JGP Journal of General Physiology

COMMENTARY

Changing face of contractile activation in striated muscle at physiological temperature

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Calcium binding to troponin, with subsequent displacement of its linked tropomyosin molecule on the thin filament surface, cooperates with myosin binding to actin in the contractile regulation of striated muscle. The intertwined role of these systems is studied in the present issue of *JGP* by Ishii et al. (https://doi.org/10.1085/jgp.202313414). A particularly interesting feature of the paper, except for studying both skeletal and cardiac muscle proteins, is that the experiments unlike most other similar studies are performed at physiological temperature (35–40°C).

Contractile activation means transition of a muscle from a relaxed state to a state where active crossbridge cycling produces force and motion. The importance of increased cytosolic [Ca^{2+}] to initiate this process in striated muscle was recognized in the 1950s–1960s (Ebashi and Endo, 1968), and soon Ebashi and coworkers (reviewed in Ebashi and Endo [1968]) identified the thin filament regulatory proteins, tropomyosin and troponin (with three subunits, troponin I, troponin C, and troponin T). This, together with other biochemical data and muscle fiber X-ray diffraction paved the way for the steric blocking model of muscle contraction (Spudich et al., 1972; Vibert et al., 1972; Parry and Squire, 1973). The latter model states that activation is initiated by Ca^{2+} binding to troponin causing the troponin–tropomyosin complex to move on the actin surface in the thin filaments to expose binding sites for myosin heads.

The steric blocking model was later expanded into a three-state model (McKillop and Geeves, 1993) to reconcile structural data with biochemical results. This, now widely accepted model (Fig. 1), assumes three states of the thin filaments in equilibrium with each other that block myosin head binding to different degrees. Almost complete blockage is attributed to the "Blocked state" (B-state) where tropomyosin quite effectively covers the myosin binding sites on actin. Less blockage is attributed to the "Closed state" (C-state) allowing weak binding of myosin heads and least blockage in the Open (myosin) state (O-state or M-state) where strong myosin head binding is possible. The B state dominates in the absence of Ca^{2+}. When Ca^{2+} binds to the troponin C subunit, this releases binding of troponin I to actin, allowing tropomyosin to move freely over the actin surface and more extensively populate the C-state, to a greater degree in cardiac than skeletal muscle (Houmeida et al., 2010; Risi et al., 2017). In order to significantly populate the O-state, strong myosin binding is required (Fig. 1). Structural correlates of the states in the three-state model have been published both for skeletal (Vibert et al., 1997) and heart (Risi et al., 2017) muscle with different twists, e.g., explaining higher degree of activation by Ca^{2+} alone in cardiac muscle. The model has also been expanded with details (Mijailovich et al., 2012) to explain a range of cooperative mechanisms (reviewed in Geeves et al. [2019]; Lehman et al. [2020]). For instance, binding of one Ca^{2+} to troponin affects the myosin affinity of several myosin binding sites and the strong binding of one myosin head displaces tropomyosin to expose even more myosin binding sites related to end-to-end linkages of neighboring tropomyosin molecules. Additionally, there is negative cooperativity between actin binding of myosin and of troponin I (Geeves et al., 2019; Brunello et al., 2023).

In contrast to striated muscle, the main regulation of actin–myosin II interactions in smooth muscle and non-muscle cells is not thin filament based but rather relies on phosphorylation of the myosin regulatory light chains upon increased cytosolic [Ca^{2+}] (Craig et al., 1983). Such phosphorylation disrupts an interacting head motif (IHM), with the myosin heads parked at well-defined helical positions along the thick filament backbone where the two heads of each myosin molecule interact with each other as well as with the thick filament backbone. Phosphorylation of the RLC of myosin has long been known to also modulate striated muscle contraction without being a critical on/off switch as in smooth muscle (Sweeney and Stull, 1986). More recently, increasing evidence has emerged for a more central role of myosin and the thick filaments in achieving full contractile activation also in striated muscle (Irving, 2017; see also Brunello et al., 2023). These developments have been catalyzed by structural findings that an IHM, similar to that underlying

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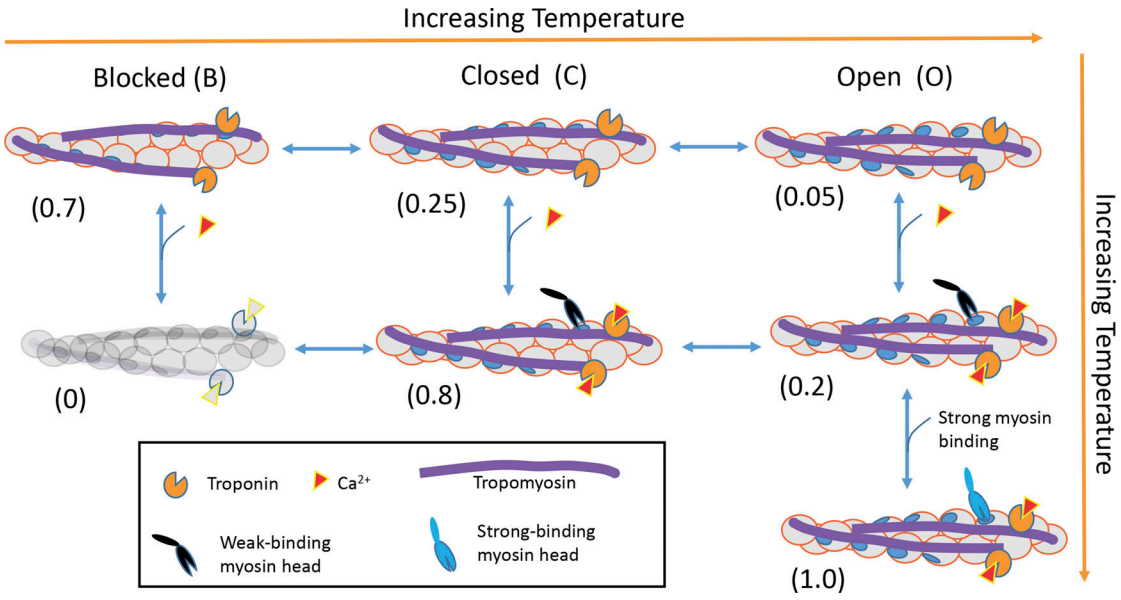

Figure 1. **Schematic illustration of three state model (**McKillop and Geeves, 1993**) of thin filament activation in relation to** Ishii et al. (2023)**.** The equilibrium distribution between the three thin filament states (B, C, and O) is modified by binding of Ca^{2+} to troponin (troponin C) and strong-binding myosin crossbridges (rigor-like) to actin. Numbers in parentheses give proportional occupation of different states based on results from skeletal muscle (Geeves et al., 2019). According to most models of muscle contraction, both high-tension development and high actin–myosin sliding velocity, require the capability of crossbridges to enter strong-binding states. Therefore, the results in Ishii et al. (2023), together with data from some of the papers they cite suggest that increasing temperature in the physiological range shifts the equilibrium from the B and C states to the O state. Additionally, it seems likely that the intervention also shifts the equilibrium of crossbridge binding to the strongly bound state.

the switched off state in smooth muscle and non-muscular myosin II, is a universally conserved mechanism for myosin II inhibition (Jung et al., 2008; Lee et al., 2018).

There is now quite broad acceptance of the three-state model (Fig. 1), considering both thin filament components and myosin motor domains in the thin filament regulation (Geeves et al., 2019; Brunello et al., 2023). Also, as outlined in the preceding paragraph, the importance of thick filament-based regulation has become increasingly recognized. Limited attention has been given to the quite major effects of increased temperature on the regulation and most previous studies have been performed at temperatures appreciably lower than the physiological. However, remarkably, a limited number of studies, including that of Ishii et al. (2023), in the current issue of *JGP*, have presented evidence that temperatures in the range 35–40°C initiate contractile activation that produces significant shortening under virtually Ca^{2+}-free conditions (pCa = 9; $[Ca^{2+}]$ = 1 nM). It is pertinent in this context to mention that human muscle temperature, while normally, 37°C may increase to 39°C after exercise (reference in Ranatunga [1994]). Moreover, the physiological body temperature of rabbits, one species used in Ishii et al. (2023) as well as many other studies, is close to 40°C. The warming-induced activation studied by Ishii et al. (2023) expands previous results dating back to Hill (1970) and references therein, showing that resting tension in living frog skeletal muscle increase with increased temperature. In the 1990s, similar findings were reported for rat skeletal muscle fibers (Ranatunga, 1994). Importantly, Ranatunga showed that the behavior was quite similar in intact (living) and skinned muscle fibers with increase in resting tension upon warming from 25°C to 40°C by an amount corresponding to about 10% of the isometric tetanic tension. The reproduction of the effect in skinned fibers demonstrates that it was not due to increased Ca^{2+} release from intracellular stores because pCa was held constant at 8 in the skinned fibers. Certain characteristics of the resting tension (e.g., delayed stretch activation and reduction by increased concentration of inorganic phosphate) suggested that it was attributed to cycling crossbridges (Ranatunga, 1994). Evidence for Ca-independent activation by heating to physiological temperatures were later found in intact and skinned isolated rat cardiomyocytes (King et al., 2011; Oyama et al., 2012). More recently, the activating effects of increased temperature were observed in the in vitro motility assay (Ishii et al., 2019) where surface adsorbed myosin motors or motor fragments propelled actin filaments that were reconstituted with cardiac troponin and tropomyosin. This suggests that a significant part of the warming effect on activation resides in the thin filaments themselves along with effects of their interaction with myosin motor domains. Interactions with the thick filament backbone, absent in the in vitro motility assays, does not seem critical, suggesting that the activation due to heating may be possible to interpret in terms of the three-state model for thin filament regulation (Fig. 1).

In their current study, Ishii et al. (2023) expand the in vitro motility assay experiments to investigate heating-induced activation with different combinations of myosin and thin filament components from either heart or skeletal muscle and at pCa 5 (full activation) and 9. The most interesting part of the data are those derived under "relaxing conditions" (pCa 9). First, they show temperature dependencies (Q_{10} values) that vary markedly

depending on how troponin and tropomyosin from either cardiac and/or fast skeletal muscle is combined with either cardiac ventricular myosin or fast skeletal muscle myosin. This suggests intertwined effects due to properties of the regulatory thin filament proteins and the myosin isoform with fine-tuning of the activation mechanism and, specifically, the temperature effects. Possibly this reflects fine-tuning of one of the two positive feedback loops proposed (Brunello et al., 2023) to ensure fast cooperative activation according to a recent model for interactions between the thin and thick filament based mechanisms. One may also speculate that it is related to the negative cooperativity between myosin head binding and binding of troponin I to actin (Geeves et al., 2019; Brunello et al., 2023) being modified by switching protein isoforms.

However, these interesting reflections aside, what at first sight is most striking is that the results of Ishii et al. (2023) seem to indicate almost full contractile activation at physiological body temperature in the virtual absence of Ca^{2+} (pCa 9). One would expect that such a property is, among other problems, a clear disadvantage from an energy conservation perspective and by compromising diastolic filling in the heart. However, looking in greater details into the results, one first notices that the maximum activation level, as indicated by the in vitro sliding velocity at pCa 9 with proteins from the heart, is less than half that seen with fast skeletal muscle proteins. Second, one must bear in mind that sliding velocity is likely to overestimate the activation level compared to tension development. Thus, maximum velocity is expected to be reached at a lower number of available crossbridges than required to generate maximum force. This is inherent in models of muscle contraction with independent force-generators (Huxley, 1957). As long as at least one crossbridge is available to propel a thin filament at each point in time, the velocity is determined primarily by the crossbridge detachment rate (detachment limited regime) rather than the number of available myosin binding sites on the thin filaments. This view accords with the previous results (Ranatunga, 1994) showing a resting tension of only 10% of the maximum tetanic force; this would suggest an activation level of only 10% of maximum. Such a low number may not be detrimental, neither with regard to energy consumption, nor with regard to diastolic filling. Instead, it may be advantageous by allowing for a very fast increase in force upon nerve stimulation in a living skeletal muscle, particularly after a warming-up period (Ishii et al., 2023). Based on findings that the observed stretch activation in relaxed muscle is higher at more extended sarcomere lengths, Ranatunga (1994) also suggested that extended sarcomeres become more activated upon stretch at low activation levels which may help to maintain sarcomere length uniformity.

One might think that the activating effects of temperature (Ishii et al., 2023) in the in vitro motility assay, is counteracted by thick filament-based regulation. However, arguing against this idea, Ishii et al. (2023) report that rabbit psoas myofibrils exhibit similar temperature dependence of the activating effect on shortening in the 30–40°C range as suggested by the in vitro motility assay data. It would be of interest to repeat these experiments using cardiac myofibrils.

Ishii et al. (2023) also discuss possible molecular mechanisms that could explain the heating-induced activation. For instance, they note that lower affinity of the troponin–tropomyosin complex for actin has been seen at high temperature and experimental results based on phosphorescence anisotropy suggested increased flexibility of tropomyosin on the actin surface at increased temperature and with a difference between cardiac and skeletal muscle tropomyosin (see Ishii et al. [2023] for references). Additionally, even without Ca^{2+}, recent high-speed–atomic force microscopy movies revealed quite substantial flexibility of tropomyosin on cardiac thick filaments. There is also evidence for increased population of strongly binding force-generating crossbridges upon increased temperature (Offer and Ranatunga, 2015). All these findings are consistent with the idea that, warming within the physiological range shifts the population of the states of the three-state model from the blocked to the open state even in the absence of Ca^{2+} (Fig. 1). This idea is not far-fetched considering that the Blocked:Closed:Open states in skeletal muscle myosin are populated in the ratios 0.7:0.25:0.02–0.05 (McKillop and Geeves, 1991; Geeves et al., 2019) even at 20°C. To the best of my knowledge there are no detailed investigations using biochemical methods (McKillop and Geeves, 1993; Geeves et al., 2019) of how this distribution varies with temperature up to the physiological range.

Aside from the direct physiological relevance of the results of Ishii et al. (2023), it is of interest to note that a majority of previous biophysical and physiological experiments of contractile regulation have been, and still are, performed at temperatures below the physiological range. Therefore, they do not capture key effects observed in the current paper (Ishii et al., 2023). Actually, a significant portion of previous studies are performed at room temperature and below (to preserve protein and structural integrity of, e.g., skinned muscle cells). This does not only miss the type of effects seen by Ishii et al. but also effects of thick filament-based activation that is not well developed below 26°C (Brunello et al., 2023). Clearly, much remains to be learned about contractile regulation at physiological temperatures, exemplified by interesting questions raised by the current work (Ishii et al., 2023).

Acknowledgments

Henk L. Granzier served as editor.

Funding is acknowledged from The Swedish Research Council (grant # 2019-03456) and the Linnaeus University, Faculty for Health and Life Sciences.

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