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The art of the deal in myofilament modulation of function

Margaret V. Westfall

Department of Cardiac Surgery, University of Michigan School of Medicine, NCRC Building 26, Room 263S, 2800 Plymouth Road, Ann Arbor, MI 48109, USA

Both early and long-term remodeling of the heart following myocardial ischemia (MI) includes structural and functional changes within the working cardiac myocyte [1]. One early response during acute MI is a transient elevation in myofilament protein phosphorylation [2,3], and a major target is the molecular switch protein, cardiac troponin I (cTnI) [2–5]. MI initially increases cTnI phosphorylation levels, while these levels often return toward and dip below basal levels with longer periods of ischemia and/or the initial reperfusion [2,6,7]. Similar cTnI phosphorylation patterns develop in tissue adjacent to an infarct and in more distant regions [7], which indicates that this dynamic response is not restricted to a small region of ventricular tissue. The initial elevation in cTnI phosphorylation levels during MI is mediated, at least in part, by PKA and PKC signaling [8,9], and thus, likely results in the phosphorylation at multiple sites within cTnI. PKA and PKC activation can each phosphorylate cTnI Ser23/24 (S23/24) in the N-terminus of cTnI [10,11] (Fig. 1A), and PKC activation also phosphorylates Ser43/45 and Thr144 in cTnI ([10]; using rat sequence, uniprot P23693). Alterations in the phosphorylation pattern at specific residues have yet to be identified and/or investigated for their functional role during acute MI. Additional MI-induced signaling pathways which are likely candidates to contribute to elevated cTnI phosphorylation are the AMP-activated protein kinase (AMPK) and p21-activated kinase 3 (Pak-3) cascades. MI activates the AMPK signaling pathway (see [12]), and this pathway can phosphorylate Ser150 (S150), and also target S23/24 in cTnI [13]. The same S150 residue is phosphorylated by p21-activated kinase 3 (Pak-3, see [14]) and there is evidence MI activates Pak signaling [15]. However, Pak-3 is primarily localized in the brain [16], and therefore, is a less likely candidate than AMPK to target cTnI S150 in the myocardium. The AMPK-mediated phosphorylation of cTnI S150 during MI increases myofilament Ca^{2+} sensitivity and enhances the amplitude of cardiac contraction while slowing relaxation [17,18]. This response is noteworthy because it is in the opposite direction from the reduced myofilament Ca^{2+} sensitivity and accelerated cardiac relaxation rate produced by S23/24 phosphorylation (reviewed in [19]). An open question now addressed in a recent study by Nixon and colleagues is whether MI increases cTnI phosphorylation at S150, presumably via the activation of AMPK [20]. In this work, MI enhances phosphorylation of both S23/24 and S150 in cTnI. The development of acidosis also is an early and often major response during MI [21], which profoundly influences myofilament function within myocytes [22]. Perfusion of hearts with an acidotic buffer

similar to the pH reached during MI also triggers elevated myofilament phosphorylation in hearts [23]. While the specific cTnI residues phosphorylated during acidosis are not yet known, the pattern is expected to be comparable to the MI response. Nixon et al. now utilize this relationship to demonstrate that S150 in cTnI maintains steady state thin filament function in response to the acidosis associated with MI [20]. This conclusion about S150 in this study is based on the independent and combined influences of cTnI S23/24 and S150 phosphomimetics on troponin and re-constituted thin filament behavior using fluorescent probes. For each preparation, Ca^{2+} -activated responses obtained at physiological pH were compared to acidic pH results [20]. Several observations in troponin and the more complex thin filament preparations were consistent with each other, but there also were some inconsistencies. While a divergent result in protein and thin filament preparations is not new for this research area, the present work demonstrates how these similarities and differences can provide important mechanistic insights. For example, the anticipated leftward shift in the sensitivity of steady state Ca^{2+} binding with cTnISer150Asp (cTnIS150D) and the rightward shift with cTnISer23/24Asp (cTnIS23/24D) are observed in the troponin and thin filament preparations. Acidosis also reduced the Ca^{2+} sensitivity of steady state binding in both preparations. The comparable responses in both preparations provide evidence that a conformational change within troponin plays a key role (Fig. 1B). In contrast, cTnIS150D significantly attenuated the acidosis-induced decrease in the Ca^{2+} sensitivity of the thin filament, but not in troponin alone. This result indicates the fluorescence response to acidosis with cTnIS150D depends in part on the transmission of information along the thin filament, and not just within the troponin complex (Fig. 1C). *In vivo* studies to examine S150 contributions during MI are certainly needed in the future, but would be difficult to interpret without these *in vitro* insights. In addition to cTnIS150D alone, the responses in thin filament *versus* troponin preparations differed for the combined phospho-mimetic mutant, cTnISer23/24Asp/Ser150Asp (cTnIS23/24/150D). Phosphorylation of cTnI plays a critical role in fine-tuning cardiac performance, and yet there is little known about the functional consequences when multiple residues are phosphorylated on cTnI. At least 10 phosphorylation sites are present within cTnI [24], and proteomic studies on human tissue detect cTnI phosphorylation on multiple residues [24–26]. The relatively close spatial proximity of S23/24 and S150 residues within troponin raises the possibility that these sites interact and/or their combined influence could instead depend on transmission along the thin filament (Fig. 1C). Studies with cTnIS23/24/150D begin to address these questions about their combined impact on function. The Ca^{2+} sensitivity of steady state binding with cTnIS23/24/150D was midway between cTnIS23/24D and cTnIS150D under physiological conditions, but this was not the case for acidosis. Interestingly, neither the additive decrease in Ca^{2+} sensitivity produced by cTnIS23/24D and acidosis nor the attenuated pH-induced shift observed with cTnIS150D explained the response produced by cTnIS23/24/150D. Instead, the combined cTnI construct produced a response comparable to the wildtype cTnI response to acidosis alone. In troponin alone, cTnIS23/24/150D attenuated the acidosis response and Ca^{2+} sensitivity was much closer to results obtained with cTnIS150D for both pH conditions. These results suggest that the combined influence of S23/24 and S150 depends on communication within and/or along the thin filament. The kinetics of thin filament Ca^{2+} dissociation rate, which is most often the initial rate limiting step in relaxation [27], also was evaluated using the same phospho-mimetic constructs [20]. Unlike steady

state Ca^{2+} binding, this response was not pH sensitive, and the accelerated dissociation rate observed with cTnIS23/24/15D resembled cTnIS23/24D much more than the slowed rate observed with cTnIS150D alone. In additional studies with myosin S1-ADP, the individual phospho-mimetic substitutions each slowed the strongly bound rigor detachment rates in a pH-independent manner. These additional observations suggest that S150D and S23/24D operate independently for some components of thin filament function and *via* shared responses for others, depending on the mechanisms probed in each assay. Overall, the results with cTnIS23/24/150D are expected to translate into the *in vivo* maintenance of systolic function under physiological pH, and a relative preservation of the diastolic interval regardless of pH. If proven to be true, the multi-site phosphorylation examined by Nixon et al. [20] could optimize ejection and minimize changes in relaxation and/or diastolic filling for each cardiac cycle. This shift in behavior could be especially important under pathophysiological conditions such as MI. The responses observed by Nixon and colleagues are intriguing [20], although a number of critical questions remain to be answered. First, the influence of multi-site phosphorylation should be further examined using *in vivo* animal models. However, the present study also suggests that a systematic analysis of multi-site phosphorylation is needed across a range of preparations of differing complexity. The exclusive focus on one extreme, such as *in vivo* animal models or preparations of limited complexity, such as purified troponin is unlikely to provide the desired mechanistic insight. Instead, an integrated mechanism may depend on information gained from a range of preparations studied under physiological and pathophysiological conditions. As mechanistic insight into the role played by S150 evolves, this site alone and/or in combination may be a target for small molecule therapeutics, especially if S150 phosphorylation levels are associated with improved cardiac tissue survival and/or function during MI. Such a therapeutic could be particularly useful for clinical procedures with a significant risk for transient ischemia. One additional set of observations worth noting in the recent work by Nixon and colleagues [20] is the differential impact of acidosis, which modified steady state Ca^{2+} binding in a cTnI phosphomimetic-dependent manner and yet had no influence on Ca^{2+} dissociation rates. The influence of cTnIS23/24/150D on the Ca^{2+} dissociation rate also was much closer to the influence of cTnIS23/24D alone. This difference may depend on experimental conditions for steady state *versus* kinetic assays. Alternatively, these results indicate that it may be possible to develop a myofilament Ca^{2+} sensitizer with little influence on Ca^{2+} dissociation rates. Unlike many current Ca^{2+} sensitizers [28,29], a desirable property of a new generation of these small molecules would not influence the Ca^{2+} binding pocket of troponin C. Instead, their mechanism would involve modifying the molecular switch “on” functions in the thin filament without altering the Ca^{2+} dissociation rate. The current study by Nixon et al. [21] is just a start in our efforts to understand the modulation of sarcomere function by multi-site phosphorylation, and many questions still remain to be answered. Work is needed to determine whether multi-site phosphorylation is distributed across different cTnI proteins, restricted to a single cTnI, and/or localized to sub-regions within the sarcomere. A spatial distribution of multi-site phosphorylation within cTnI may produce similar or different responses than the observations made in the present study. A temporal component also may be involved in fine tuning of the sarcomere. The spatial and temporal issues could be equally important for other phosphorylated residues in addition to S23/24 and S150, other phosphorylated myofilament proteins, and/or with other

post-translational modifications. The results obtained with acidosis by Nixon and colleagues also raise questions about the importance of multi-site phosphorylation under pathophysiological conditions. Related to this issue, their results raise questions about spatial and/or temporal relationships between S150 phosphorylation and the development of cTnI degradation during MI [2]. In the future, both mechanistic and clinically relevant insights are expected to emerge as experimental work fills these and other key gaps in our understanding of modulatory signaling in the myofilament.

Acknowledgments

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Fig. 1.

A. Illustration of flexible (lines) and alpha-helical (cylinders; H1–H4) domains within cardiac TnI. Additional designations are for the N-terminal extension, which is unique to the cardiac isoform, the inhibitory peptide (IP), and the locations of the S23/24 and S150 residues. B. The troponin complex composed of troponin T (TnT, green), TnI (pink) and troponin C (TnC, orange) based on the crystal structure [30]. The amino (N) and carboxy (C) termini of proteins are shown in addition to the S23/24 and S150 locations (pink buttons). Evidence of similar responses in purified troponin and in the thin filament suggests that conformational changes in the troponin complex are responsible for the functional response. C. Illustration of a portion of the thin filament showing the coiled coils of actin (blue), tropomyosin (Tm; gray), and the troponin complex (green, pink and orange). Transmission of signals by troponin positioning could influence TnT and Tm positioning on the thin filament (white arrow) and/or be transmitted to neighboring functional units (yellow arrows).