Vertebrate striated muscle thin filaments are thought to be thermodynamically activated in response to an increase in Ca\(^{2+}\) concentration. We tested this hypothesis by measuring time intervals for gliding runs and pauses of individual skeletal muscle thin filaments in cycling myosin motility assays. A classic thermodynamic mechanism predicts that if chemical potential is constant, transitions between runs and pauses of gliding thin filaments will occur at constant rate as given by a Poisson distribution. In this scenario, rate is given by the odds of a pause, and hence, run times between pauses fit an exponential distribution that slopes negatively for all observable run times. However, we determined that relative density of observed run times fits an exponential only at low Ca\(^{2+}\) levels that activate filament gliding. Further titration with Ca\(^{2+}\), or adding excess regulatory proteins tropomyosin and troponin, shifted the relative density of short run times to fit the positive slope of a gamma distribution, which derives from waiting times between Poisson events. Events that arise during a run and prevent the chance of ending a run for a random interval of time account for the observed run time distributions, suggesting that the events originate with cycling myosin.

We propose that regulatory proteins of the thin filament require the mechanical force of cycling myosin to achieve the transition state for activation. During activation, combinations of cycling myosin that contribute insufficient activation energy delay deactivation.

The crucial, second step of a two-step process for thin filament activation of vertebrate striated muscles involves shifting of filamentous tropomyosin (Tm) on the helical backbone of F-actin from a position that blocks interaction with myosin (B-position) to a position that permits myosin crossbridge cycling (M-position) (1, 2). In the preceding step, Ca\(^{2+}\) binding to a Tm-associated protein, troponin (Tn), weakens interactions between Tn and actin that control the equilibrium distribution of Tm between B-position and a central C-position (3, 4). The equilibrium favors either B- or C-position at low (pCa 8 – 10 nM) or high (pCa 4 – 4.5) Ca\(^{2+}\), respectively, resulting in the exposure of thin filament sites for myosin interaction at the higher Ca\(^{2+}\) levels (5–8). The transition of Tm between C- and M-positions depends on the formation of ternary complexes composed of myosin, Tm, and actin (9). This second step is both necessary and sufficient to activate thin filaments (10), as shown by cooperative binding of rigor myosin in noncycling conditions (11). Even in the presence of physiological ATP, rigor myosin is a strong-binding transient intermediate of crossbridge cycling (12).

Strong-binding myosin intermediates of the crossbridge cycle, including rigor, are essential to proposals that mass action explains cooperative activation of thin filaments (13, 14). However, Ca\(^{2+}\) binds noncooperatively to thin filament proteins with or without myosin if conditions support only mass action (15–17). Preparations supporting cycling crossbridges, which have only a small fraction of myosins in strong-binding intermediate states (12), bind Ca\(^{2+}\) cooperatively (17–19) and bind myosin independently of bound Ca\(^{2+}\) (20). Measurements of crossbridge turnover provide evidence that cycling crossbridges affect Ca\(^{2+}\) sensitivity of muscle activation (21). Compliance of the thin filament (22, 23) that varies with activation (24) may transmit mechanical force cooperatively (24, 25), which is the basis for an alternative, nonthermodynamic proposal for activation (26). The contribution of mechanical energy to the activation mechanism has not been investigated directly, despite the assumption that mass action is required to induce a cooperative response. Mass action is possible only if changes in molar mass (N) changes free energy G at constant chemical potential, \(\mu = G/dN\). At equilibrium, only fluctuations in thermal forces should elicit changes in activation over time.

A flow cell of a motility assay (27) represents a closed system having constant temperature, pressure, and chemical potential. Thin filaments remain bound to the surface of immobilized myosin whether gliding continuously (run) or remaining stationary (paused) at high and low Ca\(^{2+}\) concentration, respectively (28–30). Intermediate Ca\(^{2+}\) concentrations reveal random binary switching events between runs and pauses (28–30), consistent with the moment in time a regulatory unit transitions between C- and M-positions. Binary activation of individual regulatory units is demonstrated by clusters of myosin that form around nascent pairs of myosin in foci along a thin filament (31). Also observed are fluorescent myosin molecules cycling in and out of the clusters, and fluorescent clusters moving along the filament length in waves (31).

If mass action is required for cooperative activation, thermal fluctuations drive the abrupt changes between run and pause in the motility assay. For a thermodynamic change, the Poisson distribution gives the probability of switching, and the exponential distribution gives the probability of an uninterrupted run. In this study, we test whether relatively short run times are
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most frequent for all observable intervals of time, as predicted by the exponential distribution.

Results

Extreme run times vary with potential

To provide an initial assessment of the quality of our assay, we exposed native thin filaments to Ca$^{2+}$ concentrations that bracketed the extremes of physiologically relevant Ca$^{2+}$. In high free Ca$^{2+}$, $10^{-4}$ M (pCa 4.0), thin filaments sampled the entire surface at average speed $3.67 \pm 0.128 \mu\text{m/s}$ (mean ± S.E., $n = 518$, 25°C), and their motion appeared similar to the smooth, continuous motility of unregulated F-actin (Movie S1). By contrast, thin filaments remained stationary or moved rarely in buffers containing pCa 8.0 or pCa 7.0 (Movie S1). Thus, the two extreme chemical potentials supported corresponding run time extremes.

In assays conducted between pCa 6.6 and 5.6, individual thin filaments switched clearly between runs and pauses (Movie S2). Unbiased observers routinely scored a switch with minimum precision of ~10 video frames (300 ms). A potential source of systematic error was brief pauses (<15 frames) that were difficult to distinguish from episodes of filament slowing. To reduce bias, we aggregated like data from multiple observers. The shortest runs corresponded to displacements of ~500 nm (3–4 pixels). The longest runs (~500 frames), corresponded to ~15 s and a displacement of about 90% of the field of view. Runs leaving the field of view were not counted, which effectively increased the relative density of shorter runs. This type of distortion, however, does not change the negative slope of an exponential distribution.

Relative densities of run times shift with activation potential

To compare relative densities of observed run times, we used the same binning method and temporal scale for all activation potentials. By inspection of the shortest time bins, the change in density shifted from negative to positive slope as Ca$^{2+}$ between pCa 6.6 to pCa 5.6 varied the chemical potential (Fig. 1, A and G).

We used Pearson’s chi-squared statistic to test goodness of fit between expected and observed densities based on raw run time measurements. Compared with the cumulative density of the best fit exponential, observed run time density differed significantly in the pCa 5.6 condition but not the pCa 6.6 assay (Table 1). In pCa 5.8 conditions, the statistical fit became less significant as the number of observations was increased (Table 1), consistent with diminishing confidence in the fit as the number of comparisons (degrees of freedom) increases. In summary, the shortest run times changed from relatively likely to relatively unlikely as Ca$^{2+}$ in the assay increased 10-fold from pCa 6.6 to pCa 5.6.

To address the possibility that long run times were the result of substandard regulation (30), we flushed the flow cell and added excess native tropomyosin-troponin complex (TmTn) with native thin filaments in the same assay conditions (Movie S3). Rather than restoring density to short run times expected for an exponential, the addition of excess TmTn increased the relative density of longer run times for all chemical potentials tested by varying Ca$^{2+}$ (Fig. 1, A–H). Chi-squared tests rejected all exponential fits with fewer degrees of freedom than tests conducted without added TmTn (Table 1). A maximum likelihood algorithm (32) found no improvement by using the positive sum of multiple exponentials to fit any of sample sets collected.

Previous studies showed that a gamma distribution or equivalent (33, 34) fit sample distributions with increasing slope as we observed. Distributions fit poorly by an exponential distribution had the highest probabilities of fitting a gamma distribution by chi-squared analysis (Fig. 1, F–H and Table 1). By contrast, observations that most favored an exponential distribution fit a gamma distribution poorly (Fig. 1, A and C and Table 1). Without added TmTn, the pCa 5.8 condition (Fig. 1E) appeared to mark a transition between chemical potentials that supported exponential distributions (Fig. 1, A and C) and a gamma distribution (Fig. 1G). The chemical potential given by pCa 6.2 with added TmTn (Fig. 1D) marked a similar transition between chemical potentials that supported exponential (Fig. 1B) and gamma (Fig. 1, F and H) distributed run times. Hence, neither an exponential nor a gamma process alone explained observations in all chemical potentials.

Delay events account for varying run time density

Gamma distributions arise from random waiting times between Poisson-distributed events, which we interpret as random events that delay deactivation. From a linear combination of delay and Poisson rates, we obtain a blended response function, $y(t, O_p, a)$, where $t$ is elapsed time, $O_p$ is the odds of a pause produced by a thermal switch, and $a$ is a coefficient for linear combination of the delay rate (Equation 1, see “Experimental procedures”). Given $O_p$ and $a$, $y(t)$ is the relative run time distribution expected if a delay is because of a force that opposes the thermal force for the switch. We suggest that a mechanical force during a run prevents a singular transition state required for the switch. As a result, the delay persists until the transition state returns by mechanical force. If crossbridges generate the mechanical force, then the delay should depend on the crossbridge cycling rate. The probability of a switch at the moment the transition state returns is the probability of a pause, $P_p$, which is given by $O_pP_p$, where $P_p$ is the probability of a run, $1 - P_r = P_p$, and $O_p = P_p/(P_t)$. Hence, $y(t)$ has only two parameters, $a$ and $O_p$, that can be adjusted for fitting, which is consistent with the free parameters of the gamma distribution.

Fig. 2A shows predictions of $y(t)$ for selected values of $O_p$ (Fig. 2A). The response shifts from more exponential to more gamma as $O_p$ increases, consistent with shifts in sample distributions as increasing Ca$^{2+}$ concentration varies chemical potential (Fig. 1). Increasing $a$ alone produces a more gamma-like response (Fig. 2B), consistent with samples in which chemical potential was varied by adding TmTn to the assay at constant Ca$^{2+}$ concentration (Fig. 1 and Table 1). Evident from the trend shown is that $y(t)$ accounts for run times that are too short or too long to measure, because of experimental limitations, at the extremes of Ca$^{2+}$ concentration. The difference
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\[ y(t) - y_n(t) \] (Equation 1) accounts for the mechanical work done on the system, which must, therefore, be nonthermodynamic.

To test our proposed delay mechanism, we fit observed cumulative probability density. Cumulative density of raw run times (Fig. 3) were compiled with an algorithm that computed maximum likelihood fits to exponential distribution (Fig. 1A) and gamma distribution (Fig. 1G). For the expected probability density, we normalized \( y(t) \) to the area under the curve (Fig. 2), which has an analytical solution \( \int y(t) dt \) (see “Experimental procedures”). Using \( y(t)/\int y(t) dt \), we adjusted \( a \) and \( O_p \) to fit experimental data points. Given \( a = 3 \), we obtained satisfactory fits of cumulative probability densities of pCa 6.6 and pCa 5.6 with switch rates of 19 s\(^{-1}\) and 0.5 s\(^{-1}\) (Fig. 3), corresponding to probabilities of activation \( (P_t) \) of 0.05 and 0.67, respectively. For \( P_t = 0.67 \), the fit is distinctly sigmoidal, indicative of a self-sustaining mechanism consistent with cooperativity. Because variables \( O_p \) and \( a \) interact, an independent measurement of \( O_p \) is required to determine \( a \) confidently (see “Experimental procedures”).
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Table 1
Not all sample distributions fit an exponential by goodness of fit

| Panel Fig. 1 | pCa | Addition | Events | $H_0 = \text{exponential}^b$ | $H_0 = \text{gamma}^c$ |
|--------------|-----|----------|--------|-----------------------------|---------------------------|
|              |     |          |        | p   | df | $H_0$ | p  | df | $H_0$ |
| A            | 6.6 | none     | 568    | 0.085 | 7 | accept$^d$ | 4 x 10^{-10} | 4 | reject |
| C            | 6.2 | none     | 366    | 0.505 | 5 | accept  | 0.016  | 4 | reject |
| B            | 6.6 | TmTn     | 362    | 0.0055 | 5 | reject  | 0.067  | 1 | accept |
| D            | 6.2 | TmTn     | 312    | 0.0003 | 6 | reject  | 7 x 10^{-04} | 3 | reject |
| E            | 5.8 | none     | 592    | 0.0006 | 6 | reject  | 1 x 10^{-06} | 3 | reject |
| F            | 5.8 | TmTn     | 722    | 8 x 10^{-09} | 7 | reject  | 0.0047 | 4 | reject |
| G            | 5.6 | none     | 528    |        |   |         | 6 x 10^{-09} | 3 | reject |
| H            | 5.6 | TmTn     | 277    | 8 x 10^{-07} | 7 | reject  | 0.186  | 4 | accept |

$^a$Results of Pearson’s chi-squared test comparing sample observations with events expected by the cumulative density function that best fits observed events.

$^b$Test of the null hypothesis ($p < 0.05$) for the exponential distribution.

$^c$Test of the null hypothesis ($p < 0.05$) for the gamma distribution.

$^d$Accept throughout more precisely means cannot reject.

Proposing that force contributes to the transition state

Crucial to our proposal is an adiabatic pathway through a singular transition state that requires a minimum integral force. Based on a change in thin filament compliance (22, 23, 35), we suggest that the transitional force stretches Tm along its length, resulting in a conformation change that allows Tm to transmit force along its length in the M-position (26). The requisite integral force may arise locally by a combination of ternary complex formation and ATP-driven conformational change in myosin; in the intact sarcomere, this could arise remotely by contraction of opposing sarcomeres in series. Acceleration of the mass of Tm by an integral force (Newton’s second law) is nearly instantaneous, assuming negligible inertia (at low Reynolds number). By the applied force, Tm acquires a reference momentum in the chemical potential plane (Fig. 4). Mechanical impulses of cycling crossbridges accelerate Tm relative to the reference (no crossbridges), giving rise to intermediates distinguished by momentum for each distinct combination of ternary complex $m_i$ (pathways 2 and 3 in Fig. 4). Provided that Tm remains stable in the M-position, all combinations of crossbridges incapable of generating the reference momentum of Tm prevent the formation of the transition state. The transition state is delayed until the turnover of cycling crossbridges restores the requisite combination of crossbridges. An elastic force in the Tm polymer that opposes the stretch may destabilize ternary complexes. If ternary complexes collapse by strain,
Mechanical work that opposes thermodynamic work, i.e. $y(t) - y_0(t)$ (Equation 1), requires a framework distinct from thermodynamics for detailed balance. To reconcile delay events with detailed balance, we introduce a quasi-thermodynamic free energy $G'$ (see “Experimental procedures”) in which chemical potential and kinetic energy are variables. The chemical potential, $\mu = \Delta G' / \Delta N$, which includes the transition state, is opposed by kinetic energy $1/2 \, mv^2$, which is relative to the reference momentum of the transition state. Detailed balance of a Markov Chain (paths 1–3 in Fig. 4) has an analytical solution (Equation 2) which does not include the diabatic pathway (pathway 4 in Fig. 4). Hence, Equation 2 is valid only with respect to microscopic reversibility. It is noteworthy with how we model prolonged run times. Drag forces acting on the gliding filament may also affect the relative density of run times. A potential complication that arises from the stored energy is released irreversibly in a diabatic pathway (pathway 4 in Fig. 4).

Mechanical work that opposes thermodynamic work, i.e. $y(t) - y_0(t)$ (Equation 1), requires a framework distinct from thermodynamics for detailed balance. To reconcile delay events with detailed balance, we introduce a quasi-thermodynamic free energy $G'$ (see “Experimental procedures”) in which chemical potential and kinetic energy are variables. The chemical potential, $\mu = \Delta G' / \Delta N$, which includes the transition state, is opposed by kinetic energy $1/2 \, mv^2$, which is relative to the reference momentum of the transition state. Detailed balance of a Markov Chain (paths 1–3 in Fig. 4) has an analytical solution (Equation 2) which does not include the diabatic pathway (pathway 4 in Fig. 4). Hence, Equation 2 is valid only with respect to microscopic reversibility. It is noteworthy with regard to reversibility that Equation 2 is the Hill equation (36) for the special case of $\alpha = 1$.

To test the proposal that activation responds to mechanical force, we varied the density of HMM on the surface of the flow cell. Previous results showed that complexes of actin, Tm, and Tn (regulated actin) were propelled at submaximal measured speed if the concentration of HMM was sufficiently low during immobilization on the surface of the flow cell (37). As Liang et al. did (37), we incubated surfaces with 50 and 200 $\mu$g/ml HMM. Compared with the distribution of run times obtained with 200 $\mu$g/ml HMM, the surface prepared with 50 $\mu$g/ml HMM supported relatively greater density of short run times (Fig. 5). This correlation between relative density of long run times and relative density of HMM on the surface is expected if the nonthermodynamic force that prolongs a run is mechanical, as we propose (Fig. 4). A plot of the cumulative density of run times shows that the time required to reach maximum den-

**Figure 4.** Schematic for proposed mechanical mechanism. Depicted is a quasi-thermodynamic Gibbs free energy space ($G'$). It includes probabilities of intermediates corresponding to macroscopic states of the thin filament (see “Experimental procedures”): C (red), M (green), and M (green). A saddle point representing the transition state of an adiabatic pathway (1) is depicted as having a unique combination of ternary complexes composed of myosin (ovals), Tm (green), and filamentous actin (filled circles). Other combinations of ternary complexes give rise to intermediates, $m_1 = m_2$, $m_2$, $m_3$, $m_4$, ..., have insufficient momentum and, hence, lack the transition state. Changes in the mass of stationary ternary complexes account for acceleration among intermediates (2, 3). The possibility that $m_4$ collapse by a diabatic pathway is included (4). A Markov Chain summarizes possible transitions between states.

**Figure 5.** Varying HMM alters the run time response. Only the density of HMM was varied by adding either 50 $\mu$g/ml (blue) or 200 $\mu$g/ml (green) HMM protein during the surface-forming step of the motility assay. Regulated actin filaments in the assay were a 1:1.5 molar mixture of sheared Rh-Ph-actin and TmTn complex. Motility was measured in the pCa 5.8 solution described for Fig. 1 except the ionic strength was increased from 37 mM to 50 mM by adding KCl. Distributions of relative density (blue and green) were compiled by algorithm (Matlab fitdist) from 547 and 464 raw run times determined by visual tracking of regulated actin filaments, respectively. Inset, empirical cumulative density functions of raw run times (blue and green) were compiled by algorithm (Matlab ecdf). These data were fit with Equation 1 (black), where $\alpha = 0$ (left) or $\alpha = 3$ (right) and parameters, $P_T$, $\tau_0$, and $\tau_T$ are constant (0.3, 30, 30, respectively).

Discussion

We describe a controlled laboratory study to test whether a thermodynamic mechanism fully accounts for the rate by which thin filaments switch in binary fashion. The test depends on being able to distinguish between observed and expected run time responses. A potential complication that arises from matching the expected exponential density function is that crucial contradictory observations are lost in the temporal resolution, total duration, and field of view for the recordings. However, our records include observations that fit the expected exponential density function, which serves as a positive control for contradictory observations in other conditions. The visual...
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tracking method used here to determine the duration of run times is subject to human error. We pooled data from multiple, naïve volunteers to mitigate conscious and unconscious bias. However, the most powerful control was unanticipated, namely, adding TmTn to the same sample shifted the sample distribution away from exponential density functions. Hence, internal controls raise confidence in the reliability of the results.

The dynamic clusters of fluorescent myosin dispersed along individual thin filaments (31) demonstrate a regulatory unit consisting of a focus of Tm subunits rather than the entirety of the Tm strand. We represent a regulatory unit as $S_i$ comprising both deactivated $S_1$ and activated $S_2$ states (see “Experimental procedures”). Studies showing that the density of $S_1$ states with open sites for myosin-binding increases as $Ca^{2+}$ binding shifts equilibrium distribution between B- and C-positions (5–8) are consistent with a constant rate of fluctuation. The assumption that the density of open states in C-position should be proportional to activation holds only if a delay does not introduce another rate of fluctuation. A delay in $S_2$ localizes M-position to a focal site where a cluster of myosins could bind sites held open by ternary complexes (31). The fluctuation rate of $S_1$ is important in maintaining relatively homogeneous myosin-binding at low $Ca^{2+}$ (31). If our interpretation of the transition state is valid, the impulse rate of cycling myosin must match the density of open binding sites at a given moment to achieve the state required for the switch to $S_2$. A delayed return to an even distribution of myosin along the filament causes available myosin to distribute in foci representing regulatory sites in M-position. Active redistribution of myosin to achieve activation is supported by observations that filaments in the motility assay remain attached to the myosin surface during both runs and pauses, and that varying $Ca^{2+}$ produces a large change in activation but negligible change in bound, cycling myosins (20). A turnover of myosin during a delay ($mi$, in Fig. 4) fits with dynamic cluster formation and the migration of clusters along the thin filament (31).

Mechanical force is a context for future studies based on gamma distributed run times we observed. Waiting time inherent in the gamma distribution is evidence that a thermodynamic switch from activation is prevented for a random interval of time. Chemical intermediates, which are depicted as points on the thermodynamic plane (GN) of Fig. 4, have temporal probability determined by the Poisson distribution. Hence, intermediates of the crossbridge cycle, which likely alter average run length, cannot explain how the distribution of run times deviates from exponential. Mechanical force, which we tested but did not rigorously demonstrate, may be the only nonthermodynamic force (Fig. 4) in the motility assay to explain waiting time. We propose two thermodynamic and one kinetic state as the minimum requirements for the waiting time we detected (Fig. 4, inset). Our proposal includes the possibility of microscopic reversibility, which must be demonstrated separately. We show that the Hill equation (36), which is commonly used to fit activation data, is related to our parsimonious model (Fig. 4, inset) through detailed balance (Equation 2). The existence of a mechanical component of activation depends on neither the accuracy of reaction pathways and diagrams of states (Fig. 4) nor our conjecture that a transition state of the thin filament requires integral force of cycling crossbridges to alter the structure of Tm.

The simplest proposal consistent with our results is that regulatory units activate independently. Although it follows that simultaneously active regulatory units are redundant with respect to unloaded sliding of a thin filament, redundancy can regulate isometric tension. Strong interactions among regulatory units explain how isometric tension becomes less cooperative as native Tn is replaced with a $Ca^{2+}$-insensitive mutant (38). However, Equation 2 (see “Experimental procedures”), which includes the delay mechanism described here (Equation 1), also explains these data (26). The proposals differ in the mechanism that accounts for the sigmoidal shape of response to $Ca^{2+}$. Because the sigmoidal shape could arise by Ising/allosteric mechanisms (13, 14) and also by cumulative probability density (Fig. 3), neither mechanism has an advantage in explaining the cooperative response to incremental changes in $Ca^{2+}$ concentration. However, preventing random interruptions in activation may be a selectable advantage for the investment of mechanical energy.

Independent activation of regulatory units predicts that sarcomere shortening occurs at $V_{max}$ when thin filament regulatory units are randomly unblocked (39). Maximum sliding speed of individual thin filaments in a motility assay is determined by the density of cycling crossbridges in the vicinity of the filament as it slides (37). However, speed is reported as intermediate and not maximum if $Ca^{2+}$ concentration is set within the same narrow range in which random switching is most prevalent (30). Filaments chosen for speed measurement move for a standard interval of time judged to be continuous displacement (30). Continuity based on time assumes that sampling rate excludes roughly the same fraction of pauses for all $Ca^{2+}$ concentrations. This assumption is reasonable if the frequency of pauses declines exponentially with time and if sufficient time is given for an exceptional run. However, our results show that the density of pauses giving rise to short run times varies with $Ca^{2+}$ (Fig. 1). As $Ca^{2+}$ concentration increases, the cumulative probability shifts significantly to longer time intervals and the rate of observed pauses changes from 19 to 0.5 s$^{-1}$ (Fig. 3). This 38-fold decrease also applies to intervals ($t + r$) that are too short to measure for the given sampling rate of the recording instrument. By reducing the average number of undetected pauses, the computed displacement of the filament per unit time increases with incremental change in $Ca^{2+}$ concentration. In effect, the titration with $Ca^{2+}$ measures the change in switching rate at fixed sampling rate.

Relatively unlikely short run times contradict a purely thermodynamic mechanism for thin filament regulation and reveal a role for a mechanical force to delay exit from the activated state. A model in which an applied mechanical force is essential for the transition state explains random myosin binding without proportional activation. The delayed deactivation we propose is an alternative to the mass action explanations for cooperative $Ca^{2+}$ activation of striated muscle contraction. We introduce, for future study, a quasi-thermodynamic potential to account for nonthermodynamic mechanical work that preserves detailed balance. The amplification achieved by adding
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TmTn in our assay manifests the search for other agents that prolong activation. A complete analytical description of activation appears to be a tractable opportunity.

Experimental procedures

Thermodynamic description of the system

Of the mechanism that regulates thin filaments, we are testing only whether the binary switching between run and pause in a motility assay is thermodynamic. Here we describe how a canonical ensemble of states in thermodynamic equilibrium relates to an exponential function of elapsed time, \( P'(t) = e^{-\lambda t} \), where \( \lambda \) is a rate constant. From the exponential distribution used to fit experimental observations, \( 1 - P'(t) \) is the cumulative density function and \( d(1 - P')/dt \) equals the probability density function, \( \lambda P'(t) \). The probability density function is a sample space in which each point is infinitesimal containing statistically framework is consistent with empirically derived Maxwell-Boltzmann, may apply. The Maxwell-Boltzmann i.e., and, hence, \( \Delta G \nabla \) restricts \( S \) to \( S \), causing \( P_r = 1 \) during the delay. To end a run from a delay requires that \( s' \) be restored before an equilibrium change can occur. During the delay, \( P_r = 1 \) and \( P_p = 0 \), but \( P_r \) returns with rate \( \tau_r \), which arises from the rate-limiting step of a cycle of force rather than \( \Delta G / \Delta G \). The Poisson probability of a pause (not \( O_p \)) returns by a nonthermodynamic force at constant rate given by \( d(1 - P_p)/dt = \frac{P_r}{\tau_r} \). The probability of an uninterrupted switch in interval \( t + \tau \) is a function of \( \tau \), \( P(\tau_r) = O_p e^{-\tau_r} \).

In a canonical ensemble, \( P_p \) and \( P_r \) represent probabilities of macroscopic states \( S \), embedded into a closed system having total energy, \( E \), commonly referenced as the heat bath. Because \( S \) belongs to the same thin filament, which moves as independent particle in the assay, the simplest statistical mechanics, i.e., Maxwell-Boltzmann, may apply. The Maxwell-Boltzmann statistical framework is consistent with empirically derived \( S \), such as \( S_1 \) includes all states associated with B- and C-positions of \( T \) and \( S_2 \) includes only ternary complexes in \( M \)-position. Microscopic states of \( S \), with energy, \( E_i \), are equally probable by the fundamental theorem of statistical mechanics, and, hence, \( Z = \sum_i e^{-E_i/kT} \) represents the total entropy of \( S \).

Letting \( S_1 \) and \( S_2 \) represent deactivated and activated macroscopic states, \( P_p = 1 \), \( P_r = 1 \) gives the expression for \( e^{-E_i/kT} \) in terms of the \( i \)th state. The integral, \( \int dy = \frac{\alpha}{\alpha + \beta} \left( \frac{e^{-(\alpha / \beta)}}{e^{-(\alpha / \beta)}} - e^{-(\alpha / \beta)}t \right) - e^{-(\alpha / \beta)} \) determines total power and the ratio, \( y(t)/y(t)dt \), gives the cumulative probability of a pause during a run.

Accounting for detailed balance

A previously described relationship includes the contribution of mechanical work described in Equation 1 in accounting for activation that varies with \( \text{Ca}^{2+} \) (42, 43). This relationship determines probabilities of thin filament states, \( C \) and \( M \), giving rise to eponymous positions, as reproduced here

\[
M = K_0 C (1 + (\alpha - 1) M)^n \quad \text{(Eq. 2)}
\]

where \( M \) represents the probability of activation, \( \alpha \) represents an average number delay events during activation, \( C \) represents the probability of states that arise from blocking states, \( B \), by simple mass action response to variable \( \text{Ca}^{2+} \) concentration, and \( n \) represents ternary complexes required for a single regulatory unit to activate. If \( \alpha = 0 \), \( M/C \) (Equation 2) accounts for a pure thermodynamic equilibrium in which \( \Delta G = -RT \ln (K_0(1 - M)) \). The \( M/C \) change is quasi-thermodynamic for \( \alpha > 0 \) (Eq. 2) and \( \Delta G' = -RT \ln (K_0(1 - (\alpha - 1) M)) \). Because \( M/C \) ratio (Equation 2) determines \( O_p \) of Equation 1 and \( a \) (Equation 2) determines \( \alpha \) of Equation 2, \( O_p \) and \( a \) interact in the context of \( \Delta G' \). As described, Equation 2 arises from the detailed balance of either a thermodynamic or quasi-thermodynamic free energy change.
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Preparation of native thin filaments, myosin, and acetone powder

All preparations derive from fast skeletal muscle of freshly exsanguinated rabbit. Animals were housed and euthanized in accordance with procedures approved by the Florida State University Animal Care and Use Committee (Protocols 1435 and 1533).

To prepare myofibrils, muscle was homogenized (Polytron) in 3 volumes ice-cold 100 mM potassium phosphate, 1 mM EGTA, 8 mM Mg²⁺ acetate, and 1 mM DTT, pH 7.0, and washed four times with the same buffer by sedimentation and resuspension. Native thin filaments were prepared from a small portion of the residue (40) and the preparation was stored 4°C with 1 mM Na₂SO₄ added for up to 3 days. We prepared myosin from the remainder of the residue using standard procedure (41) but without the final ammonium sulfate fractionation step (42). Purified myosin was solubilized by repeated dialysis in 0.6 M KCl, 5 mM EDTA, 0.2 mM DTT, pH 7.0, clarified by centrifugation (39,000 × g), and stored as a 50% mixture of glycerol at −20°C. Acetone powder was prepared from the myofibril residue after myosin extraction (43).

Protein preparation

We prepared actin and TmTn complex from acetone powder as described (44) and stored on ice in AB (35). Actin was polymerized and stored in the filamentous form (44). We prepared the soluble head portion of myosin, HMM, from purified myosin stored in 50% glycerol. Myosin was diluted with 10 volumes of cold 1 mM DTT and the polymerized myosin filaments were collected by sedimentation (12,000 × g). Myosin of the pellet was digested with chymotrypsin and HMM was recovered from the residue as described (45). HMM was pretreated to remove presumable dead heads as described (29). We prepared fresh HMM for each day of use.

Fluorescently labeled filaments

RhPh-actin and RhPh-native thin filaments were incubated with rhodamine phalloidin overnight on ice or 1 h at 37°C as described (42). Sheared filaments were prepared by repeatedly passing RhPh-actin through a 22-gauge needle.

Motility assay conditions

All solutions used to support and record filament movement contained 20 mM MOPS, 2 mM EGTA, 3 mM free MgCl₂, 2 mM MgCl₂-ATP, and KOH to achieve pH 7.0. Variable CaCl₂ was included to achieve predetermined free Ca²⁺ concentrations (46). We designate a motility buffer, MBx, where x represents the pCa.

We performed motility assays on nitrocellulose-coated coverslips contained in flow cells using a standard procedure (42) with modification (47) and the following customizations and clarifications. In place of AB (42), we used MB8.0. A motile surface was prepared by exposing the nitrocellulose surface in the flow cell to excess HMM (60–130 µg/ml) in MB8.0 supplemented with 2 mM ATP. Filaments were recorded in MB containing the anti-fading mixture GOC (42). To assay actin motility, sheared RhPh–actin filaments were used in place of RhPh–thin filaments.

In a preliminary study, we tested the function of TmTn preparation in a control motility assay. Sheared actin filaments (33 µg/ml) were combined with excess TmTn (100 µg/ml) and recorded in the standard motility assay containing either pCa 4.0 or pCa 7.5. Reconstituted filaments moved continuously at pCa 4.0 but remained mostly stationary at pCa 7.5 (Movie S4), confirming that the TmTn preparation can regulate actin as expected.

We conducted a second round of recording with added TmTn complex. Native thin filaments and 100 µg/ml TmTn were incubated at 30 min on ice in MB8.0/GOC. After motility was recorded in the first round, the flow cell was rinsed first with MB8.0/GOC without ATP and, second, with MB8.0/GOC supplemented TmTn for a second round of recording.

The video microscopy system was described previously (48), except that fluorescent filaments were illuminated using 532-nm laser line source. Real time images were captured at 30 frames/sec and recorded on DVD.

Image analysis and statistical analyses

DVD recordings were converted to AVI format and processed into video stacks of 1000 frames as described (48). NIH Image J (RRID:SCR_003070) was used to convert AVI to tif or MP4 file formats. We used video processing functions of Icy (RRID:SCR_010587) to track spots representing centroids of individual filaments, measure the speed along spot trajectories, and generate binary sequences of images. We used speed algorithm to determine the maximum speed of individually tracked filaments.

Undergraduate student volunteers tracked movement of individual filaments visually. Using a video display interface (NIH/Image) and a unique identifier for each filament recorded on the first frame, a volunteer followed individual filaments in a video sequence (1000 frames) of binary images. Records for each filament included the video frame of a switch between a run and pause. Frames before the first and after the last observed switch, either to a run or to a pause, were excluded from the analysis. Likewise, frames interrupted by detachment from the surface and reattachment, filament moving out of the field of view, and tracks in which a filament either did not move or moved continuously for the duration of the video sequence were excluded. Extended runs that left the field of view were not counted, and, hence, long runs are relatively undercounted. Given these rules, run times were measured as the number of video frames between pauses and run times from all filaments were combined for each free Ca²⁺ condition.

Raw observed run times were analyzed with statistical algorithms in MatLab. We used distributionFitter to calculate the probability density of observed run times, distribute density into 40 bins, and obtain the maximum likelihood fit of exponential and gamma probability density functions. Pearson’s chi-squared statistic was used to compare the densities of observed sample of run times with the density determined by the maximum likelihood fit. The program chi2gof returned the probability that sample observations came from expected
cumulative density functions for exponential or gamma distributions. We set \( p < 0.05 \) as the limit to reject the null hypothesis. We compared the outcomes of the goodness-of-fit test and visual representation of the sample distribution in the analysis as described in the experimental design.

Data availability
Data shared upon request. Send inquiries to Henry Zot (hzot@westga.edu).

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: Tm, troponymosin; Tn, troponin; R, gas constant (molar); T, thermodynamic temperature.

References
1. McKillop, D. F. A., and Geeves, M. A. (1993) Regulation of the interaction between actin and myosin subfragment 1: Evidence for three states of the thin filament. *Biophys. J.* 65, 693–701 CrossRef Medline
2. Lehman, W., Hatch, V., Korman, V., Rosol, M., Thomas, L., Maytum, R., Geeves, M. A., Van Eyk, J. E., Tobacman, L. S., and Craig, R. (2000) Tropomyosin and actin isoforms modulate the localization of tropomyosin strands on actin filaments. *J. Mol. Biol.* 302, 593–606 CrossRef Medline
3. Potter, J. D., and Gergely, J. (1974) Troponin, tropomyosin, and actin interactions in the Ca\(^{2+}\) regulation of muscle contraction. *Biochemistry* 13, 2697–2703 CrossRef Medline
4. Galińska-Rakoczcy, A., Engel, P., Xu, C., Jung, H., Craig, R., Tobacman, L. S., and Lehman, W. (2008) Structural basis for the regulation of muscle contraction by troponin and tropomyosin. *J. Mol. Biol.* 379, 929–935 CrossRef Medline
5. Vibert, P., Craig, R., and Lehman, W. (1997) Steric-model for activation of muscle thin filaments. *J. Mol. Biol.* 266, 8–14 CrossRef Medline
6. Pirani, A., Xu, C., Hatch, V., Craig, R., Tobacman, L. S., and Lehman, W. (2005) Single particle analysis of relaxed and activated muscle thin filaments. *J. Mol. Biol.* 346, 761–772 CrossRef Medline
7. Risi, C., Eijsner, J., Belknap, B., Heeley, D. H., White, H. D., Schröder, G. F., and Galkin, V. E. (2017) Ca\(^{2+}\)-induced movement of troponymosin on native cardiac thin filaments revealed by cryoelectron microscopy. *Proc. Natl. Acad. Sci. U. S. A.* 114, 6782–6787 CrossRef Medline
8. Matusovsky, O. S., Mansson, A., Persson, M., Cheng, Y.-S., and Bassign, D. E. (2019) High-speed AFM reveals subsecond dynamics of cardiac thin filaments upon Ca\(^{2+}\) activation and heavy meromyosin binding. *Proc. Natl. Acad. Sci. U. S. A.* 116, 16384–16393 CrossRef Medline
9. Eaton, B. L. (1976) Tropomyosin binding to F-actin induced by myosin heads. *Science* 192, 1337–1339 CrossRef Medline
10. Bremer, R. D., and Weber, A. (1972) Cooperation within actin filament in vertebrate skeletal muscle. *Nat. New Biol.* 238, 97–101 CrossRef Medline
11. Greene, L. E., and Eisenberg, E. (1980) Cooperative binding of myosin subfragment-1 to the actin-troponin-tropomyosin complex. *Proc. Natl. Acad. Sci. U. S. A.* 77, 2616–2620 CrossRef Medline
12. Eisenberg, E., Hill, T. L., and Chen, Y. (1980) Cross-bridge model of muscle contraction: Quantitative analysis. *Biophys. J.* 29, 195–227 CrossRef Medline
13. Hill, T. L., Eisenberg, E., and Greene, L. E. (1980) Theoretical model for the cooperative equilibrium binding of myosin subfragment 1 to the actin-troponin-tropomyosin complex. *Proc. Natl. Acad. Sci. U. S. A.* 77, 3186–3190 CrossRef Medline
14. Smith, D. A., and Geeves, M. A. (2003) Cooperative regulation of myosin-actin interactions by a continuous flexible chain II: Actin-troponymosin-troponin and regulation by calcium. *Biophys. J.* 84, 3168–3180 CrossRef Medline
15. Zot, H. G., and Potter, J. D. (1987) Calcium binding and fluorescence measurements of dansylaziridine-labelled troponin C in reconstituted thin filaments. *J. Muscle Res. Cell. Motil.* 8, 428–436 CrossRef Medline
16. Rosenfeld, S. S., and Taylor, E. W. (1987) The mechanism of regulation of actomyosin subfragment 1 ATPase. *J. Biol. Chem.* 262, 9984–9993 CrossRef Medline
17. Tobacman, L. S., and Sawyer, D. (1990) Calcium binds cooperatively to the regulatory sites of the cardiac thin filament. *J. Biol. Chem.* 265, 931–939 CrossRef Medline
18. Zot, H. G., Güth, K., and Potter, J. D. (1986) Fast skeletal muscle skinned fibers and myofibrils reconstituted with N-terminal fluorescent analogues of troponin C. *J. Biol. Chem.* 261, 15883–15889 CrossRef Medline
19. Brandt, P. W., and Poggesi, C. (2014) Clusters of bound Ca\(^{2+}\) initiate contractin in fast skeletal muscle. *Arch. Biochem. Biophys.* 552–553, 60–67 CrossRef Medline
20. Chalovich, J. M., and Eisenberg, E. (1982) Inhibition of actomyosin ATPase activity by troponin-tropomyosin without blocking the binding of myosin to actin. *J. Biol. Chem.* 257, 2432–2437 CrossRef Medline
21. Brenner, B. (1988) Effect of Ca\(^{2+}\) on cross-bridge turnover kinetics in skinned single rabbit psoas fibers: implications for regulation of muscle contraction. *Proc. Natl. Acad. Sci. U. S. A.* 85, 3265–3269 CrossRef Medline
22. Huxley, H. E., Stewart, A., Sosa, H., and Irving, T. (1994) X-ray diffraction measurements of the extensibility of actin and myosin filaments in contracting muscle. *Biophys. J.* 67, 2411–2421 CrossRef Medline
23. Wakabayashi, K., Sugimoto, Y., Tanaka, H., Ueno, Y., Takezawa, Y., and Amemiya, Y. (1994) X-ray diffraction evidence for the extensibility of actin and myosin filaments during muscle contraction. *Biophys. J.* 67, 2422–2435 CrossRef Medline
24. Martyn, D. A., Chase, P. B., Regnier, M., and Gordon, A. M. (2002) A simple model with myofilament compliance predicts activation-dependent crossbridge kinetics in skinned skeletal fibers. *Biophys. J.* 83, 3425–3434 CrossRef Medline
25. Chase, P. B., Macpherson, J. M., and Daniel, T. L. (2004) A spatially explicit nanomechanical model of the half-sarcomere: myofilament compliance affects Ca\(^{2+}\)-activation. *Ann. Biomed. Eng.* 32, 1559–1568 CrossRef Medline
26. Zot, H. G., Hashun, J. E., and Van Minh, N. (2009) Striated muscle regulation of isometric tension by multiple equilibria. *PLoS One* 4, e8052 CrossRef Medline
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27. Kron, S. J., and Spudich, J. A. (1986) Fluorescent actin filaments move on myosin fixed to a glass surface. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 6272–6276 CrossRef Medline
28. Honda, H., and Asakura, S. (1989) Calcium-triggered movement of regulated actin in vitro. A fluorescence microscopy study. *J. Mol. Biol.* **205**, 677–683 CrossRef Medline
29. Fraser, I. D. C., and Marston, S. B. (1995) *J. Biol. Chem.*
30. Homsher, E., Kim, B., Bobkova, A., and Tobacman, L. S. (1996) Calcium and Chase, P. B. (2003) Ca2⁺ sensitization on myofilament lattice spacing and cross-bridge mechanics in mouse cardiac muscle. *J. Mol. Cell. Cardiol.* **35**, 183–198 CrossRef Medline
31. Desai, R., Geeves, M. A., and Kad, N. M. (2015) Using fluorescent myosin and troponin C-mediated Ca²⁺ sensitization on myosin-coated surfaces. *Methods Enzymol.* **318**, 540–558 CrossRef Medline
32. Korobkova, E. A., Emonet, T., Park, H., and Cluzel, P. (2006) Hidden stochastic nature of a single bacterial motor. *Phys. Rev. Lett.* **96**, 058105 CrossRef Medline
33. Reck-Peterson, S. L., Yildiz, A., Carter, A. P., Gennerich, A., Zhang, N., and Vale, R. D. (2006) Single-molecule analysis of dynein processivity and stepping behavior. *Cell* **126**, 335–348 CrossRef Medline
34. Woody, M. S., Lewis, J. H., Greenberg, M. J., Goldman, Y. E., and Ostap, E. M. (2016) MEMLET: An easy-to-use tool for data fitting and model comparison using maximum-likelihood estimation. *Biophys. J.* **111**, 273–282 CrossRef Medline
35. Huxley, A. F. (1957) Muscle structure and theories of contraction. *J. Physiol.* **191**, 399–486 CrossRef Medline
36. Hill, A. V. (1910) The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. *J. Physiol.* **40**, lv–vii
37. Liang, B., Chen, Y., Wang, C. K., Luo, Z., Regnier, M. A., Gordon, A. M., and Chase, P. B. (2003) Ca²⁺ regulation of rabbit skeletal muscle thin filament sliding: role of cross-bridge number. *Biophys. J.* **85**, 1775–1786 CrossRef Medline
38. Regnier, M., Rivera, A. J., Wang, C. K., Bates, M. A., Chase, P. B., and Gordon, A. M. (2002) Thin filament near-neighbour regulatory unit interactions affect rabbit skeletal muscle steady-state force-Ca²⁺ relations. *J. Physiol.* **540**, 485–497 CrossRef Medline
39. Huxley, A. F. (1957) Muscle structure and theories of contraction. *Prog. Biophys.* **7**, 255–318 CrossRef Medline
40. Lehman, W., Vibert, P., Uman, P., and Craig, R. (1995) Steric-blocking by troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* **251**, 191–196 CrossRef Medline
41. Pollard, T. D. (1982) Myosin purification and characterization. *Meth. Cell Biol.* **24**, 333–371 CrossRef Medline
42. Kron, S. J., Toyoshima, Y. Y., Uyeda, T. Q., and Spudich, J. A. (1991) Assays for actin sliding movement over myosin-coated surfaces. *Methods Enzymol.* **196**, 399–416 CrossRef Medline
43. Spudich, J. A., and Watt, S. (1971) The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the troponymotroponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* **246**, 4866–4871 CrossRef Medline
44. Cooper, J. A., and Pollard, T. D. (1982) Methods to measure actin polymerization. *Methods Enzymol.* **85**, 182–210 CrossRef Medline
45. Lowey, S., and Cohen, C. (1962) Studies on the structure of myosin. *J. Mol. Biol.* **4**, 293–308 CrossRef Medline
46. Dweck, D., Reyes-Alfonso, A., Jr., and Potter, J. D. (2005) Expanding the range of free calcium regulation in biological solutions. *Anal. Biochem.* **347**, 303–315 CrossRef Medline
47. Gordon, A. M., LaMadrid, M. A., Chen, Y., Luo, Z., and Chase, P. B. (1997) Calcium regulation of skeletal muscle thin filament motility in vitro. *Biophys. J.* **72**, 1295–1307 CrossRef Medline
48. Loong, C. K. P., Takeda, A. K., Badr, M. A., Rogers, J. S., and Chase, P. B. (2013) Slowed dynamics of thin filament regulatory units reduces Ca²⁺-sensitivity of cardiac biochemical function. *Cell Mol. Bioeng.* **6**, 183–198 CrossRef Medline