vivo and skinned relaxed muscle (Fig. 4A). We also did the comparison with the densities of the 2.0-nm 3D map, used to fit the 3JBH IHM-based model (20) (Fig. 1 B, a) and the higher resolution (1.3 nm) (51) thick-filament frozen-hydrated 3D maps (Fig. 4A and SI Appendix, Fig. S1). The striking similarity of the layer line profiles shows that the average position of the heads above the backbone and their conformation in ex vivo and skinned muscle is very similar to the ones in both cryo-EM 3D density maps and in the 3JBH-derived IHM-based model. The correctness of the fitting of the model to the observed XRD intensities was assessed using the crystallographic R-factor (Materials and Methods). The R-factor for the IHM-based model and the ex vivo data was low (4.9%), while those for the model and the 2.0- and 1.3-nm 3D-map densities were 4.0% and 8.4%, respectively, indicative of generally good fits. There are two significant differences between the observed and calculated layer line profiles: 1) the strong, narrow M3 meridional reflection in both experimental XRD patterns, and 2) the sampling at the positions of the equatorial 10, 11, and 20 reflections in the ex vivo MLL3 (Fig. 4A, arrows), caused by the filament lattice in sarcomeres, while all calculations were performed for a single thick filament in the absence of a lattice. The first difference is because the interference of the X-rays scattered by neighboring thick filaments in the ordered lattice results in a higher meridional peak on MLL3, as explained in Koubassova and Tsaturyan (45). The second difference is because the filament lattice in ex vivo is much better ordered than in skinned muscle.

We conclude that in tarantula live muscle, the 3D-maps obtained by cryo-EM and the IHM-based model derived from these maps fully explain the recorded XRD pattern. Thus, the thick filaments in live tarantula muscle have a structure similar or identical to that observed by cryo-EM, in which myosin heads are in the IHM conformation. This contrasts with the conclusion that IHM-based thick filaments are not consistent with XRD...
patterns of bony fish skeletal muscle and Lethocerus flight muscle (52). The reason for this discrepancy is not yet clear, as cryo-EM studies unambiguously show the presence of the IHM structure in isolated, frozen-hydrated Lethocerus thick filaments, while no cryo-EM studies have yet been reported on fish thick filaments. Because studies of isolated thick filaments in relaxing conditions from a broad evolutionary range of species show the IHM as a prominent feature (16), our results suggest that it is likely that this will also be the case with all these species in vivo.

Time-Resolved XRD Suggests No Significant Change in Backbone Length in Contracting Tarantula Muscle. In SI Appendix, Supplementary Results, we show that we can use our 3D tarantula thick-filament reconstructions to demonstrate that the M6 meridional reflection contains information from both the thick-filament backbone and myosin head periodicities (SI Appendix, Fig. S1), so changes in SM3 can be used as a measure of thick-filament length changes. The time course of SM3 (Fig. 5B) shows a small, statistically not significant, decrease of 0.02 nm (0.13%) during the tetanus, while SM6 (Fig. 5C) increases by 0.02 nm (0.27%) during force development (Fig. 5A). This increase of SM6 in tarantula is much smaller than the 1.8% increase for frog muscle, associated with mechanosensing (49), whereas the 0.13% decrease in SM3 in tarantula contrasts with a 1.61% increase in frog. In frog it has been proposed that a small population of constitutively ON motors generates a mechanical stress in the thick filament that lengthens the filament and releases the remaining motors from the OFF state, allowing the development of the full isometric force (1). For tarantula this would imply that the Ser35 mono-P swaying FHs should generate a similar stress releasing the remaining FHs and BHs. However, the lengthening of the tarantula thick-filament backbone as estimated from SM6 is only 15% of the lengthening reported for frog. The very small SM6 change that we observe in tarantula suggests that the thick-filament backbone in these muscles is very rigid, consistent with the presence of a paramyosin (PM) core that is absent in frog. We conclude that the small change in the SM6 and SM3 in tarantula compared with frog suggests that a mechanosensing mechanism based on changes in backbone length would, if present, be of minor importance or at a minimum, be very different from the vertebrate mechanism.

The Myosin RLC Is Highly Mono-P and Bi-P during the Tetanic Plateau in Tarantula Muscle. Phosphorylation and dephosphorylation of the myosin regulatory light chains via MLCK, MLCP, and calmodulin has been a common feature of vertebrate and invertebrate skeletal muscle (53), including tarantula (50), since the earliest times of evolution. In tarantula, RLC phosphorylation has been demonstrated using multiple complementary techniques: SDS/PAGE gels (27, 33–35), U-G gels (27, 33–35), autoradiography (27, 33), Western blots using anti-RLC antibodies (35), iso-electric focusing (54), and MS detection of phosphorylatable serines (34–36). RLC phosphorylation was investigated in tarantula filament homogenates using U-G gel electrophoresis, which allows discriminating RLC molecules by their charge as non-P, mono-P, and bi-P, detected as three gel bands (27, 33–35), with the relative quantities of phosphorylated RLC molecules quantified by the relative optical densities of the bands. The BH and FH RLC molecules are distributed between the three bands according to the activation state of the preparation, and their numbers can be accounted for by the CPA mechanism (20). We used a similar approach to determine the phosphorylation levels in rapidly frozen tarantula muscles at the end of a tetanic plateau (SI Appendix, Fig. S2 A, b), showing a substantial increase in mono-P and bi-P and almost complete disappearance of non-P as compared with relaxed live muscles (SI Appendix, Fig. S2 A, a and B, a). These changes contrast with the ones we have seen on Ca²⁺-activation in filament

Fig. 3. XRD patterns of muscles of tarantula femurs. (A) Ex vivo pattern of resting relaxed femur muscles. Equatorial, meridional and off-meridional intensity profiles in blue (Top), orange (Left), and green (Right); 23 °C, exposure 200 ms. (B) Pattern of in vivo femur muscles recorded directly from the femur of an intact live tarantula; 23 °C, exposure 1,000 ms. (C) Pattern of a skinned femur muscle in relaxing solution; 23 °C, exposure 1,000 ms. (D) Set-up for recording patterns from ex vivo femur (seen through yellow window in chamber), allowing electrical stimulation and tension recording through a metallic linker (hook near the knee) connected to a transducer. (E) Set-up for recording patterns from in vivo femur muscle.
homogenates (35) (SI Appendix, Fig. S2 B, b vs. SI Appendix, Fig. S2 B, a), suggesting that the MLCK is more effective in the ex vivo muscle because of its higher activity and concentration in the sarcoplasm and possible structural advantages of the sarcomere lattice in vivo (SI Appendix, Fig. S3A).

Time-Resolved XRD and U-G Gels Add Key Evidence for an IHM-Based Phosphorylation Mechanism. To test the IHM-based CPA mechanism structurally (Fig. 2B), we determined the radial distance of the heads from the backbone and their degree of disorder by recording seven time-resolved XRD patterns (Fig. 5) from ex vivo leg muscles. Patterns were recorded before and during a stimulus train that elicited an initial twitch (pretetanic), followed by a tetanus, and then several posttetanic twitches (up to 13.5 s after the end of the tetanus) (Fig. 5 E–H), and finally 2 to 3, 4 to 5, and >6 min later, during recovery after the stimuli (Fig. 5 I–L). In the second strongest layer line coming from the helical organization of myosin heads was used as a measure of helical order; and a decrease in I_{10} and increase in I_{11+20} were used as indicators of movement of heads away from the thick-filament surface.

During the tetanus plateau, I_{MLL4} decreased to a low (but nonzero) residual value compared to the maximum value before the tetanus (Fig. 5J), while I_{10} decreased to a low value (Fig. 5 F and L) and I_{11} increased to a high value (Fig. 5 G and K). We conclude that the heads progressively move away from the thick filament backbone toward the thin filaments during the tetanus and, in the process, become disordered. The presence of residual I_{MLL1} intensity (~20 to 25%) during contraction has been reported in electrically stimulated skeletal muscle from frog (55, 56) and mouse (57) during tetanic contraction and has been interpreted as indicating that during the tetanus plateau ~50% of heads are disordered while ~50% remain ordered in their helical positions as in the resting muscle. Here we interpret the observed residual I_{MLL1} and equatorial changes in tarantula similarly to refs. 55 and 56, but in the context of the CPA mechanism (Fig. 2B) and the IHM-based thick-filament structural model (Fig. 1C). First, we interpreted the observed XRD pattern changes (Fig. 5) by associating the disordered heads as FHs and the ordered ones as BHs (Fig. 2 B, c–e). We then calculated the effect on I_{MLL1} and I_{MLL4} of progressively removing FHs (SI Appendix, Fig. S3A; compare with SI Appendix, Fig. S3B) for interpreting the observed I_{MLL1} and I_{MLL4} changes (Fig. 5). The purpose was to simulate the swaying of the FHs between a docked position in the ordered IHM for a given percentage of the time (t_{DHM}) and a released (disordered) position for the balance of the time (t_{DHM}); the duty cycle [t_{DHM}/(t_{DHM} + t_{DHM})] for the ensemble of heads would correspond to the fraction of FHs remaining docked in the simulation (SI Appendix, Fig. S3C), being greatest if the FH spends all of its time in the IHM and least if it spends all of its time released (SI Appendix, Fig. S3C) (19). The simulation results for I_{MLL1} and I_{MLL4} (SI Appendix, Fig. S3C) showed residual values of ~34% and 14%, respectively, when all FHs were removed (while all BHs are still present) (SI Appendix, Fig. S3B), consistent with our proposal that the residual I_{MLL1} and I_{MLL4} come from the (more stable) BH helices (SI Appendix, Fig. S3B), while all bi-P FHs are disordered during a tetanus. Therefore, we interpret the observed decrease of I_{MLL4} intensity to ~18% (Fig. 5J), as reflecting the release and disordering of most FHs while most BHs remain helically ordered. This association is supported also by the observed concomitant changes in I_{10} and I_{11+20} (Fig. 5 F–H).

We previously found by EM of isolated filaments that RLC phosphorylation induced the release and disordering of the helically ordered heads (27), as shown also by changes in the equatorial reflection intensities from skinned tarantula muscle (28). Similarly, XRD studies on skinned rabbit psoas muscle (31) showed decreases in I_{MLL1} upon RLC phosphorylation, consistent with partial loss of the helical order of myosin heads, and increases in I_{11}/I_{10}, supporting the idea that phosphorylation of myosin RLC causes the myosin heads to move away from the thick filaments toward the thin filaments, thereby enhancing the probability of interaction with actin. Our diffraction (Fig. 5) and U-G gel electrophoresis (SI Appendix, Fig. S2 A, b and B, a) results in live, intact tarantula muscle are consistent with the proposal that at the end of the plateau (Fig. 2 B, e), most BHs are Ser45 mono-P (SI Appendix, Fig. S2 A, b and B, a); that is, the filament-docked non-P BHs become swaying Ser45 mono-P BHs contributing to the X-ray pattern (Fig. 2 B, d, double curved arrows) with their own docked/released duty cycle (SI Appendix, Fig. S3D), similar to the swaying Ser35 mono-P FHs in the relaxed state (Fig. 2 B, b, double curved arrows), while a significant proportion of FHs are bi-P, released and fully disordered.

Previous studies (56, 58, 59) suggested that in frog skeletal muscle, the recovery after a short tetanus takes at least several seconds, and that there was a discrepancy between the fall of tension and the return of myosin heads to their helical paths.
Here we found that in tarantula muscle after a tetanus I\textsubscript{M3L4} and I\textsubscript{10} stayed low, and I\textsubscript{11} high until 12 to 13 s, slowly returning in >6 min to their initial resting values (Fig. 5 J–L).

This slow recovery of the original helical arrangement of the myosin heads (several minutes) correlates with the slow dephosphorylation by the endogenous MLCP of first, the Ser45 mono-P swaying BHs, which allows them to dock back helically again to the filament, together with non-P BHs already docked (SI Appendix, Fig. S3B), and second, the fully released bi-P FHs, so they can dock back as swaying heads on the previously docked BHs, both increasing their contribution to the I\textsubscript{M3L4} and I\textsubscript{10} (Fig. 5 J and L). As with the helical layer lines, the I\textsubscript{11+20} remains high after the tetanus (Fig. 5K), consistent with the bi-P FHs (and some Ser45 mono-P BHs) remaining in the vicinity of the thin filament, recovering slowly to their resting value as dephosphorylation progresses as mentioned before.

Heads that Remain Released and Disordered after a Tetanus Produce PTP. When muscle is stimulated soon after a tetanus, the twitch elicited is substantially stronger than a pretetanic twitch (PTP). It has been proposed that such PTP results from RLC phosphorylation and its accompanying structural effects, in which the myosin heads project from the filament backbone, facilitating their interaction with actin. This structural model has received experimental support from EM studies of isolated thick filaments (27, 30) and XRD of skinned vertebrate and invertebrate muscles (28, 31), but has not been demonstrated in live muscle. Here we have used ex vivo tarantula muscle to correct this deficit.

Fig. 6 shows that early after a tetanus, myosin heads remain disordered (weak I\textsubscript{M3L4}) and away from the filament backbone, facilitating their interaction with actin. This structural model has received experimental support from EM studies of isolated thick filaments (27, 30) and XRD of skinned vertebrate and invertebrate muscles (28, 31), but has not been demonstrated in live muscle. Here we have used ex vivo tarantula muscle to correct this deficit.

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compare with SI Appendix, Fig. S2 A, b and B, a). The bi-P FHs are disordered and project from the filament backbone, remaining this way until being dephosphorylated to Ser45 mono-P. The mono-P BHs are swaying, spending part of their time, defined by their $I_{110/111} (I_{110} + I_{111})$ duty cycle (see previous section and SI Appendix, Fig. S3D), on the helical tracks and thus contributing to the layer lines, similar to the swaying FHs in the relaxed pattern. When muscle is stimulated to contract during this time, these heads, already projecting away from the thick filament (reflected in the high $I_{111} + I_{110}$ and $I_{111} + I_{110}$) (Fig. 6 A and C), attach to actin (producing enhanced force, or PTP), shown by a further increase in $I_{111} + I_{110}$ and $I_{111} + I_{110}$ (Fig. 6 A and C red arrows). Thus, our experiments suggest that in live muscle a tetanus leads to phosphorylation of many heads (including FH and BH), which continue to project toward the thin filament (phosphorylation “memory”) and are therefore readily available for interaction with actin, producing a strong posttetanic twitch. During recovery from the tetanus, RLC phosphorylation gradually returns to baseline through MLCP activity, and we observe a decline in PTP. This follows a return of heads to the thick-filament backbone ($I_{111} + I_{110}$ and $I_{111} + I_{110}$ drop and $I_{110}$ increases) and restoration of their helical arrangement ($I_{MMLL1}$ regains its strength) (Fig. 5 D and J). We have observed twitch and tetanic tension in freshly dissected muscle where the helical ordering is weak or absent. Thus, helical ordering of the heads establishing the IHM is not a requirement for contraction. Rather, our results show that the highest tension twitches come from heads that are disordered (i.e., during PTP).

Proposed IHM-Based PTP Structural Mechanism for Tarantula. Based on our results and on the dual phosphorylation IHM-based CPA mechanism (Fig. 2B), we propose the structural model shown in Fig. 7 to explain force enhancement and PTP in the muscles of tarantula. This mechanism supports the general concepts for phosphorylation-based potentiation advanced previously for fast-twitch vertebrate muscle (22, 23, 43, 60–62), in which it was hypothesized that in live muscle, RLC phosphorylation releases the heads, similarly to what was found in isolated thick filaments and skinned muscle in tarantula (27, 28), Limulus (29), and rabbit (30, 31), thus facilitating their attachment to actin, providing an explanation for potentiation both during and after a tetanus (as PTP). The model, which includes the constraints of the tarantula thick-filament structure (34), suggests a specific structural mechanism that explains: 1) Force enhancement during a tetanus by recruiting additional bi-P FHs and Ser45 mono-P BHs (Fig. 7C vs. Fig. 7B), grading (i.e., potentiating) the achieved force, and 2) the PTP phenomenon (compare Fig. 7E vs. Fig. 7B), consistent with our experimental XRD (Figs. 5 and 6) and U-G results (SI Appendix, Fig. S2 A, b and B, a).

Arthropod striated muscles share similar thick-filament head arrangements and long RLC NTEs with dual PKC/MLCK phosphorylation sites. Apart from tarantula, RLC phosphorylation has been reported in Limulus (24), and PTP in scorpion (63), whose thick filaments (25, 64) are similar to tarantula. Therefore, the proposed mechanism could, in principle, be extended to the other species exhibiting long RLC NTEs with PKC/MLCK consensus sequences. RLC phosphorylation remaining after a tetanus has been reported in frog (65), rabbit (66), and mouse (62). Residual $I_{MMLL1}$ after a tetanus has been observed in frog (55, 56) and mouse (57). Here we have associated, in intact tarantula muscle, residual RLC phosphorylation (SI Appendix, Fig. S2 A, b and B, a), residual $I_{MMLL1}$ and $I_{MMLL4}$, and residual released and disordered heads after a tetanus, with PTP (Fig. 7). Can the mechanism shown in Fig. 7 be extended as well to explain PTP in vertebrate muscles? Brito et al. (34) reported that vertebrate muscle RLCs exhibit only short NTEs, with MLCK consensus sequences and only one MLCK-phosphorylatable serine. Therefore, the proposed mechanism could be extended to vertebrate muscle but modified for allowing only monophosphorylation. Mono-PKC–phosphorylated, swaying heads in tarantula would correspond to the constitutively ON (non-phosphorylated) heads in vertebrate (1). In tarantula the recovery
phase after a tetanus implies a slow dephosphorylation time $\tau$ (minutes) of the mono-P and bi-P heads (Fig. 7, Inset), that by remaining released and disordered “memorize” the previous muscle history. In vertebrates, this could be accomplished more quickly as the PTP recovery would involve the dephosphorylation time of only mono-P heads. Brito et al. (34) also reported that some species that show direct $Ca^{2+}$-activation via the RLC, like scallop (67), exhibit RLCs with only short NTEs and a single serine, with no MLCK consensus sequence, suggesting that a phosphorylation-based mechanism like the IHM-based CPA mechanism (Fig. 2) and the PTP mechanism (Fig. 7) cannot be functional for them.

**Evolution Has Resulted in Different Thick-Filament Activation Mechanisms.** Different molecular mechanisms for activation have evolved to control the release and disordering of myosin heads in striated muscles in order to actively recruit them to produce force: 1) Direct $Ca^{2+}$-binding, 2) RLC phosphorylation, 3) mechanosensing, and 4) delayed stretch activation. Mechanosensing (Fig. 8C) is a prominent feature of vertebrate skeletal muscle (1). Here we show evidence (Figs. 5 and 6 and SI Appendix, Fig. S2) that in tarantula there is a primary phosphorylation mechanism (Fig. 8B), with at most a subsidiary role for mechanosensing because the lengthening of the backbone was much smaller (and statistically not significant) than in frog. In tarantula muscle, during a twitch, the swaying heads (due to their mono-P at Ser35) are intermittently extended ready to bind to $Ca^{2+}$-activated thin filaments to produce initial force, and phosphorylation releases more heads. In frog, lacking constitutive phosphorylation, constitutively ON heads develop initial force, and inactive heads are then released by mechanosensing as force increases. In both muscles, PTP is due to RLC phosphorylation, possibly differing in how long the PTP lasts. Another important difference between the thick filaments of invertebrates and vertebrates is the presence of PM in the vertebrate filament backbone, suggesting that the lack of backbone lengthening in tarantula may be associated with the rigidity of its thick filaments, due to a core of tightly packed PM molecules (Fig. 1B, d), in contrast to frog that lacks this rigid core. Some of these mechanisms may be widespread, such as the dual phosphorylation CPA mechanism in Arthropoda, with single phosphorylation variations present in vertebrate muscle as a modulatory mechanism. Other activation mechanisms are very specific, such as the $Ca^{2+}$-binding activation mechanism, observed only in mollusks (Fig. 8A), and the stretch activation observed in the indirect flight muscles of *Lethocerus* and other flying insects. So far, mechanosensing appears to be confined to vertebrates.

We conclude that the molecular mechanisms of thick filament activation and PTP evolved differently in invertebrate and vertebrate striated muscle as adaptations to the lifestyle of the
organisms. Our results support the notion that helically ordered IHMs are the key to energy conservation in the resting relaxed state, brought about by the interactions between the heads that inhibit their activity (20). In tarantula live muscle, while the ATP turnover rates for the swaying FHs is either slow (250 to 300 s) or fast (<30 s) while being transiently detached and disordered (20, 54), an important fraction of the heads (BHs)—permanently docked forming helices on the filament—exhibit a very slow ATP turnover rate (1,800 s), an energy conserving state. Force potentiation both during and after a tetanus in tarantula muscle could be a way to increase force when needed, according to the previous muscle activation history (PTP), while saving ATP when not needed (54), which could be energetically favorable in an arthropod that spends large amounts of time immobile, with only occasional bursts of energy required for moving when capturing food or avoiding predators, among other vital activities.

A key point to note is the existence of two different relaxed states that are structurally distinct: 1) The “resting” relaxed state in which heads are helically ordered in the IHM, with minimal usage of ATP (possibly equivalent to the superrelaxed state); and 2) the “posttetanic” relaxed state, in which heads are in a disordered, preactivated, non-IHM state, near to actin. Both states are mechanically relaxed, and the thin filaments are switched off. These states, determined experimentally, may correlate with different phases of the daily life of the tarantula. The animal spends most of its time at rest, hidden and motionless, conserving energy (resting relaxed state). When the need to capture food or escape a predator arises, the muscles become active and RLC phosphorylation is initiated, leading to enhanced force production. Relaxation still occurs when the thin filaments are switched off between contractions (posttetanic relaxed state), but more force is produced upon activation, as many heads are already near actin. Energy conservation is lower during these relaxed intervals but that is less important than having high force when needed. When activity ceases, the muscles return to their resting relaxed state.

Tarantula provides an excellent model system for understanding thick-filament structure and function in terms of the BHs and FHs of the IHM. EM yields key insights into the organization of the two heads in the IHM in the static state of relaxation, including their interactions, relative stability, and accessibility of their RLC phosphorylatable serines (8, 34, 35). XRD complements these studies, demonstrating the presence of this same IHM arrangement in intact muscle, and additionally revealing dynamic changes in the heads that underlie activation and potentiation. These studies suggest very different roles and dynamics for BHs and FHs in tarantula. A similar distinction between heads might also occur in vertebrate muscle, where the IHM is also present (68). Previous studies indicate a residual population of helically ordered heads in activated muscles, but not assigned to any particular head (blocked or free) (55–57).

Our tarantula observations would suggest that the majority of residual ordered heads would be BHs, and that any constitutively active heads would come from the FH population. Our tarantula observations would suggest that the majority of residual ordered heads would be BHs, and that any constitutively active heads would come from the FH population.

**Materials and Methods**

**X-Ray Diffraction.** Legs were excised from anesthetized Texas brown tarantulas and placed in an X-ray chamber. Muscles were stimulated through platinum electrodes using an Aurora Scientific 701A stimulator. XRDR patterns from muscles in resting or activated states were obtained at the Advanced Photon Source, Argonne National Laboratory, and recorded on a Pilatus 3 M detector (Dectris Inc.) with 20-ms exposures and 50 ms between exposures. For in vivo experiments, the live tarantula was constrained in a plastic tube mounted in the X-ray beam.

**XRD Pattern Modeling.** Fourier transforms were calculated for the thick filaments assuming a myosin head spacing of 14.5 nm, a helical repeat of 43.5 nm, and fourfold rotational symmetry.

U-G gel electrophoresis was carried out according to Sulbaran et al. (35) on muscles frozen at the end of the tetanic plateau.

Full details of methods are provided in SI Appendix.

**Data Availability.** Data were deposited in the Open Science Framework, https://osf.io/anxyu/?view_only=5491c6ba8bca4add9d97930614734e0e.

**ACKNOWLEDGMENTS.** We thank Dr. Carlo Caputo for advice and Dr. Michael Previs for suggestions. Research was supported by National Institutes of Health Grants National Institute of General Medical Sciences GM103622 (to T.I.), National Heart, Lung, and Blood Institute HL139883 (to Richard Moss and R.C.), National Institute of Arthritis and Musculoskeletal and Skin Diseases AR072036 (to R.C.), AR067279 (to R.C. and D. Warshaw), Office of Research Infrastructure Programs 15100DD108090-01 (to T.I.), and Russia State Program AAAA-A19-11901299011-3 (to N.K. and A.T.). The Advanced Photon Source is operated for the Department of Energy Office of Science by Argonne National Laboratory under Contract DE-AC02-06CH11357. The content of this work is solely the responsibility of the authors and does not necessarily reflect the official views of the National Institutes of Health.


