Viewpoint

The mechanism of thin filament regulation: Models in conflict?

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Running title: Two or three states of the thin filament?

Key words: actin, myosin, tropomyosin, troponin, muscle regulation

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ABSTRACT

In a recent article in this journal, Heeley and colleagues (Heeley, White, and Taylor 2019 J Gen Physiol 151, 628-634) reopened the debate about 2 vs 3 state models of thin filament regulation. The authors review their work, which measures the rate constant of P_i release from myosin.ADP.Pi activated by actin or thin filaments under a variety of condition. They conclude that their data can be described by a 2-state model and raise doubts about the generally accepted 3-state model as originally formulated by McKillop and Geeves (*Biophysical Journal* 65: 693–701, 1993). However, in the following article, we follow Plato's dictum that "twice and thrice over, as they say, good it is to repeat and review what is good". We have therefore reviewed the evidence for the 3- and 2-state models and present our view that the evidence is overwhelmingly in favor of three structural states of the thin filament, which regulate access of myosin to its binding sites on actin and, hence, muscle contractility.

Summary Sentence: Evidence on 2 & 3-state models of the calcium regulation models of muscle contractions remain in favor of 3-states of the thin filament.

INTRODUCTION

In a recent paper in this Journal, Heeley et al. (D. H. Heeley, White, and Taylor 2019) argue that investigation of thin filament activation of myosin ATPase either by transient kinetic or by equilibrium binding studies can lead to conflicting models of muscle regulation. They proceed to provide evidence for such inconsistency derived from kinetic studies of P_i release from actin.myosin following ATP hydrolysis. From their analysis, they go on to propose that two thin filament activity states are sufficient to explain regulation of the process, in conflict with the widely accepted McKillop-Geeves 3-state model (McKillop and Geeves 1993).

Background

The argument over whether two or three regulatory states govern muscle contractile activity is reminiscent of similar differences discussed earlier in the *Biophysical Journal* in 2002 (Geeves and Lehrer 2002, Chalovich et al. 2002) and evaluated further in 2012 (Geeves 2012). Thus, the conflict between opposing interpretation of raw and modeled data is not a new one and apparently differences have not been resolved. Nonetheless, understanding the molecular steps involved in controlling myosin motor activity on muscle thin filaments, i.e. the purpose of such experimentation and modeling, is of significant biomedical importance and deserves revisiting. Here, we outline the basis of the two models before critically evaluating key elements supporting the models.

It is generally accepted that activation of the muscle contractile machinery occurs in steps. Influx of calcium ions into the muscle sarcoplasm triggers the process but is insufficient to fully switch-on the interaction of myosin and actin to result in force generation. Evidence that calcium, itself, is not enough to fully activate the thin filament, and that myosin is needed as a modulator (not just as an enzyme), came from seminal studies on the kinetics of actin-myosin ATPase (Weber and Murray 1973, Lehrer and Morris 1982). Conversely, removal of calcium, while triggering the process of relaxation, does not alone result in relaxation; in fact, relaxation lags considerably behind the fall in calcium concentration (Poggesi, Tesi, and Stehle 2005). This is usually interpreted as representing a slow decay in the number of cycling myosin crossbridges that continues to maintain the thin filament in the on-state, well after calcium has been removed. The on-state is prolonged until a critical threshold number of actin-bound crossbridges is passed, which then allows rapid complete relaxation.

The McKillop-Geeves 3-state model

The work of McKillop and Geeves (1993) yielded a formal understanding of the two-step activation/relaxation process described above. By merging the steric-blocking model of regulation proposed by Hanson and Lowy (1964), Moore et al., (1970) and Huxley (1970) with earlier puzzling biochemistry on myosin binding to regulated and unregulated actin, McKillop and Geeves (1993) extended previous work into a more complete and understandable model. In its simplest heuristic formulation, the 3-state hypothesis has proven invaluable in explaining data and formulating new experiments. However, conceptualizing the 3-state model quantitatively can be difficult, since the states are in dynamic equilibrium and are biased in one direction or another by the effects of troponin-binding to actin.tropomyosin, Ca^{2+}-binding to troponin (Tn), myosin-binding to actin.tropomyosin, and the catalysis of ATP hydrolysis by myosin, all compounded in their complexity by interdependent cooperative and allosteric effects (Geeves and Lehrer 1994, Lehrer and Geeves 1998, Mijailovich, Kayser-Herold, et al. 2012). Still, while the McKillop and Geeves (1993) work itself is widely cited, it may not be as widely read or even understood implicitly in simplified form (Geeves 2012, Lehman 2017). This dissonance between general acceptance and overall assessment leaves the work exposed to challenge. In fact, the recent opinion piece in the journal (Heeley et al., 2019), as mentioned, questions the validity of the three-state model,

The question, as posed by Heeley et al, is itself somewhat ironic since the 3-state model was originally proposed to account for discrepancies between equilibrium and transient kinetic myosin binding results which, even when conducted in the same lab with the same proteins, resulted in different, incompatible estimates of the fraction of the actin filament turned on or off in the presence and absence of calcium (McKillop and Geeves, 1993). To explain this discrepancy, 3 actin.tropomyosin structural states which sterically block and unblock myosin binding were proposed, whose occupancy quantitatively depended on calcium and myosin occupation (McKillop and Geeves, 1993) (see Fig 1 and Table 1 for details). The three states were originally termed Blocked, Closed and Open but the names have altered slightly as the three states have been gradually become more connected to three structural states observed in X-ray fiber diffraction studies and electron microscope reconstructions (Holmes 1995, Vibert el al 1997). The Blocked or B-State is one in which the majority of the myosin binding site on actin is blocked by tropomyosin (Tpm), and this predominates in the absence of calcium, although the other states are also present in the equilibrium mix of states. The Calcium-induced, Closed or C-state is one in which calcium removes the interaction of TnI with actin.Tpm and allows Tpm to revert to its most favorable binding site on actin, thereby exposing much but not all of the myosin binding site on actin. Note that the binding site for Tpm is not a fixed, well

defined site but a shallow free energy well which allows frequent thermally driven excursions of Tpm over the surface of actin away from the shallow minima (Orzechowski et al. 2014, Kiani et al. 2019). In the McKillop and Geeves estimation, Tpm in a calcium loaded thin filament spends ~ 80% of its time in the C-state. In the C-state, myosin exhibits limited binding to actin, Tpm is in a position to block complete binding, and in some structural interpretations Tpm would impede myosin cleft closure (Lorenz et al. 1995, Poole et al. 2006). To allow myosin to bind into the well-defined rigor conformation, Tpm needs to move further away from the Blocked position to the Myosin induced (M) or Open (O) state. Whether tropomyosin accesses this site under its own thermal motion or is physically displaced by myosin binding remains an open debate. But in the McKillop-Geeves model, 20% of Tpm will be in the O-state at saturating calcium in the absence of any myosin binding.

Table 1 sets out the fractional occupation of actin sites in the three states under a variety of conditions. Note however that this simple outline neglects the degree of cooperativity between the various players, i.e. Calcium with TnC; TnC with TnI; TnI with actin; Tpm with myosin; and actin with Tpm. In addition, the calcium-TnC complex and actin compete with each other for TnI binding, while myosin and TnI compete with each other for actin.Tpm. Thus, each of the players is connected to all of the others directly or indirectly through TnI. Estimates of the cooperativity in the system suggest that TnI and one strongly bound myosin control seven actin sites (the size of a single Tpm). A single TnI binding to actin.Tpm will put seven actin binding sites into the B-state, while a single strongly bound myosin anywhere in the vicinity will displace one TnI from actin.Tpm and put seven actin sites into the O or M-state. If TnI has already been displaced by calcium binding, then a single myosin binding to an actin.Tpm will put up to fourteen actin sites into the M-state.

The Heeley et al. 2-state model.

The Heeley et al. review of their previously published work dealt with measurement of the rate of P_i release from the myosin crossbridge once rapidly mixed with an excess of actin (either pure actin or troponin-Tpm regulated thin filaments). In these data, P_i release is accelerated by actin and the degree of acceleration is a function of the presence of calcium and tightly bound myosin heads. This is in agreement with the widely held view that calcium alone is insufficient to fully activate the thin filament. Crucially, however, the data of Heeley et al. show that strongly bound myosin heads alone are also unable to fully activate the thin filament, and this

observation contradicts expectations of the 3-state model as well as earlier biochemistry. Such a surprising observation deserves careful evaluation to determine if, indeed, the P_i release data does undermine the 3-state model.

Heeley et al. go on to propose that the thin filaments regulate P_i release directly but not by controlling access of myosin to its binding site on actin, as proposed in the original steric blocking model and incorporated in to the 3-state model. This revives arguments from the 1980's and 1990's of Eisenberg and Chalovich (Chen et al. 2001, Chalovich and Eisenberg 1982), who also favored models in which P_i release was a regulated step in the myosin ATPase pathway, but at the time were formulated without detailed structural data available. Following up on the Eisenberg-Chalovich interpretation and with their own new kinetics, Heeley et al. present a 2-state actin model in which actin notionally is either active, A, (able to promote Pi release from myosin) or inactive, I, (unable to promote Pi release). Both calcium and myosin can bind to both active and inactive actin sites and rebalance the A/I equilibrium of actin towards the active form. See Fig 3 for the 2-state model and Table 2 for a comparison of the occupancy of the states predicted in the Heeley and Eisenberg and Chalovich models.

While the 2-state model of Heeley et al. incorporates many of the features of the earlier 2-state models developed by Hill and Eisenberg (Hill, Eisenberg, and Greene 1980) and developed further by Chalovich and Eisenberg (1982), the model as proposed here does not attempt to address well-established complexities of the cooperative nature of the interactions of calcium and myosin with the filament. The cooperativity of the thin filament was a core element of both the Hill 2-state model and the McKillop-Geeves 3-state model. Similarly the 2-state model as presented by Heeley et al. does not address a structural basis for the regulatory mechanism. It is noteworthy that Heeley et al. have quite distinct estimates for the on (A) and off (I) states from those of the earlier Hill et al model, and the reason for this discrepancy is not addressed (Table 2). In the Heeley et all model the thin filament is 95% in the inactive state 5% in the active state. Calcium or rigor bridges reduce the fraction in the inactive form but not to zero.

DISCUSSION

Any analysis of contrasting kinetic models will inevitably concentrate on two major issues: the details of the experimental evidence, and the model proposed. We will focus first on the nature of the Heeley et al. 2-state model since this is relatively straightforward. Evaluation of the

experimental data of Heeley et al. is more nuanced, which may not be fully appreciated by a non-specialist audience and requires explanation with greater attention to detail.

2-state vs 3-state models

Heeley et al. (2019) suggest that two thin filament activity states are sufficient to explain calcium regulation of the myosin-ATPase activity and thus muscle contraction. However, they do so without acknowledging the wider experimental support for three states or providing a structural basis for their proposed regulatory mechanism. In the following section, we will discuss the structural, physiological and biochemical evidence for the 3-state model. We contend that the experimental data in question can easily be incorporated in a standard 3-state model with modestly adjusted equilibria.

The need for a 3-state regulatory model becomes logically imperative once the accumulated evidence for three structural states of the thin filament is considered. Fiber-diffraction studies of Lorenz et al. (1995) showed that addition of calcium to intact fibers only partially activates the thin filament to produce a closed-state like configuration of tropomyosin on actin that still, in fact, partially obstructs myosin-binding. Further myosin-binding is then necessary to fully activate the filament by producing the open-state structure (Lorenz et al. 1995). EM reconstructions of Vibert, Craig, and Lehman (1997) confirmed these results and later Poole et al. (2006) consolidated results of these approaches to show a strict correspondence between the diffraction studies on fibers and the EM studies on isolated filaments. These structural studies, identifying static configurations presumed to be in the force generation pathway, are not mentioned by Heeley and colleagues. More recently Bershitsky et al. (2017) presented muscle fiber diffraction evidence for the open state in contracting muscle preparations – countering the argument that the open structure is an artefact of rigor-type crossbridges binding actin.

Three identifiable structural states of Tpm on actin with two partially blocking myosin binding (Vibert, Craig, and Lehman 1997, Poole et al. 2006) to actin are not obviously compatible with a two activity state model of the thin filament. More recently, significant progress has been made in defining the dynamics of Tpm movement on actin, which are broadly compatible with the Tpm being in an equilibrium mixture of at least two states under all conditions, except when thin filaments are saturated with myosin crossbridges, as predicted in the 3-state model (cf.(Geeves 2012, Lehman 2016). Such a view is supported by results of EM-reconstructions of thin filaments preserved in the absence and presence of calcium (Pirani et al. 2005, Risi et al. 2017)

and confirmed by assessment of energy landscape plots of tropomyosin transitions across actin (Orzechowski et al. 2014, Kiani et al. 2019).

Given the lack of identifiable structural support for the 2-state model, it is difficult to understand and to formulate a convincing and detailed framework for how myosin binds to actin and then how P_i is released. Conceptualizing how troponin-tropomyosin then regulates the process is even more difficult without a structural context. These events are not a just simple single step binding phenomenon on actin or on regulated thin filaments. Instead, they involve a complex process of docking at the interface between the two proteins, not the least of which is the requirement for a) the major cleft on myosin to close to form the full rigor like interface and b) the docking with actin to be transmitted to the nucleotide pocket to trigger P_i release and the myosin power stroke on actin. These two steps in the actin-myosin cycle may be the same event or represent distinct phenomena. However, in the closed, i.e. calcium-induced, position, Tpm appears to act as a molecular gag sitting between the jaws of the upper and lower 50 kDa domains of myosin (Lorenz et al. 1995, Poole et al. 2006)), incompatible with the closing of the myosin cleft, which means that the calcium-induced C-state cannot be the same as either the Blocked or Open state.

In addition to the structural data just outlined, substantial evidence from mechanical studies of single muscle fibers also requires three thin filament states to fully explain corresponding data. Again, simple observation shows that both myosin crossbridges and calcium are required to fully activate the thin filament not only *in vitro* but also *in situ* (Gordon, Homsher, and Regnier 2000). For example, physiological studies with muscle fibers equivalent to the biochemical studies of Weber and Murray 1973 produced similar force vs pCa curves with a comparable $pCa_{50\%}$ - i.e. a mid-point usually referred to as the calcium sensitivity. This sensitivity is not just found to be an inherent property of the thin filament. It also depends upon the number of crossbridges actively cycling on actin; thus, any treatment of the fibers with an agent (e.g. low ATP or high ADP concentrations) that increases the number of crossbridges attached to actin in the steady-state will increase the calcium sensitivity (i.e. less calcium is required to activate the contraction) and vice-versa for agents that reduce the number of cross bridges (e.g. inhibitors of P_i release during ATP hydrolysis). Complementary calcium dependent kinetics of rigor-like myosin binding to thin filaments shows precisely the same calcium sensitivity (McKillop and Geeves 1993, Boussouf, Maytum, et al. 2007; Boussouf, Agianian, et al. 2007) . Here, the rate of myosin binding to actin varies with calcium concentration, while the $pCa_{50\%}$ parallels that expected for calcium affinity to TnC in the filament which can be altered by treatments that

affect TnI or TnC behavior or, alternatively, by preloading the filament with small amounts of rigor myosin heads bound to actin.

These studies taken as a whole gave rise to a view of Ca^{2+} activation of thin filaments as a classic allosteric system (Lehrer and Geeves 1998) in which myosin and TnI compete for binding to actin.Tpm. In the presence of ATP and low calcium**,** TnI dominates and the system is *off* (actinTpm in blocked-state*)*. In the presence of calcium and ATP, myosin can bind and the system is on (actinTpm in open state). Anything that changes the balance between the TnI and myosin competition will alter the calcium sensitivity. Mutations in myosin or troponin provide a way to explore the rebalancing in relative affinities.

An elegant example of this balance between TnI induced inhibition and myosin-induced activation is a study in *Drosophila* flight muscle by Kronert et al. (1999) on mutations in muscle proteins: for example, a mutation in TnI (*heldup 2*) disrupts the regulation of contraction and causes the muscle to be hypercontractile. This is consistent with the mutant TnI no longer being able to prevent myosin from binding to actin. A second mutation, *D45,* this time in myosin showed a two-fold weaker than normal affinity for actin was hypocontractile, but then in combination with *heldup2* reverted to wildtype phenotype, i.e. the *Drosophila* could fly. Thus, the balance between TnI and myosin binding for actin was restored when both modulators had a similar reduction in affinity for actin.

The evidence for such an allosteric relationship between TnI and myosin accordingly is quite robust. Moreover, Mijailovich, Li, et al. (2012) demonstrated that these studies can be modelled using the 3-state paradigm. Calcium binding to TnC alters the equilibrium between Blocked and Closed states with smaller effects on the equilibrium between Open and Closed states. Other allosteric effectors operating via actin.Tpm.Tn or myosin can be readily incorporated in to this computational system. Current versions of this modeling with three states can generate sarcomere force-pCa curves and twitch contractions under a variety of conditions (Mijailovich, Prodanovic, et al. 2019, Campbell, Janssen, and Campbell 2018). In contrast Mijailovich, Li, et al. (2012) were unable to model the calcium dependence of myosin binding to thin filaments using 2-state thin filament models without imposing unrealistic calcium dependencies on the model parameters~~. Nonetheless, the 3-state model required no modifications to deal with a wide range of pCa values from 9 to 4.5~~. Heeley and colleagues have not presented any data on the calcium dependencies of their own parameters, but based on attempts to by Mijailovich, Li, et al. (2012 to model the calcium dependency of related 2-state models this is unlikely to be successful.

Phosphate release

While the work of Heeley et al. (2019) does raise some important questions requiring serious appraisal, the authors appear to raise the 3-state model as a straw man to be taken down without adequately addressing the entirety of the outstanding evidence. Their presentation relies on measurement of the rate of P_i release from M.ADP.P_i when the complex is rapidly mixed with actin or thin filaments. Figure 3 of Heeley et al. (2019), documenting the cardiac system, indicates that the rate constant of P_i release can be increased from 0.49 s^{-1} in the absence of actin to 36 s^{-1} at high thin filament concentration when both rigor crossbridges and calcium are present (a similar value was seen using pure actin). If just calcium is present, the acceleration is only to 27 s^{-1} or 24 s^{-1} for only rigor bridges present. This result is compatible with the view that both calcium and strong binding myosin bridges are required to achieve maximum acceleration of P_i release, a feature common to both 2- and 3-state models. However, the authors' evidence that rigor bridges alone cannot fully activate the thin filament needs to be considered carefully, since it contradicts the original 3-state regulatory scheme. Two factors complicate a simple interpretation of the effect of strong binding myosin rigor bridges: 1) Regulatory protein motions in each state of the 3-state model should be considered as oscillating back and forth across low energy-barriers (Maytum et al. 2008, Orzechowski et al. 2014); and 2) The cooperativity of the system needs to be defined to allow an assessment of how many myosin bridges are required to bind an actin filament in order to fully activate the system. This latter point is important because in a cooperative system in solution, the myosins will tend to cluster at open-state actin sites. This constraint is different in muscle fibers where filament geometry limits the number of myosin that can bind to each section of an actin filament.

We bear in mind that the experiments of Heeley et al are technically challenging and to date have not been repeated by any other laboratory group. Their approach requires working at very low ionic strength and high actin concentrations (as do most steady-state ATPase assays). The low ionic strength used will affect the stability of electrostatic interactions between either Tpm or TnI and actin, and again between myosin and actin. As we have stated above, this can alter the balance between the cooperative competitive interactions of TnI and myosin with actin**.** In addition, the rates of individual steps in the ATPase cycle and the stability of the regulatory states of the thin filament can change at the very low salt concentrations required in their assays. The very high actin concentrations also can influence the homogeneity of proteins mixed in a highly viscous solution of actin and therefore the homogeneity of myosin binding to actin. As alluded to by Heeley et al. – it is important to be careful about non-physiological

conditions. The requirement for high actin concentrations means that, in most cases, Heeley et al. were only able to use actin concentrations up to ~twice the value of that predicted to produce a half maximal ATPase rate (the effective Km or K_A for actin binding in the steady state). This will lead to uncertainty in the extrapolated values for the maximum value of the P_i release rate constant. Such estimates are normally understood to require 3 to 4 times the effective Km for reliable estimates of the maximum rate constant. The lack of error analysis or the statistical significance of the differences in the values presented by Heeley and colleagues is also conspicuous.

In essence, evidence related by Heeley et al. (2019) is that P_i release can be activated 30% by calcium over that achieved by rigor cross-bridges (the ~2-fold effect is a little larger for the skeletal system). They assert that this is not predicted by the 3-state model and is consistent with direct activation of the P_i release step. However, the two-fold change is rather small on the scale of the changes in ATPase or P_i release rates normally noted during actin activation (up to 1000 fold). We again note that measuring the maximum P_i release rate with high precision is challenging. In fact, thin filaments in the *in vitro* system examined by Heeley et al. may not be fully activated by rigor-like crossbridges under their set of conditions and the rigor bridge activation thus actually underestimated. Even if the P_i information provided is correct, a wholesale rejection of the 3-state mode is not required or prudent. The perspective offered in McKillop and Geeves (1993) 25 years ago was based on the best available evidence at the time. A small effect of calcium on the P_i release could be easily accommodated within the 3-state model, but we doubt the 2-state model proposed by Heeley et al. could easily account for the broader data set discussed here.

Heeley et al. argue that the McKillop & Geeves data and modeling are flawed because the rigor-like crossbridges in that study (i.e. using apo-myosin or with ADP, pyrophosphate, or other analogs bound) were fundamentally different in their interaction with thin filaments compared to typical nucleotide association in the steady-state of the ATPase cycle, either because of some intrinsic difference in the nature of the interaction or because of the transient nature of the interaction in the presence of ATP. Heeley et al. refer to rigor crossbridge conformations as in a *non-physiological form*. While this is hard to prove convincingly, it would require all of the evidence on structural states of the crossbridge based on crystallography or cryo-EM to be similarly dismissed as non-physiological.

Finally, we consider a principal problem with the Heeley et al. 2-state model to be the lack of correlation with any obviously defined or predictable structural states, other than nebulously

referenced active and inactive conformations. Understandably, kinetic data alone cannot provide unambiguous structures or conformations. That means that any possible sub-states in this model due to the effects of calcium and myosin binding remain undefined structurally, making the 2-state model appear to be a semantic artefact. In our opinion, lacking obvious structural correlates, the 2-state hypothesis has little apparent predictive power. In contrast, defining three structural states of actin.Tpm and in actin.TpmTn, whose equilibrium is affected by calcium, myosin and other factors (ionic milieu, temperature, pH, etc.) provides a malleable model that has stood the test of time. Unlike the 2-state hypothesis, the more pliable 3-state model continues to evolve to incorporate ancillary regulatory effects of myosin-binding protein C (Mun et al. 2014), nebulin and titin (Mijailovich, Stojanovic, et al. 2019) as well as adjust to separate modulation of thick filament responsiveness (W Lehman 1978, Irving 2017).

Conclusion

The 3-state model as formulated by McKillop and Geeves in 1993 is not the last word on how muscle contraction is regulated by calcium. Indeed the model has undergone several revisions since first proposed. These include: 1) the size of the cooperative unit differs for the B to C and C to M transitions (Geeves and Lehrer 1994); 2) the incorporation of a worm-like-chain model of Tpm on the surface of actin (Smith and Geeves 2003);. 3) the addition of a 4^{th} state, a substate of the open or M-state with myosin bound in the absence of calcium, implicated hypertrophic cardiomyopathy (Lehrer and Geeves 2014). Each of these has required significant adaptations of the original model. Future modelling will need to include the influence of myosin binding protein C on thin filament activation (Mun et al. 2014) (Irving 2017). The ability of the model to adapt to such new experimental data or ways of thinking is tribute to the utility of the original formulation of the basic model. Recent work on the roles of thick filament strain and myosin binding protein C in the activation of contraction will require further developments to overall models of how contraction is regulated. As always new evidence needs to be carefully evaluated to understand if it truly is a paradigm shift in how we understand the mechanism of regulation or just more precise data that requires a new tweak to a well-defined system.

Acknowledgements

W.L is supported by NIH grant RO1H036153.

The authors declare no competing financial interests

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Table 1. Properties and occupancy of the three states in the McKillop & Geeves model as assayed by different methods.

Tpm position as defined in EM and X-ray fiber diffraction data. Inner and outer refer to the position of Tpm on the inner subdomains (3 and 4) or outer subdomains (1 and 2) of actin. Myosin binding refers to measurements of myosin binding to actin following rapid mixing of myosin S1 with thin filaments (Kinetic) or in titration studies where myosin S1 is titrated slowly into a solution of thin filaments (Equilibrium). In both cases the binding can be followed using the fluorescence of a pyrene label attached to actin. Actin-Tpm occupancy is the interpretation of the occupancy of the three actin.Tpm.Tn states from the myosin binding studies.

TF states		**Blocked**	**Closed**	**Open**
Tpm position on actin		Outer domain	Inner/Outer domain	Inner domain
ATPase/activity		Off/low	Off/low	On/high
Myosin Binding	Transient Kinetic	Off	On	On
	Equilibrium	Off	Off	On
ActinTpm Occupancy	-Tn	0	0.8	0.2
	+Tn -Ca	0.7	0.25	0.05
	+Tn +Ca	0	0.8	0.3
	+Myosin (1:1 ratio of myosin: actin) (+/-Tn, +/-Ca)	0	0	1.0

Table 2. Properties and occupancy of the two activity states in the Heeley et al 2-state model.

The occupancy is based on the K_{eq} values given in Scheme 2 of Heeley et al (2019). The values in brackets are the occupancies predicted by the earlier 2-state model of Hill et al.

States		Inactive	Active
ATPase		Low	High
Occupancy	+Tn -Ca	0.95 (0.95)	0.05 (0.05)
	+Tn + Ca	0.25 (0.8)	0.75 (0.2)
	+myosin (ratio of myosin: actin, 1:7)	0.33 (0)	0.67 (1.0)

Figure 1

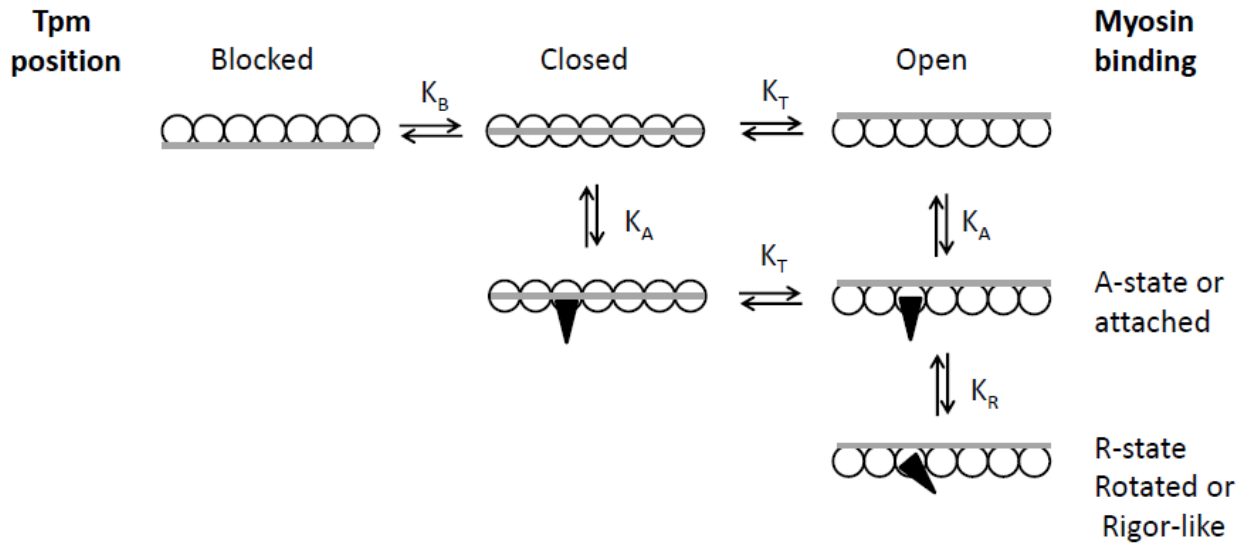

Figure 1 Diagrammatic version of the 3-state model as originally envisaged by McKillop & Geeves (1993). Tpm on a single strand of seven actin monomers can sit in one of three positions on the actin surface Blocked, Closed or Open. In the Blocked position the major binding sites of myosin on actin are blocked by Tpm and no significant binding of myosin is possible (weak electrostatic interaction may be possible). In the Closed position myosin can bind to some of its binding site to form the relatively weakly Attached or A-state but rotation into the Rigor-like-state is prevented by Tpm. More recent structural interpretations of the transition from A- to R-state would suggest that the A-state is formed by the lower 50 kDa domain of myosin binding to actin. The R-state requires closure of the cleft between the upper and lower 50 kDa domains (linked to switch 1 opening) allowing the upper 50 kDa to access its binding site on actin. In the C-state of the thin filament the position of Tpm would sit between the upper and lower 50 kDa domains forming a *molecular gag* preventing cleft closure. See Table 1 for the occupancy of the different states under different conditions

Figure 2

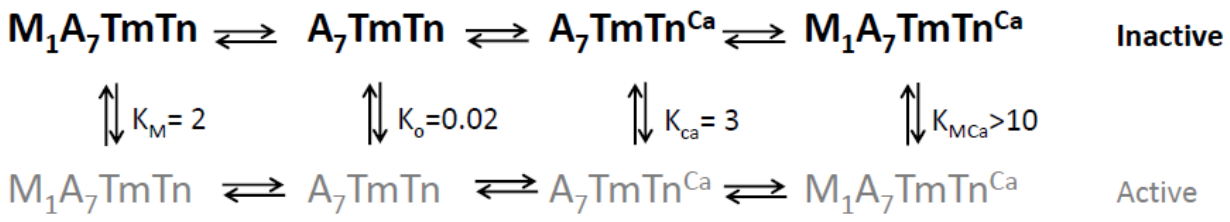

Figure 2 Two-state model of the thin filament based on Scheme 2 of Heeley et al (2019). In this model the thin filament has two activity states Inactive and Active. The linkage between structural transitions of thin filament complex and activity states are not detailed by Heeley et al. To avoid any assumptions about the structural transitions the two activity states are shown as black (**A_7TmTn**) and grey (A_7TmTn) respectively. The thin filament is predominantly in the inactive form in the absence of both calcium and myosin. The binding of either calcium (Ca^{2+}) or a single strongly bound myosin (M) will bias the system towards the active state but neither is sufficient on its own to switch the system totally to the active form. See Table 2 for the fraction of the system on under different conditions.